CORPUS LUTEUM FUNCTION AND UTERINE LUMENAL FLUID PROTEIN IN EWES DURING THE ESTROUS CYCLE AND EARLY PREGNANCY

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ABSTRACT

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In the ewe, luteal maintenance during the third week of pregnancy requires maternal recognition of that pregnancy. Since the mechanism of this recognition is unclear, the objectives of the present study were to assess the antiluteolytic capacity of the conceptus and determine if the early maternal recognition of pregnancy involved either qualitative or quantitative changes in uterine lumenal fluid protein.

Ewes given 250 μg 17β-estradiol per day on days 11 and 12 or 12 and 13 following mating to a vasectomized ram had less serum progesterone (ng/ml) on day 14 than did similarly mated control ewes (.4 ± .1 vs 1.9 ± .6; $P < .01$). However, estradiol treatment caused a decline in serum progesterone in only 6 of 12 ewes mated to fertile rams. Of ewes mated to fertile rams in experiment II, four of eight and one of eight given 125 or 250 μg estradiol per day, respectively, on days 11 and 12 post mating were pregnant on day 16. Pregnant ewes had higher ($P < .05$) serum progesterone on day 15 (1.6 ± .8) than nonpregnant ewes (.3 ± .1). In experiment III, five of 17 ewes mated to

fertile rams and given 125 μg estradiol on days 11 and 12 were pregnant on day 20. These pregnant ewes had higher progesterone ($P < .01$) on day 14 than ewes similarly mated but not pregnant on day 20 (4.1 ± .3 vs 1.3 ± .3 ng/ml). Serum progesterone in estradiol treated ewes that were pregnant on day 20 did not differ from levels in normal pregnant ewes.

In experiments IV and V, recoverable uterine lumenal protein increased ($P < .05$) from 1.84 ± .21 mg on day 3 (five ewes/day) to 4.97 ± 1.20 mg on day 9 of an estrous cycle and remained at that level until day 14. Uterine protein recovered on day 14 of pregnancy did not differ from day 14 of an estrous cycle (6.61 ± .76 vs 5.1 ± 1.60 mg, respectively). Up to 35 protein bands were detected after isoelectric focusing of uterine protein collected on day 14 of an estrous cycle; only two faint bands (pI 7.2 to 7.6) were not present also in blood serum. With the exception of the two bands focused between pH 7.2 and 7.6, protein collected on days 3 and 9 of an estrous cycle focused a similar number of bands. During pregnancy, uterine protein differed from that collected during an estrous cycle as follows: (1) after day 14, a pregnancy-specific protein migrated toward the cathode at pH 4.5; (2) after day 13 of pregnancy, increased staining intensity of a protein of ≃ 9500 MW; and (3) decreased proportions of proteins focused between pH 5.4 and 7.0 occurred on day 14 of pregnancy.

Compared to blood serum, uterine protein collected from ovariectomized ewes in experiment VI (five ewes/treatment) had a higher proportion of proteins focused at less than pH 4.7. After 10 days of

progesterone replacement, the proportion of proteins that focused at less than pH 4.7 decreased while recoverable protein increased (1.20 ± .26 vs 3.48 ± 1.20 mg; $P < .05$). Twenty-one protein bands were detected after progesterone treatment and only one of these (pI 7.9) was not present also in serum. Estradiol replacement increased recoverable uterine protein (4.98 ± 2.37 mg) but profiles of protein collected after estrogen treatment were not different from those observed in control ovariectomized ewes. Progesterone plus estradiol replacement increased the proportion of lumenal proteins focused between pH 4.7 and 5.8.

Estradiol (125 μg/day; experiment VII) on days 11 and 12 of an estrous cycle increased the proportion of proteins focused at less than pH 4.7 in three of five ewes and decreased the proportion focused between pH 5.2 and 6.9 when compared to day 14 of a normal estrous cycle and the two uterine-specific proteins (pI 7.2 to 7.6) were not detected. Estradiol administration on days 11 and 12 of pregnancy did not alter uterine protein collected on day 14.

The present study had demonstrated that (1) the conceptus had a definite antiluteolytic capacity and was able to exert that capacity between days 11 and 13 of pregnancy, (2) the majority of proteins in uterine lumenal fluid were present also in blood serum, and (3) progesterone influenced the presence of proteins in uterine fluid.

CORPUS LUTEUM FUNCTION AND UTERINE LUMENAL FLUID PROTEIN IN EWES DURING THE ESTROUS CYCLE AND EARLY PREGNANCY

By

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INTRODUCTION

In the ewe, normal regression of the corpus luteum during the late luteal phase of an estrous cycle requires the presence of the uterus. Evidence accumulated from many studies indicates that a factor(s) emanates from the uterus, transverses a local venoarterial pathway to the ovary and causes the demise of the corpus luteum. Much of this evidence would suggest that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the uterine luteolysin in the sheep (Goding, 1974).

Presence of an embryo in the ovine uterus on day 12 after estrus results in maintenance rather than regression of the corpus luteum. The mechanism by which the conceptus prolongs luteal life may be through support of the corpus luteum (luteotropic effect) and/or through protection of the corpus luteum (antiluteolytic effect).

In the ewe, premature luteal regression can be induced by daily injection of estradiol after day 8 of an estrous cycle or by administration of $PGF_{2\alpha}$. Estradiol's luteolytic effect can be blocked by administration of gonadotropins and hysterectomy. The luteolytic effect of exogenous $PGF_{2\alpha}$ can be blocked by gonadotropins and pregnancy.

Estradiol-induced luteolysis is probably mediated through release of $PGF_{2\alpha}$ from the uterus. Estradiol has been implicated

in a similar role in luteolysis during the late luteal phase of an estrous cycle. The luteolytic effect of exogenous $PGF_{2\alpha}$ can be blocked by pregnancy, however, assessment of the antiluteolytic capacity of the conceptus during an estrogen mediated luteolytic challenge has not been reported.

The first objective of the present study was to assess the antiluteolytic action of the conceptus when pregnant ewes were given doses of estradiol capable of causing premature regression of the corpus luteum in cycling ewes.

The present study also involved investigation of the means by which the conceptus was capable of preventing luteolysis. Literature reports had indicated the factor involved was a protein and was active prior to day 13 after mating. This factor may be produced by the conceptus or by the uterus in response to the presence of the conceptus. It was assumed that this protein factor would be in the fluid secreted into the lumen of the uterus because: (1) if the conceptus was flushed from the uterus, the factor was also removed and was contained in a homogenate of the embryo; and, (2) if the factor was produced by the embryo, it must pass through the lumenal fluid to leave the uterine lumen since placental attachment does not begin until day 15 in the sheep.

Since adequate preliminary information was not available, the present study was to describe the uterine lumenal fluid protein in the ewe. Specifically, the objectives were to determine if proteins in uterine lumenal fluid change qualitatively or quantitatively

during the estrous cycle, and to determine the contribution of the conceptus to uterine lumenal fluid protein.

REVIEW OF LITERATURE

Uterine Influence on Corpus Luteum Function in Sheep

Complete hysterectomy between days 3 and 8 of an estrous cycle in ewes prolonged the life-span of the corpus luteum to the termination of the experiment at 100 days after pre-surgery estrus (Wiltbank and Casida, 1956). Kiracofe and Spies (1966) observed luteal maintenance in hysterectomized ewes for at least 160 days; however, corpora lutea regressed within the next 40 days and new ovulations were observed at 200 days after the pre-operation estrus. Hysterectomy on day 15 of an estrous cycle interrupted luteal regression provided the luteal tissue was still capable of progesterone secretion (Moor et al., 1970).

The corpus luteum was prolonged to at least 34 days in five of six ewes after removal of the uterine horn ipsilateral to the ovary containing the corpus luteum on day 6 of an estrous cycle; the sixth ewe was observed in estrus on day 24 (Inskeep and Butcher, 1966). Moor and Rowson (1966b) observed that on day 8 of a cycle unilateral hysterectomy ipsilateral to the ovary containing the corpus luteum lengthened 22 of 31 observed estrous cycles. Removal of the uterine horn contralateral to the corpus luteum did not effect cycle length (Inskeep and Butcher, 1966; Moor and Rowson, 1966b).

