PROGESTERONE SYNTHESIS BY THE PERFUSED BOVINE OVARY OF EARLY AND LATE PREGNANCY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Progesterone is apparently required for the maintenance of pregnancy in many species of animals, including the cow. The corpus luteum is the prime source of progesterone during the estrous cycle and during early pregnancy and ovariectomy during early pregnancy causes immediate abortion. However, in contrast to many species, yet similar to some species, the ovaries of the cow may be removed during late pregnancy without causing abortion. It is not known if (a) the corpus luteum is producing less progesterone and is no longer essential to late pregnancy, (b) other endocrine organs, such as the adrenal glands and placenta, are producing enough progesterone to maintain pregnancy in the absence of the corpus luteum, (c) the requirement for progesterone to maintain pregnancy has decreased, or (d) other progestins from other endocrine organs are sufficient to maintain pregnancy.

The functional ability of ovaries during late pregnancy has not been determined and such information would assist in clarifying the role of the corpus luteum during late pregnancy. The corpus luteum of late pregnancy may have lost its functional ability to synthesize progesterone or may be fully capable of progesterone synthesis but may be nonfunctional due to an absence or shortage of the proper gonadotropins.

The objective of this study was to determine the functional ability of corpora lutea of late pregnancy in comparison to corpora lutea of

early pregnancy with respect to progesterone synthesis with and without added gonadotropin.

CHAPTER II

LITERATURE REVIEW

The corpus luteum is a transient structure whose secretions play an important role in the control of the female reproductive processes. Corpora lutea are normally formed from all ruptured follicles, but they soon degenerate if fertilization of the ovum and implantation do not occur. If fertilization and implantation do occur, the corpus luteum becomes transformed into a corpus luteum of pregnancy and does not regress until parturition. The primary function of the corpus luteum is to produce progesterone. The secretion of progesterone by the corpus luteum is essential to the orderly regulation of the sex cycle. Although the length of time the corpus luteum is essential for pregnancy varies among species, the requirement for progesterone or compounds with similar activity remains the same (27).

Progesterone is predominantly a hormone of pregnancy and is required for the preparation of the endometrium for implantation of the fertilized ovum. Further, it is necessary for the maintenance of the embryo during the time it is lying free in the uterus. It also causes growth of the uterine glands, modulates the contractions of uterine muscle, and is necessary for maintenance of the endometrium. Because of these effects, it is essential for the continuation of pregnancy. The fetus apparently dies within hours when the level of progesterone becomes inadequate (27). Progesterone also prevents ripening of more follicles and ovulation, and

prevents the occurrence of estrus. It stimulates the growth of the alveoli of the mammary glands and depresses luteinizing hormone (LH) production by the anterior pituitary (50).

Development of the Corpus Luteum

The formation of the corpus luteum follows the discharge of the ovum from the follicle and its development proceeds to a certain point regardless of whether or not pregnancy occurs. It is generally accepted that LH provides the stimulus for ovulation and formation of the corpus luteum in the ruptured follicle (65).

Immediately after ovulation, the ruptured follicular cavity shrinks and the granulosa and theca interna layers collapse into folds which nearly fill the cavity. Normally, in the bovine, there is no blood clot present in the cavity after rupture of the follicle, but slight hemorrhage may occur at the point of rupture (50).

The primary cells of the corpus luteum are termed lutein cells. The lutein cells develop solely from the granulosal cells according to Grady and Smith (16), but Salisbury (50) indicates that the lutein cells are formed from both the granulosa and the theca interna. Nalbandov (42) agrees that the theca interna cells may participate in formation of the gland. Blood vessels and connective tissue from the surrounding ovarian tissue also grow into the cavity. When fully developed the corpus luteum is compact and is composed largely of lutein cells. The lutein cells contain large, well defined, granular nuclei and have large amounts of protoplasmic material which have an affinity for eosine stain (9). The individual lutein cells are surrounded by a thin layer of connective tissue and capillaries are abundant throughout the tissue in close

association with each cell (49). This well developed network of blood vessels is probably required for the proper function of the corpus luteum and is highly important in the rapid regression of the corpus luteum at later stages (9).

Foley and Greenstein (11) have performed an extensive histological examination of the bovine corpus luteum of early pregnancy (16-33 days of pregnancy). They described the bovine corpus luteum of early pregnancy as an eccentric oval body varying in color from a deep yellow through various shades of orange to a cocoa brown and ranging in weight from 3.2 to 9.3 grams. They found that there was an outer connective tissue capsule which was well vascularized and that there were trabeculae with abundant fibroblasts growing inward towards the center, dividing the tissue into well defined segments.

Their (11) study described the types of lutein cells present during various stages of early pregnancy which were classified into five groups. These cell types were designated as Types I, II, III, IV, and V and were considered to represent different stages of growth, regression, or secretory activity of a single basic lutein cell. Type I cells represented "immature" lutein cells and Type II cells represented mature cells which had reached their maximum size and development (20 microns, dia.). Type III cells were believed to be in the initial stage of regression which continued through Type IV cells and terminated as Type V cells. During this stage of pregnancy, there were about 83% Type I and II cells, 10% Type III, and 7% Type IV and V cells.

The Luteotropic Process

Rothchild (49) defined the luteotropic process as "one which promotes

the growth of the corpus luteum and a rate of progesterone secretion at least sufficient to prevent ovulation and/or to permit implantation to occur". A number of substances have been found to have luteotropic activity in the bovine. Hansel (22) tested the luteotropic activity of bovine growth hormone, equine LH, ovine prolactin, human chorionic gonadotropin (HCG), and a crude aqueous extract of bovine anterior pituitary glands by their ability to overcome inhibition of progesterone synthesis in bovine corpora lutea by oxytocin. The crude extracts of bovine anterior pituitary tissue, HCG, and purified bovine LH preparations were all capable of overcoming the inhibitory effects of oxytocin on corpus luteum weight and progesterone content of the corpus luteum. None of the other anterior pituitary hormones tested gave evidence of a luteotropic effect in this system (22). Hansel (22) concluded that "LH is the major luteotrophic principle in cattle". This conclusion is well documented.

The effects of ovine LH, bovine LH, equine LH, HCG, porcine follicle stimulating hormone (FSH), ovine FSH, ovine prolactin, ovine growth hormone, and adrenocorticotropic hormone (ACTH) upon progesterone synthesis in bovine corpus luteum slices were reviewed by Marsh and Savard (32). The LH from the various species, HCG, and FSH were found to stimulate progesterone synthesis while none of the other tropic hormones had any effect. The FSH effects were attributed to LH contamination.

Bartosik et al. (5) perfused the bovine luteal ovary with citrated blood and reported that prolactin and LH both augmented the progesterone secretion rate. Prolactin did not augment progesterone secretion from the non-luteal ovary, whereas LH did. These results contradict those reported by Marsh and Savard (32) and Hansel and Seifart (24). It was pointed out by Bartosik et al. (5) that the luteotropic effects of

prolactin in the bovine have been demonstrated only in *in vitro* perfusions, or *in vivo* infusions, where cellular integrity was maintained. They (5) suggested that the stimulatory effect of prolactin depends either on cellular integrity or on an interaction between the luteal cells and surrounding ovarian tissue which would account for negative reports from experiments in which only corpus luteum slices or homogenates were used.

Considering this evidence, one can conclude that both LH and prolactin have luteotropic capabilities in the bovine, but the role of each and/or their synergistic activity is not clearly understood. As Rothchild (49) pointed out, other hormones may also need to be included in the luteotropic process.

Corpus Luteum Maintenance

As indicated earlier, the bovine corpus luteum forms from a ruptured follicle and apparently develops to a certain functional state regardless of whether or not pregnancy ensues. If fertilization and implantation do not occur, the corpus luteum regresses, but is maintained in the event of implantation.

According to Mares *et al.* (30), the functional activity of the corpus luteum of the estrous cycle of the cow appears to increase to about Day 15 and then declines as is shown by a decrease in corpus luteum weight, progesterone concentration and content, RNA-DNA ratio, and per cent of Type I and II luteal cells. The abrupt drop in progesterone concentration reported between Day 15 and Day 17 is accompanied by a lesser drop in corpus luteum weight. Mares *et al.* (30) concluded that the mechanism for maintenance of the corpus luteum would need to be

initiated by at least Day 15 of pregnancy. Ovarian vein plasma levels (14) and peripheral vein plasma levels (57) of progestins lend support to this conclusion.

In some species, there is little doubt that at least one stimulus is of neural origin at estrus. In rats and mice, stimulation of the cervix results in pseudopregnancy which is characterized by prolonged maintenance of the corpus luteum. Deep anesthesia or cervical innervation destroys this effect (42). No comparable effect is known to occur in the bovine. Uterine dilations or infusions of raw semen or preputial fluids into the bovine uterus at estrus did not lengthen the estrous cycle but instead caused shortening of the cycle (23).

When the entire uterus is removed from the heifer during the luteal phase of the estrous cycle, estrous behavior does not occur and the corpus luteum persists for at least 270 days which approaches the life span of the corpus luteum of pregnancy (2). The maximum time of luteal persistence in hysterectomized heifers is not known.

However, Malven and Hansel (29) found that hysterectomy did not impair the mechanisms of follicular growth, ovulation, and corpus luteum formation and concluded that removing the source of uterine stimuli does not markedly affect the ability of the hypophysis to secrete the hormones required for follicular growth, ovulation, and corpus luteum formation.

Anderson et al. (2) reported that a portion of the uterus must remain in the animal for regression of the corpus luteum to occur. The presence of an anterior half or one fourth of both horns was sufficient for luteal regression and continuation of luteal cycles.

If one accepts the argument of Hansel (22) that LH is the major luteotropic hormone in the bovine, the question arises as to whether

luteal regression is due to a decrease in the plasma level of LH or the intervention of an active luteolytic mechanism capable of causing regression in the presence of a continued high level of LH (24). A reduction in LH secretion can cause regression of bovine corpora lutea, but data available suggest that pituitary LH content increases during the time of luteal regression in the cow (24). Corpus luteum slices obtained at 19 days post estrum or later synthesized minimal amounts of progesterone and failed to respond to LH when compared to corpora lutea taken prior to Day 19 (3). This observation lends support to the idea that regression occurs as a result of an active luteolytic process and in the presence of relatively high levels of LH. Hafs and Armstrong (19) found that capacity for maximal luteal function may be retained from mid-cycle to as long as Day 18 or 20 of a 21-day cycle by addition of NADPH but not by addition of NADPH-generating systems. They suggested that NADP or some substrates (e.g. glucose-6-phosphate) capable of oxidation by NADP-dependent enzymes may be rate limiting to steroidogenesis in aging corpora lutea. Histological observations revealed an altered appearance of blood vessels in aging corpora lutea which provided morphological evidence of circulatory changes which could result in deficiences of oxidizable substrates, or indeed, of oxygen, which in turn could account for decreased steroidogenesis in these corpora lutea (3,19,56).

