Estrogens and progesterone in the posterior vena cava and estrogens in the urine of cattle
by Pran Nath Varman

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Animal Science (Animal Physiology)
Montana State University
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Abstract:
Cannulation of the posterior vena cava of four Jerseys and one Holstein (non-gravid) and one Jersey (gravid) was performed. Blood samples were collected every other day until the tubing plugged* Urine was also sampled at the same time* The samples of blood were analyzed for the estrogens and progesterone and of urine for the estrogens* Two peaks in the total blood estrogens occurred in all cows sampled* In the majority of the cows the first peak occurred during the period of days 6 to 8 and the second during the period of days 12 to 17 after heat* Similarly, the quantities of the excretion of estrogens in the urine reached two peaks* In most of the cows the first occurred during a period between days 3 and 8, whereas the second peak occurred between days 15 to 20, after heat* The levels of progesterone reached a peak level on day. 17 Of the estrous cycle* During thirty-four days postconception only one peak of the total estrogens in the blood and urine, corresponding to the first peak observed during estrous cycle, was found to occur. The possibility of the interplay of the gonadotropic hormones, progestins, estrogens and the estrogen-inactivating mechanism has been postulated.

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ABSTRACT

Cannulation of the posterior vena cava of four Jerseys and one Holstein (non-gravid) and one Jersey (gravid) was performed. Blood samples were collected every other day until the tubing plugged. Urine was also sampled at the same time. The samples of blood were analyzed for the estrogens and progesterone and of urine for the estrogens.

Two peaks in the total blood estrogens occurred in all cows sampled. In the majority of the cows the first peak occurred during the period of days 6 to 8 and the second during the period of days 12 to 17 after heat. Similarly, the quantities of the excretion of estrogens in the urine reached two peaks. In most of the cows the first occurred during a period between days 3 and 8, whereas the second peak occurred between days 15 to 20, after heat.

The levels of progesterone reached a peak level on day 17 of the estrous cycle.

During thirty-four days postconception only one peak of the total estrogens in the blood and urine, corresponding to the first peak observed during estrous cycle, was found to occur. The possibility of the interplay of the gonadotropic hormones, progestins, estrogens and the estrogen-inactivating mechanism has been postulated.

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INTRODUCTION

It has been recognized for many years that one of the larger problems concerned in the physiology of reproduction of animals (especially the cattle) is low fertility rather than sterility. Many factors contribute to low fertility and thus cause great economic loss to the animal industry. Disorder of the endocrine glands is probably one of the most important factors.

Another problem being faced by research workers interested in the reproductive physiology is inconsistant results of synchronization of the estrous cycle of domestic animals. Similarly the physiologists engaged in the study of ova-transplantation in cattle have not as yet completely succeeded.

It seems evident that there is a lack of knowledge concerning the physiological levels and the interactions of various hormones concerned with reproduction. This lack of knowledge may be responsible for the Veterinarians not being able to satisfactorily diagnose or treat some of the endocrine disorders of the animals. This may also very well be responsible for the unsatisfactory responses in synchronization and ova-transplantation.

The levels of the estrogens circulating in the blood, as well as being excreted in the urine of normal women during menstrual cycle, have already been established. Whereas there has been no report which has shown the day-to-day changes in the levels of these hormones during the estrual cycle of domestic animals.

In contrast to relatively higher concentration of estrogens in

the urine, the quantities of these hormones in the blood obtained from the jugular vein of the cattle are extremely low.

It is known that the posterior vena cava drains blood from the hind portion of the body and also receives blood from the ovaries, uterus (also placenta in pregnant individuals) and the adrenals. Collection of the blood from this vein before much dilution and inactivation of the sex hormones has taken place can possibly be advantageous.

Considering the problems mentioned above, the purpose of this study was:

(1) To cannulate the posterior vena cava at a site close to all the points of secretion of the sex hormones.

(2) To determine the day-to-day changes in the levels of the endogenous estrogens and progesterone in the blood and estrogens in the urine of cattle during the estrous cycle.

(3) To determine the relationship of the secretion and urinary excretion of the estrogens.

I. DEFINITIONS OF ESTROGENS AND PROGESTINS

A. Estrogens:

Estrogens, a Greek word, is made up of two words, oistros, meaning "mad desire" and "Gen" referring to "to beget" (Stedman's Medical Dictionary). It is, therefore, evident that an estrogenic compound refers to any substance which produces estrus whether derived from the ovary or not. In general, estrogenic compounds may also produce growth of secondary sexual characteristics, changes in vaginal mucosa, and proliferation of the endometrium. A hormone is defined as a physiologic, organic substance liberated by living cells of a restricted area of the organism which diffuses or is transported to a site in the same organism where it brings about an adjustment that tends to integrate the component parts and actions of the organism (Zarrow, 1962). Estrogenic substances found in animals are referred to as natural estrogenic hormones because these compounds do fit the preceeding definition of hormones.

Chemically, estrogens are steroids and are characterized by an aromatic Ring-A and a hydroxyl group at carbon atom-3. Estrane is considered to be the parent compound of the estrogens. The major estrogens of mammals are estrone, 17α and 17B-estradiol, and estriol (Fieser and Fieser 1959).

The nomenclature of the two isomers of estradiol refers to the "cis" or "trans" position of the hydroxyl group at carbon-17 position.

The isomer with "cis"-hydroxyl group is designated by the prefix "B"

while that with "trans" hydroxyl group as the "α" (Turner, 1961).