Pathway of Uterine Luteolysin to Ovary

Bilateral ligation of uterine arteries and veins prior to day 7 of an estrous cycle prolonged luteal maintenance to at least 25 days after pretreatment estrus in seven of eight ewes (Kiracofe et al., 1966). However, ligation of uterine arteries alone did not affect cycle length.

On days 7 to 9 of an estrous cycle Ginther et al. (1973) anastomosed the uterine vein from the intact side of unilaterally hysterectomized ewes to the uterine vein on the operated side and observed luteal regression on the hysterectomized side in three ewes. Also, anastomosis of the ovarian artery on the intact side to the ovarian artery on the hysterectomized side decreased corpus luteum weight in six ewes. These data indicated that the uterine vein and ovarian artery were the proximal and distal components, respectively, of a local venoarterial pathway between the uterine horn and its adjacent ovary.

Reconstituted uterine venous plasma from day 14 of an estrous cycle decreased plasma progesterone 50 percent within six to eight hours when infused into the ovarian artery of seven recipient ewes (Caldwell and Moor, 1971); jugular venous plasma from day 14 or uterine venous plasma from day 8 had no effect. In cross circulation experiments, McCracken et al. (1972) reported that uterine venous blood from donors on day 15 of an estrous cycle decreased plasma progesterone more than 50 percent in five ewes with ovarian transplants on the neck. Progesterone decreased less than 20 percent when recipients received uterine venous blood from donors on days

2, 6, 10 or 13 of an estrous cycle. Baird et al. (1973) observed a 33 percent decrease in progesterone secretion from seven ovarian transplants upon infusion of uterine venous plasma collected on days 14, 15 and 16 of an estrous cycle, whereas, plasma collected on days 9, 10 and 11 decreased progesterone secretion only 10 percent and infusion of jugular venous plasma from day 14 had no effect on progesterone secretion.

The ovarian artery lies adjacent to the uterine vein and this contact area is increased by the tortuous path of the artery over the surface of the vein (Del Campo and Ginther, 1973a; 1973b). Coudert at al. (1974a) found that this contact between the ovarian artery and uterine vein allowed diffusion of xenon from the vein to the artery, but there was no direct shunt to allow transfer of proteins (human serum albumen).

When the ovarian artery and uterine vein were dissected on day 2 or 3 of an estrous cycle and folds of peritoneum interposed between them, the corpus luteum was maintained until day 25 (Barrett et al., 1971). As further evidence of counter-current exchange, McCracken et al. (1972) reported increased $^{3}H\text{-}PGF_{2\alpha}$ in the ovarian artery within 20 minutes after the start of a one hour infusion of $^{3}H\text{-}PGF_{2\alpha}$ into the uterine vein. Radioactive $PGF_{2\alpha}$ peaked in the ovarian artery after 80 to 90 minutes and this transfer accounted for approximately two percent of the total $^{3}H\text{-}PGF_{2\alpha}$ infused. Land et al. (1976) also observed increased levels of $^{3}H\text{-}PGF_{2\alpha}$ in the ovarian artery within 40 minutes of the start of a uterine vein infusion.

However, Coudert et al. (1974b) were unable to detect transfer of $^{3}H\text{-}PGF_{2\alpha}$ from the uterine vein to ovarian artery within 80 minutes after 11 ewes were infused for 40 to 60 minutes with $^{3}H\text{-}PGF_{2\alpha}$ into a uterine vein.

Prostaglandin $F_{2\alpha}$ Induced Luteolysis

Luteolysis has been reported after administration of $PGF_{2\alpha}$ in various doses and via several routes. Plasma progesterone gradually decreased in four ewes infused (ovarian artery) with $PGF_{2\alpha}$ at the rate of 2 µg/hr for 9.5 to 18 hours, however only one ewe exhibited estrus (Chamley et al., 1972). Increased rate of infusion (10 to 110 µg $PGF_{2\alpha}$/hr for three to seven hours) decreased progesterone secretion to at least 30 percent of control values within 24 hours or less from the start of infusion and induced estrus behavior within 48 to 72 hours (McCracken et al., 1970; Barrett et al., 1971; Thorburn and Nicol, 1971; Chamley et al., 1972; McCracken et al., 1972).

Uterine vein $PGF_{2\alpha}$ infusion required doses of 20 µg/hr for nine hours (Goding et al., 1972) or 40 µg/hr for six hours (Thorburn and Nicol, 1971) before a consistent decrease in progesterone secretion and estrus were observed.

Infusion of 200 µg/hr $PGF_{2\alpha}$ for three hours into the uterine horn ipsilateral to the corpus luteum caused luteolysis, estrus and ovulation in one ewe, while 50 µg/hr for nine hours resulted in luteolysis in one of two ewes with no signs of estrus

(Goding et al., 1972). Douglas and Ginther (1973) reported reduced corpus luteum weight (96 vs 725 mg) after a 2 mg intrauterine bolus of $PGF_{2\alpha}$.

On day 8 of an estrous cycle, the minimum intramuscular dose of $PGF_{2\alpha}$ to shorten the interval from treatment to estrus was 6 mg (Douglas and Ginther, 1973).

Prostaglandin $F_{2\alpha}$ Secretion During the Estrous Cycle

Many reports describe the pattern of production and/or release of $PGF_{2\alpha}$ from the uterus during an estrous cycle in the ewe. Different methods of quantitation do not allow comparison of absolute values, however, most investigators have observed increased $PGF_{2\alpha}$ during the late luteal phase of the cycle compared to the early luteal phase. Also, some investigators have not eliminated the possible contribution of $PGF_{1\alpha}$ to reported values and have expressed their results in terms of prostaglandins of the F series (PGF).

Wilson et al. (1972) observed both increased content and concentration of $PGF_{2\alpha}$ in endometrium on day 14 of an estrous cycle compared to values found on days 3, 5 and 11.

Vena cava plasma PGF was less than 100 pg/ml until four days prior to estrus in five ewes and peaked (1260 pg/ml) 72 hours prior to estrus in four of five ewes; 2705 pg/ml was observed 84 hours prior to estrus in the fifth animal (Fitzpatrick and Sharma, 1973).

In uterine venous plasma, Bland et al. (1971) estimated 3.4 to 8.0 ng/ml $PGF_{2\alpha}$ on days 14, 15 and 16 of an estrous cycle, but

concentrations were undetectable (<2.9 ng/ml) between days 2 and 13. Thorburn et al. (1972) reported basal concentrations of less than 1.0 ng/ml PGF in uterine venous plasma between days 13 and 17 of an estrous cycle but during this period, a series of peaks of PGF (4 to 22 ng/ml) were observed with individual peak duration of less than two to three hours. Nett et al. (1976) measured uterine venous plasma between days 11 and 17 of an estrous cycle and observed 32 peaks averaging 41.3 ± 5.1 ng/ml $PGF_{2\alpha}$ per peak with basal levels of 9.6 ±.6 ng/ml.

Although variation in uterine venous plasma estradiol occurred throughout an estrous cycle, it was not until day 13 or 14 that increases in estrogen (12 to 132 pg/ml) were associated with peaks of $PGF_{2\alpha}$ (3 to 8.5 ng/ml; Barcikowski et al., 1974). Luteal regression was associated with 9 to 23 ng/ml $PGF_{2\alpha}$ peaks lasting two hours or less on day 15. Beginning on days 12 to 14 of five estrous cycles, Baird et al. (1976) observed transient increases in uterine venous plasma $PGF_{2\alpha}$ (peak height 2 to 10 ng/ml) and maximum $PGF_{2\alpha}$ (10 to 16 ng/ml) released on the day prior to onset of estrus. In most cases, estradiol increased (50 to 180 pg/ml) within six hours before each increase of $PGF_{2\alpha}$.