Ginther (12) has reviewed the local utero-ovarian relationships in a number of species. For cattle he cited results from hysterectomy, intrauterine devices, and oxytocin administration experiments and concluded that the data provided good evidence that local utero-ovarian mechanisms were at least partly involved in the changes in estrous cycle length associated with these studies.

Williams et al. (62) found that acetone dried powder preparations of late luteal and early estrual bovine uteri induced regression of corpora lutea, development of follicles, and a depression of acetate incorporation into progesterone when injected intraperitoneally into pseudopregnant rabbits. The same dosage of a similarly prepared abdominal muscle powder was without effect. It was proposed that these uterine preparations contained a protein luteolytic hormone which affected ovarian function without direct involvement of LH. The physiologic importance of these results will not be fully recognized until this bovine uterine powder has been shown to have an effect upon the bovine corpus luteum.

The foregoing experiments indicate that there is a luteolytic agent in the uterus of the cow which shortens the life of the corpus luteum in a nonpregnant animal. In pregnancy, the uterus in some way is apparently caused to lose its luteolytic ability, thereby allowing the corpus luteum to remain functional.

Ovarian Steroidogenesis

Four organs are known to secrete steroid hormones into the circulation. They are the adrenal cortex, ovary, testis, and the placenta. Synthesis and/or release of steroid hormones by these organs is controlled by "tropic" hormones, many of which originate in the anterior pituitary. Secretion of the adrenocorticoids, with the exception of aldosterone, by the adrenal cortex is controlled by ACTH. Steroidogenesis by the gonads is controlled by FSH, LH, and prolactin and, in some species, by gonadotropins produced by the fetoplacental unit of pregnancy. It is not known whether placental secretion of steroid hormones is controlled by pituitary or placental gonadotropins or both.

In these steroidogenic tissues, the biosynthesis of steroid hormones is thought to follow the same general pathways. Although cholesterol is generally considered an obligatory intermediate in the steroid biosynthetic pathway, it has become apparent that not all of the cholesterol in the adrenal gland, corpus luteum, or the testis (all cholesterol-rich tissues) is involved in steroid biosynthesis. Two or more separate pools of cholesterol appear to exist, and only a part of the total cholesterol content of these three steroidogenic tissues appears to be involved as an intermediate in steroid synthesis (52). Beyond cholesterol, double isotope techniques have demonstrated simultaneous utilization of Δ^4-and Δ^5-pregnene pathways (28). The availability of more than one pool of cholesterol and more than one pathway for steroid synthesis may be part of a mechanism for regulating hormone production. The pathway utilized for steroidogenesis would be a characterisitc of the endocrine organ and there is apparently some variation between species with respect to the same organ. This can be shown by comparing the secretion of steroids in the corpora lutea of women and cows. There appears to be a deficiency of the aromatizing enzyme complex and of 17-hydroxylase in the bovine corpus luteum and estrogens or androgens are not produced in the corpus luteum of this species. The human corpus luteum, on the other hand, synthesizes large quantities of both androgens and estrogens (52).

There is increasing evidence for a "unifying theme" in the control of steroidogenic tissue. In 1960, Haynes et al. (25) put foth a hypothesis for the mechanism of action of ACTH on adrenal steroidogenesis. In general, the hypothesis is that ACTH initially acts upon adenyl cyclase to form increased amounts of the cyclic nucleotide, adenosine 3',5'-monophosphate (3',5'-AMP), which in turn activates the phosphorylase

activity of the adrenal cortex. The increased phosphorylase activity breaks down adrenal glycogen to produce glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P), and the G-6-P, when metabolized via the pentose phosphate pathway, produces increased amounts of the nucleotide, reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH stimulates corticoid production through its cofactor role at many points in the steroidogenic pathway between acetate and cholesterol and from cholesterol to the final corticosteroids (52).

Observations on steroidogenesis in the corpus luteum of the ovary have indicated that its control by LH may be very similar to the hypothesis presented above for ACTH action in the adrenals. Marsh and Savard (31) reported that addition of LH to incubating slices of bovine corpus luteum significantly stimulated phosphorylase activity. ACTH, epinephrine, and glucagon did not stimulate phosphorylase activity, indicating a rather specific response to LH. In this study there was a high correlation between LH-phosphorylase stimulation and progesterone synthesis. However, 3',5'-AMP had no effect on phosphorylase activity but stimulated progesterone synthesis.

In a later study, the same workers (33) again reported a stimulation of steroidogenesis upon addition of 3',5'-AMP. Other structurally related nucleotides were ineffective. Use of labeled precursors revealed that both LH and 3',5'-AMP yielded products having the same specific activity. LH had no additive effect on corpora lutea maximally stimulated by 3',5'-AMP. Likewise, 3',5'-AMP had no additive effect on corpora lutea maximally stimulated by LH. These two facts added strength to the theory that the effect of LH was mediated via 3',5'-AMP.

It has since been shown (32) that addition of LH to incubating

slices of bovine corpora lutea brought about a striking increase in the endogenous concentration of 3',5'-AMP as well as an increase in progesterone synthesis. ACTH, epinephrine, glucagon, prolactin, and peroxide inactivated LH were completely ineffective. The level of 3',5'-AMP was relatively high after only 7.5 minutes while an effect on progesterone synthesis was not evident until between 15 and 30 minutes. This time relationship is consistent with the theory that 3',5'-AMP is the mediator of LH activity.

When NADP and G-6-P were added to incubating slices of corpora lutea, there was a marked increase in the amount of progesterone formed (32). Also when acetate-1-^{14}C was used, LH caused an increase in both the amount of progesterone formed and the incorporation of radioactivity into progesterone. Exogenous NADPH on the other hand did not cause an increase in the amount of progesterone formed. It was concluded that the progesterone formed in the presence of NADPH could not have come from the same pool of labeled precursors that gave rise to the progesterone synthesized in the presence of LH. When radioactive cholesterol was used as a precursor for progesterone, a difference in the effect of exogenous NADPH and LH was again observed. NADPH increased the conversion of cholesterol-7-^{3}H into progesterone to a far greater extent than did LH while both brought about equal increases in the amount of progesterone formed. From this it was concluded that the effects of LH and NADPH are separate and independent.

It has been found that puromycin and actinomycin D inhibit steroidogenic stimulation by LH in the corpus luteum and testis (28) in a manner similar to that reported for the adrenal. It was therefore concluded that protein synthesis plays some role in the action of the various

tropic hormones on their respective glands. There are other similarities between the actions of ACTH and LH on their respective target organs. Both ACTH and LH appear to stimulate steroidogenesis at a point after cholesterol (21,58) and both cause a fall in the ascorbic acid content of their target organs (44,53). Also, both cause an increase in the concentration of 3',5'-AMP in their target organs (25,32) and in turn, 3',5'-AMP stimulates steroidogenesis in these tissues (25,33). Furthermore, agents known to cause mitochondrial swelling and alterations of mitochondrial permeability have been shown to stimulate pregnenolone synthesis in mitochondrial fractions from adrenal cortex and corpus luteum of the bovine (26). It was suggested that ACTH and LH may regulate steroidogenesis via changes in mitochondrial permeability in their respective target organs (26). It must be pointed out, however, that the control of steroidogenesis in the testis is apparently different from that of the ovary and adrenal since neither 3',5'-AMP nor pyridine nucleotides enhance steroidogenesis in testicular slices (28).

Progesterone Biosynthesis

Since Edgar (8) first identified progesterone in the bovine corpus luteum, there have been numerous approaches developed to determine the ability of the corpus luteum to synthesize progesterone, pathways involved, and control mechanisms.

Homogenates of bovine corpora lutea were found by Tamaoki and Pincus (60) to be steroidogenic by their capacity to cleave the side chain of cholesterol or 20α-hydroxycholesterol with the formation of progesterone via pregnenolone. Additions of HCG, FSH, or pregnant mare serum gonadotropin (PMS) did not affect the conversion of cholesterol to progesterone.

Hall and Koritz (20) have found that acetone-dried powder of mitochondria from bovine corpora lutea converted cholesterol and 20α-hydroxycholesterol to pregnenolone and progesterone, however, NADPH had to be added to achieve this conversion. LH had no demonstrable effect on the reaction.

Mason, Marsh, and Savard (36) found it necessary to use tissue slice techniques to study steroidogenesis in the bovine corpus luteum because homogenization abolished the stimulatory effect of gonadotropins both on de novo progesterone synthesis from endogenous sources and on the activation of phosphorylase in this tissue (35). Their studies concerning the role of 3',5'-AMP in the synthesis of progesterone (32,52,31,33) have been reviewed in the preceding section. They have reported a wide range in progesterone concentration (from 10 to 100 μg/g of fresh tissue) in unincubated individual cow luteal tissues (37). The synthesis of progesterone in the absence of added gonadotropins varied from 0 to 98 μg/g of luteal tissue. There was no correlation between the level of progesterone synthesis and the amount present in the unincubated tissue. The levels of synthesis of progesterone, when gonadotropin was added, varied from 12 to 451 μg/g of luteal tissue and only about one-half of the corpora lutea from either pregnant or nonpregnant animals responded to gonadotropins. In a later study, Savard and Casey (51) found that the incorporation of acetate-1-^{14}C into progesterone by bovine corpus luteum slices resulted in varied specific activities ranging from 1,190 to 22,250 cpm/μg. This variability was thought to reflect the individual variabilities in steroidogenesis in corpora lutea from different cows (37). Corpora lutea from cows of early pregnancy (embryo length 5.0 to 30 cm) as well as from nonpregnant cows were found to vary greatly in

their response to graded doses of LH although progesterone synthesis levels in the absence of LH were comparable (34).

