PROGESTERONE CONTENT OF BOVINE REPRODUCTIVE ORGANS DURING PREGNANCY

by

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INTRODUCTION

The importance of progesterone in the physiology of reproduction has been emphasized during the last few years with special reference to implantation of the embryo and maintenance of pregnancy. A great deal of information has supported the fact that progesterone is required for the preparation of the uterus prior to and during the process of implantation of the embryo. The survival of the embryo during the early uterine life has been considered to be under the direct control of progesterone with the progesterone and estrogen ratio being of great importance in most species. Reproductive tissues of various species have been assayed for progesterone content. However, few investigations have been carried out on bovine reproductive organs. The earliest significant work with organs from domestic animals was done using sow corpora lutea, Elden (1934). Progesterone content of bovine reproductive organs has not been studied during different stages of pregnancy. The purpose of this investigation was to determine the presence and make a quantitative study of progesterone content during pregnancy, of bovine corpus luteum, residual ovarian tissue following corpus luteum removal, adrenal gland, placenta, amniotic and allantoic fluids, and blood.

Information on the general relationships existing between levels of progesterone and physiological processes taking place during pregnancy would be useful in elucidating the mechanisms of action of this hormone. The content of the various organs, tissues, and fluids may be suggestive on the various sources of progesterone, rate of production, storage, and relationships with other hormones such as estrogens and ganadotrophins

from the placenta and pituitary. It is realized that the rate of production and release of progesterone may or may not be highly correlated with tissue content. Of the biological functions of progesterone, the maintenance of pregnancy has been the sole function which exhibited a relation to the mode of conversion of this hormone to pregnanediol, Guterman (1952). This indicated that maintenance of pregnancy might be looked upon as a major function of progesterone in the reproductive mechanism. Of its many relationships with other hormones and biological effects on tissues, the mechanism of pregnancy maintenance has been the most complex.

LITERATURE REVIEW

Historical

The bovine corpus luteum has long been studied and noted for the presence of yellow color that the name implies. This body was named by Reijnier de Graaf in 1672. By way of suggestion, Fallopio (1584) described the follicle and corpus luteum.

Work on the corpus luteum prior to the latter part of the 19th century was morphological in character with little knowledge of physiological function or correlation to specific action at different stages of the reproductive cycle. If we followed the background progressively, according to Corner (1942), Fraenkel's early work in 1903 was the first that actually suggested progestational proliferation was due to the corpus luteum. Although Fraenkel made these interpretations, Bouin and Ancel (1910) made the important morphological contribution to the concept of progestational proliferation. This was the fundamental investigation that led to the discovery of the corpus luteum hormone. Loeb (1907) conducted classical work on the induction of decidual tissue by placing pieces of glass in the uterus of the guinea pig on the 7th day following unfertilized ovulation. He found that deciduomata could be produced only during the first week after ovulation and that cauterization of the corpus luteum prevented development of artificial deciduomata. In 1909 he proved the inhibitory effect of corpora lutea on follicular development.

Morphological Changes Induced by Progesterone

Corner (1919) working with corpora lutea of swine with special reference to their origin, concluded that there was good evidence that some of the theca interna cells persisted throughout pregnancy as distinct elements of the corpus luteum. He also stated that the membrana granulosa was retained intact after the rupture of the follicle, with its cells becoming laiden with lipoid substances. This early work indicated that the corpus luteum originated from both membrana granulosa and theca interna.

Loeb (1923) continued to work on the mechanism of the sexual cycle of the guinea pig, dividing it into major catagories of the follicular, ovulation and lutein phases. Loeb (1928) reported work on the interaction between follicular and corpora luteal extracts, in which he proved that the lutein phase was controlled by the corpus luteum and that it was a gland of endocrine secretion with the hormone elaborated being distinctly different from the follicular hormone. He also reported the proliferating effect of corpora luteal extracts on the mammary gland and the antagonism between persistant corpus luteum and effects of injected follicular extracts. The corpus luteum prevented ovulation; expression of the corpus luteum hastened ovulation. He showed that the corpus luteum was necessary for implantation and sensitization of uterine mucosa which lead to the production of placentomata. He noticed a gradient effect where a small corpus luteum did not show the inhibitory effects of the large persistant corpus luteum. Although Loeb advanced the idea of antagonism, the first worker to show antagonism between estrogen and a progestational substance was Smith (1926). Smith terminated gestation by administrating estrogen to rats during the

first 5 days of gestation. She suggested that the estrogens have an antagonistic effect on progesterone. She also demonstrated that estrogen was responsible for both uterine growth and estrus.

Macht et al., (1929) confirmed earlier work on the inhibitory effect of the corpus luteum extracts on estrus and showed that extracts of the placenta also inhibited estrus. These experiments have lead to further work on the production of hormones by placental tissue and their role in the maintenance of pregnancy. Some of the early work on the gonadotrophic complex was conducted by Zondek and Aschheim (1927) where they put forth the idea of periodic liberation of gonadotrophic hormones as controlling the ovarian cycle, but had no suggestion as to factors controlling this periodic liberation.

Mc Nutt (1924) has described the histological changes during ovulation and the development of the corpus luteum in the bovine ovary. Papanicolaou (1926) conducted work with cattle, where the expulsion of the corpus luteum brought about immediate estrus. He showed inhibitory effect of luteal tissue upon follicular development. From this early experimental work it seemed evident that the corpus luteum controlled the length of the estrous cycle, and that other factors acted indirectly upon the corpus luteum. During pregnancy however, when the corpus luteum remained large, the rhythm of the cycle was suppressed. Anatomical studies have shown that the natural involution of the corpus luteum was associated with the growth of the largest graafian follicle.

In Corner's work (1926) on the physiology of the corpus luteum, he proposed the name of progestin (favors gestation) to the hormone of the corpus luteum. It was stated that the progestational effects were distinct

from the estrogenic effects. The progestin was differentiated from estrin by destruction with alkalis. Fevold and Hisaw (1932a) called the hormone of the corpus luteum "corporin" and reported further purification. Fevold and Hisaw (1932b) while working on extraction and further purification were unable to extract corporin from fetal gonads. This being one of the bases for the theory of low progesterone production by the fetal gonads.

Allen (1932) in his work on further purification of progestin suggested the idea that there were other possible sources of the corpus luteum hormone such as placenta, fetal fluids and urine.

In 1934 several groups of workers crystallized and characterized pure progesterone. Butenandt (1934) was the first to crystallize progesterone from 56,000 ovaries of swine with a yield of 20 mg. Later in 1934 Wintersteiner and Allen crystallized fractions with the fraction of highest activity melting at 128 degrees.

Kimura and Cornwell (1938) were the first workers to study the variations of progestin content of corpora lutea of the sow during the estrous cycle and pregnancy. The assay was that of Allen using adult rabbits. The progestin activity of the corpora lutea were high early in the life of the corpora lutea. The progestin content of the corpora lutea during the estrous cycle was maximum at the 10th day decreasing until the next follicle started rapid growth. During pregnancy the first maximum amount of progestin was reached at 20 days with a further increase to 105 days. There was a sharp decline after the 105th day with no activity detected during the last 10 days. In 1938 Astwood and Greep reported the first work on the function of the placenta in secreting a substance responsible for maintenance of the function of the corpus luteum. They concluded that

the function of the corpus luteum of the rat during the first half of pregnancy was under the control of the hypophysis with the fetal placenta secreting a substance responsible for maintenance of function of the corpus luteum during the latter half of pregnancy. Early work has been reported on the presence of a material in the adrenal showing progestational activity, but the first conclusive proof came from the work of Beall and Reichstein (1938) when they isolated progesterone and allopregnanolone from the bovine adrenal.

Work on the pregnanediol excretion was conducted before progesterone was known, Marrion (1929). With further studies on pregnanediol, it was maintained that pregnanediol was a metabolic product of progesterone, Venning and Browne (1940). The thinking was that progesterone secretion caused a fluctuation in the pregnanediol excretion in certain species. Dorfman (1948) reported data that suggested pregnanediol to be the principal metabolite of progesterone in the human female. Work by Stevenson (1947); and Hill et al., (1954) has indicated that pregnanediol is absent from the urine of goats, cattle and horses. Short (1957) presented evidence that pregnanediol was present in the urine of pregnant mares. This has eliminated the possibility of using urinary pregnanediol excretion to study progesterone metabolism in these animals. Miller and Turner (1955) studied androgen content of feces from sexually active bovine. A significant increase was demonstrated in the excretion of fecal androgen in the male and non-pregnant female following injection of progesterone. It was suggested that this androgen was a metabolite of progesterone.

Zaffaroni et al., (1949) was the first to apply partition paper chro-

matography to steroids. This method depended upon differences in relative solubilities of compounds in the components of a 2 phase solvent system. The stationary phase was formed by water bound to the cellulose fibers on filter paper. The mobile phase was an organic solvent immiscible with H_2O. These workers were credited with working out many of the basic problems on chromatography of steroids using a petroleum ether; propylene glycol system known as the Zaffaroni system. Bush (1953) had developed the system using petroleum ether: methanol: water which was known as the orthodox method with volatile solvents.

Physiological Actions of Progesterone

Introduction

It has been demonstrated that progesterone functions in (1) growth and secretion of the glands in the uterine mucosa preparatory to implantation of the ovum, (2) nutrition and protection of the embryo during the time when it lies in the uterus, (3) development of the placenta and membranes after implantation, (4) lobule-alveolar mammary growth, (5) maintenance of a quiescent uterus, and (6) inhibition of ovulation and estrous. Pregnancy has been shown to continue in the guinea pig, mare, cow, ewe, man and monkey with removal of the corpora lutea after mid pregnancy; Raeside and Turner (1951); Uren and Raeside (1951); Mc Donald, Nichols, and Mc Nutt (1953); and Short (1957).

Uterus

Corner and Allen (1937) described the process of progestational proliferation as an enlargement of the uterus with hyperemic, superficial and glandular epithelium undergoing proliferation. The crypts and glands increased their complexity of ramification. Decidual tissue was formed by the stimulation of the rabbit uterus. This formation of decidual tissue was prevented by removal of the corpus luteum. Implantation did not occur if ovaries were removed the 1st week of pregnancy. Progestational proliferation was not produced by estrogen alone. The corpus luteum seemed to be a temporary organ of internal secretion acting upon the endometrium in behalf of the embryo. Cystic corpora lutea extracts failed to produce progestational proliferation. They were successful in recovering progestin from human placental tissue. Hisaw and Leonard (1930) studied the relationship between gonadotrophins and the corpus luteum, concluding that a balance between FSH and the corpus luteum hormone was necessary for progestational proliferation. Shelesnyak (1953) proved the uterine response to be dependent on both estrogen and progestin and was affected without aid of the pituitary stimulation. Makepeace et al., (1936) working with the rabbit uterus in vitro, proposed pituitrin desensitization in the rabbit as a physiological property of progesterone.

Reynolds and Ginsburg (1942) showed that progestational proliferation was dependent on the presence of optimal amounts of estrogen and progesterone. They also showed that large doses of estrogen alone or in combination with large doses of progesterone inhibited progestational proliferation. Mc Ginty et al., (1939) applied progesterone locally to the rabbit

uterus. Progestational proliferation was produced. His work showed that 0.0005 mg. progesterone in the lumen was equivalent to 0.5 mg. progesterone injected intramuscularly.

Evans et al., (1941) was unable to obtain progestin production with artificially induced corpora lutea as shown by the placentoma test. Lactogenic hormone was shown to stimulate production of progestin by luteal tissue. Elden (1934) while working with two ovariectomized human females observed that bleeding of the uterine mucosa could be induced with estrogen administration and delayed with progesterone administration. Searle and Yanow (1953) produced secretory endometrial changes and withdrawal of bleeding with buccal and intramuscular administration of progesterone.

Salvatore (1950) summarized myometrial growth during gestation and endometrium growth during the proliferative phase as accomplished by nuclear interphasic growth cycles. The nuclei were thought to be of great importance and related to preparation of the endometrium for nidation of the ovum. Implantation of the ovum resulted in an interphasic nuclear disintegration with the fall of progesterone. Hooker (1940) was able to stimulate cell growth with 25 mg. progesterone alone or in combination with estrogen in the mouse.

Huggins et al., (1955) in studying the chemical structure of steroids in relation to promotion of growth of the vagina and uterus found that the number and site of functional groups, geometry of the molecule, and state of oxidation determined the activity of steroids on these sites of action. Sakiz (1957) in a study to further elucidate a mechanism of action found that although cortisone interfered with normal development of rat fetuses,

it did not interfere with development of decidumatas provoked by uterine trauma plus injection of progesterone.

Venning and Browne (1940) while studying the factors affecting the pregnanediol excretion found that in the human, endometrium was not necessary for excretion of pregnanediol but endometrium facilitated the conversion of progesterone to pregnanediol. Hisaw and Velardo (1951) found that administration of pregnanediol inhibited progesterone action.

Ovarian function

Smith and Engle (1927) were able to increase ovarian weight by pituitary implants. Superovulation occurred, the uterine weight exceeded that of untreated. In the adult there was an increase in number of follicles and corpora lutea with small and large cysts formed. The mechanism held back production of gonadotrophic hormone or blocked its action. A follicular-luteal interaction did not satisfactorily explain the mechanism. Rhythmic ripening of follicles could have been explained by inhibitory affect of corpora lutea on follicular development.

Hammond (1927) stated that in the follicle the active principle entered the blood only from the network of capillaries in the theca; however in the corpus luteum the luteal cells were in close connection with the capillaries so the secretion was not normally stored but passed into the blood stream. This secretion probably acted on uterine blood vessels causing their dilation and bringing about hypertrophy of the uterine mucosa in preparation for pregnancy. The sudden removal of the secretion caused a decrease in lumen size of the blood vessels and extravasation of blood with disintegration of the mucosa.

Makepeace et al., (1936) stated that progesterone suppressed ovulation by interference in the chain of the ovulation-provoking mechanism. He was able to inhibit the estrous cycle in the rat with 1.5 mg. of progesterone. Dempsey et al., (1936) experimentally induced estrus in the normal and ovariectomized guinea pig. Their conclusions were that after conditioning levels of estrogen, estrus was caused by the action of progesterone, produced under the influence of lutenizing hormone during the preovulatory enlargement of the follicle. Dempsey (1937) observed that pregnancy and injection of progesterone prevented preovulatory swelling and ovulation; he attributed this to inhibition of luteinizing hormone. Astwood and Fevold (1939) were able to inhibit luteinization and decrease ovarian weights by injection of follicle stimulating hormone and progesterone. In hypophysectomized rats no inhibition was obtained. The conclusions were that progesterone suppressed release of luteinizing hormone from the pituitary and this suppression was responsible for absence of estrous cycles during active luteal function, and some hormone other than luteinizing hormone was responsible for continued function of formed corpora lutea.

In 1941 Astwood reported that anterior pituitary luteotrophin maintained the functional activity of the corpora lutea in rats. He explained the end of the luteal phase by the method that the corpus luteum had an inherent life span under stimulus of luteotrophin and that the prolonged luteal function of pregnancy required further stimulus of a cyonin. He maintained the suppression of luteinizing hormone by progesterone and that estrogen conditioned the production of luteotrophin. Everett (1948) stated that recent studies have made it apparent that progesterone facilitated release of luteinizing hormone. He found that progesterone suppressed

ovulation under certain conditions, and that under elevated levels of estrogen, progesterone facilitated ovulation in the rat. This may have been accomplished by modification of the threshold of the luteinizing hormone release mechanism to estrogen. Hansel and Trimberger (1952) hastened the ovulation process in dairy heifers by subcutaneous injection of 5-10 mg. of progesterone at the beginning of estrus. Both length of estrus and time from end of estrus to ovulation were reduced. Histochemical changes suggested that progesterone produced by the ovary before ovulation normally played a role in release of luteinizing hormone and ovulation in the cow.

Everett (1943) presented data to support the theory that progesterone began to be secreted in the preovulatory phase of the cycle. The repair of interstitial cells in the ovary which accompanied successful corpus luteum formation indicated that progesterone caused momentary release of luteinizing hormone.

Selye and Friedman (1940) studied the action of various steroid hormones on the ovary of the rat. Progesterone administered at 2 mg. per day caused atrophy of stroma and prevented maturation of follicles, at 10 mg. per day the same results were seen with the formation of a single set of pregnancy type corpora lutea.

Moore and Nalbandov (1955) investigated the maintenance of the corpus luteum in sheep. They found that prolactin would maintain the corpus luteum longer than usual, it was not known if the action was on the luteal tissue or mediated through the pituitary. Gonadotrophins may have been closely related to this mechanism and may have modified or determined the type of response from luteotrophin.

Barraclough (1955) prevented corpora lutea formation in 5 day old mice with androgen but was unable to succeed with 20 day old mice. Androgenic activity may have had a function in the suppression of the corpus luteum under certain conditions. The corpus luteum has been shown to have estrogenic function during the menstral cycle and pregnancy up to the 16th week by Furuhjelm (1954). The corpus luteum did not develop if all follicles were removed from the ovary.

Pregnancy

Aimé (1907) investigated the interstitual cells of fetal ovaries and suggested that internal secretion of these cells might cross the placental barrier and establish an equilibrium with the maternal luteal tissue. He also suggested that the secretion of the fetal gonads might be supplementing that of the maternal corpus luteum. Upon investigation he found the fetal gonads to be negative for progestin.

Corner (1926) proved that the special action of the corpus luteum was to produce progestational proliferation. Although other workers had observed progestational proliferation they did not conduct long term experiments to correlate the corpus luteum to this action. Corner observed in rabbits, ovariectomized 18 hours after mating, the ova developed to the early blastocyst stage and died upon entering the uterus. With the removal of 1 ovary and half the other, fetal development was normal. Nelson et al., (1929) contributed to the function of the corpus luteum by prolonging pregnancy in rats with extracts of the corpus luteum.

Cole et al., (1931) indicated that removed ovaries were

gesterone might be needed for parturition. Cole and Hart (1933) found that during the last one third of pregnancy mare ovaries were devoid of large follicles and corpus luteum. The ovary appeared to be quiescent. In early pregnancy the luteinizing process was taking place in maternal ovaries, due to the high concentration of gonad stimulating hormone in the serum. The stage of greatest development of the corpora was when the crown rump measured 30 cm. When the crown rump measurement equaled 45-60 cm. maternal ovaries were devoid of lutein tissue.