B. Progestins:

Another major group of hormones of reproduction is known as

Progestins. "Pro" (Greek) means "in favor of" and gestatio (Latin),

from gastatus, meaning "to bear" (Stedman's Medical Dictionary). This

generic term was suggested for substances regardless of chemical

purity, which cause changes in the endometrium, adapting it for the

reception of the fertilized ovum (ova in multiparous animals).

Progestins also, may partially, regulate the periodicity of the sexual

cycle, stimulate the mammary glands, inhibit uterine musculature and

relax the pelvic ligaments (Turner, 1961).

Progesterone, also a steroid, is the most potent natural

progestin. Theoretically, allopregnane and pregnane are the parent

substances for the progestins.

II. METABOLISM OF ESTROGENS AND PROGESTERONE

A. BIOGENESIS

Estrogens:

The estrogens have been isolated and identified from various

tissues and endocrine glands of the body. These hormones, in the

female, are produced by the ovary, placenta and adrenal cortex and in

the male by the testis and the adrenal cortex. Like other steroid

hormones and unlike nitrogen containing hormones, estrogens are not

stored in the organs concerned with their biogenesis (O'Donnell and

Preedy, 1961).

It is generally thought that estradiol-17B is the principal estrogenic hormone produced by the ovary and is the mother substance of estrone and estriol (Boscott, 1962, Harrow and Mazur, 1962). To determine the sites of biogenesis of estrogens, extraction and identification of these hormones from various tissues have been performed. However, Nyman et. al. (1959) have pointed out that the presence of a steroid in an endocrine organ is no proof of its synthesis at that site, nor does the ability of the tissue to metabolize a steroid prove that the products are released into the blood stream in physiologically significant amounts.

Human ovaries obtained from patients primed with FSH are able to convert acetate into estrone, estradiol, and cholesterol (Ryan and Smith, 1961a). Similarly, Heard et. al. (1956), and Werthessen et. al. (1953) have reported the conversion of acetate to the estrogens in pregnant mare and sow ovaries respectively, but Hollander and Hollander (1958) during in vitro studies with dog ovaries could not demonstrate the conversion of acetate-1-C^{14} to C^{14}-estradiol-17B and instead some radioactive cholesterol was isolated. Conversion of acetate to cholesterol in the rat has also been reported (Caspi et. al. 1962).

Bloch (1945) pointed out that the animal organism is capable of synthesizing the steroids which it normally requires. It was, therefore, thought that the compounds possessing the cyclopentanophenanthrene structure arise either individually by synthesis or by degrada-

tion from a common precursor. Cholesterol has been suggested to be the precursor of the steroid hormones (Bloch, 1945, Ryan and Smith, 1961c, and Werbin et. al. 1957).

Huffman et. al. (1940) proposed that progesterone could function as an obligatory or main intermediate in normal estrogen formation. Ovaries from already primed humans are able to convert progesterone-C^{14} into radioactive estrone and estradiol (Ryan and Smith, 1961b). Although estrogens have been isolated from the human placenta, Ryan and Smith (1961b) were unable to detect any in vitro conversion of progesterone to estrogens in human placental system. Very recently Aasted and Stakemann (1963) have reported that the estrogens of human pregnancy are produced neither by the placenta nor by the fetal adrenal alone but by a complex procedure involving both tissues. It has, therefore, been suggested that fetal adrenals produce a steroid precursor which is metabolized into estrogens by the placenta.

Nathanson and Towne (1939) demonstrated that the use of testosterone propionate in human female castrates leads to vaginal cornification and increased urinary estrogen levels, decreased follicle stimulating hormone titres and increased urinary androgens. The in vivo conversion of testosterone propionate by ovariectomized and andrenalectomized women, to estrone and estradiol-17B has been reported by West et. al. (1956). Estrone and estradiol are promptly synthesized when testis of the gonodotropin primed stallion are perfused with horse blood and sodium acetate (Nyman et. al. 1959). This may be due to the presence

of a relatively high proportion of Leydig's cells in stallion testis.
Similarly pregnant mares have also been found to be able to convert
testosterone into the estrogens (Heard et. al. 1956), and for the con-
version of testosterone to estrogens in human ovarian tissue (Bagget
et. al. 1956, Wotiz et. al. 1956), in placental tissue (Ryan, 1959a)
and in rat ovaries (Stitch et. al. 1962).

Ryan (1959b) demonstrated that 16α-hydroxyestrone (a metabolite
product of androstenedione) is metabolized by the placental system to
form estriol. It was suggested that aromatization of an already 16-
hydroxylated androgen may also give rise to estriol.

It is known that 19-nortestosterone when administered to humans
is converted to estrone and the magnitude of conversion is similar to
the one observed on the administration of testosterone. These findings
indicate that the absence of the angular methyl-group (C-19) does not
significantly influence the extent of the conversion (Engel et. al.
1958).

Progesterone:

Allen and Wintersteiner (1934) were the first to isolate pro-
gesterone from extracts of corpus luteum. It is generally believed
that progesterone is synthesized by all the steroid-producing glands
via the acetate→cholesterol→pregnenolone→progesterone pathway (Samuels
1955).