Armstrong and Black (3) examined corpora lutea from cows of known reproductive histories and at known stages of the estrous cycle in an attempt to determine possible causes of failure of some tissues to respond to LH, as well as the variability between tissues in the absolute amounts of progesterone synthesized. Tissues were obtained by laparotomy under local anesthesia, or at autopsy, immediately after slaughter. Incubations were begun within 20 minutes after collection of tissues. Progesterone synthesis rates per gram of tissue did not appear to differ significantly with stage of cycle prior to 14 days post estrus and it was concluded that the principal factor determining the total progesterone production during this part of the cycle was size of the corpus luteum. Progesterone production per gram of tissue declined slightly after 13 days post estrus and fell precipitously to undetectable levels at 18 days. The addition of LH to the incubation medium resulted in an increased rate of <u>in vitro</u> progesterone synthesis at all stages prior to 18 days. LH had no effect upon corpora lutea obtained on Days 19-21 post estrus even though these tissues responded to additions of exogenous NADPH. The lack of response of these tissues to LH was attributed to their inability to form pregnenolone since these tissues readily convert added pregnenolone to progesterone. Holding tissues at anaerobic conditions at 37^{o} C for a period of 40 minutes reduced, but did not abolish, the ability of corpora lutea to synthesize progesterone and/or respond to additions of LH or NADPH. No conclusions were drawn as to why other workers (37) did not get consistent stimulation with LH in bovine corpus luteum slices.

Functional ability of corpora lutea from nonpregnant heifers at Day 14 of the estrous cycle has been compared to that of heifers pregnant 28 days by Wickersham and Tanabe (61). The average de novo progesterone production by corpora lutea from Day 28 pregnant heifers during in vitro incubation was 68.5% higher than the de novo production by corpora lutea from Day 14 of the estrous cycle. This increased functional activity of the corpus luteum of pregnancy is consistent with the general concept that progesterone is essential for the continued development and secretory activity of the endometrium associated with pregnancy. The progesterone concentrations in the incubated samples were considerably more variable than that of the unincubated samples, possibly reflecting a wide normal range in the metabolic activity of luteal tissue. However, some of this variability may be attributed to conditions under which the animals were handled prior to slaughter or surgery. The discrepancies among data from various investigations were attributed to (a) the differences in parity and breed of experimental animals, (b) variations in the methods of removing and handling of glands, and (c) varying efficiencies of steroid extraction procedures (61).

Romanoff (47) has used yet another approach to study progesterone synthesis in bovine corpora lutea. Bovine luteal ovaries from nonpregnant cows were perfused four at a time with citrated blood. The venous effluents were pooled so that data reported were from "an 'average' ovary". Synthesis rates were reported on a weight per minute basis and varied from 2.78 to 11.23 μg/minute during the control period (no LH) and from 3.72 to 29.18 μg/minute after addition of LH. In a later publication (5) the same data were reported on a basis of volume of venous effluent. The progesterone concentrations from three perfusions were

2.8, 8.3, and 17.0 μg/100 ml of venous effluent during the control period (no LH) and 3.9, 75.6, and 55.9 μg/100 ml, respectively, after LH addition. There was no statistical consideration of the data from either of these studies.

Other approaches to the study of progesterone synthesis in the bovine are the quantitations of progesterone in ovarian venous effluents *in vivo* and peripheral (jugular) vein levels of progesterone. Short (55) was apparently the first to chemically quantitate peripheral progesterone levels in the bovine. During pregnancy the level ranged from 0.74 to 0.98 μg/100 ml plasma during the 32nd-256th day period, and decreased after this time. The day before calving the level of progesterone was 0.1 to 0.4 μg/100 ml plasma.

Melampy *et al.* (40) measured progesterone concentrations in peripheral blood of pregnant cows and reported that levels increased from 0.9 to 4.0 μg/100 ml of whole blood during the first eight months of pregnancy and then declined to 3.1 μg/100 ml of whole blood during the ninth month. Levels near parturition were not determined.

Progestin levels in ovarian vein plasma during late pregnancy were measured by Gomes *et al.* (13). At 250-254 days of gestation the progesterone level was 2.59 μg/ml and declined to 1.40 μg/ml at 281-282 days. In a later experiment, Gomes *et al.* (14) determined progestin levels in jugular and ovarian venous blood in the nonpregnant bovine. Ovarian venous plasma levels of progesterone increased from 0.9 μg/ml at Day 2 of the cycle to 6.2 μg/ml at Day 15 and decreased to 0.7 μg/ml at Day 21. Dobrowolski *et al.* (7) reported that the lowest ovarian vein level of progesterone in the nonpregnant bovine was 5.6 μg/100 ml found on Days 1 to 4 of the cycle. On the 4th day the hormone levels began to

rise and increased to about 125 μg/100 ml by the 8th day of the cycle and on Days 9 to 11 of the cycle a decline lasting 2 to 3 days was observed. On Days 10 to 11 a second, very rapid and continuous rise in the level occurred which reached its peak at 166 μg/100 ml on the 14th to 15th day of the cycle. This level was maintained for 24 hours only, then a continous decline was observed until the day of ovulation. The differences between these ovarian vein plasma values and those reported by Gomes et al. (14) were attributed to methods of collection.

Jugular progesterone levels reported by Gomes et al. (13) during the estrous cycle of the cow were 3.2 mμg/ml at estrus, increased to a peak of 22.0 mμg/ml at Day 14, and then decreased to 1.8 mμg/ml at Day 21. Plotka et al. (46) found peripheral levels of progesterone ranging from 9.9 mμg/ml at Day 2 of the cycle to a peak of 25.8 mμg/ml which occurred at estrus. Progesterone concentration in jugular venous plasma during the bovine estrous cycle has also been examined by Stabenfeldt (57). Average values from 6 cows ranged from 0.3 mμg/ml at estrus to a peak of 7.0 mμg/ml at Day 17.

The wide variations observed among the various experiments can probably be attributed to methods of collection and analysis and the breeds of animals.

Essentiality of the Bovine Corpus Luteum of Pregnancy

During pregnancy, large amounts of progesterone are elaborated, but the source of this hormone apparently varies with the species. In man, horse, cat, and guinea pig, for example, ovariectomy can be performed after a certain stage of gestation without interrupting pregnancy (18). The hormonal functions of the ovary, in these species, are supposedly

taken over by the placenta. In other species, for example, the rabbit, the ovary is indispensible at all stages of pregnancy, and its removal results in abortion within one to two days (18).

McDonald et al. (38) have shown that the corpus luteum is necessary for the maintenance of pregnancy in some cows as late as Day 236 which is over four-fifths of the way through pregnancy. However, in sheep, Neher and Zarrow (43) have found that ovariectomy may be performed as early as the 66th day of a 148-day gestation without causing abortion. Levels of progesterone in the blood continued to rise and remained comparable to that of pregnant sheep with intact ovaries. Bioassays were used to determine progesterone levels. It was suggested that, in the ewe, the placenta assumes the function of the corpus luteum during the last two trimesters of gestation. In the ewe, the corpus luteum shows definite morphological signs of involution by the 18th week (126th day) of pregnancy which is further evidence for the transitory role of the corpus luteum in this species.

Melampy et al. (40) have reported that the bovine placenta contains up to 200 μg of progesterone/kg. However, results of experiments conducted by Short (54), Gorski et al. (15) and Bowerman and Melampy (6) suggest that the bovine placenta is not a source of progesterone or 20β-hydroxy-Δ^4-pregnen-3-one. Yet the fact remains that pregnancy is maintained after ovariectomy during late pregnancy in the cow (38).

Stormshak and Erb (59), after studying the levels of progestins in bovine corpora lutea, ovaries, and adrenal glands during pregnancy, suggested that the corpus luteum as a source of progesterone was most critical during the first 3 months of pregnancy and thereafter declined in function. This decline may indicate that (a) after the third month,

lower levels of progestins are required by the bovine in maintaining pregnancy to normal birth; (b) a combination of progestins elaborated by the adrenal gland and ovaries is at least partially sufficient for pregnancy maintenance; (c) an extraovarian source of these hormones exists (but as yet has not been found); or (d) other progestational compounds exist in the bovine. They suggested that the corpus luteum must remain functional throughout getation for normal maintenance of pregnancy in the bovine.

It has been shown recently, by Ainsworth and Ryan (1), that bovine placental preparations convert pregnenolone to progesterone and that the progesterone is rapidly metabolized to 20α-hydroxy-Δ^4-pregnen-3-one, 20β-hydroxy-Δ^4-pregnen-3-one, 5β-pregnane-3α,20β-diol, and 5β-pregnane-3α,20α-diol. The possibility that other metabolites could be present in smaller amounts was recognized. These results may also be used to explain why pregnancy is maintained in some cows beyond ovariectomy.

Estergreen et al. (10) found that following ovariectomy, extraovarian sources appear generally inadequate to support normal gestation length and normal parturition including expulsion of the fetal membranes. Gestation was shortened and fetal membranes were retained in nearly all animals after corpus luteum removal or early hormone withdrawal. These results were in agreement with previous results reported by McDonald et al. (38).

In summary, removal of bovine ovaries during early pregnancy results in abortion, whereas ovariectomy during late pregnancy does not cause abortion but causes a shortened gestation period and retention of fetal membranes. From this it appears that progesterone secretion by the corpus luteum of late pregnancy is essential for normal pregnancy

maintenance. The fact that pregnancy is maintained beyond ovariectomy indicates that there are extraovarian sources of progestin or that the requirement for progestin has decreased. However, the level of progesterone in the peripheral blood has been found to remain high until near term. The functional ability of ovaries during late pregnancy has not been determined. Such information would assist in clarifying the role of the corpus luteum during late pregnancy.

The experiment reported herein was designed to determine the functional ability of corpora lutea of late pregnancy in comparison to corpora lutea of early pregnancy with respect to progesterone synthesis with and without added gonadotropin.

CHAPTER III

MATERIALS AND METHODS

Techniques for the collection, cannulation, flushing, and perfusion of bovine ovaries have been previously described (41) and have been modified to increase precision and efficiency. The techniques as modified are described below.

Collection of Ovaries

The uteri of cows with unknown reproductive histories were observed as they passed through the abattoir processing line about thirty minutes after the cows had been slaughtered. Ovaries were taken from cows which appeared normal and in early pregnancy (less than 90 days) or late (pregnancy (over 210 days) as determined by embryo length (64). It was necessary to remove a large portion of the broad ligament along with the reproductive tract to allow proper cannulation of the ovarian artery. The uterine artery was located and traced to the point of bifurcation where the ovarian artery originated. A hemostat was placed on the ovarian artery at the point of bifurcation and the uterus and excess tissue were trimmed away. The ovarian artery is highly convoluted in this area and was difficult to cannulate. Best results were obtained by removing the connective tissue which held the artery in its convolutions and straightening the artery as much as possible. Cannulations closer to the ovary usually resulted in only partial flushing of the ovary due to

subsequent branching of the ovarian artery as it approached the ovary. Cannulas were made of polyethylene tubing (I.D. 0.034" x O.D. 0.050") about 22 inches long, beveled on one end, and the other end attached to a twenty guage hypodermic needle.