Adler et al., (1934) considered other sources for progesterone besides the corpus luteum. He was able to show that both the bovine and human placenta possessed progestin.

Wislocki and Dempsey (1955) observed the syncytial trophoblast as the site of localization of placental steroid hormones. The hormone content of the trophoblast was always high. From this histochemical study the lipoid droplets in the syncytial trophoblast appeared to contain the steroid hormones. Diczfalusy (1952), using counter current distribution for isolation of progesterone from placental tissue, suggested that progesterone was present in full term human placental tissue. Noall et al., (1952) isolated 1 mg. of progesterone per kilogram of human placental tissue. This work suggested a protein interaction with progesterone.

Pearlman and Cerceo (1952) advanced the idea of the placenta playing an important role in the elaboration of progesterone, especially in later stages of pregnancy. The data supporting this theory were: (1) pregnanediol excretion in pregnant women was marked after the 2nd month, (2) ovariectomized pregnant women continued to excrete pregnanediol, (3) the corpus luteum and ovaries had been removed after the first few weeks

of pregnancy in the human, and (4) there was definite progesterone activity in placental extracts. They were able to isolate 0.93 mg. of progesterone per kilogram of human placental tissue. Pearlman and Cerceo (1953) isolated 1-1.5 mg. of progesterone per kilogram of placental tissue. Salhanick et al., (1956) isolated progesterone from human placenta. They stated that 35 percent of the steroid isolated was thought to come from the blood present in the tissue.

Wislocki and Dempsey (1955) studied the human placenta with the electron microscope. Cytoplasm structure of the syncytial trophoblast seemed to play a role in the regulation of placental exchange of metabolites, synthesis of placental hormones, and maintenance of the placenta. This was good evidence in support of the biosynthesis of hormones by the placenta.

Averill et al., (1950) were able to maintain pregnancy in hypophysectomized rats with placental implants. It was suggested that the placenta was the source of the gonadotrophin produced. Pregnancy was not maintained by similar treatment in ovariectomized rats. Bradbury et al., (1950) have suggested that a luteolytic substance is produced by the endometrium which hastens the involution of the corpus luteum in nonfertile cycles, while inactivation of this substance may allow corpus luteum to persist in an active state for a period of time during pregnancy. In certain species placental hormones or lactogenic hormone may have been the active luteolytic agents which maintained the corpus luteum. In primates the chorionic gonadotrophin appears to maintain the corpus luteum in early stages of pregnancy. Fried and Rakoff (1952) suggested that lactogenic hormone in synergism with luteinizing hormone had luteotrophic

properties in the human. They were able to prolong the function of the corpus luteum by injecting 2,000 I.U. of chorionic gonadotrophin and 200 units of lactogen. There was no effect with smaller doses alone or in combination. Meites et al., (1951) studied the effects of corpora lutea removal in pregnant goats. Progesterone therapy was used to maintain pregnancy. Ten mg. of progesterone per day were required to maintain pregnancy. If one corpora lutea was excised normal pregnancy resulted. If all corpora lutea were excised abortion occurred in 2 days.

Hain (1941), observing the role of progesterone in pregnancy, concluded that abortion occurred at both high and low levels of pregnanediol excretion. There was no pregnanediol excretion in early pregnancy in the human. He stated that combined estrogen and progesterone were essential for maintenance of pregnancy but utilization varied between individuals. The peak of output of both hormones occurred during the last mongh of pregnancy.

Bruner (1951) found the highest concentration of chorionic gonadotrophin in the placenta, next in concentration was the mother's blood and the uterine endometrium. Some choronic gonadotrophin was thought to have crossed the placental barrier and entered the fetal system across the wall of the chorionic vessicle. All tissues investigated decreased in concentration of choronic gonadotrophin after the 25th week. These data supported the view that the hormone was produced by the embryonic trophoblast.

Higher progestational activity of the blood in the umbilical arteries than the umbilical vein suggested to Forbes (1955) that progesterone may be secreted by the fetus and removed by the placenta. Berstine and Meyer (1953) while working on the origin of rabbit amniotic fluid found that

chloral hydrate given to the mother was catabolized in part by the fetus, excreted in fetal urine and recovered in fetal fluid. The opinion was that the fetal fluid was a transudate preceeding fetal kidney function then becoming of more renal origin in later fetal life. Biochemical analysis of amniotic fluid has been made. The protein constituents and amino acids were qualitatively the same as for blood. Both pyruvate and a-keto glutarate were isolated from amniotic fluid. He concluded that amniotic fluid was secreted by the amnion and the composition modified by fetal micturition.

Mammary gland

Loeb (1907) conducted work on the physiological changes of the mammary gland in the guinea pig. He observed that extirpation of the corpus luteum prevented the secondary proliferation of the mammary gland. The cycle of the mammary gland corresponded closely to that of the ovary and uterus. Gardener and Hill (1936) studied the effect of progesterone upon the mammary glands of mice. Progesterone alone promoted tubular and acinar development in castrate mice. Duct growth and some lobule-alveolar growth was stimulated with estrogen. The growth was related to the dose and length of treatment. Progesterone alone was without effect, in combination with estrogen it increased lobule-alveolar growth. Effective progesterone-estrogen ratios were 1;3000-5000.

Whitten (1955) made endocrine studies on delayed implantation in lactating mice where he found ovaries and uteri from non-lactating mice on the 7th day after parturition were more active than those of lactating mice. Evidently during early lactation the ovary was not refractory to

gonadotrophin. Implantation was blocked by tubal lock when serum gonadotrophin was injected. Delay in implantation in lactating mice was thought to be due in part to reduced secretion of gonadotrophin by the pituitary and a decrease in the production of estrogen.

General Theories of Hormone Function

Dorfman and Goldsmith (1951) have advanced the theory that endocrine secretions exerted regulatory influences on growth and metabolism by modifying the activity of specific enzyme systems.

Stadie (1951) suggested that hormones, like drugs, might alter the permeability of the target tissue or cell by orientation at its surface or interacting with metabolic systems responsible for maintaining the state of the cell membrane. The ability of hormones to form bonds with protoplasmic components has been demonstrated. Steroid hormones appeared to exert strong affinity for protein, this combination could preceed its action. Estrogens were more strongly bound to proteins than progesterone, which may have been related to polarity.

Dorfman (1952) has pointed out that effects of hormones on enzyme systems may have been due to (1) changes in tissue concentration, (2) hormones functioning as coenzymes, (3) inhibition or acceleration of enzyme activity or, (4) direct or indirect effects on accelerators and inhibitors of enzyme systems. In 1941 Green presented the "trace substance-enzyme" hypothesis. Any substance biologically affecting an organism in trace amounts did so by altering an enzyme system. Hechter (1955) stated that one basic concept has governed the efforts on the study of hormone mechanism, that is, hormones as a group of biologically active trace substances

constitute an important set of enzyme-regulating factors. However, no hormone has been shown to be an essential component of an enzyme system and the described hormonal effects on enzyme systems have not explained the physiological action of any hormone.

Gold and Sturgis (1953) have shown that steroids alter the nucleic acids prepared by the human and rat uterus. There is a variation in the cytosine and thymine content of DNA. This could direct the activities of a cell.

Metabolism of Progesterone

There have been many investigators dealing with various aspects of progesterone metabolism in mammals. Many workers have used microbiological transformations, in vitro, and in vivo methods in elucidating the precursors and metabolic products of progesterone.

The in vitro work of Taylor (1954) with rabbit liver brought out the first stage in enzymic reduction of progesterone as involving non-stereospecific addition of 4 hydrogens in ring A to form pregnanolones. All pregnanedione was formed by subsequent re-oxidation. Five crystalline steroids were isolated after progesterone incubation. In 1955 Taylor was able to increase the metabolism of progesterone by adding diphosphopyridine nucleotide or nicotinamide. He stated that methods of using urinary pregnanediol excretion as an index of endrogenous progesterone production must be interpreted with caution due to fecal excretion of catabolic products of progesterone. In 1956 Savard et al., using in vitro studies with human testicular tissue worked out the pathway of progesterone to 17, alpha-hydroxy progesterone to 4, androstene-3,17-dione to testesterone.

Progesterone has been indicated as a precursor to androgens under certain conditions. Slaunwhite and Samuels (1956) incubated testis from hypophysectomized animals with labeled progesterone, labeled testosterone was produced.

Hayano et al., (1956) incubated bovine corpora luteal tissue with progesterone and recovered delta-4, pregnene-20 beta-ol-3one. The corpus luteum tissue contained 6, beta-hydroxylase and 20, hydrogenase activities.

Pearlman and Cerceo (1948) isolated pregnane steroids from the bile of pregnant cows, which implicated the liver in the metabolism of progesterone. Shen et al., (1954) administered carbon-14 labeled progesterone to rats; 15 percent of the carbon-14 was in the expired air, 30 percent in the urine, and 15 percent in the feces. The bile was the major route of excretion when cannulated. With a ligated bile duct the kidney was the major route of excretion. Wiest et al., (1955) administered labeled progesterone to man and found that 64 percent was excreted in 24 hours; 65 percent of this amount through the bile fistula and 35 percent in the urine.

Klopper et al., (1957) showed that the adrenal contributed to the urinary excretion of pregnanediol but pregnanediol was not present in the urine of cattle and goats. They found that cows excreted androgens in feces, whereas males and immature females did not. Injection of progesterone gave an increase in fecal androgen in non-pregnant cows but no increase in pregnant animals.

Everett (1945) studied lipids of the cyclic corpora lutea in the rat. This work indicated cholesterol as a precursor to progesterone. Lever (1956) with further microscopy studies found the mitochondria of all corpus luteum cells to be sites of lipid accumulation or formation.

Hechter et al., (1951) stated that the placenta did not possess a 11-hydroxylation system in postulating that anti-arthritic effects of pregnancy may result from the circulation of progesterone or closely related steroids produced by the placenta and transformed to 17-hydroxy-corticosterone by the adrenal.

Gallagher et al., (1954) determined that steroid hormone metabolites occurred in the urine as conjugates of sulfuric acid and glucuronic acid. One third of the total dose of progesterone injected was recovered as urinary metabolites. The recovery was higher following intravenous administration than after intramuscular injection. The relative slow elimination of the intramuscular dose may have been a reflection of slow absorption from oily depot.

Rock et al., (1957) inhibited the development of corpora lutea by administering synthetic progestins (17-alpha-ethinyl-5-(10)-estraeneolone and 17-alpha-ethyl-19-nortestosterone) for three cycles in the human. The inhibition of corpora lutea was indicated by low excretion of pregnanediol. Davis and Poltz (1957) discussed delta 4-3keto-pregnene-20,alpha-ol and delta 4-3keto-pregnene-20,beta-ol as naturally occurring gestagens. These workers administered labeled progesterone to pregnant humans. A highest percent of the labeled progesterone went into the fat with the corpus luteum and placenta being the second in concentration at the 17th week. At the 18th week the placenta increased in content of injected progesterone. The placenta seemed to play a dominant role in the dramatic hormonal changes during human pregnancy. It appeared almost certain that this organ could synthesize progesterone during later stages of pregnancy. When the corpus luteum was removed the placenta had produced

progestins on the 35th day. The intact corpus luteum of pregnancy remained functional especially in cholesterol synthesis even after most morphologists classed it in the regressional state. Progesterone produced by the placenta was thought to be a precursor to the other steroid hormones produced by the placenta. If the placenta failed, hormonal support to pregnancy was weak. A defective trophoblastic development may have produced inadequate amounts of chorionic gonadotrophin necessary to maintain the activity of the corpus luteum in early pregnancy. Powerful compensatory mechanisms existed between the placenta and ovary. This was one factor that added to the complexity of understanding mechanisms of pregnancy maintenance.

Riegel, Hartop, and Kittinger (1950) found that progesterone was not stored in blood, rapid metabolism and excretion took place. The methyl group was shown to be oxidatively excreted as carbon dioxide. Most radioactivity was found in the neutral unsaponifiable fraction of the feces. The pituitary and adrenal of mice contained large amounts of activity. The adrenals of rats contained none. Marker (1938) studied the steroid content of bovine urine. He claimed that 5 beta-pregnane-3 alpha:20 alpha-diol was the principal diol component of the neutral steroids of bull or cow urine. Heard, Bauld, and Hoffman (1941) demonstrated that pregnanediol was the major metabolite of progesterone in the rabbit. Dorfman (1954) gave evidence that both 21 carbon and 19 carbon metabolites have been isolated from 21 carbon steroids using <u>in vivo</u> studies.

Jurgen and Davis (1957) injected labeled progesterone intramuscularly into pregnant women scheduled for abortion. The highest concentration went directly from the circulation to the maternal fat compartments of the

body. Only moderate amounts were found in the myometrium and deciduas. There was low to moderate concentration in the corpus luteum and placenta. There was a small amount in all fetal tissues except the brain and testes.

Everett (1947) investigated the deposition of cholesterol in the corpus luteum of the rat. In hypophysectomized rats lutenizing hormone caused cholesterol accumulation in the corpus luteum. Lutenizing hormone appeared to have action of increasing cholesterol stored in luteal tissue. The degree of luteotrophic stimulation determined the rate at which cholesterol was utilized. Cholesterol was indicated as a precursor of progesterone. Woodward and Block (1953) supported the theory that squalene (tri-terpenoid hydrocarbon) was an intermediate in cholesterol synthesis. Acetic acid has been shown to be a carbon source for both squalene and cholesterol. Hechter (1953) reported the pathway of cholesterol to progesterone which may go to the hydroxy progesterones or desoxycorticosterone. Heard et al., (1956) reported that the pathway of acetate to squalene to cholesterol to progesterone was widely accepted with other hormones such as adrenal cortical thyrotrophic hormone exerting a profound effect on certain conversions. Block (1945) using deuterium in the study of cholesterol catabolism concluded that the conversion of cholesterol to pregnanediol was a normal process in the pregnant human. In 1954 Pearlman isolated progesterone with a 12 percent yield from homogenated human placenta incubated with pregnenolone. Hayano (1956) attributed this transformation to dehydrogenase enzyme. Besides being a precursor to progesterone, cholesterol has been shown to be a precursor to the androgens (11-ketoetiocholonolone and tetrahydrocortisone).

Biological Assay

Elden (1934) employed the first large scale biological assay on the progestin content of the corpus luteum of the sow. The rabbit was the test animal used for assay. The corpora lutea were divided into groups as follows: (1) six to seven days pregnant, (2) middle pregnancy, (3) degenerating corpora lutea, and (4) corpora albicans. The progestin contents were respectively 4.1, 3.1, 1.6, and 0.25 rabbit units per 100 grams of luteal tissue. The estrogen content of this luteal tissue was determined with the respective values of 2.7, 3.4, 3.0, and 1.3 rabbit units per 100 grams of tissue. Haskins (1939) developed an intrauterine application method of assay for progesterone using the rabbit. A much smaller dose of progesterone was effective as compared to other methods of administration.

Hooker (1945) studied a criterion of luteal activity in the mouse. He described large spherical, vesicular, stromal nuclei due to the effect of progestin on the cells of the uterine wall in ovariectomized mice. In 1942 Hooker and Forbes reported a bioassay for minute amounts of progesterone utilizing the former observation of Hooker. The minimum effective dose was calculated as 0.0002 mcg. Forbes (1954) tested the specificity of this bioassay further. The nuclei were more precisely described as having a smooth, plump, elongated oval outline; a conspicuous nucleoli; and fine evenly distributed chromatin particles. Hisaw and Velardo (1951) showed that steroid compounds inhibited progesterone in decidual development. Olsen (1952), Zarrow and Neher (1953) found that estradiol-17-beta inhibited the positive response elicited by progesterone in the Hooker-

Forbes assay when the estrogen-progesterone ratios were 1:200 or greater. Desoxycorticosterone acetate, stilbesterol, and testosterone also prevented a typical response.

Chemical Assay

Slotta et al., (1934) worked on the purification of the hormone of the corpus luteum. They were the first to utilize the fact that progesterone has a maximum absorption of 240 mμ. Allen and Goetsch (1936) made progress in the preparation of progesterone from pig ovaries whereby 25 percent was recovered in the pure state. Reynolds and Ginsberg (1942) made the first approach to the problem of chemical assay of progesterone in biological materials. His method was based on the quantitative estimation using ultraviolet spectroscopy at 240 mμ. The measurable concentration range was from 1-20 mcg. per ml. of ethanol. Herget and Shorr (1941) made an analysis of the spectrophotometric methods of studying molecular configuration. They concluded that visible and ultraviolet absorption spectra reflected the electronic state of the molecule. The infra-red absorption was determined by atomic groupings. This method of analysis was found to be applicable to biological materials. Loofbourow (1943), while working with the absorbancy of progesterone, determined that more precise readings were at 0.43 with the error being ± 1 percent using a cell 1 cm. in width. The Beckman photomultiplier was used to increase the sensitivity of the Beckman quartz spectrophotometer. A maximum of 240 mμ. was used with the reading at 280 mμ. giving an idea of the contaminating pigments. Williamson and Craig (1947) worked out a mathematical method of calculating the theoretical curves expected in the countercurrent distribution

of organic compounds. This equation has been applied to progesterone and has worked using extracts of biological materials. Bush (1953) used the thiosemicarbazide in the microestimation of steroid ketones. The thiosemicarbazide of delta-4, 3-keto steroids had maximum absorption at 299-301 mμ. This part of the spectrum was better suited for accurate measurement, with a cleaner and more accurate absorption band. The disadvantage was that 100 mcg. of progesterone were required for accurate determination. The Cary recording spectrophotometer was used in these analyses. Levy and Kushinsky made a study of the methods used for isolating corticosteroids. They divided them into classes as follows: (1) dialysis was used for highly polar steroids, (2) absorption on charcoal was adaptable to very large volumes of biological material, (3) solvent extraction had the greatest virtue in combination with absorption on partition chromatography, and (4) countercurrent technique had the greatest future possibilities according to these workers. Savard (1954) has been a foremost worker in the field of chromatography of ketosteroids. He suggested reducing the polar properties of the mobile solvent, thereby reducing the rate of the non-polar ketosteroid movement.

Physical-chemical factors were involved in the determination of the migration rate of a ketosteroid, with the movement depending directly upon its distribution coefficient between 2 phases, with the determining factors lying in the carbonyl functions present. There were hydrogen bondings between the carboxyl groups and the paper with the ketone groups less likely to H-bond than hydroxyl groups with equatorically-oriented steroids, which were less hindered than polar oriented substances. In the chromatography of steroids, long exposure to natural and ultraviolet light had

to be avoided because of molecular transformation that could occur. It was determined that complete elution was obtained in 30 minutes. The short elution time helped minimize molecular transformation.