Although there is no direct evidence, a general agreement exists
that the cells of the theca interna in the graafian follicle are

responsible for the estrogen production. The granulosa cells, after

they have hypertrophied to form the corpus luteum, are responsible for

the production of progesterone (Short 1961). Teledy et. al. (1963)

have reported that in dogs, during estrous cycle, no progesterone could

be detected in ovarian venous blood when the corpus luteum was not

present. Similarly, Huang and Pearlman (1962) have indicated that the

rat's corpus luteum in contrast to human's does not form estrogens and

instead is responsible for the formation of progesterone. It is

interesting to note that cow adrenals are capable of secreting as much

as 1.5 mg. progesterone per hour (Balfour and Comline 1957).

B. INTERCONVERSION OF ESTROGENS

Following the isolation of estrone from the urine of pregnant

women by Doisy et. al. (1929), this ketonic estrogen was considered to

be the hormone of the ovarian follicle. In 1936, however, MacCorquodale

et. al. were able to identify 17α-estradiol as the principal estrogenic

hormone of the liquor folliculi of the swine ovary. From time to time

various workers have demonstrated the interconversion of estrogens.

The conversion of α-estradiol into estrone in man (Heard and Hoffman

1941) and into estrone and B-estradiol in guinea pigs and rabbits

(Fish and Dorfman 1941) has been demonstrated. Similarly the conversion

of estrone to estriol and estradiol-17α in rats (Wotiz et. al. 1958b)

and in humans (Pearlman and Pincus 1943) has been demonstrated. It

has been suggested that both estradiol-17α and estriol may very well be

the metabolites of estrone. Intestinal microorganisms have also been

shown to be able to reduce estrone to estradiol (Beck 1950).

The conversion of estradiol-17B to other estrogens has also been reported by many workers. Engel et. al. (1959) demonstrated that the human fetal liver slices are capable of converting estradiol-17B into estriol. The conversion in man of estradiol-17B into 16-Ketoestradiol-17B (Levitz et. al. 1956), and 2-methoxyestrone (Kraychy and Gallagher 1957) have also been demonstrated.

C. INACTIVATION OF ESTROGENS AND PROGESTERONE

1. ESTROGENS

The maintenance of circulating estrogens at a physiological level is dependant to a considerable extent on the inactivation of the estrogens by metabolic conversions. A highly effective mechanism for the capture of the free estrogens is in existance in the body tissues. It has been demonstrated in rats (Oakey et. al. 1962) and in humans (Fishman et. al. 1960) that within 30 minutes after the administration of exogenous estrogens, about 98 percent of the total free hormone cannot be recovered from the blood.

If an ovary is transplanted into the mesentery, or estrogen pellets are implanted intrasplenically, the hormone would be inactivated by its passage through the liver prior to its entrance into the systemic circulation. This shows that the liver is a very important organ for the inactivation of the estrogens (Coppedge et. al. 1950).

Inactivation of estrogens may take the form of conjugation and elimination from the body, and metabolism to known and some unknown

compounds, and/or oxidation (Eccles et. al. 1962). Very little is known about the nature of the inactivation products of estrogens but the evidence exists that estrone and estradiol are attacked by an oxidative enzyme system (Heller, 1940).

The liver is thought to form non-steroid estrogen metabolites as degradation products of estrone (Jellinck, 1959). Similarly, Werthessen et. al. (1950) have demonstrated that estrone on incubation with blood medium loses its 17-Ketosteroid group as well as its estrogenic activity. The possibility of the presence in blood of an enzyme capable of inducing oxidation of estrone to a form whose nature is unknown has therefore been suggested.

Conjugated and Bound Estrogens:

The term conjugated means a probably chemical union such as sulfuric acid or glucuronic acid with a hydroxyl group on a steroid molecule to form an ester. Whereas binding means any association of a steroid with a protein (Antoniades et. al. 1957).

In 1938, Schachter and Marrian reported the isolation of potassium salt of estrone sulfate from the urine of pregnant mares. Similarly the occurance of estrone-hydrogen sulfate in the urine of pregnant women (McKenna et. al. 1961) and estriol in women (Levitz et. al. 1958) has also been established. Conjugation of diethylstilbestrol with sulfuric acid was suggested to be occurring in rat, cat and dog but not in rabbits (Starnes and Teague 1949), whereas its conjugation with glucuronic acid in the rabbit had already been demonstrated by

Mazur and Shorr (1942). Zimmerberg (1946) indicated that the conjuga-

tion of diethylstilbestrol is perhaps the preferred method of inactiva-

tion. Oxidation of this synthetic estrogen occurs only when the liver

cannot handle it by conjugation.

Diczfalusy (1953) reported that a large proportion of estrogens

in the pregnancy blood or plasma occurs as conjugated, presumably

glucuronides and sulfates. Both conjugated and unconjugated radio-

activity in extracts of various protein fractions of plasma have been

demonstrated by Antoniads et. al. (1957). Similarly Purdy et. al.

(1961) found that plasma obtained two and one-half hours after the

administration of C^{14}-estradiol to women, contained C^{14}-estrone sulfate

as the principal radioactive metabolite. It was, therefore, suggested

that estrone sulfate may be an important transport form of estrogens

in human plasma. DeMeio et. al. (1958) have shown that Mg^{++} and

adenosine triphosphate are essential for the biosynthesis of the

sulfates of estrone and estradiol-17B.

The presence of estrogenic lipoprotein complex in the circula-

tion of several species has been reported by many workers. Estrogen-

serum protein binding in the presence of surviving rat liver tissue

occurs selectively with albumin (Szego and Sidney, 1956) and this

mechanism is correlated with the functional state of the liver (Szego,

1953). It has also been reported that the enzymetically catalyzed

association of estrone or its metabolites with albumin is inhibited or

abolished by increasing the concentrations of cortisol. This shows a

competitive phenomenon exhibited by the estrogens and certain cortico-
steroids in vivo (Szego and Sidney, 1956).