Evidence that endogenous $PGF_{2\alpha}$ causes luteolysis was provided by Scaramuzzi and Baird (1976). Four of six ewes failed to exhibit regular estrous cycles after active immunization against $PGF_{2\alpha}$ protein conjugates.

Prostaglandin $F_{2\alpha}$ Response to Steroid Hormone Administration

Estradiol injections (500 µg/day) on days 9 and 10 of an estrous cycle increased uterine venous plasma PGF on day 11 over control values (8.4 ± 2.4 vs 1.3 ± .4 ng/ml; Ford et al., 1975). However when 500 µg/day estradiol was given on days 4 and 5, PGF did not increase on day 6 unless progesterone was given (10 mg/day) on days 1 to 5 (2.8 ± 1.3 vs .5 ± .2 ng/ml, progesterone/estradiol treatment vs control, respectively).

Infusion of estradiol (1.0 ng/min) for six hours into the arterial blood supply of an autotransplanted uterus increased $PGF_{2\alpha}$ in the uterine vein from 1.4 to 102.2 ng/hr within 90 minutes (Barcikowski et al., 1974). Systemic infusion of a similar amount of estradiol had no effect on uterine venous $PGF_{2\alpha}$ in two ewes.

After 11 days of progesterone replacement (20 mg/day on alternate days) in ovariectomized ewes, 50 µg estradiol increased jugular venous plasma PGF from .3 to 1.0 ng/ml within 24 hours (Caldwell et al., 1972). However, if ovariectomized ewes were immunized against estradiol, the above replacement of steroid hormones had no effect on PGF; less than .05 ng/ml PGF was detected.

In ovariectomized ewes, uterine venous plasma PGF did not respond to an intra-arterial infusion of sufficient estradiol to increase plasma estradiol to 5 pg/ml (Scaramuzzi et al., 1974). After 14 days of progesterone replacement (10 mg/day), estradiol infusion increased PGF over control levels (22 to 110 ng/ml vs .1 to 3.0 ng/ml, respectively). However, Ford et al. (1975)

reported increased uterine venous plasma PGF in ovariectomized ewes within 12 hours of 12.5 μg estradiol, whether or not (10.5 ± 2.2 and 9.9 ± 3.3, respectively, vs .1 ± .1 ng/ml) the ewes were given five days of progesterone replacement (3 mg/day).

Progesterone replacement (3 mg/day) in ovariectomized ewes for five days (Ford et al., 1975) or 11 days (20 mg/day on alternate days; Caldwell et al., 1972) had no effect on uterine or jugular venous PGF, respectively. However, 10 mg/day progesterone for 14 days increased uterine venous plasma PGF from basal secretion (.1 to 3.0 ng/ml to 1.0 to 25.0 ng/ml; Scaramuzzi et al., 1974).

Estradiol-Induced Luteolysis

Daily injection of estradiol beginning after day 8 of an estrous cycle results in premature luteolysis in intact ewes. Many reports have demonstrated premature luteal regression from various doses given on different days of an estrous cycle: 500 and 750 μg/day on days 11 and 12 (Stormshak et al., 1969); 500 μg/day on days 8 to 11 (Ginther, 1970); 250 and 750 μg/day on days 9 and 10 or days 11 and 12 (Hawk and Bolt, 1970); 500 and 1000 μg/day on days 11 and 12 (Akbar et al., 1971); 100 to 1000 μg/day on days 10 and 11 (Bolt and Hawk, 1971), and 1000 μg on day 10 (Bolt et al., 1971).

Luteal regression was not observed in ewes hysterectomized five days after estrus and given 750 μg/day estradiol on days 11 and 12 (Stormshak et al., 1969). Bolt and Hawk (1975) also observed no luteal regression in ewes hysterectomized on day 9 after estrus and given 1 or 10 mg/day estradiol on days 9 and 10, 1 mg/day estradiol

on days 9 to 13, or 1 mg/day estradiol on days 9 to 24.

In six unilaterally hysterectomized ewes, Akbar et al. (1971) reported premature luteal regression from 1 mg/day estradiol given on days 11 and 12 regardless of whether the corpus luteum was adjacent or opposite the removed side (446 ± 50 vs 688 ± 61 mg, treated vs control, respectively). However, in ten unilaterally hysterectomized ewes with bilateral ovulations, estradiol reduced corpus luteum weight to a greater extent on the ovary adjacent to the intact horn.

Inhibition of Luteolysis by Gonadotropins

Daily injection of 2 mg/day ovine luteinizing hormone on days 9 to 13 blocked the luteolytic effect of 500 µg/day estradiol on days 10 and 11 (Akbar et al., 1971). Ginther (1970) observed 1500 I.U./day human chorionic gonadotropin (HCG) given with 500 µg/day estradiol from days 8 to 11 prevented premature luteal regression. Bolt et al. (1971) demonstrated HCG (750 I.U.) blocked estrogen-induced (1 mg on day 10) luteolysis if given with estradiol on day 10 or seven days earlier; a commercial pituitary preparation, Vetrophin (equivalent to 1 mg FSH and 1 mg LH; Abbott Lab., North Chicago, Ill.), prevented luteolysis if given seven days prior to estradiol but not if given at the same time as estradiol. Neither HCG nor the commercial preparation altered corpus luteum weight when administered alone.

Human chorionic gonadotropin (HCG), given on days 9 and 10 of an estrous cycle, prevented the luteolytic effect of 10 but not of 20 mg $PGF_{2\alpha}$ given on day 10 of that cycle (Bolt, 1973). The activity of endogenous $PGF_{2\alpha}$ may be blocked by continuous infusion of ovine luteinizing hormone starting prior to day 13 of an estrous cycle, since this treatment was capable of prolonging the life-span of the corpus luteum to day 30 after estrus (Karsch et al., 1971).

Luteal Maintenance Prolonged by Embryo

Histologically, Deane et al. (1966) observed early signs of luteal regression in luteal cells on day 12 or 13 of an estrous cycle. However, corpus luteum maintenance is prolonged if an embryo is present in the uterus between days 12 and 13. Transfer of day 12, 13 and 14 embryos to recipients on respective days of an estrous cycle resulted in 67, 22 and zero percent of the recipients becoming pregnant, respectively (Moor and Rowson, 1964; 1966d). Failure to maintain pregnancy when transfers were on days 13 and 14 of a cycle was attributed to recipient ewes since the transfer of day 13 and 14 blastocysts to day 12 recipients results in pregnancy. Surgical removal of embryos on day 5, 7, 9 or 12 did not affect return to estrus (cycle length of 18.0 ± .3 days); removal of embryos on day 13, 14 or 15 of pregnancy extended the estrous cycle to 24.5 ± .8 days (Moor and Rowson, 1966c). Rowson and Moor (1967) prolonged the length of an estrous cycle with daily intra-uterine infusion of day 14 and 15 embryo homogenates; infusion began on day 12 and ewes received the equivalent of one embryo per day.

Following synchronous transfer of day 5 or 9 embryos to the uterine horn either ipsilateral (12 ewes) or contralateral (eight ewes) to the corpus luteum, pregnancy rate did not differ (75 and 63 percent, respectively) provided the uterus was intact (Moor and Rowson, 1966a). Luteal regression occurred in all 15 ewes after transfer of day 5 embryos to an isolated horn contralateral to the corpus luteum, while the transfer to an isolated horn ipsilateral to the corpus luteum resulted in luteal maintenance in eight of ten ewes (Moor and Rowson, 1966a).