Pearlman (1954) reported on methods of estimation and isolation of progesterone. With counter-current distribution technique using 8 transfers, a value of 0.9 mg. progesterone per kg. of human placental tissue was obtained; whereas in human maternal peripherial blood 24 hours after parturition, less than 0.1 mcg. per ml. plasma was found. Butt (1951) found 0.1 mcg. per ml. pregnancy plasma. The values obtained using the Hooker-Forbes bioassay are about 50 times higher. Edgar (1953), working on the efficiency of the chemical assay, recovered 75 percent from blood samples. He determined the absorption peak of different concentrations of progesterone agreeing with the work of Loofbourow (1943). Diczfalusy (1955) verified the assumption underlying the color correlation equation of Allen in the correction for non-specific chromogen contamination in progesterone determination.

$$\text{Ab. (corrected)} = 2 \times \text{ab. max.} - \left[(\text{ab. max.}-k)+(\text{ab. max.}+k)\right] ,$$

where k is the constant determined by the absorption spectrum of the material being read.

Bernstein and Lenhard (1954) examined the absorption spectra of steroids in 97 percent concentrated sulfuric acid. If a steroid exhibited a selective maximum absorption between 279-300 mμ, in all probability it contained an alpha, beta-unsaturated ketone moiety. Plantin (1955) worked out a method of applying small amounts of steroids dissolved in chloroform on salt plates where polyethylene tubes were used, the solvent evaporating

as the solution ran out on the salt plate. The concentration on the salt plate was then used for infra-red analysis.

Loy, McShan and Casida (1957) reported a rapid method for purification and quantitative estimation of progesterone from luteal tissue. The method used was that described by Pearlman (1954). Recovery values reported were 77-92 percent.

Progesterone in Blood and Tissues

The Hooker-Forbes bioassay for progesterone has been used by several workers to assay blood extracts. Neher and Zarrow (1950) assayed the progesterone in serum of the ewe during different stages of reproduction. They found 2 mcg. per ml. of blood serum at estrus which increased to 6 mcg. per ml. the 22nd to the 40th day of pregnancy. At the 100th day, the concentration increased to 10-12 mcg.; 36 hours postpartum, 5-6 mcg.; and at 10 days postpartum, 2 mcg. per ml. These workers, in 1954, found 2 mcg. per ml. blood serum from an animal in the estrous phase and 6 mcg. per ml. from an animal in the luteal phase. In this study of pregnancy in the ewe, serum levels increased from 2 mcg. per ml. at mating to 8-12 mcg. per ml. at parturition. In all sheep, parturition took place prior to the drop in serum progestin. Removal of the ovaries between the 66th and 114th day of pregnancy had no effect on the level of progestin in the blood of the ewes. The progestin increased the same as that seen in intact ewes. It was suggested that the placenta was the major source of progestin during the last periods of pregnancy in sheep.

Hooker and Forbes (1949) studied the transport of progesterone in the blood of various species. They found 90 percent to be in the free

form, with 10 percent bound to protein or in the conjugated form. Sandberg and Slaunwhite (1957), while investigating steroids and steroid conjugates to human plasma proteins considered the binding of steroids as a mechanism of transport, being a weak and reversible reaction. There was little evidence toward the presence of a chemically bound protein steroid complex in the blood. Erythrocytes were believed to play a role in the transport of steroids. Salhanick (1954) worked on the progesterone content in human pregnancy plasma. He used two chromatographic purifications; no quantitative data were presented. Raeside and Turner (1955) detected progesterone in blood leaving an ovary, of a pregnant goat, that contained a corpus luteum, and also from one containing a graafian follicle. It could not be detected in the venous drainage of the uterine horns, gravid or non-gravid, nor in the peripheral blood of several large domesticated animals. After a single subcutaneous injection of 1 gram of progesterone, the rate of disappearance was fast with the peak concentration being 2 hours after administration. A level of 1 mcg. per ml. of blood was maintained in a young dairy bull after injection of 1 gram per day for 3 consecutive days. Forbes (1956) injected progesterone intraperitoneally and determined blood levels as being at their peak in 1 hour. Edgar (1953) was unable to detect progesterone in the peripheral blood of the pregnant or non-pregnant ewe, mare, sow, or cow.

The ewe has been ovariectomized as early as the 50th day of pregnancy with a successful term. The progesterone present in the peripheral blood of sheep was thought to be produced by an extra-ovarian source, Short (1957). Progesterone was not detected in the placental tissue of sheep but 20 alpha-hydroxypregnenone was present on the chromatograms. The

progesterone levels in the blood of pregnant sheep averaged 0.32 mcg. per 100 ml. at the 98th day of pregnancy. It was thought that the placenta of the ewe contributed little progesterone to the circulation during pregnancy.

Adler et al., (1934) showed progestational activity in the rabbit uterus after administering cow placental extracts. Short (1957) analyzed cow placentae at term or near term. No progesterone was found. A 30 ml. sample of blood from the uterine vein was assayed from a cow 224 days pregnant. No progesterone was detected in this small sample. According to the work of Short the progesterone level of the cow blood did not change appreciably with the average being 0.22 mcg. per 100 ml. for periods when positive results were obtained in both the cycle and pregnancy.

Zander and von Münstermann (1954) isolated progesterone from human fetal fluids, in all cases finding 0.05 mcg. to 0.01 mcg. per ml. of amniotic fluid. They found the concentration of progesterone in 1 kg. of placental tissue to be 4.15 mcg. at the 2nd month and decreasing to 1.5-2.2 mcg. up to the tenth month of pregnancy.

Hagopian et al., (1956) isolated an unknown substance and 6-ketoprogesterone from profusates of human placentae using bovine blood. The unknown compound had the same mobility as progesterone on the chromatogram. The maximum absorption was 241 mμ. in methanol and 294 mμ. in sulfuric acid. The major bands on infra-red absorption were 1676 $cm.^{-1}$ (alpha, beta unsaturation) and 1706 $cm.^{-1}$ (heracyclic or c-20 carbonyl). One mg. of the unknown was isolated. There was also another compound that could have been confused with progesterone (pregn-4: 11 diene-3: 20 dione). Zander and Simmer (1954).

The mare placenta has been analyzed for progesterone by Short (1957). The progesterone content was 73 mcg. per kg. tissue for those pregnant 120 days and 250 mcg. per kg. tissue for those pregnant 270 days. A sufficient amount of progesterone was assumed to diffuse across the placental barrier to exert a direct progestational effect on the endometrium, without a significant amount appearing in the maternal blood. Short had recovery values of 50 percent. He assumed most of the loss during elution from the chromatogram.

Savard administered labeled acetate intravenously to a mare. Radioactive cholesterol from the maternal side was lower in activity than cholesterol from the fetal side of the placenta. This indicated that fetal and maternal systems handled hormones differently.

Pearlman (1954) using 16-^{3}H labeled progesterone was able to demonstrate the rapid rate at which the hormone undergoes metabolic transformations into biologically inactive products. Very little hormone accumulated in the body except in the fat where it remained for about 1 hour. Recent work by Herrmann and Silverman (1957) indicated another steroid derivative excreted by the pregnant human (pregnanetriol). There was strong evidence that the placenta was the site of production of the precursor to pregnanetriol.

EXPERIMENTAL PROCEDURE

Collection and Preparation of Samples

Reproductive tracts from pregnant cows were collected immediately after slaughter of the animals at the Iowa Packing Company in Des Moines, Iowa. The apparent breed and weight of each cow was recorded. The reproductive tracts were brought immediately to the laboratory at Iowa State College where the samples and data were collected. Ovaries were dissected and placed in a small sample jar. Allantoic fluid was drained from the allantois and amniotic fluid from the amnion, and the weight of each was determined, allowing a 5 percent correction in amniotic fluid for fluid contained in fetal hair in the case of pregnancies later than 7 months, Swett, Mathews, and Fohrman, (1948). Physical characteristics of both fluids were observed. The fetus was removed from the amnion, the crown rump measurement was taken, and certain morphological characteristics were observed to help establish the age of the fetus. A logistic curve was fitted to the data on crown rump measurements to facilitate determination of the age of each fetus. The horn of the pregnancy and the sex of each fetus were recorded. The weight of placental tissue was determined after separating the cotyledons of the placetomes from the caruncles. The fetal membranes were weighed and a sample of this tissue was collected. Characteristics of the fetal membranes and cotyledons were observed. The weight of the uterus was recorded. The left adrenal gland was also collected for analysis. All samples were frozen and stored in a deep freeze until time of analysis.

In addition, blood was collected from the jugular vein of Holstein

cows during different stages of pregnancy. Some samples were treated with 200 units of bacterial beta-glucuronidase per ml. for 24 hours at 37°C.

Solvents Used

The following solvents were used in this investigation: methyl alcohol, acetone free, anhydrous, reagent grade (Mallinchrodt); ethyl acetate, anhydrous, reagent grade (Baker and Adamson); benzene, thiophene free, analytical reagent (Mallinchrodt); diethyl ether (Mallinchrodt); Skellysolve B, redistilled (B.P. 80-100°C.); 95 percent ethyl alcohol and absolute ethyl alcohol not redistilled. The ethyl alcohol used did not show absorbancy over the range from 240 to 280 mμ.

Homogenization of Tissue Sample

Tissues were only partially frozen while processing to minimize blood loss. The corpora lutea were removed from the ovaries, and the residual ovarian tissue, corpus luteum, and the left adrenal were weighed. These tissues were homogenized individually in a Lourdes homogenizer[1]. Luteal and adrenal tissues were homogenized in 95 percent ethyl alcohol; ovarian tissue was homogenized in absolute alcohol to prevent foaming caused by substances present in the follicular fluid. Diethyl ether was added to the extraction mixture to make 20 ml. of 1:3 V/V diethyl ether: ethyl alcohol. Placental tissue was homogenized in a Waring blendor in 95 percent ethyl alcohol.

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[1]Obtained from Lourdes Laboratory Instrument Corp., Brooklyn, N.Y.

Extraction Procedure

The homogenized residual ovarian tissue, corpora lutea, and adrenal glands were extracted with the 20 ml. of 1:3 V/V diethyl ether: ethyl alcohol for 2 hours using a Goldfish extractor. After cooling, the solid residues were filtered and washed with the extraction solvent. Placental tissue was extracted with 10 volumes of 1:3 V/V diethyl ether: ethyl alcohol by boiling on a steam bath for 1 hour; after cooling, the solid residue was washed with 50 ml. of the solvent. The combined extracts for each tissue were concentrated to about 50 ml. over a steam bath, and finally to 20 ml. using a Rinco vacuum rotary evaporator[1] with a bath temperature of 50°C. When foaming occurred the bath temperature was lowered to 40°C. Phospholipids accumulating on the sides of the evaporating flask on cooling were discarded. Amniotic and allantoic fluids and blood were treated in the same manner as placental tissue.

Partition of Extracts Between Organic Solvents

The various extracts were transferred to 250 ml. separatory funnels. Forty ml. portions of distilled water were added to the 20 ml. concentrates, and insoluble material was removed by filtration. These aqueous solutions were extracted 6 times with 50 ml. portions of ethyl acetate. Most of the emulsions encountered broke on standing overnight, but in a few cases centrifugation was necessary. The combined ethyl acetate extracts were concentrated *in vacuo* at 50°C. until ethyl acetate vapors could no longer be detected. The residues were transferred to 10 ml.

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[1]Obtained from Aloe, Scientific Division, St. Louis, Mo.

centrifuge tubes with 3 washings of 3 ml. portions of 70 percent aqueous methanol, and the tubes placed in a deep freeze at -12 to -15°C. for overnight storage to allow cholesterol to precipitate. Cholesterol was removed by centrifugation at 40,000 rpm at -15°C. for 15 minutes in a Spinco model L refrigerated centrifuge using sealed Teflon tubes in a sealed type 40 rotor[1] when the extracts were high in cholesterol or by centrifugation in a regular laboratory centrifuge when cholesterol content was low.

The supernates were decanted into 250 ml. separatory funnels, diluted with 20 ml. of distilled water, and extracted 3 times with 30 ml. portions of redistilled Skellysolve B (B.P. 60-70°C.) The combined extracts in each case were washed twice with 50 ml. portions of distilled water. The washed Skellysolve B extracts were evaporated to dryness in vacuo. The evaporating flasks were rinsed 3 times with 3 ml. portions of Skellysolve B, and the washings transferred to a watch glass. The Skellysolve B was removed in vacuo in a desiccator containing calcium chloride.

Chromatographic Separation

Two methods of chromatographic separation were used. Preliminary work was conducted using the propylene glycol: petroleum ether system. It was found that a more consistant paper blank was obtained when using the petroleum ether: methanol: water system. This latter system was used in obtaining the data and will be described in detail.

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[1] Obtained from Beckman-Spinco Division, Palo Alto, Calif.

Propylene glycol: petroleum ether system (preliminary work)

The paper chromatographic method of Zaffaroni, Burton and Keutmann (1949), originally designed for separation of corticosteroids using a toluene: propylene glycol solvent system, was modified to deal with less polar steroid derivatives by Savard (1953). Use of petroleum ether as the mobile phase permitted the chromatography of less polar steroids. In the original system, less polar steroids moved too rapidly.

Paper strips previously washed for 72 hours in methanol were impregnated with the stationary solvent (propylene glycol) by dipping in a freshly prepared 50 percent solution in methanol and removing the excess by blotting. Variation in the amount of solvent left in the strips was found to influence greatly the mobilities of steroids. During application of the samples, the strips were left between sheets of blotting paper with only the ends projecting. The samples on the watch glasses were dissolved in 2 µl of benzene. Spreading of the zone was minimized by the backsurge of propylene glycol after the benzene had evaporated. The strips were air-dried and placed in a round chromatographic jar (8 x 16 in.), arranged for descending chromatography and allowed to equilibrate in the presence of both solvents. Saturation of the vapor space was aided by lining the walls with filter paper sheets dipping into the solvent in the bottom of the jar. The temperature was maintained at 34°C. for the 24 hour equilibration period. Mobile solvent was added to the trough and allowed to run 3 hours. The strips were removed, dried, and scanned for fluorescent spots with a Mineralight SL-2537[1]. The Mineralight was not as efficient for detecting

[1] Purchased from Ultra-Violet Products, Inc., San Gabriel, Calif.

spots as a scanner apparatus later constructed by the author.

Spots were eluted with the aid of a preliminary capillary tube apparatus constructed in the laboratory. Eluting the intact strips was shown to introduce less contamination than methods involving grinding the paper and extracting the pulp. Much work was done to improve the efficiency of eluting the paper strips with minimum contamination and maximum recovery. The final elution apparatus was designed by the author and constructed in the glass blowing shop of the Chemistry Department of Iowa State College, and will be described in a separate section on elution. Elution was found to be most efficient when the eluting solvent (1:3 V/V diethyl ether, ethyl alcohol) flowed over the paper at a rate of 5 ml. in 30 minutes, giving 93 percent recovery. A paper blank was eluted from a strip spotted with the chromatographic solvent at the same distance from the origin as the progesterone spot. The absorbancy of the blank gave an indication of solvent contamination and was subtracted from the absorbancy of each sample. There was considerable variation in the blanks from different runs.

A manner of expressing the chromatographic mobility of a compound in a particular solvent system was provided by Cosden, Gordon, and Martin in their original work on amino acids. The term RF refers to the ratio of the distance traveled by the compound to that simultaneously traveled by the solvent front. It has been convenient to express steroid mobilities on paper chromatograms as a function of distance traveled in unit time, RT (in cm. per hour). The relative mobilities, RT, have not varied over ±8 percent. Relative mobility of the steroid was established by measuring the distance in cm. from the starting line to the center of the steroid zone and relating this value to that of the standard steroid run concur-

rently with it. RT was calculated from the following formula:

$$RT = \frac{\text{distance traveled by the unknown steroid}}{\text{distance traveled by the standard}} \times \text{rate of movement of the standard}$$

Sow corpora luteal tissue was used in developing the techniques of determination of progesterone. These values were compared with those reported by Gawienowski (1956). For this tissue recovery experiments were run using half by weight of the luteal tissue from an ovary plus added progesterone, and the other half without added hormone. Recovery was calculated as the percent of added progesterone recovered as determined by spectrophotometric analysis. Recovery was also determined in the absence of biological tissue as a check on the analytical procedures. Preliminary work was also carried out using frozen bovine luteal tissue collected from cows in different stages of pregnancy. Recovery of added progesterone was also investigated using the bovine reproductive tissues.

Petroleum ether: methanol: water system

Samples in preliminary studies were chromatographed as previously described using a round pyrex jar 18 x 6 in. In order to provide space for more chromatograms, later samples were chromatographed in a square pyrex jar[1]. Descending chromatography was used in both cases. Large sheets of filter paper were placed along the inside of the square jar to help maintain a saturated atmosphere. A sheet of filter paper 9 x 12 in. was placed in a solvent trough held by the spring support to help saturate

[1]Obtained from Research Specialties Company, Berkeley 7, Calif.

the atmosphere with stationary phase. The jar was kept at constant temperature in an incubating oven. A temperature of 34°C. was chosen as optimum for this system. The ratio of solvents was varied until a suitable ratio was obtained, consisting of 10:8:2, Skellysolve D (B.P. 80-100°C.): methyl alcohol: distilled water.

Preparation of filter paper for chromatographing. Whatman filter paper (no. 1) was cut in 17 x 60 cm. sheets. These sheets were rolled, placed in a Soxhlet extractor, and extracted with absolute methyl alcohol for 72 hours to remove impurities, Butt et al., (1951). After drying at room temperature, each sheet was cut lengthwise into 1 cm. strips spaced 1 cm. apart and still attached to each other at one end.

Transfer of extracts to chromatographic paper. The chromatographic strips were placed on a clean sheet of filter paper for application of the samples. A line was drawn across the strips 6 cm. below their point of attachment and samples were spotted on this line. Leaving the strips attached facilitated placing them evenly in the trough. The strips were supported by glass rods of a non-siphoning type, which touched the paper 4 cm. above the origin.