Following cannulation, the ovary was flushed with a cold (12° C) dilute sodium citrate, citric acid, sodium chloride, and dextrose solution (dilute ACD solution) adjusted to pH 7.4 (see Appendix). Flushing was continued until all superficial blood vessels appeared clear and the ovary was blanched and chilled. Leaks that became apparent were tied off during the flushing process. Gentle massaging sometimes aided flushing of vessels that did not clear readily. If the ovary did not take on a blanched appearance, it was discarded and another collected since it was assumed that some of the blood vessels were occluded.

The cannulated, flushed ovaries were placed in plastic bags and covered with cold dilute ACD solution. The bags were closed and placed in an ice chest with several layers of wrapping paper placed over the ice to prevent excess chilling of the ovaries. The ovaries were transported to the laboratory in the ice chest.

Perfusate

The blood for the perfusions was collected at an abattoir from cows or heifers. A concentrated ACD solution adjusted to pH 7.4 (see appendix) was used as the anticoagulant. Each quart of blood collected contained 160 ml of the anticoagulant, 400,000 units of penicillin, and 0.5 mg of dihydrostreptomycin sulfate. The citrated blood was chilled as quickly as possible and was transported on ice to the laboratory where it was kept chilled at 5° C for 7 or 8 hours prior to each perfusion.

Just prior to the beginning of each perfusion, the citrated blood was filtered through several layers of glass wool to remove debris or clots. Three liters of blood were used for each perfusion. Ten grams of dextran (av. mol. wt. 75,000), 90 mg of sodium acetate, and 3 gm of pilocarpine hydrochloride were dissolved in sufficient saline solution to make 100 ml total volume. The pH was adjusted to 7.4 with sodium hydroxide (NaOH) and the solution was added to the 3 liters of filtered blood. The blood was then warmed to 37° C and placed in the reservoir-oxygenator of the perfusion apparatus where it was oxygenated with 95% oxygen and 5% carbon dioxide for several minutes. One hundred milliliters were removed for flushing purposes and the remaining 3 liters were started to circulate through the previously warmed (37° C) apparatus. One millicurie of sodium acetate-1-^{14}C was added to the 3 liters of blood immediately after the ovaries had begun perfusing. A 100 ml fraction of the blood was removed from the reservoir-oxygenator at the beginning of the perfusion for analysis to determine the level of endogenous progesterone in the blood at the beginning of the perfusion.

Perfusion Apparatus

The organs were perfused four at a time in an apparatus diagrammed in Figure 1. The four organ chambers were made from stainless steel instrument trays (4½" wide x 8½" long x 2" deep). A ¼" hole was drilled in the bottom near one corner at the lower end of each tray. A section of a 5 ml pipette was cemented outside the tray over the ½" hole using Translucent Silicone Rubber[1]. The section of pipette served as a flow

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[1]General Electric, Waterford, N. Y.

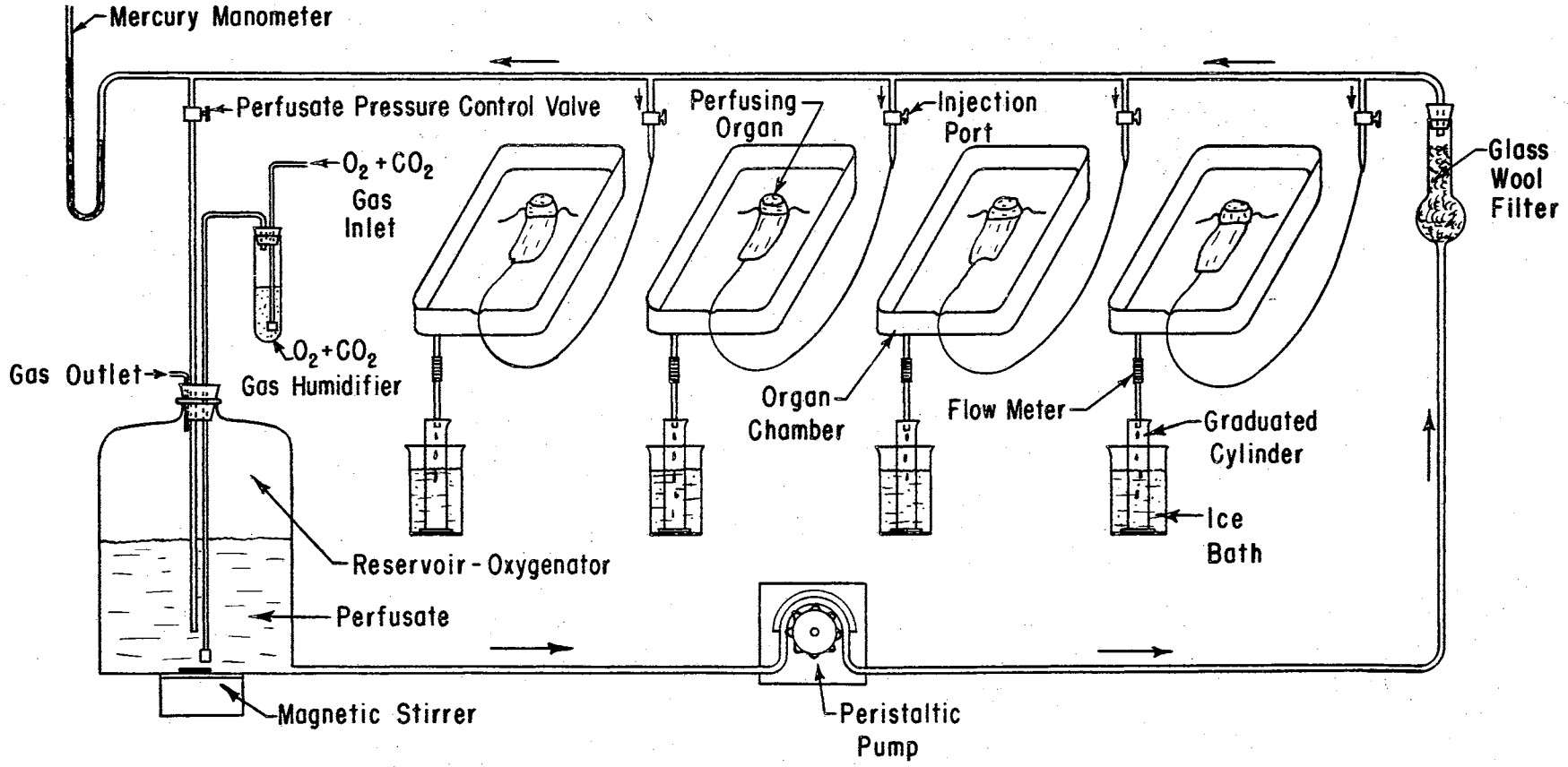

Figure 1. Perfusion Apparatus

meter. Three small cones of the silicone rubber placed about 2 inches from the upper end of the tray prevented the organs from sliding down during the perfusions. Each organ chamber was equipped with a clamp to allow individual mounting on a cross bar between two ring stands. A piece of double strength glass 5½" x 9½" was used to cover each organ chamber. An 8 liter aspirator bottle with a side tube served as the reservoir-oxygenator. Oxygen and carbon dioxide (95:5) were humidified by bubbling through water prior to bubbling through the perfusate in the reservoir-oxygenator. A magnetic stirrer was used to gently agitate the perfusate in the reservoir-oxygenator throughout each perfusion to prevent settling of erythrocytes. Two New Brunswick Peristaltic Metering Pumps[2] in a parallel arrangement were used to pump the perfusate and maintain a perfusion pressure of 100 mm Hg.

A drying tube with the bulb filled with glass wool was used to filter the perfusate before the perfusate passed through the organs. Three-way valves provided injection ports as well as attachment sites for the cannulas. Adjustment of the perfusate pressure control valve retarded or allowed the return flow to the reservoir-oxygenator and thereby controlled the perfusion pressure. The perfusion pressure was maintained at 100 mm Hg and was monitored with a mercury manometer throughout each perfusion.

Ovarian venous effluents were collected in graduated cylinders which were kept in ice baths to chill the perfusate immediately.

The perfusions were performed in a constant temperature cabinet maintained at 38° C.

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[2]New Brunswick Scientific Co., Inc., New Brunswick, N. J.

Perfusion of the Ovaries

After returning to the laboratory with the flushed and chilled ovaries (1.5-2 hours after collection), each ovary was flushed with 10 ml of the perfusate using a 10 ml syringe. Each ovary was observed carefully and all leaks appearing before the perfusate reached the ovary were tied off. The superficial vessels of each ovary and corpus luteum were checked to ensure complete circulation. If the perfusate flowed through all the superficial vessels and the ovary lost its blanched appearance, it was then placed in an organ chamber and the cannula was connected to the system. This process was repeated until four ovaries, two from early pregnancy and two from late pregnancy, were perfusing. The flow rate of the venous effluent was measured and recorded repeatedly for each ovary for a five-minute period to determine if each ovary was perfusing properly. Flow rates were measured by timing the filling of the flow meter after placing a pinch-clamp below the flow meter. If any of the ovaries showed signs of vasoconstriction, as indicated by a decrease in venous flow rate, 5 ml of pilocarpine hydrochloride (30 mg/ml) were injected into the ovary via the injection port. If any ovary did not appear to be perfusing satisfactorily, it was discarded and replaced.

Perfusion pressures were monitored continuously and the venous flow rates were checked frequently throughout all perfusions.