Blood-Borne Factor of Pregnancy

Mapletoft et al. (1975) observed corpus luteum regression in all five ewes on day 20 when the uterine vein ipsilateral to the corpus luteum contained blood from only the nongravid uterine horn, whereas the corpus luteum was maintained when the ipsilateral uterine vein contained blood from a gravid horn whether or not it also contained blood from a nongravid horn (three ewes/treatment). Mapletoft et al. (1976b) demonstrated the transfer of this blood-borne factor from uterine vein to ovarian artery via a venoarterial pathway. In order to maintain the corpus luteum, arterial blood to the ovary containing the corpus luteum was required to pass through the segment of the ovarian artery in contact with the uterine vein draining the gravid horn. In unilaterally pregnant ewes with bilateral ovulations, anastomosis of the ovarian artery of the gravid horn to the ovarian artery supplying the ovary of the nongravid side prior to day 7 after estrus resulted in the prolonged maintenance of the

corpus luteum ipsilateral to the isolated nongravid horn until day 20 in four ewes (Mapletoft et al., 1976b).

Prostaglandin $F_{2\alpha}$ During Pregnancy

Barcikowski et al. (1974), in one pregnant ewe, observed suppression of peaks of $PGF_{2\alpha}$ normally occurring on days 15 and 16 of an estrous cycle but small peaks of $PGF_{2\alpha}$ were still observed on days 13 and 14.

However, Pexton et al. (1975) did not observe any differences in uterine venous PGF in pregnant and non-pregnant ewes sampled once on day 15 after estrus (5.2 ± .9 vs 7.1 ± 3.4 ng/ml, respectively). Nett et al. (1976) observed no differences in uterine venous plasma $PGF_{2\alpha}$ from pregnant and non-pregnant ewes sampled at three hour intervals between days 11 and 17 after estrus (8.1 ± .1 vs 9.1 ± .6 ng/ml, respectively). Non-pregnant ewes had more variation than pregnant ewes as reflected by more peaks of $PGF_{2\alpha}$ (32 vs 12).

Uptake by ovarian and luteal tissues did not differ between pregnant and non-pregnant ewes on day 15 after estrus, although increased blood flow (4.5-fold) exposed the pregnant ovary to more PGF (2.1 ± .5 vs .5 ± .2 ng/min.) than the ovary of the non-pregnant animal (Pexton et al., 1975).

Prostaglandin $F_{2\alpha}$ Administration During Pregnancy

Serum progesterone declined similarly in mated and non-mated ewes after $PGF_{2\alpha}$ was injected on day 12 after estrus into a follicle on the same ovary as the corpus luteum; however, within 24 hours

progesterone recovered to pretreatment concentrations in mated ewes carrying embryos on day 17 after estrus (Inskeep et al., 1975).

Infusion of 200 μg $PGF_{2\alpha}$ into the ovarian artery on day 13 after mating was luteolytic in six of seven ewes hysterectomized before day 7, however, this dose caused luteal regression in only two of seven pregnant ewes (Mapletoft et al., 1976a).

Proteins in Uterine Lumenal Fluid

A functional relationship is required between embryonic and maternal systems after fertilization. Each of these systems is composed of many compartments and are significant in several ways. One area of importance is uterine environment and its provision of nourishment and shelter for the conceptus. Prior to implantation, fluid in the uterine lumen may provide not only nutrients, but also means of regulation of embryonic development and/or maternal premonition of pregnancy.

Several early reports documented electrophoretically and immunologically the presence of proteins in uterine lumenal fluid of estrous rabbits (Stevens et al., 1964) and rats (Junge and Blandau, 1958; Albers and Castro, 1961; Ringler, 1961) that were undetectable in blood serum of the respective species.

Uterine Lumenal Fluid Proteins In The Rabbit

In the pregnant rabbit, Beier (1968b) observed uterine lumenal fluid proteins to differ from serum proteins in the prealbumen, postalbumen, β-globulin and macroglobulin electrophoretic regions. Independently, Krishnan and Daniel (1967) and Beier (1968a; 1968b)

reported the time related occurrence of a uterine-specific protein that migrated as a postalbumen. The protein was named "blastokinin" by Krishnan and Daniel (1967) and "uteroglobin" by Beier (1968a; 1968b), however, in this review, blastokinin will be used exclusively. Blastulation and onset of implantation were associated with the initial detection and diminution, respectively, of blastokinin.

Effect of Uterine Proteins on Embryonic Development.--Krishnan and Daniel (1967) demonstrated addition of uterine lumenal fluid protein to synthetic culture media increased the incidence of initiation of morulae blastulation (78 vs 4 percent). In a subsequent experiment, similar results were observed after supplementation of media with a partially purified blastokinin preparation.

Although uterine proteins may be essential for blastocyst formation in utero, the in vitro requirement was challenged by Kane and Foot (1970). Chemically defined synthetic media was sufficient for 42 percent of two- and four-cell rabbit ova to develop to the expanded blastocyst stage.

Thymidine incorporation was not affected by blastokinin in day 3, 4, or 5 rabbit embryos, but uridine incorporation was stimulated by blastokinin in day 5 blastocysts (Gulyas et al., 1969). El-Banna and Daniel (1972a) also observed increased uridine incorporation (40+ percent) in day 5 blastocysts cultured in the presence of uterine proteins.

Steroid Binding by Blastokinin.--Uterine proteins may be steroid hormone carriers. El-Banna and Daniel (1972b) reported

increased growth (150 percent) of day 5 blastocysts cultured in progesterone-containing media after addition of uterine proteins. Urzua et al. (1970) observed uterine lumenal protein to bind more progesterone on day 5 post-coitum (p.c.) than on day 1 p.c. (74 vs 5 percent). Progesterone binding on day 5 p.c. was limited to the blastokinin containing fraction and exhibited a binding constant of 5.95×10^8 (Arthur et al., 1972). Arthur et al. (1972) reported estradiol also binds to blastokinin, but to a lesser extent than progesterone (binding constant 2.72×10^7).

Sites of Blastokinin Production.--Johnson (1972) suggested on the basis of immunofluorescence experiments that blastokinin was synthesized in the cytoplasm of uterine crypt epithelial cells. Maximum intensity of the fluorescence was observed between days 3 and 7 of pregnancy. During that period, immunofluorescence was associated with the surface villous endometrial epithelium as well as the supranuclear and paranuclear cytoplasm of crypt epithelial cells and crypt lumens (Johnson, 1972; Kirchner, 1972). In estrous rabbits, a faint immunofluorescence was confined to the uterine crypt areas (Johnson, 1972).

Passive Immunity to Blastokinin.--Requirement of blastokinin during early pregnancy was affirmed by passive immunity. Krishnan (1971) observed a reduction of implantation sites on day 11 (8.4 vs 1.4 sites per rabbit) after three alternate day injections (days 2 to 6) of IgG fraction from antiserum against blastokinin. Anti-blastokinin injections reduced offspring from 6.7 to .7 per female.

Occurrence of Blastokinin.--Refined techniques allowed Bullock and Connell (1973) and Mayol and Longenecker (1974) to observe a trace of blastokinin in uterine lumenal fluid of estrous rabbits. Originally, Krishnan and Daniel (1967) could not detect the protein until day 3 p.c. Maximum amounts of blastokinin were recovered from the uterine lumen on day 5 p.c. and by day 9 p.c., the protein was undetectable (Krishnan and Daniel, 1967; Urzua et al., 1970; Bullock and Connell, 1973; Mayol and Longenecker, 1974) except by the sensitive radioimmunoassay technique of Mayol and Longenecker (1974). By this technique, blastokinin was observed in very low amounts in uterine lumenal fluid until at least day 12 p.c.

Urzua et al. (1970) reported a secretion pattern in pseudopregnant rabbits similar to that observed in pregnant animals. Krishnan and Daniel (1967) and Beier (1968b) detected blastokinin in pseudopregnant rabbits on days 6 and 7, respectively, the only days investigated in those studies.

On day 6 of pregnancy and pseudopregnancy, 8.0 and 3.2 mg of total protein, respectively, was recovered from the uterine lumen. Blastokinin made up 22 and 32 percent, respectively, of the protein (Beier, 1968b). Arthur and Daniel (1972) observed uterine lumenal fluid protein to be 40 percent blastokinin in pregnant animals on day 5 p.c.