The residues on the watch glasses were dissolved in 0.2 ml. of benzene (described on page 36), and the benzene solutions of progesterone transferred to the chromatographic strips after the benzene had evaporated to a volume of 20µl. Absolute methanol and ethanol have also been used for this purpose. The 20µl samples were spotted on the strips in 5µl portions using a micro-pipet and a screw-type suction pipet filler. The area covered was about 1 cm. in diameter. When a progesterone sample was spread

over a larger area, it was difficult to detect after the run because of the diffuse nature of the developed spot. The papers were thoroughly dried after spotting because of the strong absorbancy of benzene at 240 mμ. Extreme care was taken to prevent contamination by other compounds, especially those of an aromatic nature. Chromatographic paper was handled with clean tongs which were used only for this purpose to prevent unnecessary contamination. A paper blank strip was run in order to subtract the absorbancy due to solvent contamination. Progesterone standards were run on alternate strips to help locate the spots in the samples from progesterone tissue extracts.

Chromatographic procedure. The spotted strips of chromatographic paper prepared for descending chromatography were placed in a jar that had been prepared as previously described. The glass rod holder was placed on the paper strips. The lid was placed on the jar with a silicone grease seal. The jar was placed in an incubator where the temperature was maintained at 34°C. The mobile solvent was added through a hole in the lid at the end of the 24 hour equilibration period. Fifty ml. of the mobile phase was added when 1 sheet was being run or 100 ml. was added when 2 sheets were run from the same trough. Three hours later the sheets were removed and dried.

Location of progesterone on chromatograms

The most sensitive procedure for locating small amounts of alpha, beta-unsaturated ketones is that of scanning the paper strip with a source of ultraviolet light which delineates these compounds as dark areas against

the purple fluorescing background of the paper. A scanner apparatus was constructed for this purpose utilizing a 12 in. desk lamp[1] with a fluorescent germicidal type A filament. Light of wave lengths other than the near ultraviolet was filtered out by use of a Corning no. 9683 filter. This filter[2] was not optimum for transmission of ultraviolet light at 240 mμ, but was suitable for detection of spots. The lamp was covered with cardboard except where the filter was located. Strips were passed over the filter and the spots marked with a pencil. No progesterone was detected in some extracts of tissues and fluids. When the solvent front was straight, as indicated by the RF values of progesterone, it was possible to draw a straight line across the strips to aid in identification. The strips were cut at 45 degree angles below the progesterone spot and at right angles above the spots. These small strips were placed in an eluting apparatus.

Elution

The elution apparatus used to elute the progesterone from the chromatogram was designed after that described by Zander (1951). This apparatus is pictured in Fig. 1. A piece of 0.25 mm. bore pyrex capillary tubing was sealed in one end of a 55/50 pyrex joint. A number 2 pyrex stopcock and a 10 ml. reservoir were sealed on to the capillary tube. The lower end of the capillary tube was bent at a 90 degree angle. The end was ground flat in a vertical position at a point 8 cm. below the top of the

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[1]Obtained from Arthur H. Thomas and Company, Philadelphia, Penn.

[2]Obtained from Corning Glass Company, Corning, New York.

joint. A rectangular glass bar 0.5 x 1 cm. was suspended from a hook on the capillary to hold the paper in place for elution. The entire elution apparatus was 40 cm. high with the paper suspended slightly higher than a 5 ml. glass stoppered volumetric flask. The bottom cylinder of the apparatus was placed on a 17.5 cm. square plate glass.

The paper strips were placed between the capillary face and the glass bar for elution. The capillary was dampened with elution fluid to hold the strip in place. The top joint was placed in position on the lower joint. A 5 ml. volumetric flask was placed directly under the paper strip. Five ml. of eluting fluid (1:3 V/V diethyl ether: absolute ethanol) was placed in the storage chamber. The stopcock was regulated to deliver 1 drop each 11 seconds. This gave an elution time of 25 to 30 minutes. Liquid was allowed to run through the capillary until exactly 5 ml. of solution was obtained. The flasks were stoppered and stored in the refrigerator until read in the Beckman DU spectrophotometer.

Spectrophotometric analysis

The solutions were poured into a set of matched quartz 1 cm. cells for reading in the spectrophotometer. The elution solution was used for standardizing the instrument. Readings of absorbancy were taken on both sides of the maximum which was at 240 mμ. These readings were used to establish the shape of the curve of each sample. Readings were also taken on paper blank solutions in order to subtract the contamination due to solvents. The maximum absorbancy was corrected for non-specific chromagen contamination using the formula of Allen (1936).

Progesterone solutions of concentrations of 4-80 mcg. were read at

240 mμ to establish a standard curve. Progesterone solutions obeyed the Lambert-Beer law with absorbancy being directly proportional to the concentration.

Qualitative analysis

A modification of the Zimmerman reaction (Savard 1953) was used as a qualitative test for determining the presence of the delta-4, 3-keto group, a purple color indicating a positive reaction. Pooled extracts were spotted on filter paper which was treated by dipping in a 2.5 normal solution of potassium hydroxide in ethanol (freshly prepared), blotting to remove excess, dipping in a 2 percent solution of m-dinitrobenzene in ethanol, and blotting again to remove excess. The strips were developed by gently warming to 65°C. in an oven.

Infra-red analysis of purified extracts

Several methods were tried during this investigation to obtain an infra-red analysis on various purified extracts of luteal, residual ovarian, placental and adrenal tissues as well as amniotic and allantoic fluids and blood. Region I (1800-1580 $cm.^{-1}$) has been studied and has proved useful in identifying carbonyl functions and unsaturated centers from the positions of the band maxima (Dobriner, Katzenellenbogen, and Jones, 1953). The main finger printing regions are at 1670 $cm.^{-1}$ (alpha, beta-unsaturation) and at 1707 $cm.^{-1}$ (carbon-20 carbonyl). The first samples were dissolved in carbon tetrachloride and evaporated on the salt plates for analysis. This method eliminated the influence of solvent and gave indications that the material being isolated was a delta-

4, 3-keto, carbon-20 carbonyl compound. In the next series of analyses the microcell method was used in which a column of solution was aligned in the light beam. Difficulties were encountered with this method such as maintaining the vertical column of liquid.

In the next series of analyses the isolated progesterone was pooled in order to obtain a larger quantity. These pooled extracts were stored in ethyl alcohol in a deep freeze until time of analysis. The samples were dried in 5 ml. beakers on a steam bath. The residues from pooled extracts of corpora lutea, residual ovarian tissue, placentae, adrenals, allantoic and amniotic fluids, and blood were mixed with mineral oil and brushed on the salt plates for analysis. The oil painting method was the most successful for holding the unknown in place on the salt plates. Insufficient recovery of the extracts from the salt plates limited the possibility for further detailed examination in the fingerprint region.

Pooled extracts from 12l. of whole blood were further purified by subjecting them to the counter-current distribution technique used by Pearlman (1954). Eight transfers were used with a solvent system of Skellysolve B (B.P. 60 to 70°C.): 70 percent methanol, 1:3 V/V. Upper layers from the third, fourth, fifth, and sixth separatory funnels were pooled and dried for infra-red analysis. Most of the contaminating pigments remained in the upper layers of the first, second, seventh, and eighth separatory funnels. Residues from purified extracts were dissolved in 2 drops of carbon tetrachloride and placed in the microcell for analysis.

RESULTS

Morphological Changes During Pregnancy of the Cow

The weight changes in bovine uterus, placenta, corpus luteum, residual ovarian tissue, and allantoic and amniotic fluids are presented in Figs. 6 through 11. Certain physical characteristics of placenta and reproductive fluids are presented in Table 12.

Standard Curve and Recovery of Progesterone

A standard curve was prepared by using concentrations of progesterone varying from 4 to 80 mcg. per 5 ml. of solvent. This solvent was a 1:3 V/V mixture of diethyl ether and ethyl alcohol (Fig. 2). The curve was linear with a regression coefficient of 0.0103 absorbancy units change for each increase of 1 mcg. per 5 ml. solution.

Recovery of progesterone from tissue extracts was evaluated by adding a known amount of the hormone to the extract from one half by weight of a corpus luteum and comparing the corrected absorbancy of this extract with that of the other one half of the gland which did not contain added progesterone. The same procedure was followed for other extracts. Recovery values ranged from 74.7 to 80.0 percent. Edgar (1953) reported an average recovery of 75 percent and Short (1957) reported a 50 percent recovery of progesterone added to extracts.

Quantitative and Qualitative Analyses

The absorbancy of extracts was plotted against wave length (Fig. 3) to establish the point of maximum absorption. This point of maximum absorption in 1:3 V/V diethyl ether:ethyl alcohol was between 240 and 242 mμ. for all tissue extracts which indicated the presence of a delta-4, 3-keto group according to Reynolds (1942). The absorbancy range from 220 to 280 mμ. was used to correct for non-specific contaminating pigments utilizing the formula of Allen (Loofbourow 1943). Absorption spectra of extracts in 97 percent sulphuric acid solution had maxima near 300 mμ. which suggest an alpha, beta-unsaturated ketone moiety which would correspond to the delta-4, 3-keto group, according to Bernstein and Lenhard (1954).

The concentrations of progesterone in mcg. per gm. of tissue are presented in Table 1 and Figs. 12 and 13. Statistical analyses of these data are shown in Tables 3 and 5.

Data on the progesterone concentration in mcg. per 100 ml. citrated whole blood collected at different stages of pregnancy from Holstein cows are reported in Table 8 and Fig. 15.

Total progesterone in mcg. per organ and per total quantity of fluids are presented in Table 2 and Fig. 14. Statistical analyses of these data are in Tables 4, 6, and 7. Summary of results obtained by other investigators on progesterone concentration are shown in Tables 10 and 11.

Pooled extracts from corpora lutea, residual ovarian tissue, placenta, adrenal, allantoic and amniotic fluids and blood gave positive tests with the Zimmerman reaction as shown in Fig. 4. The modified Zimmerman reaction

is specific for an alpha, beta unsaturation which would correspond to the delta-4, 3-keto group of progesterone.

Infra-red absorption curves for extracts of corpora lutea, residual ovarian tissue, placenta, and blood were characteristic for a carbon-20 carbonyl as shown by the peak at 1707 $cm.^{-1}$ and a delta-4, 3-keto grouping as shown by a peak at 1670 $cm.^{-1}$. A satisfactory infra-red analysis was not obtained on extracts of allantoic and amniotic fluids and adrenal glands because of an insufficient amount of progesterone in the extracts investigated. The recovery of oil smears from the salt plates was low, therefore close examination in the fingerprint region was not obtained.

Table 1. Concentration of progesterone in reproductive organs and fluids

Stage	Duration of pregnancy in days	No. of animals	Micrograms progesterone per gram					
			Corpus luteum	Residual ovarian tissue	Placenta	Allantoic fluid	Amniotic fluid	Left adrenal gland
1	10-49	9	2.3±0.7	1.1±0.5	0.08±0.04	0.06±0.01	0.02[a]	
2	50-89	3	3.6±1.5	1.0±0.4	0.08±0.03	0.08±0.01	0.01±0.003	1.5[a]
3	90-129	13	5.0±1.2	2.7±0.6	0.16±0.09	0.07±0.01	0.04±0.01	1.7±0.04
4	130-169	14	3.8±0.9	1.6±0.5	0.20±0.06	0.07±0.01	0.04±0.01	1.5±0.09
5	170-209	9	3.9±0.7	1.9±0.5	0.20±0.07	0.06±0.01	0.01±0.002	2.1±0.09
6	210-249	9	1.5±0.5	0.7±0.1	0.05±0.02	0.03±0.01	0.02±0.01	0.4[a]
7	250-280	2	1.1±0.3	0.8±0.5	0.03±0.01	0.04±0.01	0.02[a]	0.7[a]

[a]one sample

Table 2. Micrograms of total progesterone in reproductive organs and fluids during pregnancy of the cow

Stage	Duration of pregnancy in days	No. of animals	Corpus luteum	Residual ovarian tissue	Placenta	Allantoic fluid	Amniotic fluid	Left adrenal gland
1	10-49	9	9.8±3.5	6.0±2.3	1.3±0.7	38.6±8.8	1.6[a]	
2	50-89	3	18.9±9.3	7.5±1.4	5.2±2.2	57.0±12.1	1.7±0.8	17.0[a]
3	90-129	13	22.6±4.9	16.5±3.4	71.3±10.4	137.5±37.7	36.4±5.2	15.4±3.9
4	130-169	14	16.8±3.8	15.7±4.7	92.1±49.6	253.4±59.8	253.4±53.7	16.2±7.0
5	170-209	9	15.6±5.2	13.3±5.1	402.7±145.4	346.6±60.2	320.0±78.9	20.0±1.0
6	210-249	9	7.9±2.3	7.1±1.7	99.6±29.7	276.9±39.1	330.6±77.8	16.3[a]
7	250-280	2	6.8±1.3	9.7±3.3	114.9±30.0	313.6±46.2	72.7[a]	8.5[a]

[a]One sample

Table 3. Analysis of variance of progesterone concentration in bovine reproductive organs and fluids

Source of variation	Degrees of freedom	Mean square				
		Corpora lutea	Residual ovarian tissue	Placenta	Allantoic fluid	Amniotic fluid
Total	58	---	---	---	---	---
Between stages	6	19.142[a]	5.538[a]	0.005[b]	0.0076[c]	0.0020[b]
1 vs. 5, 6	1	2.847[b]	0.782[b]	0.093[a]	0.0010[b]	0.0010[b]
1 vs. 3	1	85.420[d]	16.695[c]	0.036[b]	0.0021[b]	0.0002[b]
2 vs. 4	1	0.600[b]	1.114[b]	0.013[b]	0.0001[b]	0.0004[b]
5 vs. 6	1	20.846[a]	6.601[a]	0.110[a]	0.0041[a]	0.0001[b]
6 vs. 7	1	0.276[b]	0.013[b]	0.002[b]	0.0003[b]	0.0072[b]
Within stages	52	7.819	2.931	0.029	0.0013	0.0028

[a](P=0.10)

[b]not significant

[c](P=0.01)

[d](P=0.05)

Table 4. Analysis of variance of total progesterone in bovine reproductive organs and fluids

Source of variation	Degrees of freedom	Mean square				
		Corpora lutea	Residual ovarian tissue	Placenta	Allantoic fluid	Amniotic fluid
Total	58	---	---	---	---	---
Between stages	6	277.93[a]	174.40[b]	158,362.94[c]	109,726.92[b]	20,359.90[b]
1 vs. 5, 6	1	54.41[b]	140.48[b]	424,004.01[c]	47,351.23[b]	47,351.23[b]
1 vs. 3	1	1,017.44[d]	658.18[a]	20,623.85[b]	6,384.00[b]	6,384.00[b]
2 vs. 4	1	0.03[b]	165.92[b]	86,526.02[a]	63,529.28[b]	63,529.28[b]
5 vs. 6	1	26.45[b]	177.35[b]	503,673.30[c]	1,406.25[b]	22,440.04[b]
6 vs. 7	1	2.16[b]	11.11[b]	2,909.51[b]	22,440.04[b]	1,406.25[b]
Within stages	52	195.84	166.26	39,940.10	71,163.10	12,765.19

[a](P=0.10)

[b]not significant

[c](P=0.01)

[d](P=0.05)

Table 5. Analysis of variance of composite data on progesterone concentration

Source of variation	Degrees of freedom	Mean square
Total	173	
Between extracts	4	78.87[a]
Between stages within extracts	24	35.95[a]
Between cows within stages within extracts	145	1.36

[a](P=0.01)

Table 6. Analysis of variance of composite data on total progesterone in reproductive organs and fluids

Source of variation	Degrees of freedom	Mean square
Total	173	
Between extracts	4	361,105.71[a]
Between stages within extracts	24	91,474.43[a]
Between cows within stages within extracts	145	14,180.82

[a](P=0.01)

Table 7. Analysis of variance of progesterone content between breeds

Source of variation	Degrees of freedom	Mean square
Total	58	
Between breeds	2	1,187.11[a]
Within breeds	56	405.57

[a]not significant

Table 8. Concentration of progesterone in whole blood collected from pregnant cows during different stages of pregnancy

Stage	Duration of pregnancy in days	Number of samples	Mcg. progesterone per 100 ml. whole blood
1	10-49	3	0.9 ± 0.6
2	50-89	3	1.4 ± 0.6
3	90-129	11	0.4 ± 0.1
4	130-169	6	3.1 ± 1.1
5	170-209	5	3.4 ± 1.6
6	210-249	3	4.0 ± 1.3
7	250-280	7	3.1 ± 1.4

Table 9. Analysis of variance of blood progesterone during pregnancy

Source of variation	Degrees of freedom	Mean square
Total	37	
Between stages	6	11.78[a]
3 vs. 6	1	4.34[a]
1 vs. 6	1	14.72[a]
1 vs. 4	1	18.06[a]
Within stages	31	63.08

[a]not significant

Table 10. Summary of investigation of progesterone in body fluids

Species	Stage of reproduction	Body fluid	Concentration mcg. per ml.	Reference
Sow	Unknown	Blood	0.1	Block (1936)
"	Diestrus	Follicular fluid	8	Duyvené de Wit (1938)
"	--	Blood	0.04	Duyvené de Wit (1941)
"	Diestrus	Follicular fluid	4-8	Hooker, et al. (1947)
"	Irregular cycle	Blood from ovarian vein	0.1-0.4	Edgar (1953)
"	"	Peripheral blood	0.4	"
"	66 days pregnant	"	0.4	"
"	Pre-ovulation	Follicular fluid	8	"
"	Luteal phase	Peripheral plasma	0.0083	Short (1957)
"	90 days pregnant	"	0.0034	"
Cow	Diestrus	Follicular fluid	5	Duyvené de Wit (1938)
"	56 days pregnant	Peripheral blood	0.1	Edgar (1953)
"	210 days pregnant	"	0.02	"
"	"Mid-cycle"	"	0.1	"
"	Pre-ovulation	Follicular fluid	3	"
"	24 hours before ovulation	Peripheral plasma	0.002	Short (1957)
"	4.5-6.5 mo. pregnant	"	0.0024	"
"	9 mo. pregnant	"	Not detectable	"
Ewe	Estrus	Serum	2	Neher and Zarrow (1950)
"	40 days pregnant	"	6	"
"	End of pregnancy	"	10-12	"
"	10 days post partum	"	2	"
"	90-105 days pregnant	Blood from ovarian vein	1.0-2.0	Edgar (1953)
"	8 days post estrum	"	0.5	"
"	28 days post partum	"	0.1	"
"	90-105 days pregnant	Peripheral blood	0.1	"
"	8 days post estrum	"	0.1	"
"	Pre-ovulation	Follicular fluid	16	"