None of the venous perfusate was allowed to recirculate through any of the ovaries and the perfusions were therefore termed "monocyclic". The first 100 ml of venous effluent from each ovary was discarded, as this was considered to be a "warm-up period". The following two 100 ml fractions of venous effluent were collected on ice and each was retained

for analysis to determine the level of progesterone synthesis in the absence of added gonadotropin. When these two 100 ml fractions had been collected from all four perfusing ovaries, the total volume of venous effluent from the four ovaries was tallied and subtracted from 2900 ml to determine the remaining amount of perfusate. Bovine luteinizing hormone (NIH-LH-B_4) was added to the remaining perfusate in the reservoir-oxygenator to achieve a concentration of 1 μg/ml. The following 100 ml of venous effluent from each ovary was discarded, as during the control period, and the next two 200 ml fractions were collected individually on ice. Each ovary was removed from its organ chamber after the last 100 ml fraction had been collected. All connective tissue was trimmed away and each ovary was labeled and frozen at -20° C to be thawed and the corpus luteum extirpated and weighed later. After all four ovaries had been removed from their organ chambers, a 100 ml volume of the perfusate remaining in the reservoir-oxygenator was removed to be analyzed to determine the endogenous level of progesterone in the perfusate at termination of the experiment. All of the perfusate fractions were centrifuged at 7500 times gravity at 5° C and the erythrocytes were discarded. The plasma was then frozen at -20° C to await analysis.

Progesterone Extraction and Analysis

Progesterone concentrations in the perfusates were determined using the method described by Stabenfeldt (57). The perfusate plasma was thawed and a 20 ml aliquot was removed for analysis. To allow estimation of recovery of progesterone, 5.6 mμc of 7α-^{3}H-progesterone[3] were added

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[3]Nuclear-Chicago Corporation, Des Plaines, Ill.

to the 20 ml aliquot. Two and one-half volumes of methylene chloride were added to the sample. After shaking vigorously, the emulsion was broken by centrifugation for 10 minutes at 10,000 x g at 10° C. The methylene chloride (lower layer) was transferred to a 500 ml round bottom flask. This process was repeated twice with 2½ and 2 volumes of methylene chloride, respectively, and the three methylene chloride fractions were combined in a round bottom flask. The remaining plasma was discarded. The methylene chloride was evaporated to dryness using a Rotovapor[4] at 45° C. Three 5 ml volumes of ether were added to the dry residue and each was successively transferred to a 35 ml glass stoppered conical tube. To the 15 ml of ether were added 5 ml of 1 N NaOH to saponify the saponifiable lipids. After shaking, the emulsion was broken by centrifugation. The NaOH was removed and discarded and the ether was washed twice with 5 ml volumes of water. Centrifugation was required after each wash to break the emulsion. After removing the second 5 ml of water, the sample was centrifuged again to remove the last drop of water. The ether was then evaporated to dryness under a nitrogen atmosphere at about 40° C. The dried residue was resuspended in a small amount of tetrahydrofuran and transferred quantitatively to a silica gel[5] plate. The silica gel with a fluorescent indicator was layered on the glass plate at 0.25 mm thickness. A progesterone marker was also spotted on each plate. The spotted plates were developed in benzene-ethyl acetate solvent (4:1). When thoroughly dry, the plates were observed under

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[4]Model VE 50 GD, Rinco Instrument Co., Inc., Greenville, Ill.

[5]Silica Gel G-HR/UV, Machery, Nagel & Co., Brinkman Instruments, Inc., Westbury, N. Y.

ultraviolet light and the fluorescing band with a retention time corresponding to the progesterone marker was marked off. This band was then eluted with 3 ml of ether by using suction and a porous glass filter. The ether was evaporated to dryness under a nitrogen atmosphere at 40° C. The dried residue was reconstituted with 50 µl of tetrahydrofuran. Five microliters were placed in a scintillation vial for liquid scintillation counting to determine recovery and acetate incorporation. Another 5 µl were injected into the gas chromatograph for determination of the amount of progesterone in the sample. Liquid scintillation counting was done using a Packard Tri-Carb Liquid Scintillation Spectrometer (Series 314 E)[6]. The scintillation cocktail consisted of 4 gm of 2.5-diphenyloxazole (PPO) and 0.3 gm of (p-Bis(2-(5-phenyloxazole)))-benzene (POPOP) in one liter of analytical reagent grade toluene. Ten milliliters of this scintillation cocktail were used per sample. Corrections were made for background, machine efficiency, and for quinching. Gas-liquid chromatography was performed using a Barber-Coleman Series 5000[7] instrument equipped with a Model 5121 hydrogen flame detector. Three-foot glass columns (4 mm I.D. x 6 mm O.D.) packed with 1% XE-60 on 80/100 mesh Gas-Chrom Q[8] were used. Nitrogen was used as the carrier gas with a flow rate of 48 ml/min from the column. The injection port, column bath, and detector temperatures were 255°, 220°, and 225° C, respectively. Peak areas were measured by planimetry and quantitation was accomplished by comparing peaks of unknowns with peaks of known amounts of injected

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[6]Packard Instruments, La Grange, Ill.

[7]Barber-Coleman Company, Rockford, Ill.

[8]Applied Science Laboratories, College Station, Penn.

standards.

The mass spectra of the progesterone standard and several samples of progesterone isolated by the above techniques were determined. Several samples were injected into an 8 ft., 1% OV-1 column prior to entering the mass spectrometer[9] and several others were placed in the mass spectrometer by direct probe. Column, flash heater, separator, and ion source temperatures were 220°, 240°, 243°, and 310° C, respectively. Helium was used as the carrier gas with a flow rate of 21 ml/minute. Probe temperature was 30° C for the direct probes.

Data were punched into IBM cards and were analyzed statistically by the Department of Statistics using an IBM 7040 Computer. The statistical analysis was performed using the abbreviated Doolittle method (17) and the within variation was found by the high analysis of variance method (17).

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[9]LKB-900 Mass Spectrometer-Gas Chromatograph, LKB Produckter, Carolina Institute, Stockholm, Sweden.

CHAPTER IV

RESULTS

A total of 44 bovine ovaries of early and late pregnancy were perfused with citrated bovine blood as described in Chapter III. Of the 44, 22 were from early pregnancy and 22 were from late pregnancy. Six ovaries from early pregnancy and 3 ovaries from late pregnancy failed to synthesize progesterone in this system. Also, data from two perfused ovaries from early pregnancy were incomplete and were omitted to simplify the statistical analysis. Therefore, data from 14 of the 22 perfusions of early pregnancy and 19 of the 22 perfusions of late pregnancy are reported.

The ovarian and corpora lutea weights are shown in Table I. The average ovarian weights were 10.51 and 11.42 grams for early and late pregnancy, respectively, and the average corpus luteum weights were 4.12 and 4.47 grams for early and late pregnancy, respectively. There was no significant difference between either ovarian or copora lutea weights of early and late pregnancy.

The amounts of progesterone synthesized by each ovary during the control period and after addition of LH are shown in Table II. There was no significant difference between the level of progesterone synthesized by ovaries of early and late pregnancy during the control period. Addition of LH significantly ($P < 0.005$) increased the levels of progesterone synthesis by ovaries of both early and late pregnancy. However,

TABLE I

OVARIAN AND CORPUS LUTEUM WEIGHTS FROM COWS OF EARLY AND LATE PREGNANCY

Early Pregnancy			Late Pregnancy		
Ovary Number	Total Ovarian Weight (g)	Corpus Luteum Weight (g)	Ovary Number	Total Ovarian Weight (g)	Corpus Luteum Weight (g)
1	9.85	3.72	3	13.60	6.23
2	6.62	2.72	4	9.11	4.06
5	15.37	4.18	8	15.08	5.40
6	11.75	4.28	11	13.07	5.69
7	12.73	5.59	12	10.02	4.97
9	11.79	5.00	14	14.17	5.25
10	7.99	3.54	15	11.07	2.84
13	10.94	4.27	16	13.41	4.07
17	8.92	3.61	19	10.13	3.90
18	11.82	4.61	20	12.43	4.52
25	9.90	4.00	27	12.08	7.67
26	12.38	4.09	28	9.30	2.72
29	8.19	5.45	31	15.20	5.27
30	6.99	3.43	32	13.07	3.43
35	5.39	3.21	33	9.46	3.41
36	10.47	4.78	34	9.65	3.32
37	12.76	3.95	40	7.54	3.30
38	9.88	2.82	42	9.02	3.66
39	11.50	5.55	43	11.51	5.74
41	14.97	3.62	44	9.55	3.96
Average	10.51	4.12	Average	11.42	4.47

TABLE II

PROGESTERONE SYNTHESIZED BY PERFUSED BOVINE OVARIES OF EARLY AND LATE PREGNANCY

Early Pregnancy					Late Pregnancy				
Ovary Number	Pre-LH		Post-LH		Ovary Number	Pre-LH		Post-LH	
	I	II	III	IV		I	II	III	IV
	(μg/20 ml plasma)					(μg/20 ml plasma)			
1	0.62	0.66	0.55	0.68	4	28.8	27.9	38.9	35.2
2	0.22	0.24	1.15	1.22	8	13.0	16.4	26.6	33.5
5	74.8	91.3	170.6	150.9	11	6.9	9.60	7.70	2.70
7	2.73	2.19	9.98	5.42	12	15.4	26.5	17.8	19.3
9	52.9	54.8	72.8	59.6	14	33.6	30.2	49.4	43.0
10	9.14	5.97	11.6	16.6	15	15.8	9.30	31.7	36.6
17	20.7	24.1	39.4	33.3	16	6.90	6.6	12.8	6.60
18	38.7	44.1	50.7	49.6	19	16.4	11.4	57.4	70.5
22	63.0	83.6	106.1	88.6	20	29.6	31.0	74.6	99.4
29	0.55	0.23	0.39	0.25	23	42.0	48.0	145.4	116.9
36	53.2	70.2	98.3	102.2	24	39.2	57.8	220.9	94.9
37	42.5	43.7	47.3	60.6	31	57.0	50.1	149.9	97.5
39	7.20	15.77	28.8	55.1	32	1.76	0.38	1.29	2.43
41	34.3	53.9	102.0	156.4	33	112.3	137.90	127.4	146.8
					34	29.1	28.80	56.9	62.1
					40	46.0	25.90	27.7	28.6
					42	17.4	26.90	50.4	50.8
					43	6.00	3.30	3.70	4.20
					44	42.9	66.2	68.6	39.9
Average	28.6	35.0	52.8	55.8	Average	29.5	32.3	61.5	52.2

there were no significant differences between the levels of progesterone synthesis by ovaries of early and late pregnancy after addition of LH.

The data for the incorporation of acetate-1-^{14}C into progesterone by perfused ovaries are presented in Table III. There was no significant difference in the incorporation of acetate-1-^{14}C into progesterone between ovaries of early and late pregnancy. LH did not significantly alter the incorporation of acetate-1-^{14}C into progesterone by ovaries from either early or late pregnancy.