Steroid Control of Blastokinin.--After progesterone replacement, blastokinin and other uterine-specific proteins could be detected in considerable amounts in lumenal fluid of ovariectomized

rabbits; and after sufficient replacement, protein profiles were essentially identical to intact day 5 p.c. rabbits. Arthur and Daniel (1972) observed .5 mg/kg/day progesterone for four days was the minimum dose required to elicit a blastokinin response. A dose of 1.0 mg/kg/day progesterone doubled (as a percentage of total uterine protein) blastokinin response and appeared to effect a maximum dose response. In ovariectomized rabbits, total uterine lumenal protein was 32 percent blastokinin after five days of progesterone (1 mg/kg/day) replacement (Bullock and Willen, 1974). Prior exposure to estradiol was not required to achieve a blastokinin response to progesterone replacement (Urzua et al., 1970; Arthur and Daniel, 1972; Bullock and Willen, 1974).

Blastokinin could not be detected in ovariectomized rabbits after estradiol replacement (Urzua et al., 1970; Arthur and Daniel, 1972).

Uterine Lumenal Fluid Proteins in the Pig

Murray et al. (1972) observed no difference in amount (<6 mg) of uterine lumenal fluid protein recovered between days 2 and 9 of an estrous cycle. Total uterine protein increased to 14 ± 3 mg by day 12 and to 45 ± 8 mg by day 15. Thereafter, secretion decreased rapidly and values on day 18 were similar to those prior to day 9 of an estrous cycle.

On day 15, five fractions were eluted by gel filtration chromatography of uterine lumenal fluid protein (Murray et al., 1972). Three of these protein fractions (I, MW<200,000;

II, MW ≅ 200,000; and, III, MW ≅ 90,000) were observed throughout the estrous cycle. Fraction IV (MW ≅ 45,000) was observed in uterine fluid only on days 12 to 16; and fraction V (MW ≅ 20,000) was detected on days 9 to 16.

The primary component of fraction IV was demonstrated to be a basic purple-colored protein which migrated toward the cathode (-) at pH 8.0 (Squire et al., 1972). On days 14 to 16, fraction V was composed of six proteins which migrated in the postalbumen and posttransferrin electrophoretic regions.

Progesterone Control.--Qualitative and quantitative changes in uterine lumenal fluid proteins are influenced by progesterone. Knight et al. (1973) reported an increase in total uterine protein (13.7 vs 77.5 mg) from ovariectomized gilts after 12 days of progesterone replacement (2.2 mg/kg/day). Estradiol (1.1 µg/kg/day) in addition to progesterone resulted in 167 mg uterine protein; estradiol alone had no effect. After 12 days of progesterone replacement, fractions I, II, III, IV and V were present in uterine lumenal protein from ovariectomized gilts, whether or not estradiol was also given (Knight et al., 1973). Uterine flushing from non-treated ovariectomized gilts consisted of fractions I, II and III; and estradiol replacement had no effect of the protein profile.

Knight et al. (1973) observed only fractions I, II and III after four days of progesterone (2.2 mg/kg/day) in ovariectomized gilts; after six days of progesterone, fraction IV was detected and after ten days of replacement, all five fractions were observed. Knight et al. (1974) reported a correlation of .88 between total

uterine lumenal fluid protein and amount of progesterone replacement (0 to 3000 mg/45.4 kg/day) in ovariectomized gilts.

Purple-Colored Protein.--Uterine lumenal fluid collected between days 12 and 16 of an estrous cycle had a purple color (Murray et al., 1972; Squire et al., 1972). This color was attributed to a basic protein (Squire et al., 1972) eluted as fraction IV during gel filtration chromatography (Murray et al., 1972) which comprised approximately 15 percent of the total uterine protein on day 15 of an estrous cycle and was not detected in serum (Chen et al., 1973). Chen et al. (1973) further characterized the protein as a 32,000 MW glycoprotein (12.5 percent carbohydrate) with an isoelectric point of 9.7. Schlosnagle et al. (1974) associated acid phosphatase activity with this protein.

Sites of Purple-Colored Protein Production.--Based on immunofluorescence experiments, Chen et al. (1975) suggested the purple-colored protein was synthesized and secreted by uterine surface epithelial and glandular epithelial cells. Although activity was observed throughout the estrous cycle, fluorescence increased after day 9. Between days 12 and 15, fluorescence was also present in the lumen of uterine glands. During pregnancy, fluorescence was detected in placental arealae, as well as the above tissues.

The purple-colored protein was immunologically detected in allantoic fluid only after day 30 of gestation (Chen et al., 1973). Bazer et al. (1975) demonstrated the presence of a protein of

identical physical and chemical properties as the purple-colored protein in allantoic fluid collected on days 30 to 100 of gestation.

Passive Immunity to Purple Protein.--Chen and Bazer (1973) passively immunized gilts with repeated injections of antiserum against the purple-colored protein on days 7, 11, 13 and 15 of gestation and observed decreased placental length and allantoic fluid protein concentrations on day 30. Injection of antiserum on days 34, 36, 38, 40 and 42 of gestation resulted in decreased placental length and weight, fetal wet weight and crown-rump length on day 50.

Uterine Lumenal Fluid Proteins in the Cow

In uterine lumenal fluid, Palubicki and Hunter (1974) detected three antigens not present in serum; these proteins were observed throughout the estrous cycle. One of these proteins migrated electrophoretically as an α_2-globulin and the other two as β-globulins. Mills et al. (1973) also detected a uterine-specific protein which migrated as a β-globulin on days 15 and 16 of an estrous cycle. Utilizing antiserum against bovine serum, immunoelectrophoresis of uterine lumenal protein formed ten precipitant lines (Roberts and Parker, 1974; serum and anti-uterine lumenal proteins yielded only seven precipitant arcs. Roberts and Parker (1974) used two dimensional immunoelectrophoresis to detect differences between uterine protein and serum in the α- and β-globulin regions. Uterine lumenal fluid and serum proteins reacted with antiserum against bovine serum to yield 14 and 16 precipitant arcs, respectively.

Immunoelectrophoresis provided no detectable differences in uterine lumenal fluid protein from pregnant and non-pregnant cows (Roberts and Parker, 1974).

Electrophoresis of uterine lumenal proteins at pH 4.5 revealed the transient appearance of three proteins which migrated more rapidly than albumen (Roberts and Parker, 1974). The slowest migrating of the three was detected on day 7 of pregnancy but not on day 14. The two other bands were observed on day 14, but not on day 20 of pregnancy. On day 20, a protein was observed with mobility similar to the protein on day 7. These proteins were not detected on day 14 of an estrous cycle.

Of the 35 protein bands detected by isoelectric focusing of uterine lumenal fluid from pregnant cows on day 14, all but two (pI 7.5 to 8.5) were also present in serum (Roberts and Parker, 1974).

Uterine Lumenal Fluid Protein in the Ewe

Lumenal fluid protein collected via cannulation did not differ in electrophoretic profiles during estrus, diestrous or anestrous periods (Wales, 1973). Although comparable proteins were detected in both uterine lumenal fluid and serum, the α_1-globulin of lumenal protein migrated slightly ahead of serum α_1-globulin. Wales (1973) observed albumen to make up a smaller proportion of the total protein in uterine lumenal fluid than in serum ($52 \pm .7$ vs 60 ± 1.8 percent); the opposite was true of β-globulin (31 ± 1.4 vs 22 ± 1.7 percent; uterine protein vs serum, respectively).

Uterine Lumenal Fluid Protein in Primates

Human.--Bernstein et al. (1971) detected two uterine-specific proteins in lumenal fluid by immunoelectrophoresis; however, the samples had been collected at random throughout the menstrual cycle. Shirai et al. (1972) observed a uterine-specific protein migrating between albumen and transferrin in uterine lumenal protein collected six to ten days after the rise in basal body temperature. Also during that time period, protein bands in the α-globulin region were more prominent than those in samples collected either immediately before or after that period.