Table 10, Continued

Species	Stage of reproduction	Body fluid	Concentration mcg. per ml.	Reference
Ewe	Ovariectomized 98 days pregnant	Peripheral plasma	0.0031	Short (1957)
"	99 days pregnant	"	0.0034	"
Ram	Castrated	Peripheral blood	0.1	Edgar (1953)
Mare	Non-pregnant	"	0.1	"
"	63-111 days pregnant	"	0.1-0.2	"
"	200 days pregnant	Allantoic fluid	Not detectable	Short (1957)
"	"	Amniotic fluid	Not detectable	"
Goat	134 days pregnant	Plasma from ovarian vein 1 corpus luteum in ovary	2.3	Raeside and Turner (1955)
"	"	Plasma from ovarian vein with Graafian follicle	1.8	"
"	112 days pregnant	Peripheral plasma	0.0071	Short (1957)
Rabbit	6 days pseudopregnant	Plasma	4.3	Hooker, et al. (1947)
"	"	Blood	4-8	Hooker, et al. (1949)
Mouse	5 days post coitum	"	4-8	"
Monkey	Diestrus	"	4-8	"
Woman	Pregnant	Serum	2.0	Block (1936)
"	"	"	0.1	Haskins (1941)
"	Diestrus	Plasma	0.3-1	Forbes (1949)
"	8 weeks pregnant	Blood	4-8	Hooker, et al. (1949)
"	Pregnant	"	0.1	Haskins (1950)
"	"	"	0.1	Butt, et al. (1951)
"	--	Placental blood	1.3 and 0.3	"
"	Parturition	"	0.4	Pearlman (1954)
"	Late pregnancy	Peripheral blood plasma	0.14	Zander, et al. (1954)
"	--	Amniotic fluid	Positive	Zander (1956)
"	--	"	0.01-0.05	Zander, et al. (1954)

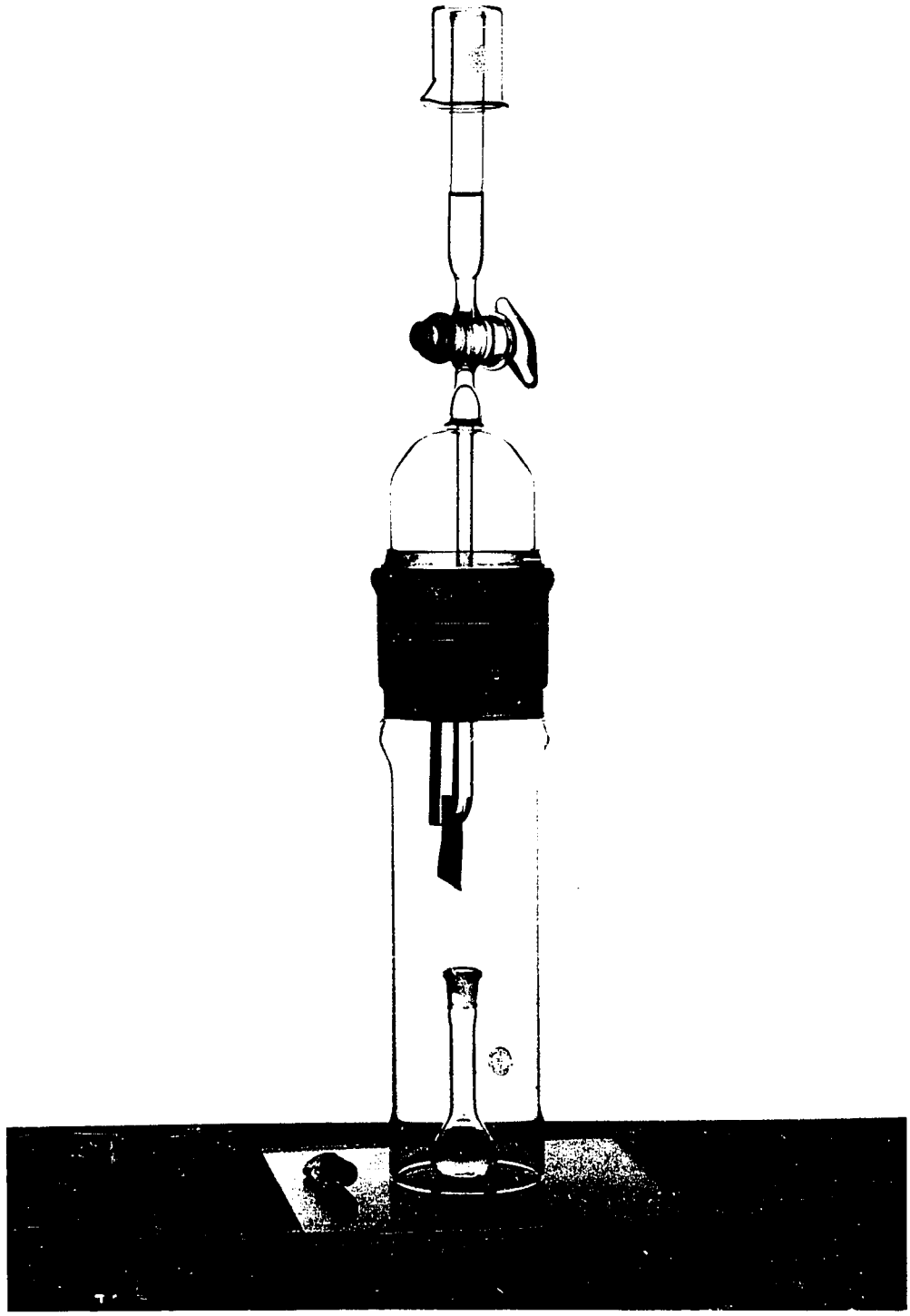
Table 11. Summary of investigations of progesterone in reproductive organs

Species	Stage of reproduction	Body organ	Progesterone	Reference
Sow	6-7 days pregnant	Corpora lutea	4.1 R.U. per 100 gm.	Elden (1934)
"	Mid-pregnancy	"	3.1 R.U. per 100 gm.	"
"	Late pregnancy	"	1.6 R.U. per 100 gm.	"
"	--	Corpus albicans	0.2 R.U. per 100 gm.	"
"	15 days pregnant	Corpora lutea	0.22 mg. per kg.	Kimura and Cornwell (1938)
"	75 days pregnant	"	0.27 mg. per kg.	"
"	10 days prepartum	"	Not detectable	"
"	55 days pregnant	"	5.9-37.9 mcg. per gm.	Gawienowski (1956)
"	"	Ovarian tissue	16-23.9 mcg. per gm.	"
"	--	Placenta	0.09 mcg. per gm.	Ehrhardt and Hardt (1937)
"	--	Corpora lutea	29.8-103.8 mcg. per gm.	Loy, et al. (1957)
Cow	--	Placenta	Progestational activity	Adler, De Fremery, and Tausk (1934)
Ewe	--	Corpora lutea	3.3 mcg. per gm.	Edgar (1953)
Mare	200 days pregnant	Intact ovary	3 mcg. per ovary	Short (1957)
"	Luteal phase of estrus cycle	Corpora lutea	46 mcg. per gm.	"
"	120 days pregnant	Placenta	0.073 mcg. per gm.	"
"	270 days pregnant	"	0.250 mcg. per gm.	"
Human	Full term	Placenta	1-1.5 mcg. per gm.	Pearlman (1954)
"	60 days pregnant	"	4.15 mcg. per kg.	Zander, et al. (1954)
"	Parturition	"	1.5-2.2 mcg. per kg.	"

Table 12. Some physical characteristics of reproductive organs and fluids

Duration of pregnancy in days		10-89	90-169	170-250	251-parturition
Allantoic fluid	color	clear	clear to light brown	brownish	dark reddish brown
	viscosity	thin	thin	thin	thin
	miscellaneous			flat, oval floating bodies	flat, oval floating bodies
Amniotic fluid	color	clear	greenish	white	white
	viscosity	thin	thin	medium	very thick
	mucus	none	sparce	sparce to abundant	abundant
	miscellaneous			feces present	feces present
Placental tissue	placetome shape	rounded	rounded	rounded, oval	flattened, oval
	placetome size	small		5 cm. diameter	5-7 cm. diameter
	no. of placetomes observed	40	120-140	120-140	140-200
	miscellaneous			focal areas of epithelial hyperplasia present	

Figure 1. Apparatus for elution of chromatogram.



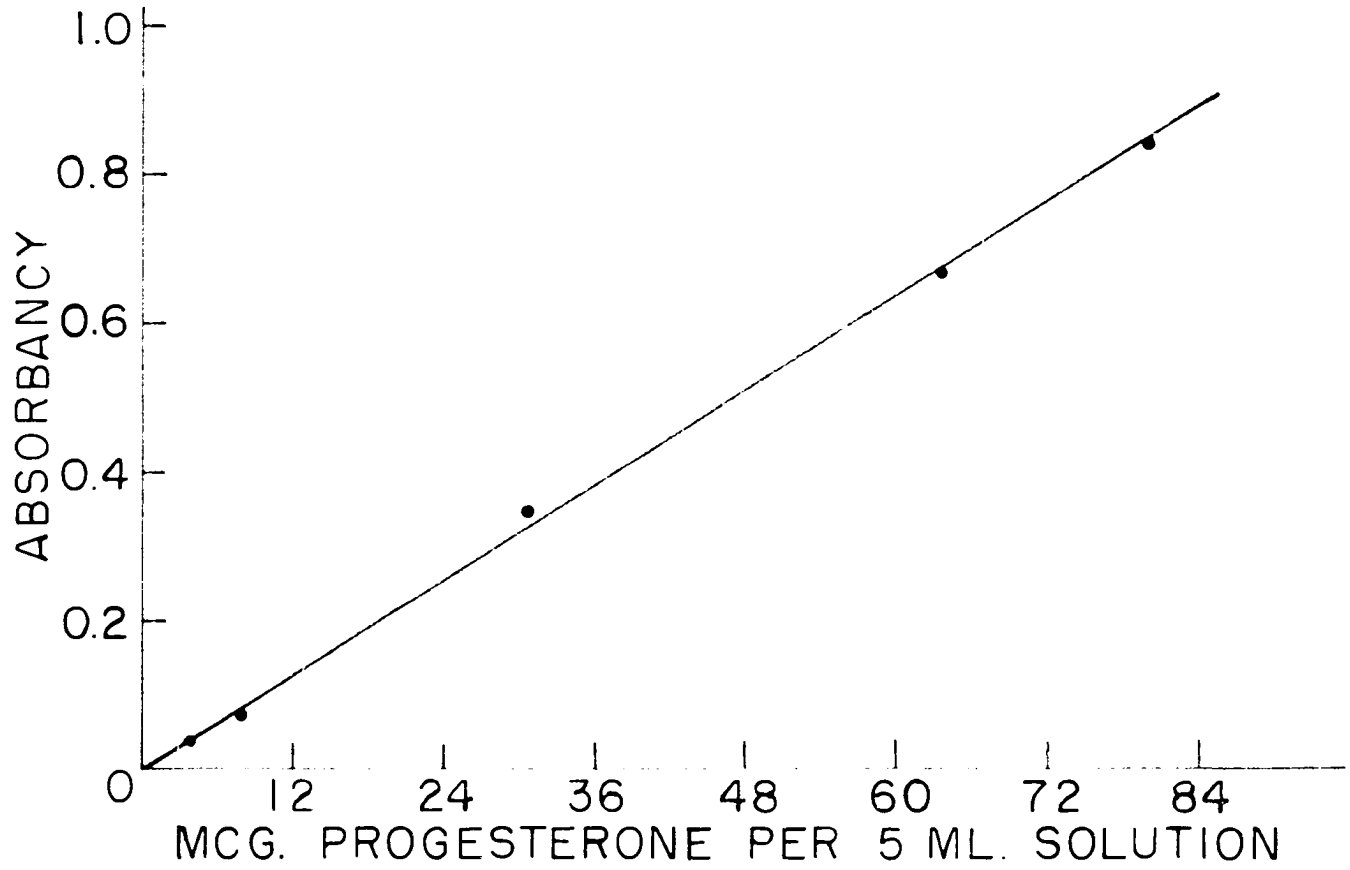


Figure 2. Absorbance at 240 mμ. for various concentrations of pure progesterone.

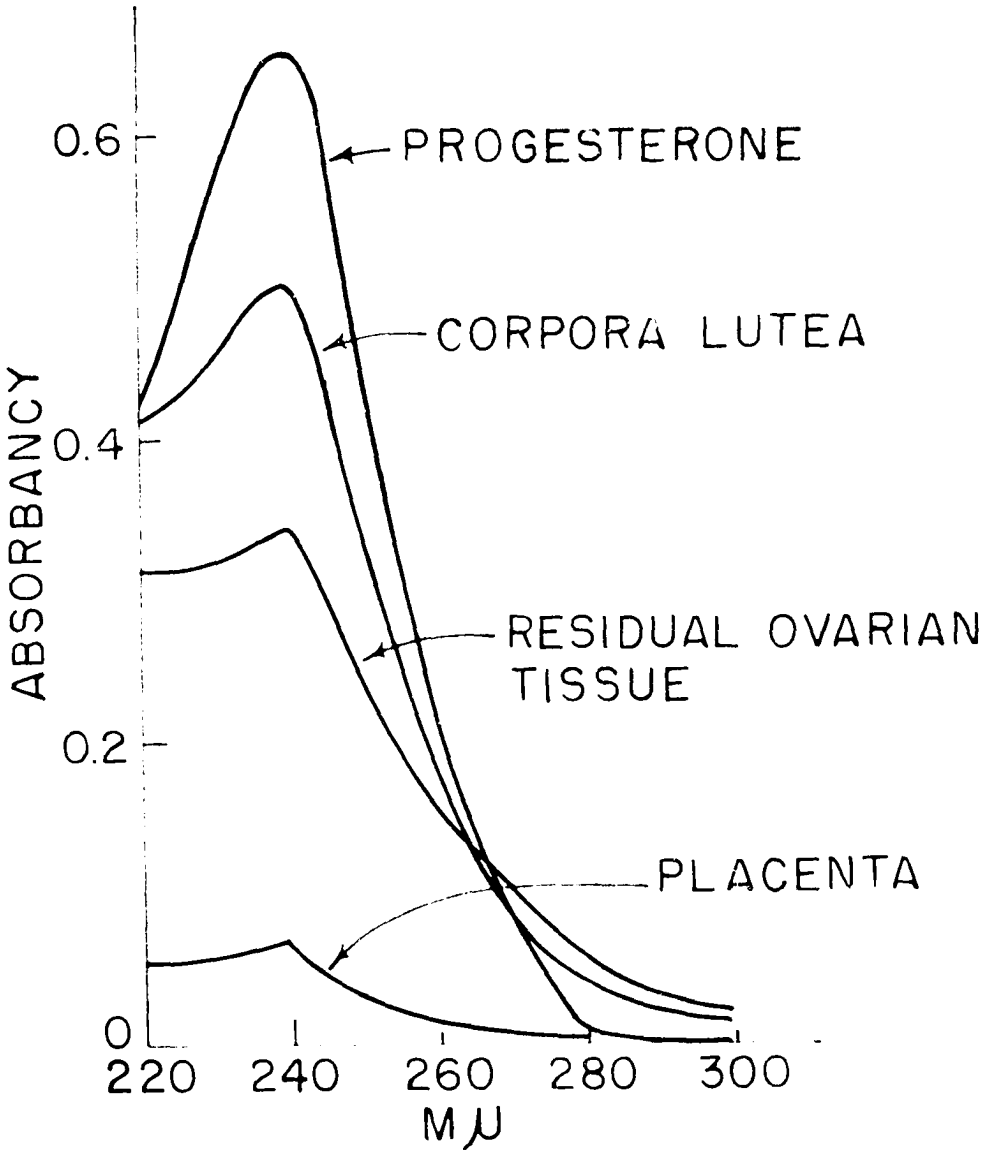
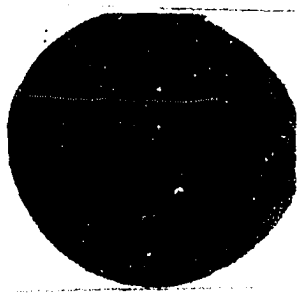
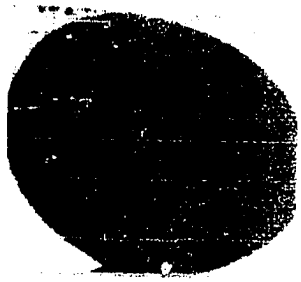


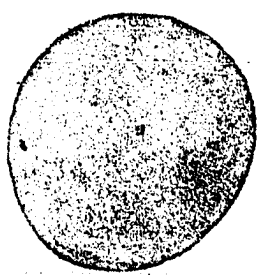
Figure 3. Absorbancy of pure progesterone and tissue extracts in ethanol; diethyl ether, 1:3 V/V.

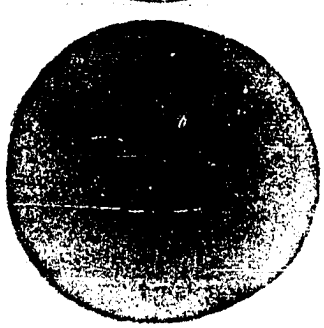
Figure 4. Results of Zimmerman reaction with tissue and fluid extracts. (1) residual ovarian tissue extracts, (2) allantoic fluid extracts, (3) placental tissue extracts, (4) corpora lutcal extracts, and (5) progesterone.

1.

2. 

3. 

4. 

5. 