Reigel and Mueller (1954) have indicated that the liver, to a
much greater extent than other tissues (uterus, submaxillary glands and
kidneys), contains an enzyme system which catalyzes the formation of a
protein-bound estrogen. This system requires an electron-donating
substrate, an electron-transporting coenzyme (TPN) and oxygen,
suggesting thereby an oxidative or perioxidative step in the binding
process. Erlanger et. al. (1959) have estimated that at least twenty
estrone residues are linked to each molecule of bovine serum albumin.

B-glucuronidase, an enzyme which can hydrolyze estrogen-
glucuronides, has been found to exist in the liver and spleen of calves
(Bernfeld et. al. 1953); spleen of calf (Bernfeld and Fishman 1953);
ox spleen (Mills 1948). The major portion of the glucuronidase
activity of the blood is concentrated in the leucocytes and platelets.
B-glucuronidase activity has also been demonstrated in saliva, gastric
juice, spinal fluid, urine and tears (Fishman et. al. 1948).

2. PROGESTERONE:

The inactivation of progesterone is brought about either by its
conversion to other metabolites or the binding with serum proteins.
Short (1961) has indicated that if an assumption is made that the
progesterone molecule can undergo reduction at C_3, C_5 and C_{20}, theoreti-
cally, twenty-six possible progesterone metabolites can be formed.
Eleven of these are already known to occur in human tissues or urine.

The generally accepted scheme for representing reduction of progesterone involves arrangement of the metabolites in order of increasing state of reduction (Taylor 1955). The scheme indicates that progesterone is reduced initially at the double bond in Ring-A to yield 5α- and 5B-pregnene-3:20-dione which undergo reduction at C-3 to the three pregnanolones, 3α-hydroxy-5α-pregnan-20-one, 3B-hydroxy-5α-pregnan-20-one and 3α-hydroxy-5B-pregnan-20-one. The latter is then reduced at C-20 to give 5B-pregnane-3α:20α-diol (pregnanediol).

Bound-Progesterone:

There has been no report which has shown that progesterone exists in the body in a conjugated form. When incubated with whole blood very little progesterone enters the red-cells and instead its major portion remains confined to the plasma (Short, 1958a).

Westphal et. al. (1955) observed that when progesterone is added into human serum albumin or blood serum and the mixture is run on paper electrophoresis, over 90 percent of the added progesterone migrates with the protein fraction. When it is added to rat serum albumin only 50 percent migrates with the protein fraction, indicating pronounced species difference. It has also been indicated that not more than one albumin molecule associates with each molecule of progesterone (Short 1961). Eik-Nes et. al. (1954) have reported that the albumin fraction of plasma proteins is responsible for the solubility of the progesterone in the blood whereas globulins have no measurable effect of such kind.

III. EXCRETION OF ESTROGENS

Between 50-80 percent of the estrogens in the blood are present
as closely bound to plasma proteins. Presumably this prevents the
bound hormone from being filtered out of the blood as it passes through
the glomerulus of the kidney (Villee 1961). Excretion of estrogens in
various excretory products has been studied by many workers. It has
been observed that the major portion of estrogens excreted in the urine
is in a conjugated form. As such the estrogens are firmly united to
such hydrophilic groups as sulfuric or glucuronic acid so that their
solubility in the urine is increased. The low binding capacity of these
conjugates with serum albumin explains their rapid filtration through
the glomeruli of the kidney into the urine (Eik-Nes et. al. 1954). In
cattle the excretion of the estrogens in urine during gestation is
lowest on the 50th day and an increase in the excretion at an accele-
rated rate occurs during the last part of gestation (Nelson and Smith
1963). Similarly the urinary estrogenic content on the 10th day after
parturition has been shown to be equivalent to 251 ug. of estrone per
liter as compared to a value of 642.5 ug. found on the 275th day of
pregnancy (Smith et. al. 1956).

Assay of urinary estrogens in primates has often shown double
peaks (Everett 1961). The occurrance of a double peak in the levels
of estrogen excretion in the urine by a female chimpanzee (Fish et. al.
1941) is shown in figure no. 1.
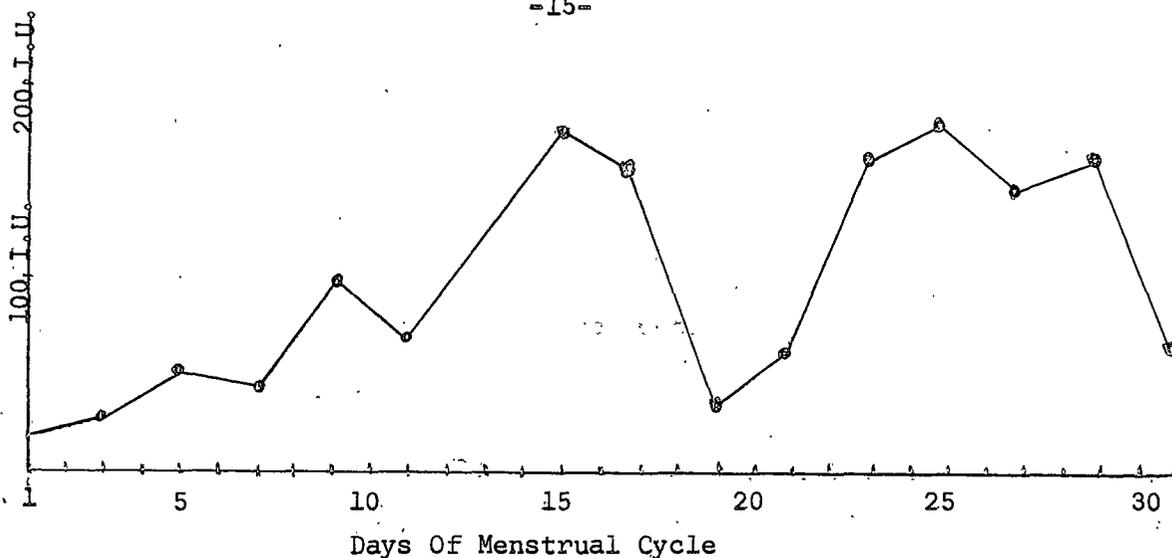
Figure 1: Total estrogen excretion by a female chimpanzee
         (from Fish et. al. 1941)