Mass spectra from a progesterone standard and sample number 41_{III} after injection into an 8 ft., 1% OV-1 column are shown in Figures 2 and 3, respectively. Mass spectra from a progesterone standard and sample number 31_{III} by direct probe into the mass spectrometer are shown in Figures 4 and 5, respectively.

TABLE III

ACETATE-1-^{14}C INCORPORATION INTO PROGESTERONE BY PERFUSED BOVINE OVARIES OF EARLY AND LATE PREGNANCY

Early Pregnancy					Late Pregnancy				
Ovary Number	Pre-LH		Post-LH		Ovary Number	Pre-LH		Post-LH	
	I	II	III	IV		I	II	III	IV
	(dpm/μg of progesterone)					(dpm/μg of progesterone)			
1	332.90	319.67	188.79	312.43	4	5.21	9.02	7.70	20.00
2	516.22	381.67	72.43	26.72	8	18.49	22.41	18.74	23.10
5	0.33	0.86	3.15	3.47	11	76.38	2.21	60.39	71.11
7	95.57	93.24	17.06	28.80	12	6.01	8.72	21.74	2.51
9	5.44	1.18	5.05	10.55	14	3.30	10.50	5.20	5.67
10	69.58	40.37	12.07	8.67	15	31.39	78.92	5.68	21.91
17	3.97	1.97	4.75	7.96	16	64.93	6.88	10.62	80.61
18	1.73	3.04	3.41	9.64	19	2.89	10.44	19.05	1.60
22	1.18	4.51	8.91	13.32	20	0.88	1.26	1.04	0.93
29	102.18	51.74	36.92	170.40	23	1.26	1.80	5.75	13.21
36	1.85	0.00	3.39	1.51	24	3.30	2.24	18.42	50.47
37	1.02	0.00	14.39	13.61	31	2.24	1.78	55.04	100.06
39	0.90	0.00	6.42	9.51	32	153.69	808.68	126.51	69.75
41	0.00	0.00	5.58	6.31	33	0.00	0.33	6.70	13.06
					34	0.00	0.46	1.58	2.69
					40	0.00	1.03	12.94	19.52
					42	4.23	0.11	14.76	17.89
					43	7.38	0.00	18.62	9.95
					44	1.07	0.33	0.77	4.53
Average	80.92	64.16	27.31	44.49	Average	20.14	50.90	21.64	27.82

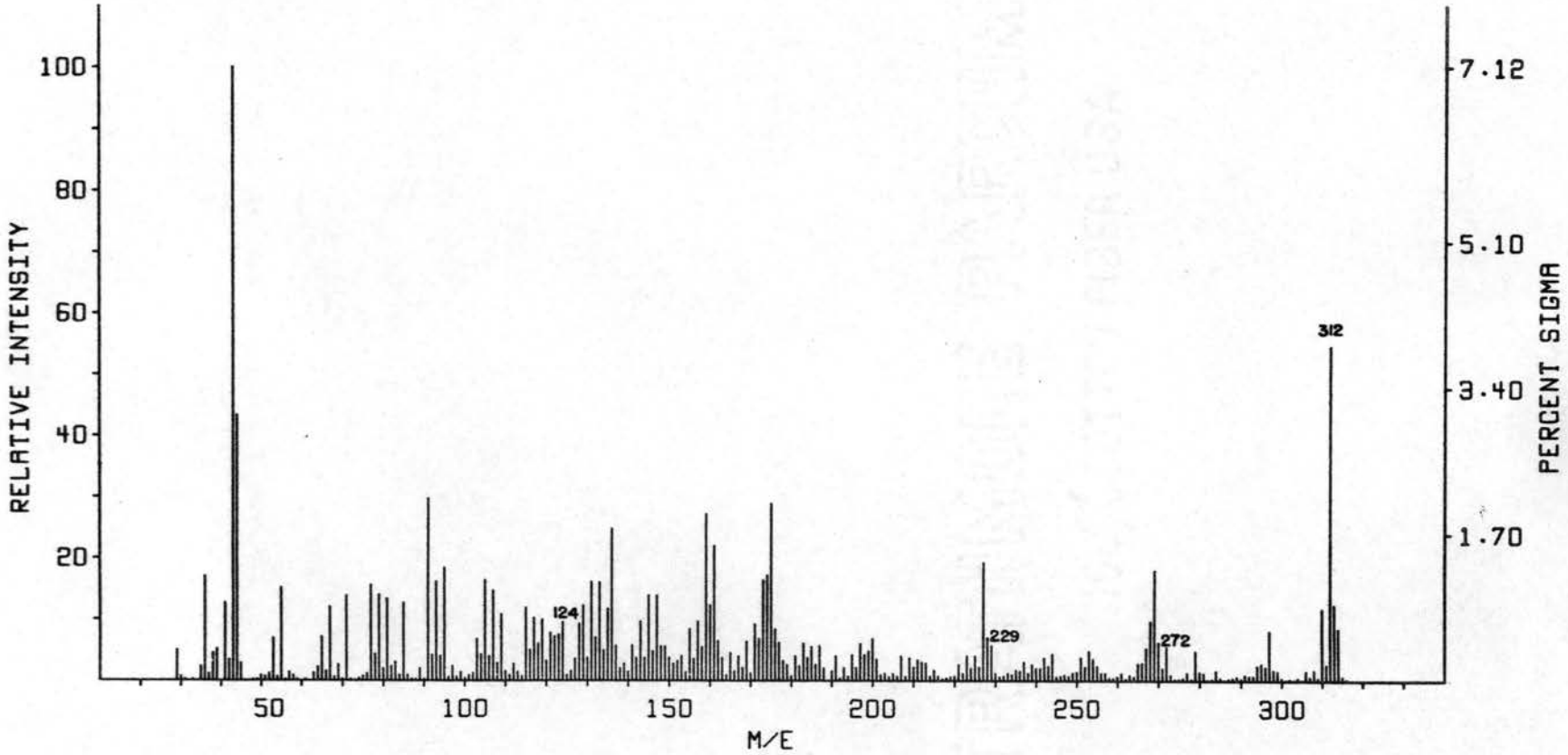

Figure 2. Mass Spectrum of Standard Progesterone After Passing Through an 8 ft., 1% OV-1 Column

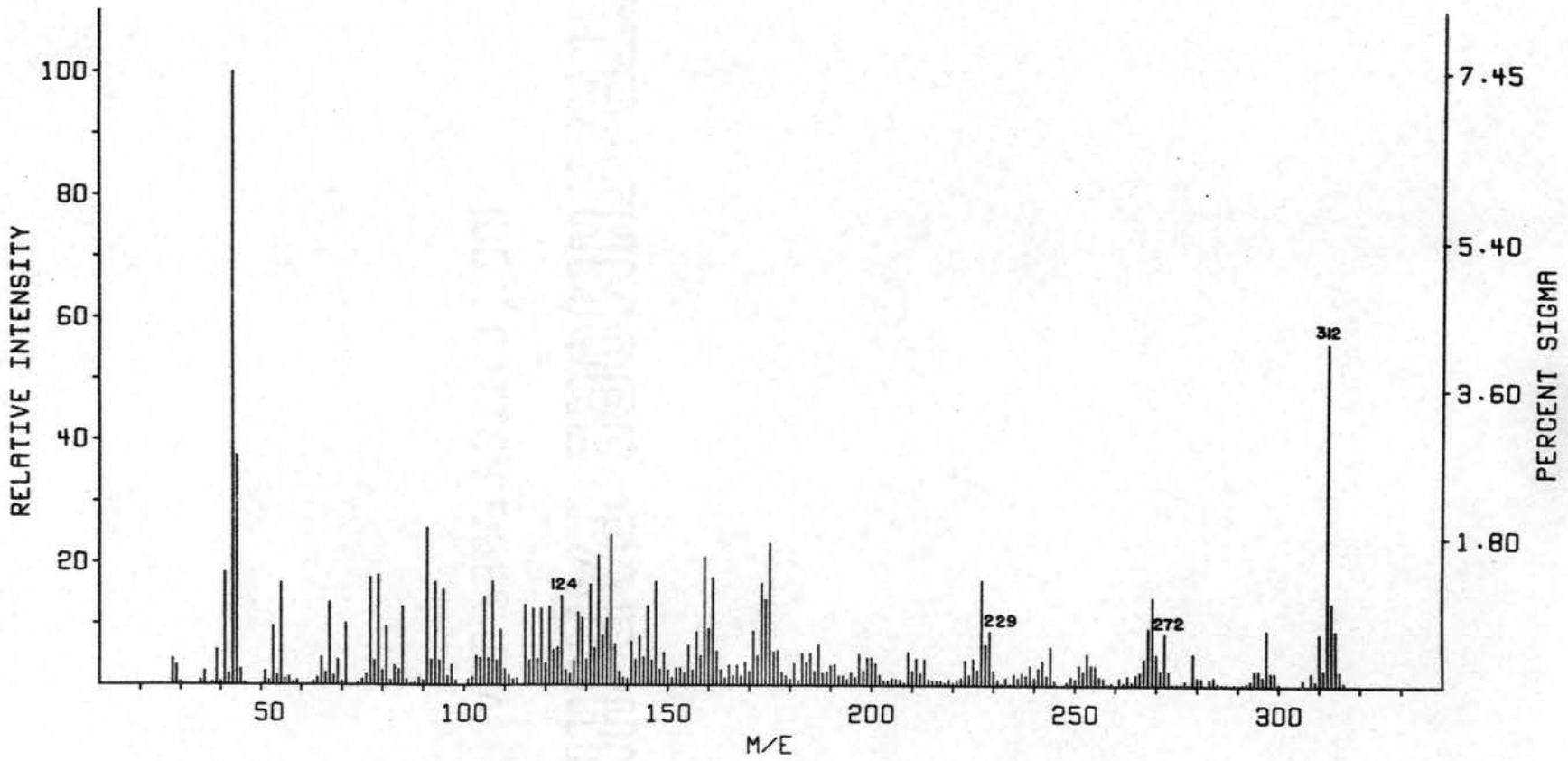

Figure 3. Mass Spectrum of Isolated Progesterone After Passing Through an 8 ft., 1% OV-1 Column