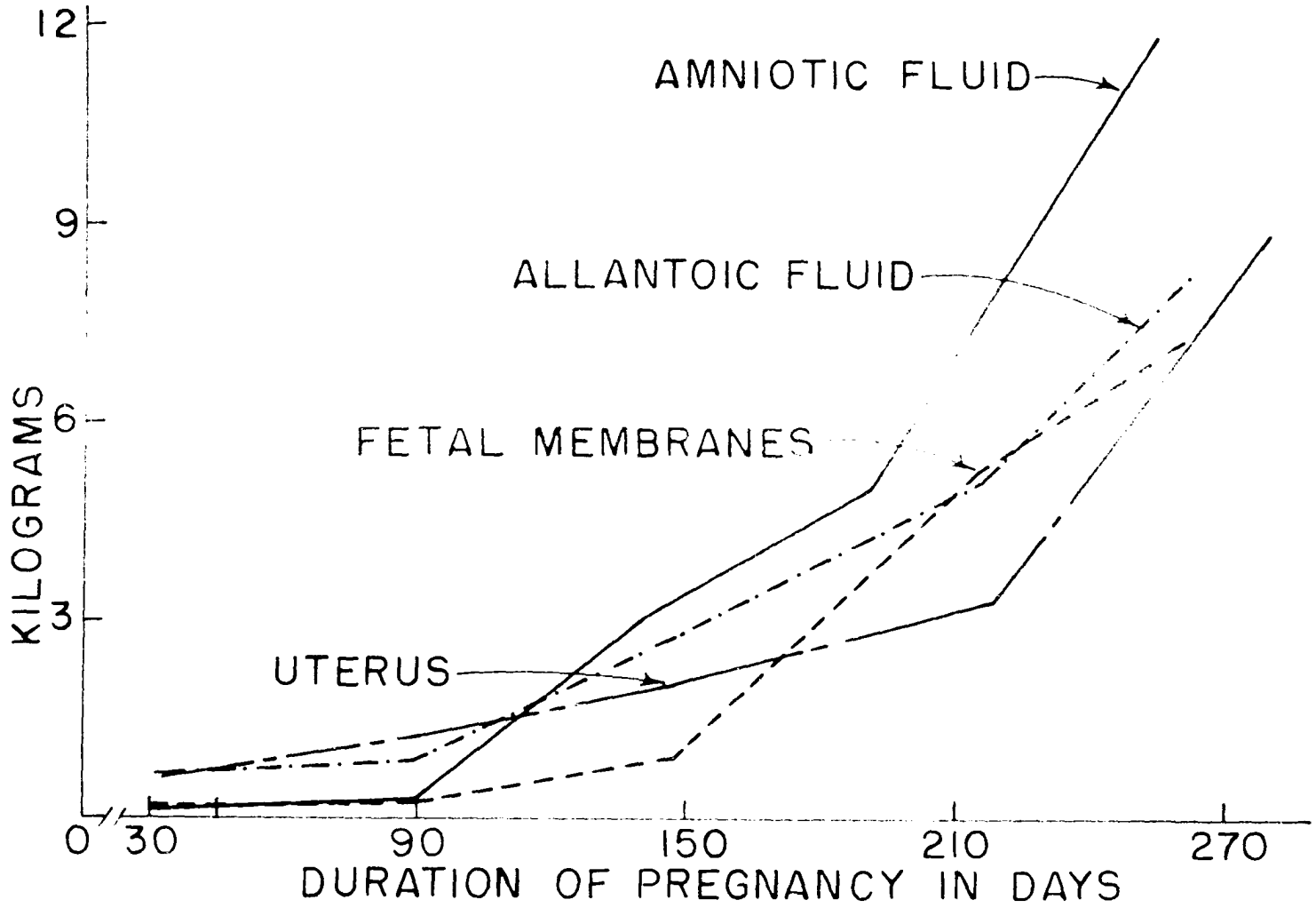


Figure 5. Weight changes in bovine uterus, placental tissue, and reproductive fluids during pregnancy.

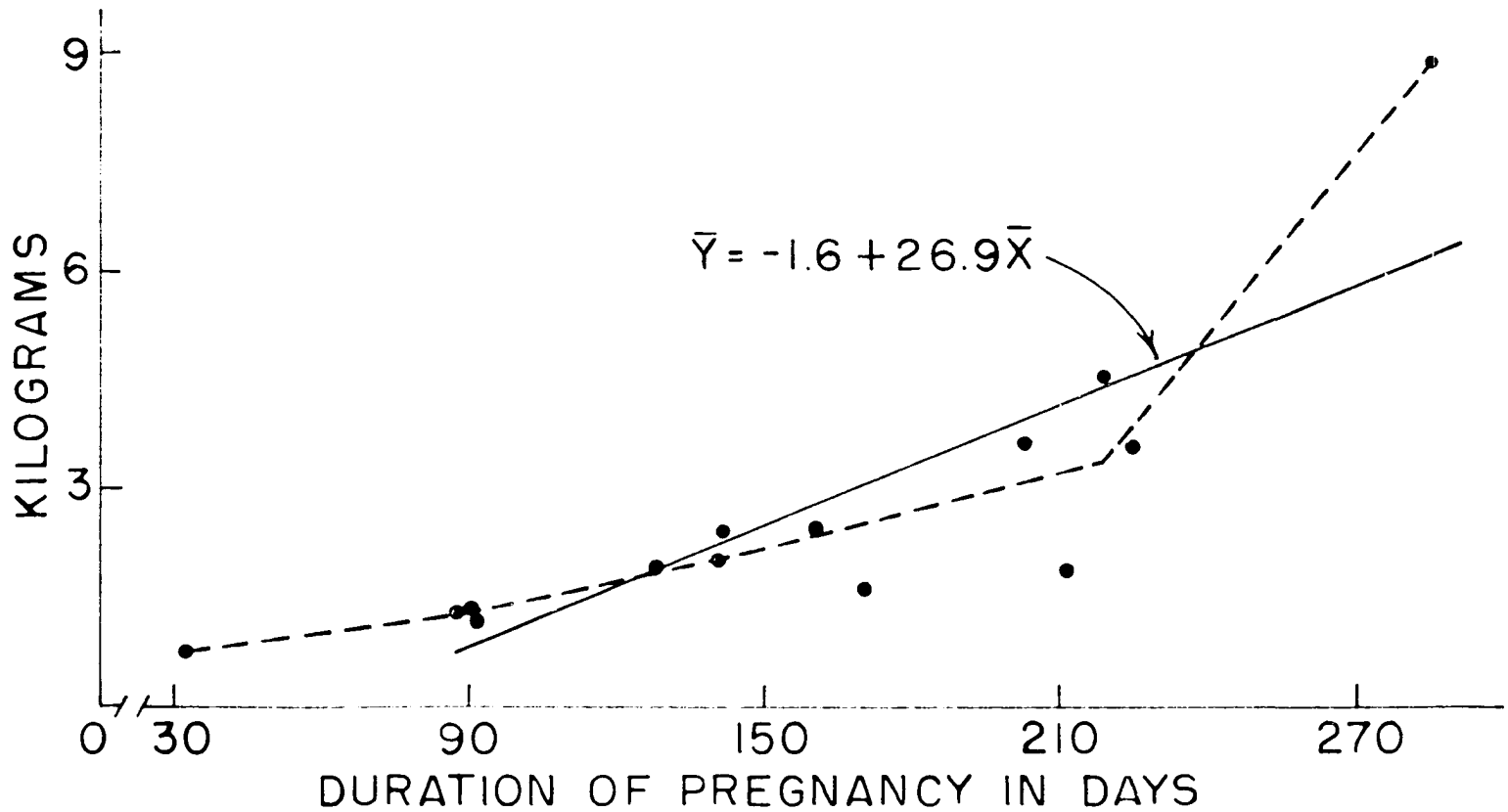


Figure 6. Weight of bovine uterus.

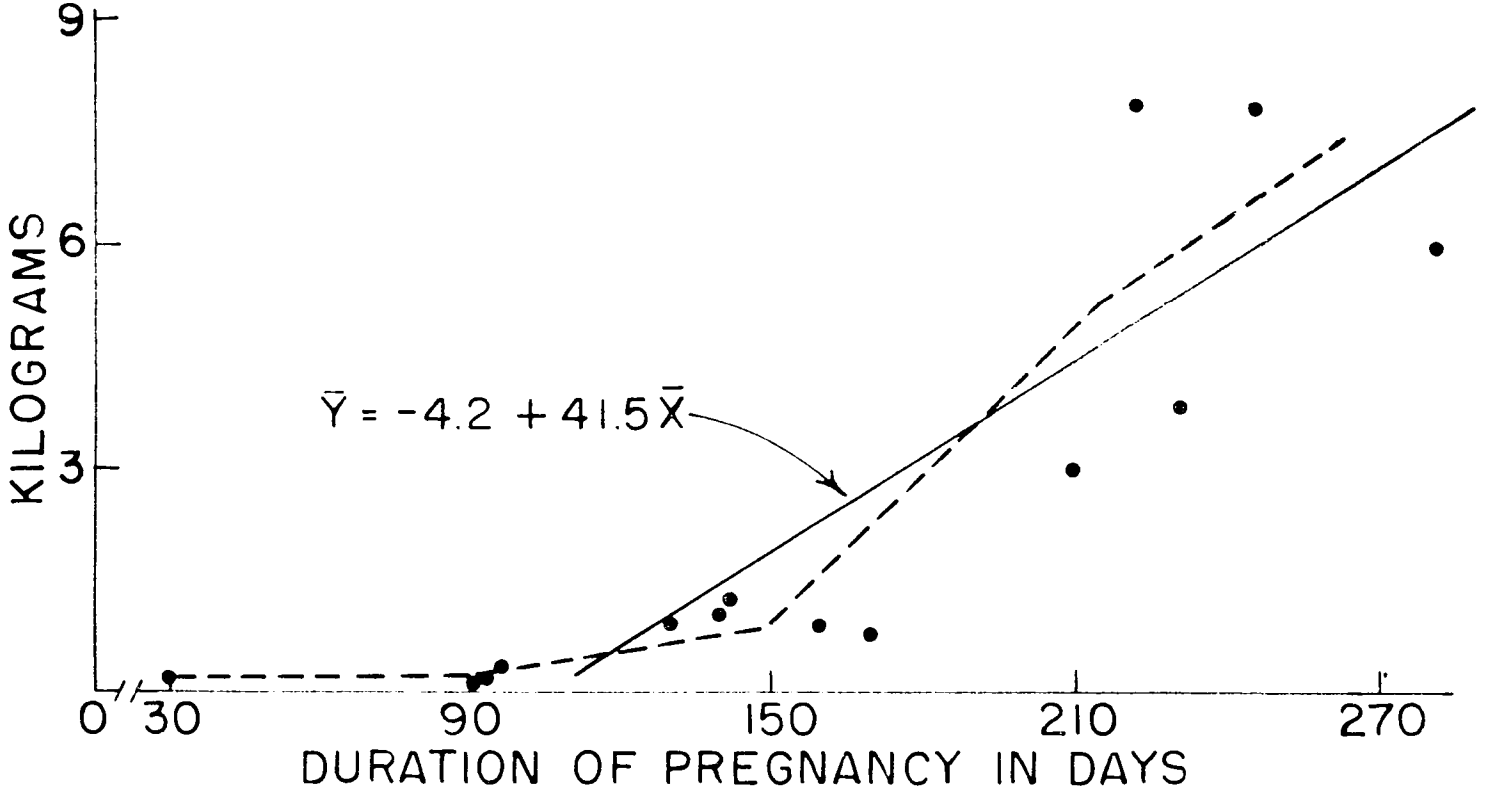


Figure 7. Weight of bovine fetal membranes.

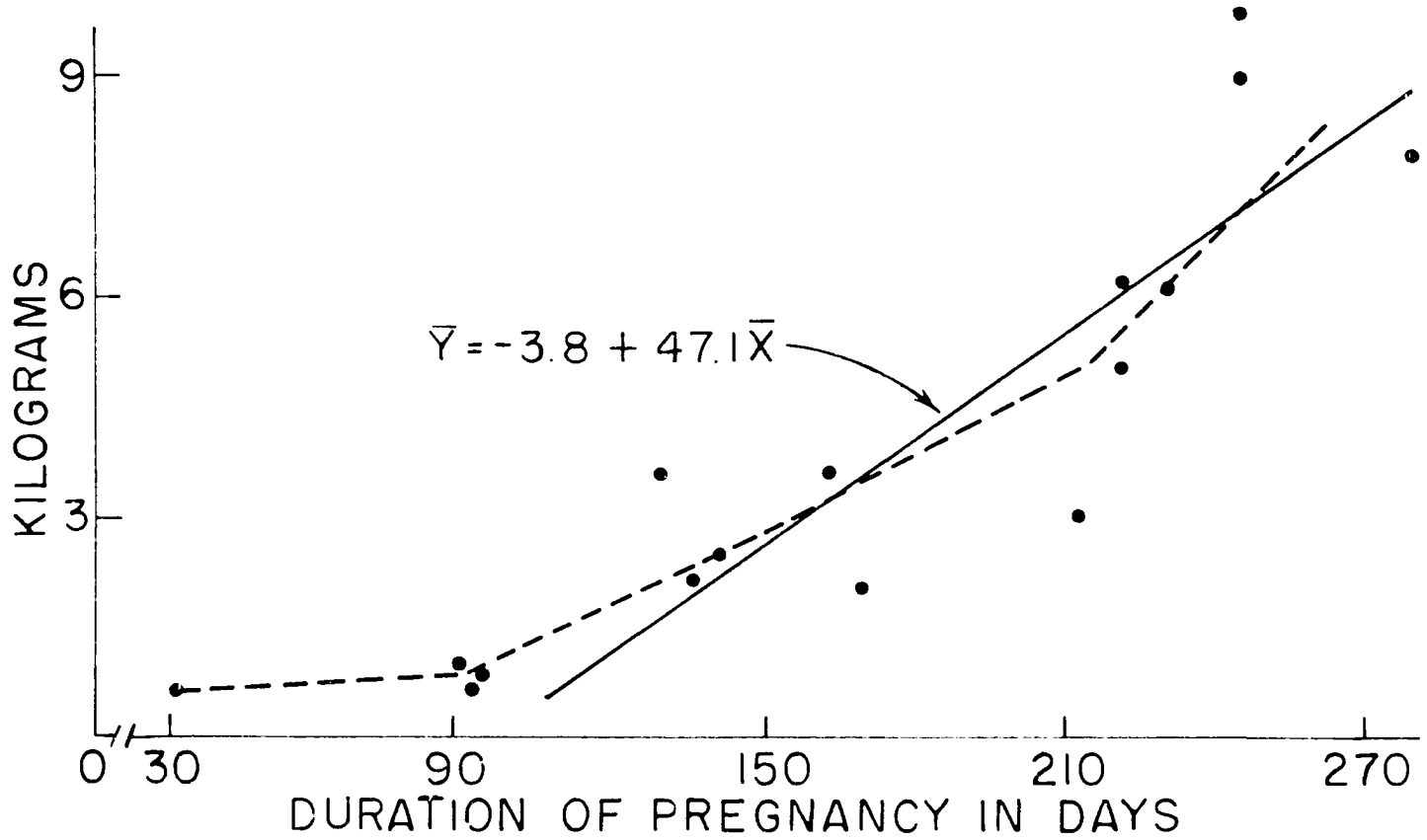


Figure 8. Weight of bovine allantoic fluid.

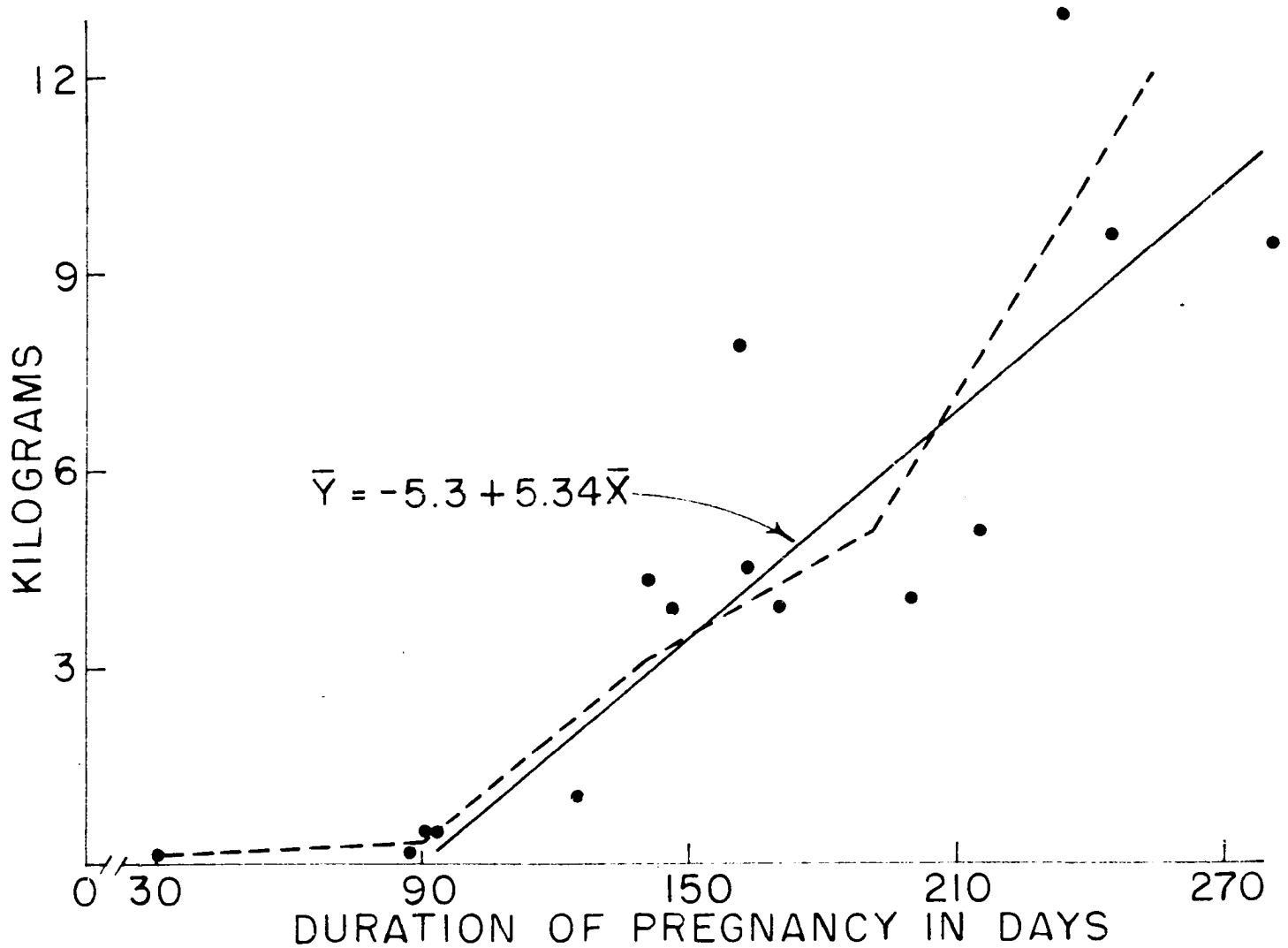


Figure 9. Weight of bovine amniotic fluid.