Daniel (1973) reported a component of uterine lumenal fluid collected eight to nine days after ovulation had the immunological and electrophoretic characteristics of blastokinin.

Baboon.--Peplow et al. (1973) detected 18 protein bands by electrophoresis of uterine lumenal fluid protein; all but one which migrated as a prealbumen were also present in plasma. Uterine fluid collected during the proliferative or secretory phases of the menstrual cycle did not differ in number of protein bands detected; however, band intensity did differ with cycle phases as well as between uterine protein and serum. Two dimensional immunoelectrophoresis of uterine lumenal proteins yielded nine precipitant arcs with antiserum against uterine lumenal protein (Peplow et al., 1974).

Uterine Lumenal Fluid Protein in Other Species

Northern Fur Seal.--Daniel (1971) reported total uterine lumenal protein increased from less than 2 mg during the period of delayed implantation to 10 mg during the time associated with expansion of the blastocyst. An additional increase (22 mg) was observed at the time of implantation. Uterine lumenal proteins migrated electrophoretically similar to albumen and blastokinin as well as several other less well-defined protein components; however, blastokinin could not be detected immunologically (Daniel, 1972).

Golden Hamster.--Noske and Daniel (1974) observed total uterine lumenal protein to peak on day 4 of pregnancy, the day of implantation (.117 ± .046 vs .055 ± .031 mg day 4 vs day 3, respectively). During the first six days of pregnancy, albumen, transferrin and two β-globulins were detected in uterine lumenal fluid. On day 3 of pregnancy, an α-globulin band was first detected and by day 6, that region resembled serum with four bands. A prealbumen was detected after day 3 of pregnancy that was not present in serum.

Ferret.--Daniel (1970) observed total uterine lumenal protein to increase at the time of blastocyst expansion (<100 μg vs 225 μg, day 0 vs day 9, respectively) and increase again at the time of implantation (2150 μg, day 16). By electrophoretic techniques, albumen was obvious in all samples but resolution of the other proteins was poor.

MATERIALS AND METHODS

Multiparous crossbred ewes confined to a dry-lot management system were used throughout the study. A mixture of grease and paint was applied to the brisket of a vasectomized ram which was allowed continuous access to the ewes. Paint markings on the rump of a ewe was considered a sign of estrus; the flock was observed twice daily (0700 and 1700 hours) and fresh markings recorded. Ewes destined to be mated to intact rams were separated from the flock when marked and pen-mated to two intact rams, once at the first observance of the paint mark and again, if still receptive, at the next estrous check. The ewe was then returned to the flock. In the present study, sample collection was extended over a three year period (Experiment I, Fall 1973; Experiments II and IV, Fall 1974; and Experiments III, V, VI and VII, Fall 1975).

Experiment I

Ewes mated (estrus = day 0) to intact rams were given (im) 250 μg 17β-estradiol in oil per day on days 11 and 12 (six ewes) or days 12 and 13 (six ewes). Control ewes mated to a vasectomized ram were given either zero (four ewes) or 250 μg estradiol per day on days 11 and 12 (five ewes) or days 12 and 13 (six ewes). Blood was collected from each ewe via jugular puncture on days 11 to 18

to monitor corpus luteum function by radioimmunoassay of serum progesterone (Louis et al., 1973; Appendix A). On days of estradiol injections, blood was collected immediately before estrogen was given.

Experiment II

Sixteen ewes mated to intact rams and eight ewes mated to a vasectomized ram were given either 125 or 250 μg estradiol on days 11 and 12. Blood samples were collected on days 10 to 16 for assay of serum progesterone. On day 16, all ewes were sacrificed, and their uteri excised and flushed with saline to ascertain pregnancy by the presence of embryonic tissue.

Experiment III

Twenty-two ewes were mated to intact rams; then 17 of these were given 125 μg estradiol (im) on days 11 and 12. Ten control ewes were mated to a vasectomized ram and five of these ewes received 125 μg estradiol on days 11 and 12. Blood was collected on days 10 to 20 for determination of serum progesterone. Ewes mated to intact rams were sacrificed on day 20 and their uteri excised and flushed with saline. Pregnancy was diagnosed by the presence of embryonic tissue.

Experiment IV

Fourteen ewes mated to intact rams and 13 ewes mated to a vasectomized ram were sacrificed on days 12, 13, 14 and 15 after mating (two to five ewes/day/mating group). The uterus of each ewe was excised within two to three minutes of death. The cervix and

tubo-uterine junctions of the uterus were clamped (Allis's forceps and hemostats, respectively) and the uterine lumen was filled with 20 ml of chilled .33 M NaCl (4 C). The tip of one uterine horn was removed, any incident blood sponged off and after the saline was exposed to the uterine lumen for five minutes, the flushing was drained through the opening. In ewes mated to intact rams, pregnancy was ascertained by the presence of embryonic tissue.

Cellular debris was removed from the uterine lumenal flushing by centrifugation (2500 g; 20 minutes; 4 C). An aliquant of the supernatant was removed for estimation of total recoverable protein from the uterus. The supernatant was dialyzed (Spectrapor Membrane #3; 3500 MW cutoff; Spectrum Med. Ind. Inc.) against distilled H_2O for 36 hours (sample: H_2O was $\geq$ 1:100; H_2O was changed at 12 hour intervals) and then lyophilized. The lumenal protein was then stored at -20 C until analysis, at which time the lyophilized protein was suspended in the appropriate buffer required for the technique utilized.

Uterine lumenal fluid protein (200 µg) from individual ewes was subjected to polyacrylamide disc gel electrophoresis at pH 4.5 (Reisfeld et al., 1962; Appendix B). The amount of protein recovered from the uterine lumen and later subjected to electrophoresis was determined as described by Miller (1959; Appendix C). Lumenal protein (300 µg) was also subjected to sodium dodecyl sulfate (SDS) electrophoresis (Weber and Osborn, 1969; Appendix D).

Experiment V

Fifteen ewes mated to a vasectomized ram were sacrificed on days 3, 9 and 14 of an estrous cycle (five ewes/day). Five ewes mated to intact rams were sacrificed on day 14 of pregnancy. Their uteri were excised, flushed with .33 M NaCl and centrifuged as in the previous experiment. After lyophilization, uterine lumenal protein (500 μg) from each ewe was subjected to isoelectric focusing on polyacrylamide columns (Bio-Rad Lab., Tech. Bull. 1030, 1975; Appendix E). In pregnant ewes, embryonic tissue was aspirated from the flushing before centrifugation and homogenized. The homogenate was centrifuged (10,000 g; 30 minutes) and the supernatant was dialyzed, lyophilized and subjected to a radio-receptor assay for gonadotropic activity (Saxena et al., 1974; Appendix F).

Experiment VI

Twenty ewes were ovariectomized. At least 14 days elapsed between surgery and random assignment to one of the following groups (five ewes/treatment): (1) control; (2) subcutaneous estradiol implant (sealed polydimethylsiloxane tubing, 4.65 O.D. 3.34 I.D. X 55 mm, packed with crystalline 17β-estradiol); (3) pessary impregnated with progesterone (polyurethane foam, 3.4 dia. X 2.5 cm; 1 gm progesterone); or (4) both estradiol implant and progesterone pessary. Blood was collected immediately before treatment (day 0) and during treatment on days 3, 6 and 9 for determination of serum estradiol (Britt et al., 1974; Appendix G) and progesterone (Louis et al., 1973; Appendix A). After ten days of treatment, all ewes

were sacrificed and uterine lumenal fluid protein obtained and processed as previously described. Lumenal protein was subjected to isoelectric focusing.