The occurrance of such two peaks in normal human females has

also been demonstrated (Markee and Berg 1944; Pedersen-Bjergaard and

Pedersen-Bjergaard 1948). The first peak has been observed to occur

on day 12 and the second on day 21 of the menstrual cycle. No such

peaks have ever been shown to occur in the bovines.

IV. EXTRACTION, ISOLATION AND MEASUREMENT OF ESTROGENS AND PROGESTERONE

A. EXTRACTION OF ESTROGENS

It is known that the urinary estrogens exist in a conjugated form.

To free these hormones some kind of hydrolysis is necessary. Stimmel

(1946) acidified urine samples (to Congo red) with hydrochloric acid

and then extracted with 0.125 volume of butyl alcohol. After the

extraction, the butyl alcohol was evaporated and the residue was taken

up in 100 ml. of 0.2 N NaOH solution which was further diluted to 400 ml.

volume with water. This mixture was then acidified with 10.0 ml. of concentrated hydrochloric acid and autoclaved at 15 pound steam pressure for 3 hours. The free estrogen content, after hydrolysis, was then extracted with ether which was ultimately washed with 9 percent $NaHCO_3$ solution and finally with water. The recoveries of estrogens added to an essentially estrogen-free urine (from castrated female) extract for estrone and α-estradiol, and estriol were 80 to 110 percent and 57 to 94 percent respectively.

Friedgood et. al. (1948) hydrolyzed the urine samples by using 30 volume percent of 6N sulfuric acid (equivalent to 15 volume percent of concentrated hydrochloric acid). The mixture was refluxed for 10 minutes and extraction was then achieved with ether. The quantitative removal of estrogens from the ethereal extract was accomplished through a reduction in their solubility by the addition of carbon tetrachloride. The estrogens were then extracted with 1N-KOH solution whereas the neutral steroids were found to remain in the organic phase (ether: carbon tetrachloride as 1:18). Potassium hydroxide phase was then acidified with 6N-sulfuric acid (to Congo red) and extracted with ether which was ultimately washed with sodium bicarbonate solution and water. The recoveries of added estrone, estradiol and estriol were found to be 91.5-99.9 percent, 91.6-100 percent and 91.0-98.0 percent respectively.

Stimmel (1949) studied the effect of the addition of 4 mg. percent zinc along with 15 volume percent hydrochloric acid on the hydrolysis of urinary conjugated estrogens. It was observed that under

such treatment estrone was converted to estradiol, whereas 15 volume

percent of hydrochloric acid alone and refluxing for 10 minutes gave

the maximum recovery of 90 percent. The next highest recovery was

obtained with 5 volume percent of sulfuric acid and refluxing for 10

minutes. Similarly Engel et. al. (1950) reported that higher yields of

estrogens are obtained after autoclaving human pregnancy urine with

15 volume percent of 12N sulfuric acid for 5-10 minutes than after

refluxing for 10 minutes with the same concentration of the acid.

In 1954, Katzman et. al. found that pregnancy urine containing

15 volume percent concentrated hydrochloric acid when boiled for 30

minutes gives the highest recovery whereas Preedy and Aitken (1961)

have reported that maximum recoveries are obtained by refluxing the

urine samples with 15 volume percent concentrated HCl for 45 minutes.

Anyway, on hydrolysis with such a concentration of acid for 30 minutes,

the free estrogens are found to be quite stable (Katzman et. al. 1954).

Even though acid hydrolysis for extraction of urinary estrogens

is so widely used, the use of some enzyme preparations for this purpose

is another alternative. Katzman et. al. (1954) have indicated that

the presence of chromogenic material in urinary extracts when acid

hydrolysis is used, can be a major obstacle to the use of the Kober

method for the estimation of estrogens. The use of enzyme preparation

has, therefore, been advocated. Beer and Gallagher (1955a) have

supported the report of Katzman et. al. (1954) that glucuronides form

the major fraction and sulfate esters a very minor fraction of estrogen.

esters in the urine. This is perhaps the most probable reason why

only B-glucuronidase enzyme has been widely used (Kinsella et. al. 1956;

Beer and Gallagher 1955a and 1955b). P-mylase, which contains

phenolsulfatase-enzyme and "glusulase", an enzyme preparation which

contains both glucuronidase and sulfatase, have also been used

(Kinsella et. al. 1956; Givner et. al. 1960; Ladany and Finkelstein

1963; Veldhuis 1953).

Mather (1942) studied the distribution of estrogens between

immiscible solvents. The distributions between benzene or ether and

0.3-M sodium carbonate solution were recommended for separating estriol

from other estrogens.