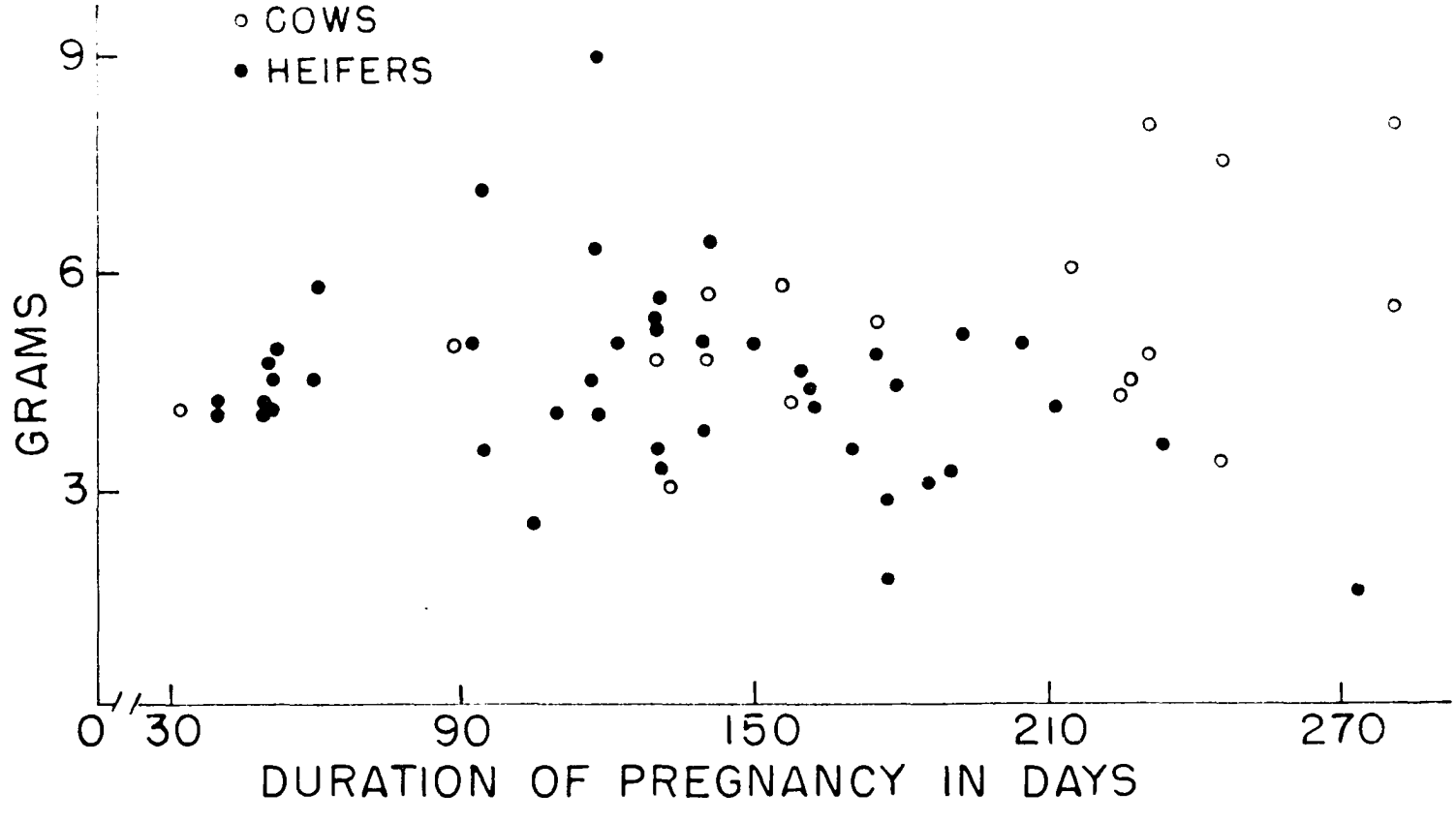


Figure 10. Weight of bovine corpora lutea during pregnancy.

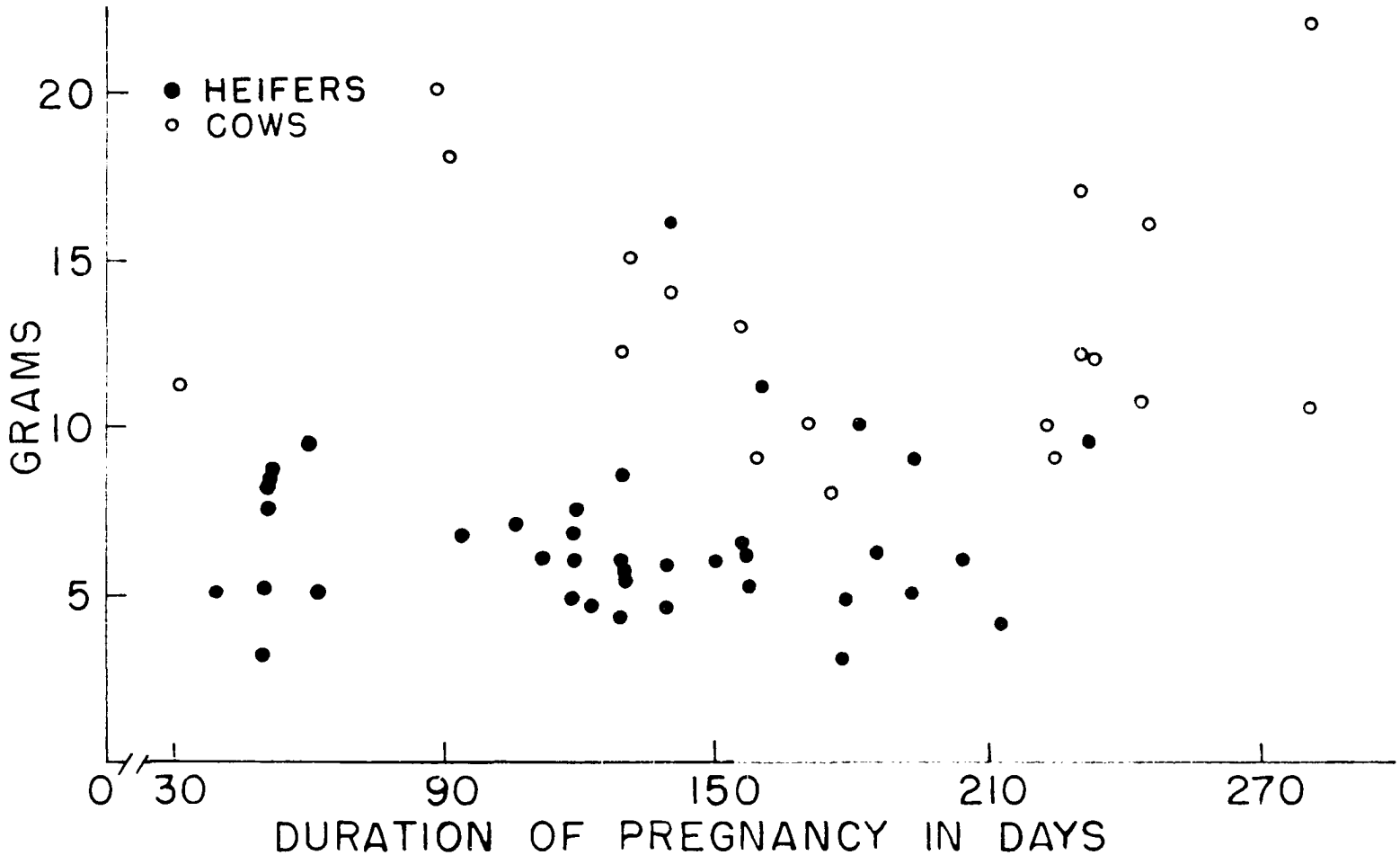


Figure 11. Ovarian weight following removal of corpus luteum of pregnancy.

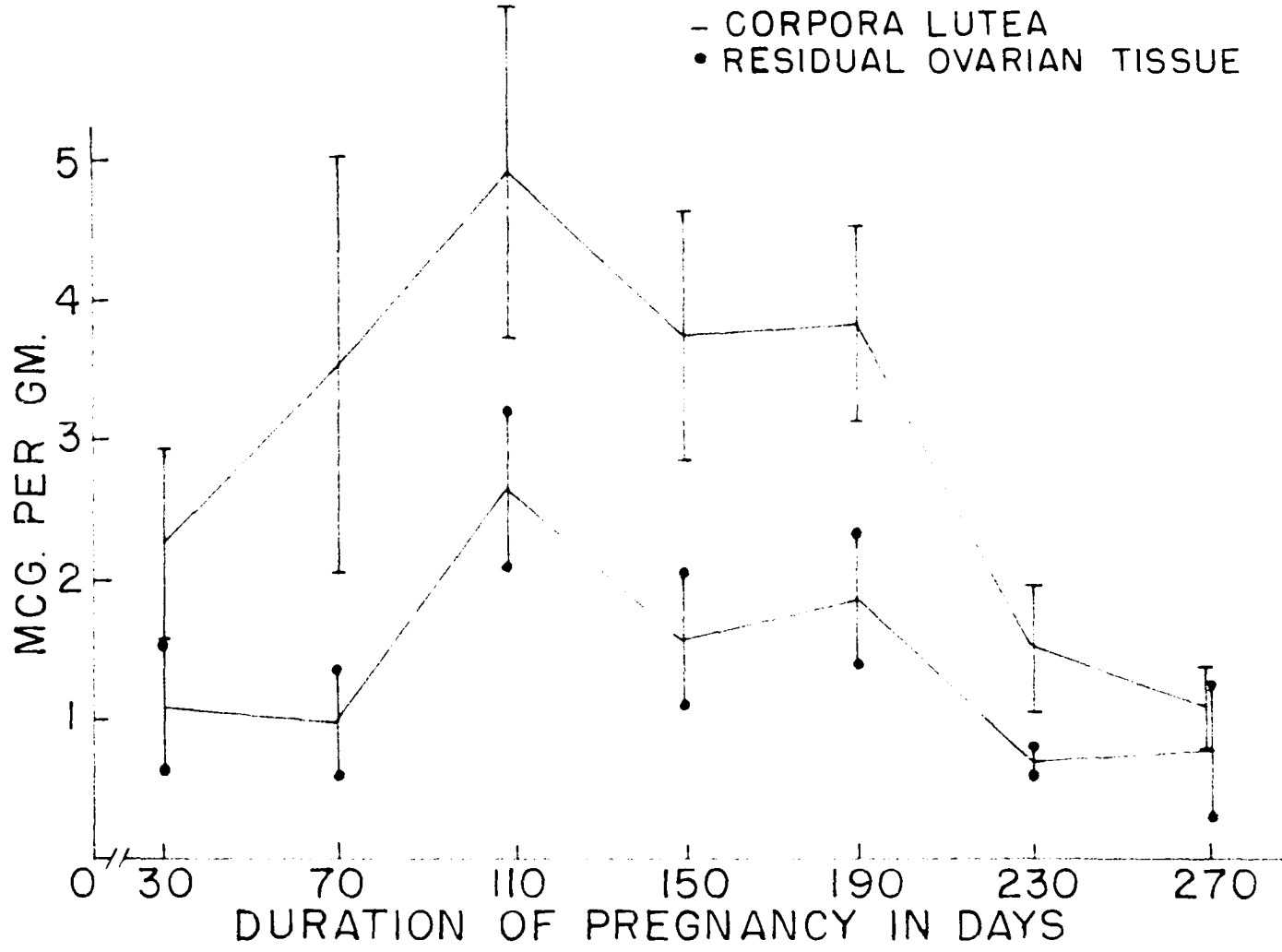


Figure 12. Progesterone concentration in corpora lutea and residual ovarian tissue during pregnancy of the cow.

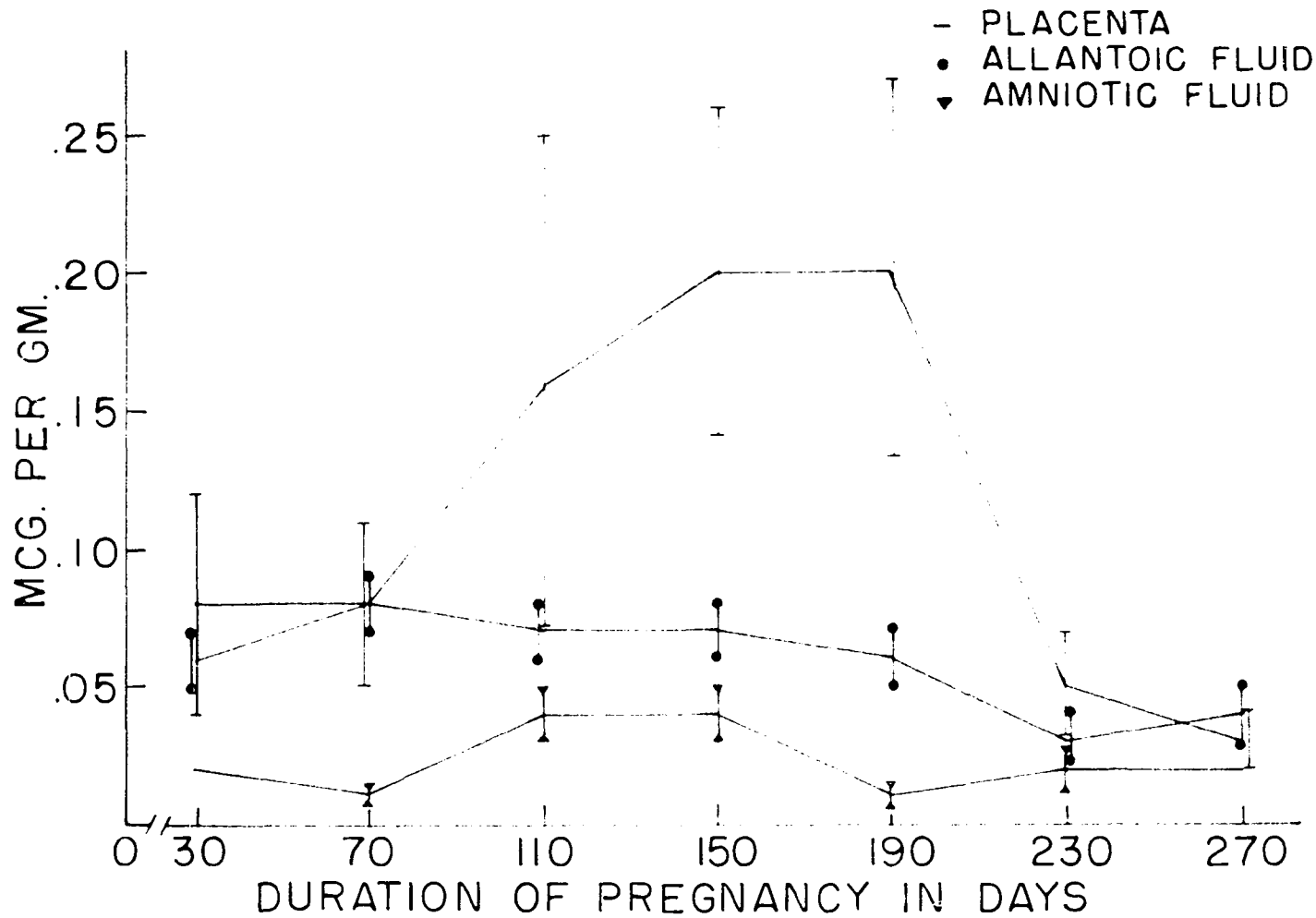


Figure 13. Progesterone concentration in bovine placental tissue and reproductive fluids during pregnancy.

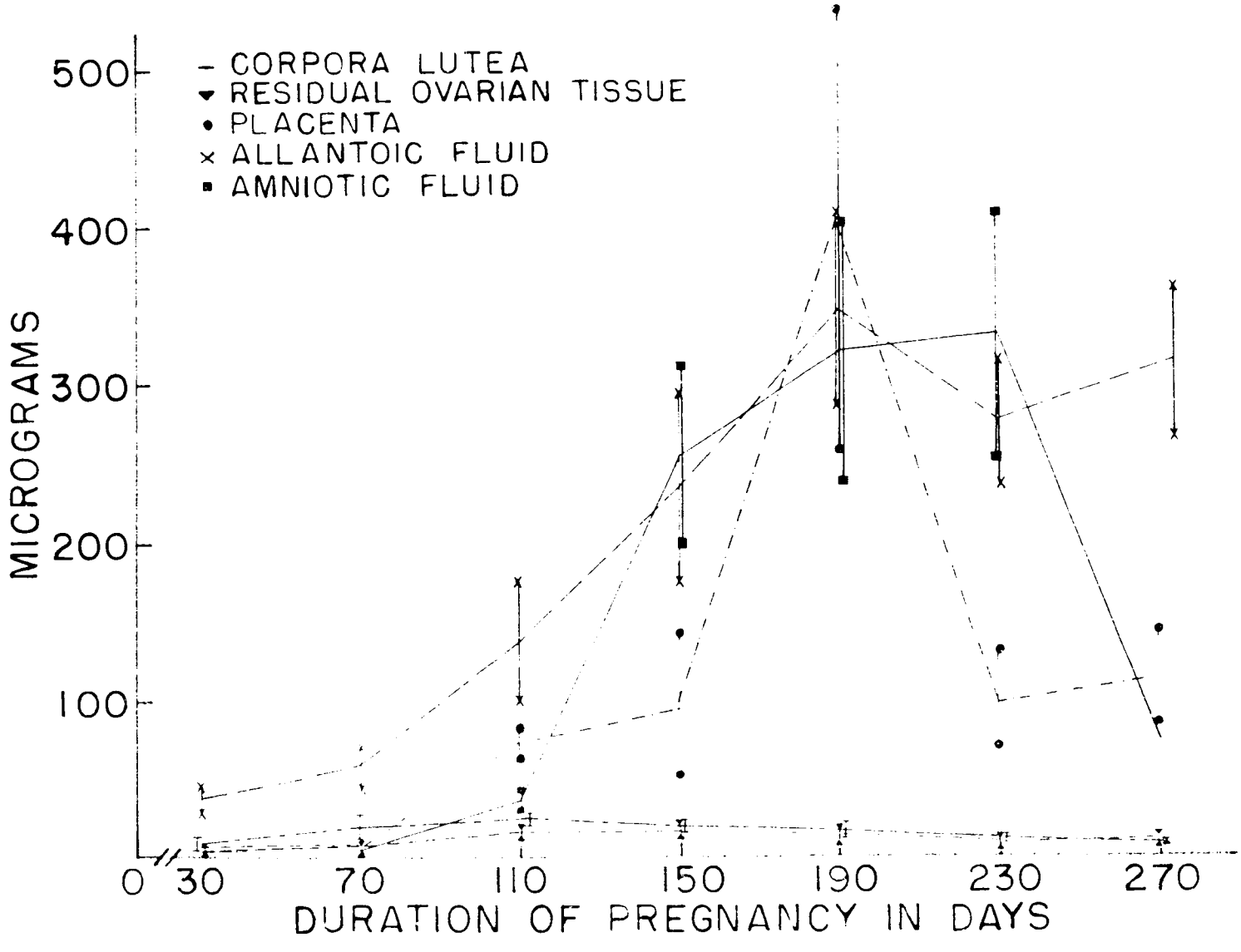


Figure 14. Progesterone content of reproductive organs and fluids during pregnancy of the cow.