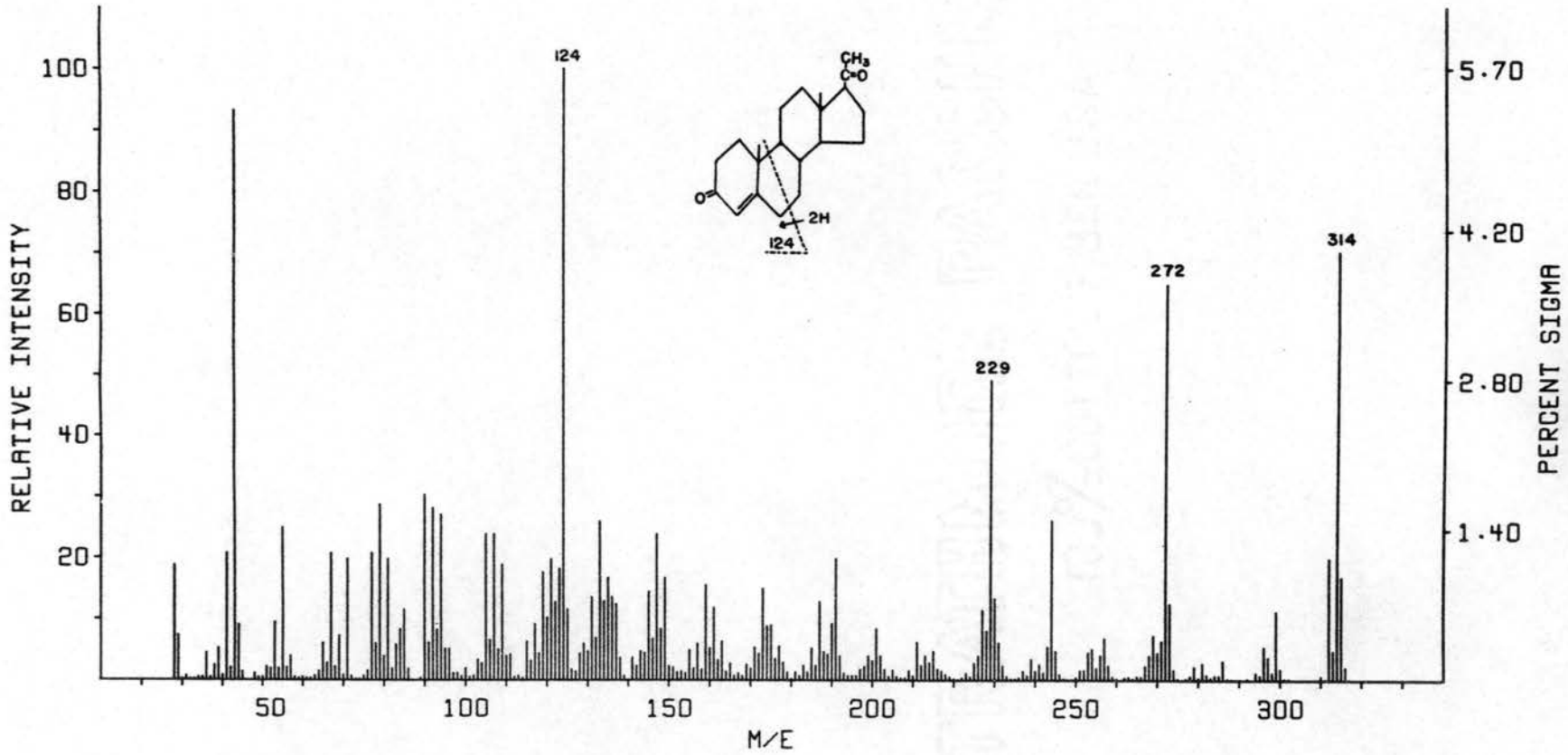

Figure 4. Mass Spectrum of Standard Progesterone Obtained by Direct Probe Into the Mass Spectrometer

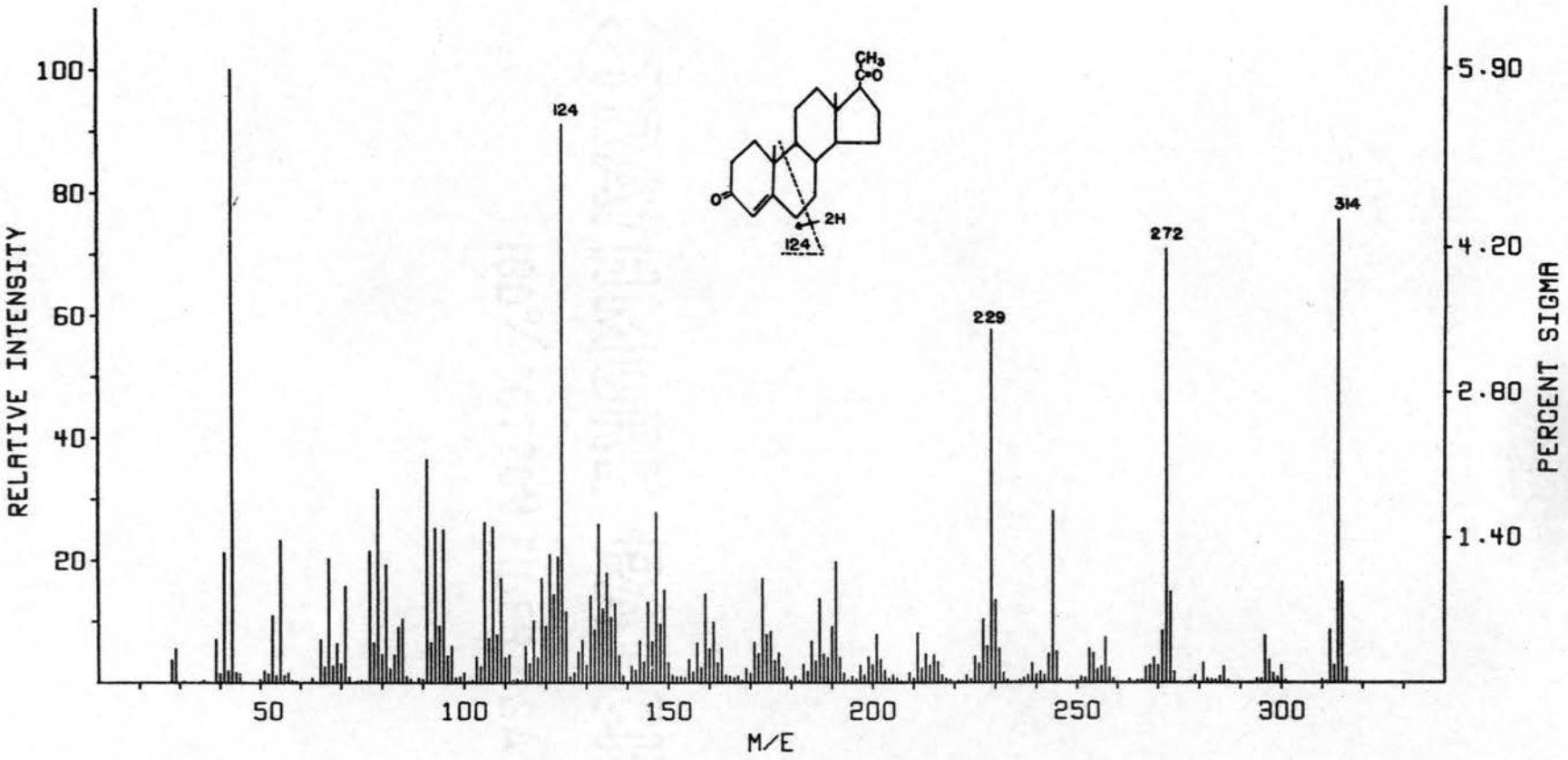

Figure 5. Mass Spectrum of Isolated Progesterone Obtained by Direct Probe Into the Mass Spectrometer

CHAPTER V

DISCUSSION

Of the 44 perfused bovine ovaries, 9 (20.4%) failed to synthesize progesterone either before or after addition of LH. Four (11.4%) of the remaining 35 ovaries failed to respond to LH with increased progesterone synthesis. Mason, Marsh, and Savard (37) found that 24 (49.1%) out of 49 ovaries collected at an abattoir, sliced and incubated for 2 hours failed to respond to LH with increased progesterone synthesis. A wide variability in levels of progesterone synthesis was also found which agreed with results reported by Mason, Marsh, and Savard (37). However, Armstrong and Black (3) obtained corpora lutea from cows at laparotomy under local anesthesia and reported that corpora lutea obtained by this method, sliced, and incubated for 2 hours, all synthesized progesterone and responded to LH with increased progesterone synthesis. From these results, Armstrong and Black (3) postulated that conditions of handling of the cows prior to slaughter and/or anaerobic conditions at body temperature for 30 to 40 minutes, after slaughter and before removal of the ovary greatly influences the ability of bovine corpora lutea to synthesize progesterone and/or respond to LH with increased progesterone synthesis.

The ovarian and corpora lutea weights of early and late pregnancy in Table I were not significantly different which agrees with data reported by Stormshak and Erb (59). These workers found that corpora lutea from early pregnancy (16 to 89 days) weighed 5.9 grams, as compared with

5.0 grams at midpregnancy (90 to 179 days), and 5.5 grams after 180 days of pregnancy. This is in contrast to observations made in sheep (43) where the corpus luteum showed definite morphological signs of involution by the 126th day of a 148-day gestation. However, these data are consistent with the hypothesis that the bovine corpus luteum of late pregnancy is functional and essential for the maintenance of "normal" pregnancy (59).

When expressed as μg of progesterone/minute (Table V in the Appendix), the average levels of progesterone synthesis were approximately 3 times greater than levels reported by Bartosik et al. (5). They obtained 5.25, 1.30, and 2.28 μg/minute during the control period of three sets of four perfused bovine ovaries of early pregnancy. When these values are divided by 4, the average synthesis per ovary was 0.77 μg/minute as compared to an average of 4.11 μg/minute reported herein. After addition of LH, the average synthesis per ovary was 2.98 μg/minute in their experiments compared to 7.30 μg/minute in this experiment (Table V). Levels of LH used by Bartosik et al. (5) were 2.7 to 3.9 times greater than the 1 μg/ml used in this study.

Levels of progesterone synthesis reported by Armstrong and Black (3) and Marsh and Savard (32) using incubated slice techniques are much greater (up to 20 times) than levels of progesterone synthesis found in this study and by Bartosik et al. (5). Levels of LH used in the incubated slice preparations (32,3) were 3 to 5 times greater than levels used in this study. The difference between the levels of progesterone synthesized by corpora lutea using the two different techniques cannot be explained.