It has well been established that a large proportion of the

estrogens circulating in the blood are in a conjugated form (Antoniads

et. al.1957; Purdy et. al. 1961). It is also known that estrogens are

transported in the blood in the form of an estrogenic-lipo-protein-

complex (Szego and Sidney 1956). It, therefore, seems evident that

some kind of hydrolysis is essential to set the estrogens free. Preedy

and Aitken (1961) have pointed out that there is no difference in the

procedure for the urinary and plasma estrogen isolations except some

minor differences in the handling.

Similarly the procedures for extraction of estrogens from ovarian

follicles (MacCorquodale et. al. 1936), endometrium (Szego and Samuels

1943), ovaries (Westerfeld et. al. 1938) and fetal cotyledons

(Veenhuizen et. al. 1960) resemble the procedure used for urine or

blood estrogens.

B. ISOLATION AND PURIFICATION OF ESTROGENS

It is possible that the unwanted substances present in the biological extracts may interfere in the quantitative analysis. It is, therefore, advisable to isolate the concerned products in their pure forms. Several chromatographic procedures which can successfully be applied for this purpose are available.

1. By Paper Chromatography:

Paper chromatography, which has been used for the separation of estrogens and other compounds, is relatively simple. By this method homogenous meterial is concentrated in one position rather than distributed over a series of fractions as in column partition chromatography. This technique has some disadvantages as: 1) large quantities of the material cannot be handled so that the plasma extracts containing much lipids cannot be chromatographed directly, 2) the resolution is poor (O'Donnell and Preedy 1961).

Many solvent systems with various solvent combinations have been reported (Oakey 1961; Axelrod 1953; Reineke 1956; Veenhuizen et. al. 1960; Burton et. al. 1951a). Burton et. al. (1951b) observed that replacement of water by certain polar organic solvents overcomes the tailing and that the bulky urine extracts can be applied in a narrower zone along the starting line when formamide rather than propylene glycol is used as the impregnating solvent. Formamide, when added to absolute methanol (1:1 by volume) is considered to be a good stationary

phase (Burton et. al. 1951b; Axelrod 1953).

2. Adsorption Column Chromatography:

Adsorption column chromatography has been used very extensively for isolation and purification of estrogens in the extracts of biological fluids and tissues. The method has the advantage that large amounts of the material can be processed. The disadvantages of this procedure are the difficulty in the standardization of the adsorbant, moisture content, tailing and relatively large volume of the eluate (O'Donnell and Preedy 1961).

Adsorbants such as celite (Bauld 1955; Givner et. al. 1960), alumina (Stimmel 1944; Umberger and Curtis 1949) and silica-gel (Beer and Gallagher 1955a and 1955b; Levitz et. al. 1956) have been used to prepare the column for this technique.

3. Thin Layer Chromatography:

Lisboa and Diezfalusy (1962) have very recently reported the use of thin layer chromatography for the separation and characterization of the estrogens. The said authors have reported the R_F values for 24 phenolic estrogens in various solvent systems. This method has also been found to be very useful by Ladany and Finkelstein (1963). The method has a favorable resolution, reproducibility and high precision. Time given for running chromatograms is less than 2 hours. When applied as a strip line, sufficiently large quantities of the extract can be handled.

Similarly, countercurrent distribution has also been used for

the isolation of the estrogens (Engel et. al. 1950).

C. QUANTITATIVE MEASUREMENT OF ESTROGENS

Various methods for the detection of estrogens in paper chromato-graphy and thin layer chromatography have been successfully used. Turnbull's blue method (estrogen spots when treated with solution con-taining equal volume of 1 percent aqueous ferric chloride and 1 percent aqueous potassium ferricyanide, give blue coloration) which in fact is a general method for the detection of phenols, has proved to be of parti-cular importance (O'Donnell and Preedy 1961). Axelrod (1954) has cautioned that even corticosteroids, androgens, urinary pigments and impurities can give positive results with this method. The technique of dipping the chromatographic strip in 15 percent fuming sulfuric acid for one minute and then observing it under an ultra-violet-lamp for fluorescence, has also been used (Axelrod 1953).

The fluorimetric (Goldzieher et. al. 1952 and 1954; Veldhuis 1953; Slaunwhite et. al. 1951; Nako and Aizawa, Nocke 1961; Purdy et. al. 1961), enzymatic (Hurlock and Talalay 1957) and bioassay techniques (Allen and Doisy 1923; Emmens 1950, Freud 1939, Lauson et. al. 1939, Evans and Varney 1941) have also been quite extensively used for the quantitative measurement of estrogens. Kober color method which has been extensively used for the estimation of estrogens is reviewed in the following section.

Kober Reaction:

In 1931, Kober discovered that when estrin is heated with con-

centrated sulfuric acid and phenol it gives rise to a yellow color. On addition of water and reheating the mixture, the yellow color is converted into a highly specific pink color (λmax, 522 mu). Anyway, the pink color thus produced is not very stable and the impurities contained in the biological extracts containing estrin produce an intense brown color which can mask the color due to estrin. Venning et. al. (1935) indicated that the light absorption of the test mixture should be measured not only at 522 mu but also at 420 mu, the latter being the wavelength of maximum absorption of brown color. By doing so the light absorption due to brown component at 522 mu could therefore be calculated from its absorption at 420 mu and thereby by subtraction, the absorption at 522 mu due to the pink component is obtained. It has also been shown that there is no other substance, except estrogens, in the urine of humans which can produce a pink color with the Kober reagent (Venning et. al. 1935). The compositions of the reagent and its amount used, the preheating time to produce yellow color, amount of water added to the yellow mixture and the reheating time are the factors which can influence the test results.