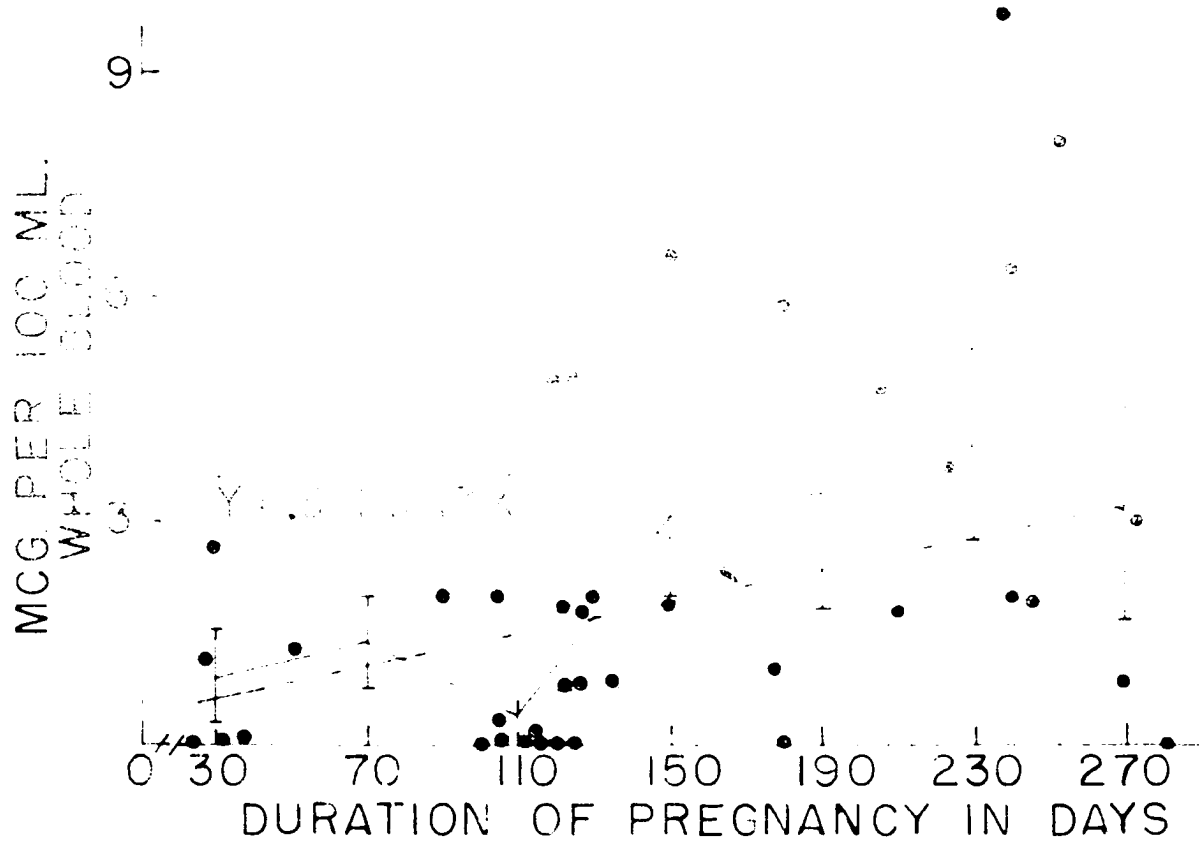


Figure 15. Blood progesterone during pregnancy of the cow.

DISCUSSION

Morphological Aspects

Linear regression lines were calculated for the data on weight changes of the uterus and its contents utilizing the equation: $\bar{Y} = a + b\bar{X}$. Straight lines were drawn to estimate the weight changes in the uterus and its contents throughout pregnancy. However, the actual observed values for a number of samples may not follow these calculated lines.

During pregnancy the average weight of the uterus increased to a maximum of 9 kg. with most of the increase in weight occurring during the last 2 months of pregnancy (Figs. 5 and 6). The regression indicated an average weight increase in the uterus of 26.9 gm. per day after the 80th day of pregnancy.

The weight of the fetal membranes increased to an average of 7.3 kg. with the greatest rate of increase between the fifth and seventh month of pregnancy. The average increase in weight of fetal membranes was 41.5 gm. per day after the 80th day of pregnancy as shown in Figs. 5 and 7. The weight of the fetal membranes increased above the uterine weight during the fifth month of pregnancy and fell below during the eighth month.

The average increase in weight of allantoic fluid was greatest during the last 2 months of pregnancy rising to an average value of 8.3 kg. near the end of pregnancy. The average daily increase in allantoic fluid after the 80th day of pregnancy was 47.1 gm. per day (Figs. 5 and 8).

The increase in amniotic fluid was greatest the last 3 months of pregnancy with the average during late pregnancy being 11.8 kg. A regression value of 53.4 indicated this amount of increase in gm. per day of preg-

nancy. The quantity of amniotic fluid was low during the first part of pregnancy but surpassed the weight of other reproductive organs and fluids during the fourth month of pregnancy as shown in Fig. 5.

The observations presented here on weight changes throughout pregnancy agree with those of Swett, Mathews, and Fohrman (1948). These workers did not measure allantoic fluid and had very few measurements of other fluids and organs near term. The values on amount of allantoic fluid agree with those of Benesch and Wright (1952).

The average weight of the individual heifer corpus luteum was 4.6 g. ± 0.2 g. and that of the cow was 5.6 g. ± 0.4 g. (Fig. 10). However, the difference between these means was not significant.

The average weight of residual ovarian tissue from heifers was 7.0 g. ± 0.4 g. (Fig. 11) and the same tissue from cows weighed 14.4 g. ± 1.3 g. The difference between these 2 means was significant (P=0.01).

The average weight of the corpus luteum and residual ovarian tissue from cows was compared and the difference found to be significant (P=0.01). The average weight of the corpus luteum and residual ovarian tissue from heifers did not differ significantly which indicated the weight of the corpus luteum in heifers to be nearer the total weight of the residual ovarian tissue than in cows.

Progesterone Concentration

Data on concentration of progesterone in reproductive organs and fluids are presented in Table 1 and Figs. 12 and 13. These data were arranged in 7 consecutive stages of 40 days each to facilitate statistical analysis. Two types of statistical treatments were used, one was carried

out on data from extracts of each organ and fluid whereas the other was carried out on a composite of data from extracts of all organs and fluids.

Analysis of the pooled data, as shown in Table 5, was used to test the variance between extracts and between stages of pregnancy. Both the variance between extracts and stages were significant (P=0.01).

The highest concentration of progesterone was found in luteal tissue as shown in Fig. 12. The residual ovarian tissue was next in concentration being higher than placenta, allantoic and amniotic fluids (Fig. 13).

The results on statistical evaluation of extracts from separate organs and fluids are shown in Table 3. Orthogonal contrasts were made to compare specific stages of pregnancy.

The average progesterone concentration in luteal tissue was found to be 2.3 mcg. per gm. during stage 1 and increased to 5.0 mcg. per gm. the third stage of pregnancy, and decreased to 0.8 mcg. per gm. during the seventh stage. The variance between stages of pregnancy was significant (P=0.10). Most of the variation was due to the difference between stages 1 and 3 (P=0.01). The difference between stages 5 and 6 was significant (P=0.10). The average hormone concentration in the corpora lutea showed a significant increase up to stage 3 and this was followed by a significant decrease after stage 5 as shown in Table 3. Investigations summarized in Table 11 indicate a higher progesterone concentration in the corpus luteum of the sow than in the cow. The level reported for corpus luteum of the pregnant ewe falls within the range reported in this investigation in the pregnant cow. Work by Gorski and Dominguez (1958) indicated a level of 4.33 mcg. progesterone per gm. of ovarian tissue taken from non-pregnant cows and heifers.

The concentration in residual ovarian tissue was 1.1 mcg. per gm. during the first stage, showing a significant increase to 2.7 mcg. per gm. during the third stage (Table 3). The decrease from 1.9 mcg. per gm. during stage 5 to 0.7 mcg. per gm. during stage 6 was significant (P=0.01). This level continued for the remainder of pregnancy. A concentration of 16-23.9 mcg. per gm. was reported for this tissue in the sow by Gaweinowski (1956).

The initial hormone concentration in placental tissue of the cow was 0.08 mcg. per gm. and increased to a maximum of 0.2 mcg. per gm. during stage 4. There was a decrease from 0.20 mcg. per gm. (stage 4) to 0.03 mcg. per gm. (stage 7). The difference observed between stage 1 and stages 5 and 6 was significant (P=0.10). The difference between stages 5 and 6 was significant (P=0.10). Previous investigations have indicated higher progesterone concentration for mare and human placenta as shown in Table 11.

The concentration of progesterone in allantoic fluid from the pregnant cow was 0.06 mcg. per gm. during stage 1 and increased to 0.8 mcg. per gm. the second stage. There was a uniform decrease in the concentration from stage 2 to stage 6 as shown in Fig. 13. This concentration continued for the remainder of pregnancy. The only significant change was a decrease from stage 5 to 6 (P=0.10).

The concentration in amniotic fluid varied from 0.01 mcg. per gm. to 0.04 mcg. per gm. These variations in progesterone concentration during pregnancy were not significant (Table 3). Zander and von Münstermann (1954) were able to isolate progesterone from human amniotic fluid with the concentration ranging from 0.01 to 0.05 mcg. per ml.

The limited data obtained on the concentration of progesterone in the extracts of the left adrenal gland of the cow were not treated statistically. The hormone concentration varied from 0.4 to 2.1 mcg. per gm. as shown in Table 1. Beall and Reichstein (1938) were able to isolate small amounts of progesterone from the bovine adrenal.

Changes in hormone concentration per 100 ml. whole blood indicated an increase from 0.9 mcg. in stage 1 to 4.0 mcg. by stage 6 as shown in Table 7. Changes throughout pregnancy were not significant (Table 9). Samples of whole blood treated with bacterial beta-glucuronidase did not show a higher average progesterone content than non-treated samples.

Blood of various species has been analyzed using chemical determination and biological assay. A summary of these investigations on the progesterone content in body fluids is presented in Table 10. Neher and Zarrow (1950) conducted a study of serum from the ewe using the biological assay technique of Hooker and Forbes. At the beginning of pregnancy they obtained a value of 2 mcg. per ml. serum in the ewe. In this study a value of 0.009 mcg. progesterone per ml. whole blood was obtained from cows in the first stage of pregnancy. The next increase in hormone concentration in serum of the ewe was about at approximately the 30th day of pregnancy when the concentration increased to 6.7 mcg. per ml. The hormone remained at this level until the 100th day of pregnancy. The comparative value in the cow during stage 6 was 0.04 mcg. per ml. The value obtained in the ewe near parturition was 10 mcg. per ml. The average value obtained in the cow during stage 7 was 0.03 mcg. per ml. Edgar (1953) using a chemical technique obtained a value of 0.1 mcg. progesterone per ml. plasma from ewes 90-105 days pregnant.

Investigations using biological assay for progesterone in rabbit serum throughout pregnancy indicated a value of 0.3 mcg. per ml. at the beginning of pregnancy as compared to 10 mcg. per ml. near parturition. An average concentration obtained by biological assay of human plasma throughout pregnancy was 2 mcg. per ml., whereas the average value obtained for the monkey was 3 mcg. per ml. as shown in Table 10.

The average concentration (mcg. per gm.) in the various organs and fluids over the entire pregnancy period of the cow were found to be as follows: corpus luteum 3.4; residual ovarian tissue 1.6; placenta 0.06; left adrenal gland 1.5; allantoic fluid 0.06; and amniotic fluid 0.03. The average concentration in whole blood over the entire pregnancy period of Holstein cows was 0.02 mcg. per ml.

Total Progesterone

Data on total progesterone content in reproductive organs and fluids were grouped in 7 stages of 40 days each as shown in Table 2. Two types of statistical treatments were applied to the data. One was calculated on results of individual extracts whereas the other was calculated using the results of all extracts together (Tables 4 and 6).

The analysis on all data, as shown in Table 6, indicated a significant variance among extracts and among stages of pregnancy ($P=0.01$).

There was little difference between progesterone content of residual ovarian tissue and that of the luteal tissue as shown in Fig. 13. The allantoic fluid contained the highest average amount of progesterone throughout pregnancy.

A summary of single statistical analyses on progesterone content in

each extract is shown in Table 4. Variance between stages of pregnancy was calculated and orthogonal comparisons were made.

The variance of progesterone content of luteal tissue between stages of pregnancy in the cow was significant (P=0.05). The total hormone content was 9.8 mcg. during stage 1, increased to 22.6 mcg. in stage 3 and then decreased to 6.8 mcg. during stage 7 (Fig. 14).

The magnitude of change in the progesterone content of residual ovarian tissue was not as great as that seen in luteal tissue. The only significant change observed was between stage 1 and stage 3 as shown in Table 6 (P=0.10). The total hormone content at stage 1 was 6.0 mcg., increasing to 16.5 mcg. during the third stage, and then decreased to 7.1 mcg. during the sixth stage.

The variation seen in the progesterone content of the bovine placenta may be attributed to changes in concentration as well as variation in organ development. The difference observed between stage 1 and stages 5 and 6 was significant (P=0.01). The comparison between stage 2 and 4 indicated a significant difference (P=0.10). The difference observed between stages 5 and 6 was also significant (P=0.01). The total progesterone content of the bovine placenta was found to be 1.3 mcg. at stage 1, increased to 402.7 mcg. at stage 5, decreased to 99.6 mcg. at stage 6 and increased to 114.9 mcg. at stage 7 (Fig. 14). Progesterone was not detected by the method used here on several samples of placental tissue. However, these zero values were included in the computed averages.

The changes in content of allantoic fluid appeared to be a steady function of the amount of allantoic fluid found. The absence of progesterone was also observed in a few samples of this fluid taken at different

stages of pregnancy. The hormone content of allantoic fluid was 36.8 mcg. during stage 1, increased to 346.6 mcg. during stage 5, decreased to 276.9 mcg. during stage 6 and again increased to 313.6 mcg. during stage 7. However, these changes were not significant as shown in Table 4.

Total hormone content in amniotic fluid was 1.6 mcg. during stage 1, increased to 330.6 mcg. at stage 6 and decreased to 72.7 mcg. by stage 7. The difference between stage 2 and stage 4 was significant ($P=0.05$).

The progesterone content of the left adrenal gland remained relatively constant throughout pregnancy of the cow with an average total value of 15 mcg. This data was not treated statistically.

The average total progesterone in mcg. in reproductive organs and fluids over the entire period of pregnancy in the cow were found to be as follows: corpus luteum, 15; residual ovarian tissue, 12; placenta, 143; adrenal, 15; allantoic fluid, 202; and amniotic fluid, 113.

Inasmuch as the reproductive organs and fluids were taken from pregnant cows of 3 apparent breeds, namely; Hereford, Angus, and Holstein; a statistical analysis was conducted to determine the variance between breeds of dams (Table 7). There was no significant difference between apparent breeds of dams which indicated more variation within the breeds than between the breeds. The major variation observed in these data presented here may be attributed to the individuality of each dam.

SUMMARY

A chemical method for the assay of progesterone was developed in this investigation to quantitatively determine the concentration and total amount of progesterone in bovine corpus luteum, residual ovarian tissue, placenta, adrenal, allantoic and amniotic fluids and blood during different stages of pregnancy. This method for the determination of progesterone utilized the methods of partition between organic solvents and paper chromatography to purify the extracts. The corrected absorbancy at 240 mμ was used to estimate the amount of progesterone present in purified extracts. Pooled extracts were further purified by the use of countercurrent distribution.

Identification of progesterone was aided by the Rf value on the chromatogram and absorption maxima: at 240 mμ in 1:3 V/V diethyl ether: ethanol; and near 300 mμ in sulphuric acid. The Zimmerman reaction and infra-red analyses were used to determine the presence of certain functional groups. A positive Zimmerman reaction indicated the presence of an alpha, beta-unsaturation which would correspond to the delta-4, 3-keto group of progesterone. Infra-red analysis was used to confirm the presence of a delta-4, 3-keto group and to indicate the presence of a carbon-20 carbonyl.

Data were obtained on weight changes in the bovine uterus and its contents during pregnancy of the cow. Samples of bovine reproductive organs and fluids were collected and assayed by the chemical method developed during this investigation. The data were grouped into 7 stages of 40 days each. The average progesterone concentration (mcg. per gm.) in

the organs and fluids over the entire pregnancy period were found to be 3.4 for corpus luteum, 1.6 for residual ovarian tissue, 0.06 for placenta, 1.5 for left adrenal gland, 0.06 for allantoic fluid, and 0.03 for amniotic fluid. Significant variations in concentration throughout pregnancy were found in all tissues except the left adrenal gland and amniotic fluid. The hormone concentration was highest in luteal tissue with concentration in residual ovarian tissue being next.

Progesterone concentration in peripheral blood collected from Holstein cows during different stages of pregnancy had an average value of 0.02 mcg. per ml. whole blood. There was an increase in progesterone concentration as pregnancy progressed although this increase was not statistically significant.

The average progesterone content (mcg.) in the reproductive organs and fluids over the entire pregnancy period of the cow were found to be 15 in corpus luteum, 12 in residual ovarian tissue, 143 in placenta, 15 in adrenal, 202 in allantoic fluid, and 113 in amniotic fluid. Significant variations in progesterone content were observed in all organs and fluids except the left adrenal gland and allantoic fluid.

The difference in progesterone content of reproductive organs and fluids of the different breeds of apparent dams (Hereford, Angus, and Holstein) used was not significant.

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