Experiment VII

Fifteen ewes were mated to intact rams; then ten of these were given 125 μg estradiol (im) on days 11 and 12. Ten ewes were mated to a vasectomized ram and five of these ewes received 125 μg estradiol on days 11 and 12. On day 14, all ewes were sacrificed, their uteri excised and flushed with .33 M NaCl. Pregnancy in the ewes mated to intact rams was ascertained by the presence of embryonic tissue. The flushings were centrifuged, dialyzed, lyophilized and subjected to isoelectric focusing.

Significant differences in serum progesterone concentrations and total protein recovered from the uterine lumen were determined by t-test if two means had homogenous variances or approximate t-test if variances were heterogenous (Sokal and Rohlf, 1969).

RESULTS

Experiment I

Serum progesterone concentrations in ewes given 250 μg estradiol per day on days 11 and 12 tended to decline more rapidly than in ewes treated on days 12 and 13; however, on day 14 serum progesterone did not differ between the two groups (Appendix H, Table 7; $P > .05$) and data were pooled (Figure 1). Injection of estradiol on days 11 and 12 or days 12 and 13 caused a decline in serum progesterone to $.4 \pm .1$ ng/ml on day 14 of an estrous cycle (Figure 1), lower ($P < .01$) than non-injected controls ($1.9 \pm .6$ ng/ml). Nine of 11 ewes given estradiol had $\leq .4$ ng/ml progesterone on day 14. However, estradiol treatment caused serum progesterone to decrease in only six of 12 ewes mated to intact rams (day 14, $.8 \pm .2$ vs $2.7 \pm .7$ ng/ml; Figure 2 and Appendix H, Table 8). The six ewes with elevated progesterone (≥ 2.0 ng/ml) on day 14 after mating were assumed to be pregnant, but this was not confirmed. After day 14, serum progesterone in estrogen treated ewes which were assumed to be pregnant was higher ($P < .01$) than in ewes mated to a vasectomized ram and treated with estrogen ($2.7 \pm .7$ vs $.4 \pm .1$ ng/ml, respectively). Progesterone concentrations in ewes assumed to be non-pregnant were not different from those in estradiol injected ewes mated to a vasectomized ram.

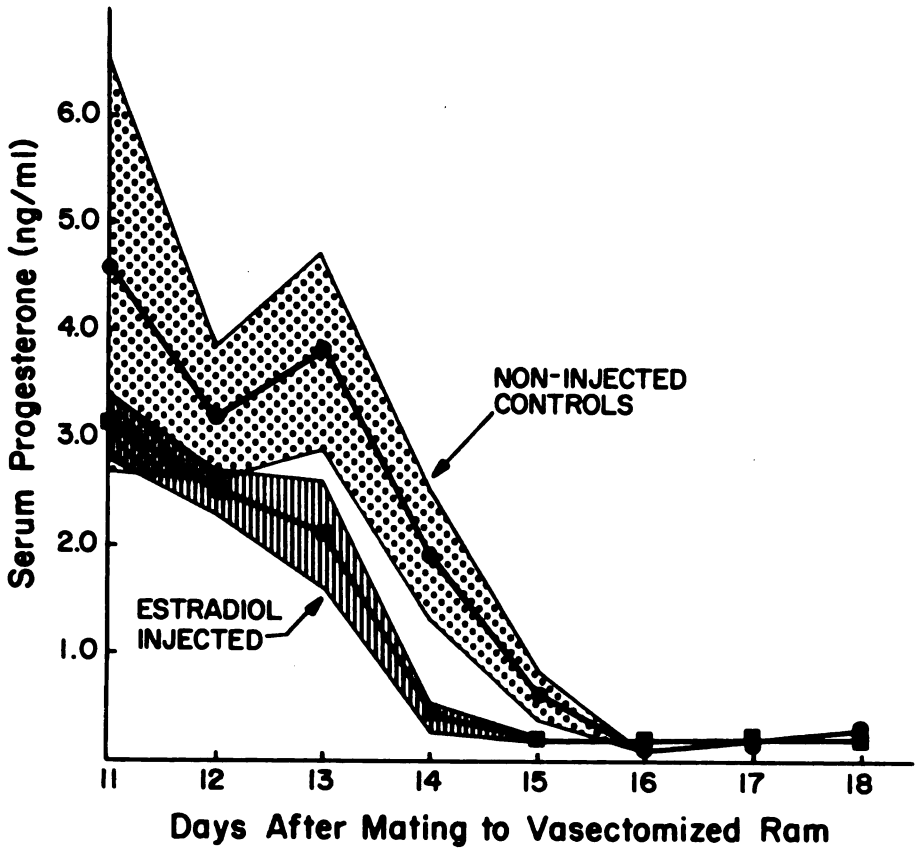

Figure 1.--Serum progesterone in ewes mated to a vasectomized ram and given 250 μg 17β-estradiol on days 11 and 12 or days 12 and 13 and in non-injected control ewes mated to a vasectomized ram (Experiment I).

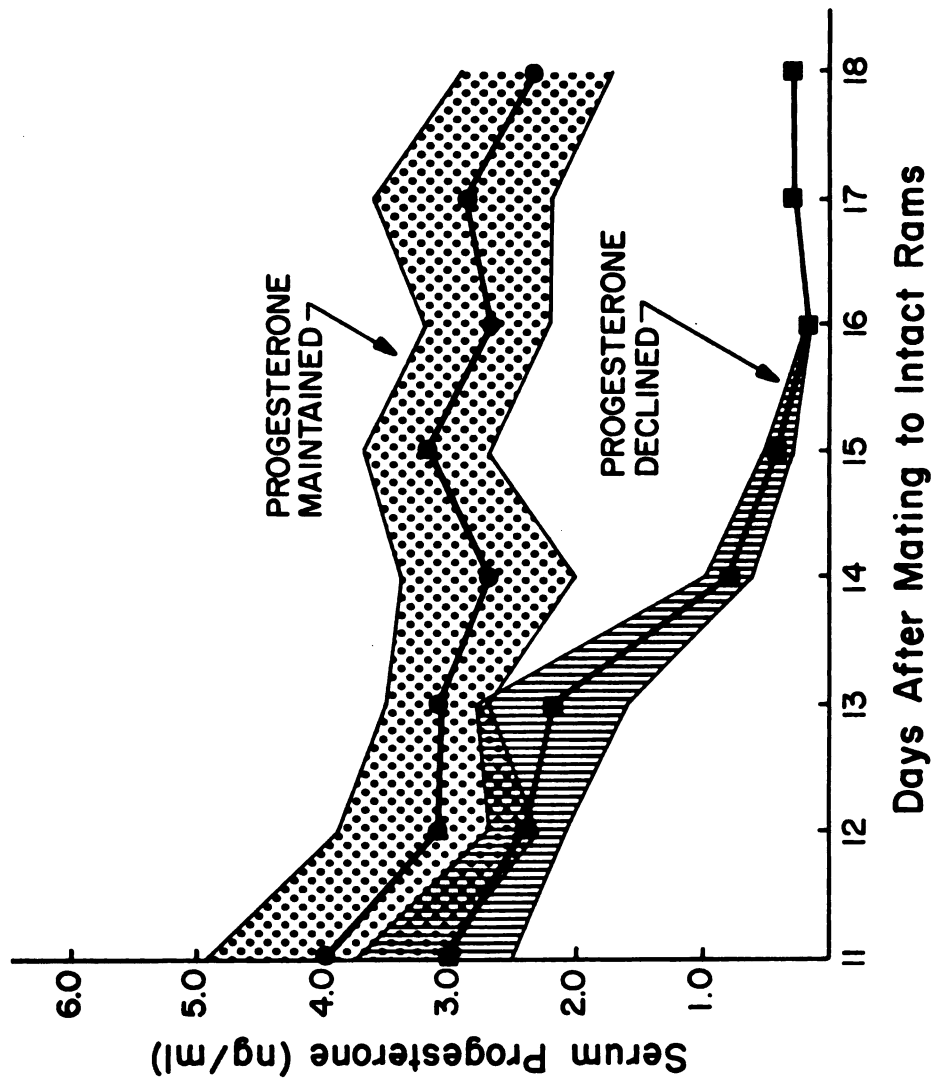

Figure 2.--Serum progesterone in ewes mated to intact rams and given 250 µg 17β-estradiol on days 11 and 12 or days 12 and 13 (Experiment I).