Bachman and Pettit (1941) found that the treatment of the final crude color product with hydrogen peroxide, as originally suggested by Kober (1938), reduces but does not eliminate the absorption due to the impurities. The said authors also pointed out that the method for the correction of absorption, due to the brown color, proposed by Venning et. al. (1935), does not permit reliable determination of the estrogens

because the spectra of the individual brown colors from different samples vary too much.

In 1950, Allen suggested a correction formula which permits the analysis of mixed absorption curves provided the absorption curves of the contaminating substances closely approximate a straight line. This has been found to be true in the case of urinary estrogenic extracts (Allen 1950). The correction equation is as follows:

$$CDX_x = OD_x - \frac{OD_a + OD_b}{2}$$

Where OD_x = observed density at wavelength "x" as the absorption

maximum

OD_a and OD_b = the observed densities at wavelengths "a" and "b",

"a" and "b" must be equidistant from "x"

CDX_x = calculated density due to substance "x" having an adsorp-

tion maximum at wavelength "x"

The actual amount of "X" is then calculated from CDX_x.

Bauld (1954) observed that a depression of about 15-40 percent in the final color produced by Kober reaction can be caused due to the presence of solvent residues. The three possible types of inhibitions which can be responsible for interference in the color development are as follows:

a) failure to form the initial yellow complex (inhibition type I)

b) failure to convert the yellow complex fully into the pink (inhibition type II)

c) instability of the pink complex (fading).

The type I inhibition in the case of estriol is due to diminished reducing power of the reagent. The second stage of the Kober reaction, involving conversion of the yellow complex into the pink one, involves oxidation (Nocke 1961) which if excessive causes fading. The Kober reagents used by Bauld (1954) for estriol, estrone, and estradiol contained 76 percent, 66 percent and 60 percent V/V of sulfuric acid respectively.

The modifications suggested by Bauld (1954) were incorporated into Brown's method for the urinary estrogens (Brown 1955). According to this method the concentration of sulfuric acid in the reagents is the same as used by Bauld (1954). Quinol at the rate of 2 percent is added into each reagent. It was suggested that addition of quinol to the estrogen fractions before evaporating the solvent, can prevent any oxidation of the hormones. Chromogenic impurities from the crude urine extracts were separated by methylating the estrogens in alkaline solution with dimethyl sulfate and then oxidizing the contaminants with hydrogen peroxide and extracting estriol methyl ether with benzene and the estrone- and estradiol-methyl ethers with light petroleum. This was based on the fact that methylation of the estrogens does not limit the estimation of the hormones by the Kober reaction (Marlow 1950). The appropriate quinol-sulfuric acid reagent (3 ml) for the particular estrogen is added to the estrogen fraction. Preheating is done for 20 minutes. After cooling, 1 ml, 0.5 ml and 0.2 ml water is added to the tubes containing estriol, estrone and estradiol respectively. The tubes

are then reheated for 10 minutes. The readings for light absorption

are taken at 480, 615, and 552 mu for estriol and estrone and 480, 518,

and 556 mu for estradiol.

In 1956, Bauld modified the method used by Brown (1955). The

composition of the reagents was kept the same whereas their amount

added to the estrogen and quinol residue, the heating periods and the

final concentration of the acid were modified. The appropriate reagents

as described by Brown (1955) (2.6 ml for estradiol-17B and estriol and

3.0 ml for estrone) were added. Preheating in a boiling water bath

was followed by cooling in water bath at 15° C. At this stage 50± 5 mg.

of quinol was added and the dilutions were carried out as follows:

estradiol-17B with 0.7 ml of the reagent, estriol with 0.7 ml of water

and estrone with 0.3 ml of water. Reheating time allowed was 15

minutes and after cooling the absorbancy was measured at 480, 512,

and 544 mu for estrone and estriol and at 480, 515, and 545 mu for

estradiol-17B. The method was used later on by Givner et. al. (1960)

and was found to be quite satisfactory.

A similar method was reported by Hunt (1957). The Kober reagents

for this procedure contain sodium nitrate, quinone and hydroquine. No

quinol is added after the preheating interval and the final concentra-

tion of sulfuric acid is 60 percent. This method has successfully been

used by Nelson and Smith (1963).

D. EXTRACTION OF PROGESTERONE FROM BLOOD AND OTHER TISSUES

As already stated, the progesterone in the blood exists as

bound to the plasma proteins. It is, therefore, evident that to iso-
late the hormone its binding with the protein part should be broken.
Zander and Simmer (1954) and Oertel et. al. (1959) have pointed out
that the protein-steroid binding is broken down even with the organic
solvents. It is also known that pretreatment of plasma with a solution
of sodium hydroxide breaks the progesterone-protein binding and while
extracting with ether prevents any emulsion formation (Short 1958a
and 1958b; Sommerville and Deshpande 1958; Sommerville et. al. 1963).
Lombardo et. al. (1955) have reported the possibility of the application
of dialysis for the extraction of progesterone from the blood.