Gomes et al. (13) have measured the progesterone levels in ovarian

vein plasma during late pregnancy (250-254 days of gestation) and found a level of 2.59 μg/ml which decreased to 1.40 μg/ml at 281-282 days of gestation. Levels of progesterone in the perfusate from ovaries of late pregnancy in this study were 1.54 μg/ml during the control period and increased to 2.84 μg/ml after addition of LH. Therefore, the data obtained in this study are comparable to data from in vivo studies.

Romanoff and Pincus (48), Romanoff (47), and Bartosik et al. (5) have all reported an increase in the incorporation of acetate-1-^{14}C into progesterone following addition of LH, by perfused bovine ovaries of the luteal phase of the cycle and of early pregnancy. Such results were not obtained in this study. Levels of acetate-1-^{14}C incorporation into progesterone were very erratic (Table III). In the studies above (48,47,5), the acetate-1-^{14}C was infused into the perfusate just prior to entering the organs, thereby minimizing the metabolism of the acetate-1-^{14}C in the blood. In this study, the acetate-1-^{14}C was added to the perfusate at the beginning of the perfusion. Therefore, the erratic data in Table III could have been due to metabolism of the acetate-1-^{14}C in the blood prior to entering the organs. The concentration of acetate-1-^{14}C may have been effectively lowered as the perfusion time increased so that levels of acetate-1-^{14}C available to the perfusing organs may have been greatly reduced near the termination of each perfusion.

The mass spectrum of progesterone isolated from the perfusate (Figure 3) is consistent with the mass spectrum of the standard progesterone (Figure 2). However, neither of these spectra correspond with the progesterone spectrum reported by Peterson (45). Peterson (45) found a base peak occurring at 124 m/e and a parent ion at 314 m/e which is consistent with the molecular weight of progesterone (314.45). Spectra of the

standard progesterone and progesterone isolated from the perfusate obtained by direct probe into the mass spectrometer (Figures 4 and 5) agree favorably with the spectrum of progesterone reported by Peterson (45). The data in Figures 2 and 3 indicate that the progesterone was broken down during its passage through the 8 ft., 1% OV-1 column or separators. The mass spectra in Figures 4 and 5, however, provide conclusive proof that the material isolated and measured in this study was indeed progesterone.

The data in Table II indicate that ovaries of late pregnancy have the same functional ability as ovaries of early pregnancy and respond with equal magnitude to gonadotropins. However, this does not provide conclusive evidence that ovaries of late pregnancy produce comparable amounts of progesterone in vivo as ovaries of early pregnancy. The levels of circulating gonadotropins during each stage of pregnancy probably control the level of progesterone synthesis during that period and may be significantly different from one stage of pregnancy to the next.

The fact that ovariectomy after about 240 days of gestation does not terminate pregnancy in the bovine (38) has been used as evidence that the progesterone secretion rate of corpora lutea of late pregnancy is lowered and is therefore not critical to late pregnancy (61,59). Data reported by Gomes et al. (13) indicate that the level of progesterone secretion by the bovine ovary declines during late pregnancy. However, ovariectomy during late pregnancy caused retention of fetal membranes (10,38). Progesterone injections until 278 days of pregnancy in ovariectomized cows allowed normal calving with expulsion of fetal membranes (39). The data reported herein provide evidence that the corpus luteum of late pregnancy is fully capable of progesterone secretion at levels

comparable to those of early pregnancy and is probably functional until near term depending on the levels of gonadotropins in the circulating blood. These results provide the basis for a postulation that the progesterone secreted by the corpus luteum of late pregnancy is essential to "normal" pregnancy. If the foregoing postulation is true, then progesterone synthesized by the placenta (1) and/or adrenal glands (4) during late pregnancy may serve only as a supplement to the progesterone secreted by the corpus luteum. Removal of the ovary during late pregnancy would therefore remove the prime source of progesterone during this period. The maintenance of pregnancy would then be dependent on the secondary sources of progesterone which apparently are not sufficient for "normal" pregnancy maintenance since gestation is shortened and fetal membranes are retained at parturition (38,10).

CHAPTER VI

SUMMARY AND CONCLUSIONS

In contrast to many species, the ovaries of the cow may be removed during late pregnancy without causing abortion. From this it appears that the bovine corpus luteum may produce less progesterone during late pregnancy and is therefore nonessential. However, fetal membranes are retained at parturition in ovariectomized cows in the absence of progesterone therapy. The role of the corpus luteum during late pregnancy is therefore not clear. This experiment was designed to establish the functional ability of corpora lutea of late pregnancy in comparison to corpora lutea of early pregnancy with respect to progesterone synthesis.

A total of 44 bovine ovaries of early and late pregnancy were perfused with citrated bovine blood containing acetate-1-^{14}C. Samples of the perfusate from each ovary were collected during a control period and after addition of LH. The samples were analyzed for progesterone content and acetate-1-^{14}C incorporation into progesterone. It was found that bovine ovaries of late pregnancy synthesized progesterone at levels comparable to those synthesized by ovaries of early pregnancy. Levels of synthesis by perfused bovine ovaries of late pregnancy were comparable to levels reported from *in vivo* ovarian venous blood studies. Ovaries from early and late pregnancy both responded to additions of LH with an equal and significant ($P < 0.005$) increase in progesterone synthesis. The incorporation of acetate-1-^{14}C into progesterone was erratic and

uninterpretable.

It was concluded that the bovine ovary of late pregnancy is capable of continued progesterone synthesis at levels comparable to levels of synthesis by ovaries of early pregnancy. The bovine corpus luteum is therefore considered functional until near parturition and any decrease in the level of progesterone secretion occurring during late pregnancy is probably due to reduced levels of gonadotropins.

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APPENDIX A

TABLE IV

PROGESTERONE SYNTHESIZED BY PERFUSED BOVINE OVARIES OF EARLY AND LATE PREGNANCY

Early Pregnancy					Late Pregnancy				
Ovary Number	Pre-LH		Post-LH		Ovary Number	Pre-LH		Post-LH	
	I	II	III	IV		I	II	III	IV
	(μg/20 ml plasma/g corpus luteum)					(μg/20 ml plasma/g corpus luteum)			
1	0.17	0.18	0.15	0.18	4	7.09	6.87	9.67	8.91
2	0.08	0.09	0.42	0.45	8	2.41	3.04	4.92	6.20
5	17.90	21.80	40.70	36.00	11	1.21	1.69	1.35	0.47
7	0.49	0.39	1.79	0.97	12	3.10	5.33	3.58	3.88
9	10.60	11.00	14.50	11.90	14	6.40	5.76	9.42	8.19
10	2.58	1.68	3.27	4.68	15	5.56	3.27	11.16	12.88
17	5.73	6.67	10.91	9.22	16	1.70	1.62	3.15	1.62
18	8.40	9.58	11.00	10.78	19	4.21	2.92	14.71	18.07
22	15.30	20.30	22.90	21.50	20	6.54	6.86	16.50	22.00
29	0.10	0.04	0.07	0.05	23	9.39	10.72	32.50	26.10
36	11.15	14.72	20.60	21.40	24	8.76	12.93	49.40	21.20
37	10.75	11.06	11.98	15.32	31	10.81	9.50	28.40	18.50
39	1.30	2.84	5.19	9.94	32	0.51	0.11	0.38	0.71
41	9.47	14.88	28.20	43.20	33	32.58	40.40	37.40	42.90
					34	8.78	8.67	17.13	18.70
					40	13.93	7.86	8.40	8.66
					42	4.76	7.35	13.78	13.89
					43	1.04	0.57	0.64	0.73
					44	10.83	16.71	17.32	10.08
Average	6.72	8.23	12.26	13.26	Average	7.35	8.01	14.73	12.83

TABLE V

PROGESTERONE SYNTHESIZED BY PERFUSED BOVINE OVARIES OF EARLY AND LATE PREGNANCY

Early Pregnancy					Late Pregnancy				
Ovary Number	Pre-LH		Post-LH		Ovary Number	Pre-LH		Post-LH	
	I	II	III	IV		I	II	III	IV
	(μg/minute)					(μg/minute)			
1	0.07	0.07	0.07	0.08	4	4.71	6.28	7.00	6.50
2	0.04	0.04	0.21	0.23	8	2.09	2.76	4.92	4.96
5	14.56	13.50	28.68	25.39	11	1.07	1.63	1.54	0.51
7	0.39	0.28	1.32	0.95	12	0.93	2.50	1.78	1.82
9	6.93	6.21	9.91	8.44	14	7.09	6.04	8.54	7.43
10	1.01	0.78	1.71	2.35	15	2.22	1.22	3.66	4.35
17	2.79	3.24	6.57	4.17	16	0.90	0.55	2.03	1.04
18	5.42	5.93	8.44	6.19	19	2.87	1.99	11.81	14.51
22	6.22	9.27	9.91	8.83	20	4.14	4.34	12.41	12.88
29	0.07	0.03	0.04	0.03	23	5.97	6.82	19.12	16.19
36	6.54	8.34	9.68	9.28	24	4.64	7.08	23.75	10.83
37	7.84	7.64	8.29	10.09	31	8.54	7.18	17.04	11.09
39	1.09	2.21	4.19	9.64	32	0.22	0.04	0.13	0.27
41	4.59	7.24	13.15	20.15	33	16.14	22.65	14.20	16.32
					34	4.18	3.97	8.18	8.93
					40	7.00	4.12	4.21	4.54
					42	2.42	3.92	7.35	7.40
					43	0.84	0.43	0.52	0.59
					44	5.75	8.54	9.58	5.57
Average	4.11	4.63	7.30	7.56	Average	4.30	4.85	8.30	7.14

APPENDIX B

TABLE VI

ACID CITRATE DEXTROSE (ACD) SOLUTIONS

Concentrated ACD Solution

Sodium Citrate	77.0 g
Citric Acid	28.0 g
Dextrose	85.7 g

Dilute to 3,5000 ml with distilled water. The pH may be adjusted to 7.4 with 1 N NaOH.

Dilute ACD Solution

Dilute 480 ml of concentrated ACD solution with 2,520 ml of 0.85% NaCl. Buffer with 60 ml of phosphate buffer. Adjust pH to 7.4 with 1 N NaOH.

Phosphate Buffer

Solution #1: KH_2PO_4 (M.W. 136). Dissolve 9.08 g in 1 L of distilled water.

Solution #2: Na_2HPO_4 (M.W. 141). Dissolve 9.47 g in 1 L of distilled water.

For 60 ml of phosphate buffer, mix 11.5 ml of Soln. #1 with 48.5 ml of Soln. #2.

VITA

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