E. ISOLATION OF PROGESTERONE

The paper chromatography method for progesterone with petroleum
ether and 80 percent methanol system as recommended by Bush (1952) has
been used quite extensively (Gomes et. al. 1963; Raeside and Turner
1956; Short 1958a and 1958b). In 1961 Short reported that decalin:
methanol:water: methylcyclohexane:acetic-acid:water and methycyclohexane:
water:methanol systems can also prove to be quite satisfactory. Simi-
larly, methanol:benzene system has been used (Oertel et. al. 1959) with
satisfactory resolution.

The adsorption column chromatography with silica-gel (Sommerville
et. al. 1963), aluminum-oxide (Noall et. al. 1953; Loy et. al. 1957) and
thin layer chromatography (Lisboa 1963; Futterweit et. al. 1963) have
also, successfully, been applied.

F. QUANTITATIVE MEASUREMENT OF PROGESTERONE

Progesterone has been described as the least polor of all the naturally occurring steroid hormones and no other B-unsaturated oxo-steroid has as yet been identified _in vivo_ with the same R_F value as progesterone in paper chromatographic system. It is also known that all steroids possessing the 4-3-ketone group absorb ultraviolet light (Short 1961). Progesterone being a member of this group of steroids and at the same time having a definite characteristic R_F-value can easily be identified.

Progesterone can also be identified from its color reaction with many reagents. Dinitrophenylhydrazine reacts with B-unsaturated ketone group of progesterone to give an orange-red color, whereas m-dinitroben-zene reacts with the 20-keto carbonyl to give a brown purple color (Raeside and Turner 1956).

Because of its unsaturated ketone grouping progesterone exhibits a characteristic absorption maximum in ethanol at 240 mu (Short 1961). This single characteristic for its quantitation has been used by many workers (Raeside and Turner 1956; Gomes et. al. 1963; Loy et. al. 1957) even though Zaffaroni and Burton (1951) have pointed out that the other lipids contained in the extract also absorb in the ultra-violet spectrum. And when the hormone is separated from the contaminants with paper chromatography, and eluted, some background material from the chromato-gram itself can enhance the absorbance. It has, therefore, been emphasized by Lombardo (1955) that the readings of the densities of

the eluate of an unused area of the chromatogram must be taken into consideration. Samuels (1947) has indicated the solution of progesterone in ethanol should possess a well defined peak at 240 mu and there should be very little or no absorption at 280 mu.

To correct the effect of the interference due to contaminents in the final progesterone eluate, the readings are generally taken at 230 mu, 240 mu and 250 mu. The corrected absorbance due to progesterone is then calculated by applying the Allen's correction formula (Gomes et. al. 1963; Loy et. al. 1957). Oertel et. al. (1959) have advocated the use of sulfuric acid-ethanol reaction. By this method the maximum absorption is obtained at 295 mu. However, this method, due to its low sensitivity, has not proved to be very useful (Short 1961).

A gas chromatographic technique for the identification and measurement of progesterone has been reported by Futterweit et. al. (1963).

EXPERIMENTAL PROCEDURE

Five Jerseys and one Holstein lactating cow from the dairy herd at Montana State College, Bozeman, were used in this study. Laparotomy from the right side of the animal was performed and the posterior vena cava was cannulated at a distance of about one inch backward from the level of the right kidney using Silastic Silicone rubber tubing. The tubing was very kindly donated by Dow Corning Corporation, Midland, Michigan. The internal and external diameter of the tubing were 0.1325 cms. and 0.2625 cms. respectively. The total length of the tubing was about 72.5 cms. The length of the tubing inside the vein was approximately 37.5 cms. whereas about 30.0 cms. portion outside the vein was anchored to the abdominal wall. The remaining 5.5 cms. was kept outside the body and a syringe adapter was fitted onto this end of the tubing. Except when the blood was actually drawn this end was always kept sealed with a metal plug. An iron ring with an internal and external diameter and thickness of 9 cms., 7.5 cms. and 0.1 cms. respectively, and having a spongy pad fixed on the under surface was sutured to the skin. An aluminum lid of the same size as that of the ring was then bolted onto the ring. The lid could thus very easily be removed at the time of the sampling of the blood.

About 250 ml. blood was collected with the help of a syringe every other day. Five ml. of sodium citrate solution in distilled water (0.5 g./ml.) was used as an anticoagulent. After the collection of the sample, the cannula was flushed with about 5-10 ml. heparin solution (200 I.U. per ml. normal saline). Heparin solution was

injected every other day even though no samples were collected. To prevent the growth of fungus or other micro-organisms in the heparin solution Terramycin was included in the stock solution (1 mg./ml.).

Urine samples were also collected immediately after the blood was drawn. The samples were immediately brought to the laboratory and the blood was centrifuged at a rate of 3000 rpm. If the samples could not be analyzed immediately the plasma was stored in the freezing chamber of the refrigerator at a temperature of about -10 C° to -5°C. The urine samples were stored at a temperature of about +5 C°.

Sample collections were made during the estrous cycle of four Jerseys and one Holstein cow and during the first 34 days post-conception period from one Jersey cow.

The urine samples were extracted using the method outlined by Nelson (1959) which is a modification of the methods of Friedgood (1948) and Stimmel (1946). The procedure followed is outlined on flow sheet no. I.

As recommended by Preedy and Aitken (1961) the extraction procedure for estrogens from the plasma was the same as for the urinary estrogens except for the following changes:

1) About 30 ml. plasma filtered through glasswool was diluted with distilled water to make 500 ml.

2) To avoid any bumping during hydrolysis with 15 volume percent of concentrated hydrochloric acid, the acidified diluted plasma was heated very gently to boiling till the bumping