Experiment II

Of 16 ewes mated to intact rams and given either 125 or 250 μg estradiol per day on days 11 and 12, four of eight and one of eight, respectively, were pregnant on day 16. Six embryos were recovered from the five pregnant ewes; one ewe had two normal embryos. Two of the six recovered embryos were retarded in development. Pregnant ewes has higher ($P < .05$) serum progesterone on day 15 than non-pregnant ewes ($1.6 \pm .8$ vs $.3 \pm .1$ ng/ml; Figure 3). In the two ewes with retarded embryos, serum progesterone averaged .2 ng/ml on day 15. Serum progesterone concentrations in non-pregnant ewes did not differ from values in estrogen treated ewes mated to a vasectomized ram (Appendix H, Table 9; $P > .05$).

Concomitant with this experiment, 18 other ewes from this flock were mated to the same intact rams and all were confirmed pregnant prior to day 16, attesting to the fertility of the flock.

Experiment III

Five of 17 ewes mated to intact rams and given 125 μg estradiol on days 11 and 12 were pregnant on day 20. Pregnant ewes had higher ($P < .01$) serum progesterone on day 14 than non-pregnant ewes ($4.1 \pm .4$ vs $1.4 \pm .3$ ng/ml; Figure 4 and Appendix H, Table 10). Estradiol injections on days 11 and 12 into ewes previously mated to a vasectomized ram caused serum progesterone to decline to $1.9 \pm .4$ ng/ml by day 13, lower than in non-injected controls ($3.2 \pm .5$ ng/ml; $P < .05$; Figure 5 and Appendix H, Table 10). On day 14, four of five ewes given estradiol had $\leq .4$ ng/ml of serum progesterone. Serum progesterone concentrations in estradiol injected ewes that

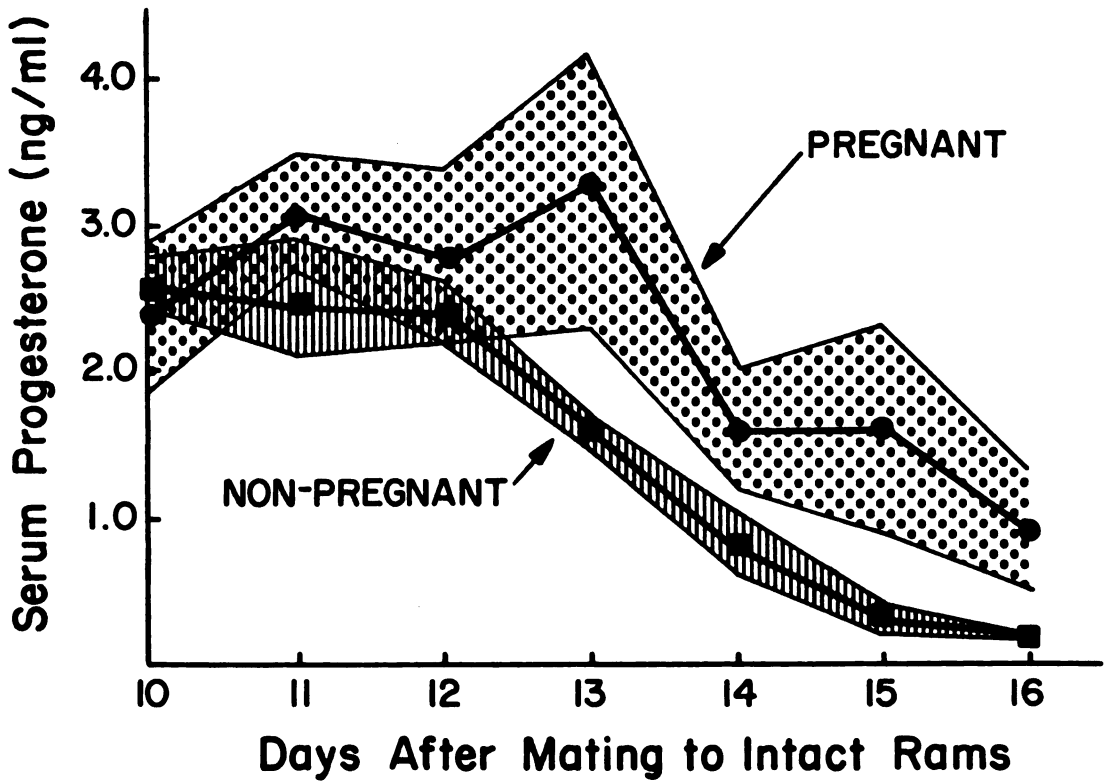

Figure 3.--Serum progesterone in ewes mated to intact rams and given 125 or 250 μg 17β-estradiol on days 11 and 12 after mating (Experiment II).

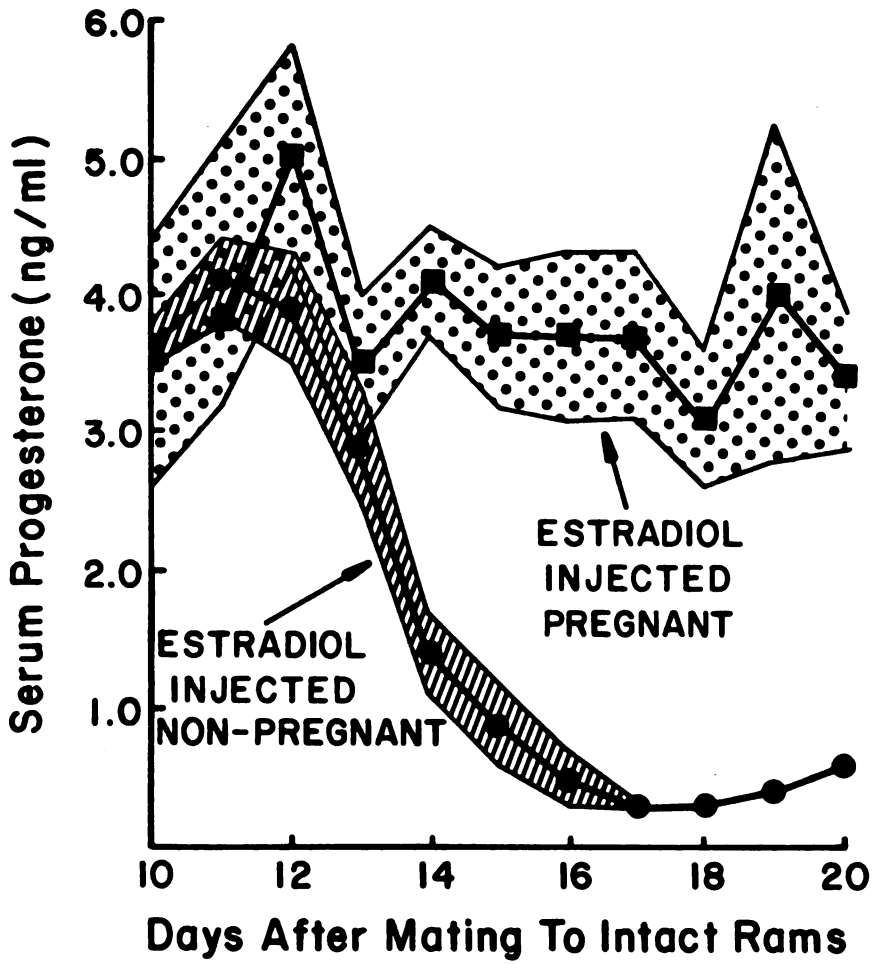

Figure 4.--Serum progesterone in ewes mated to intact rams and given 125 μg 17β-estradiol on days 11 and 12 after mating (Experiment III).

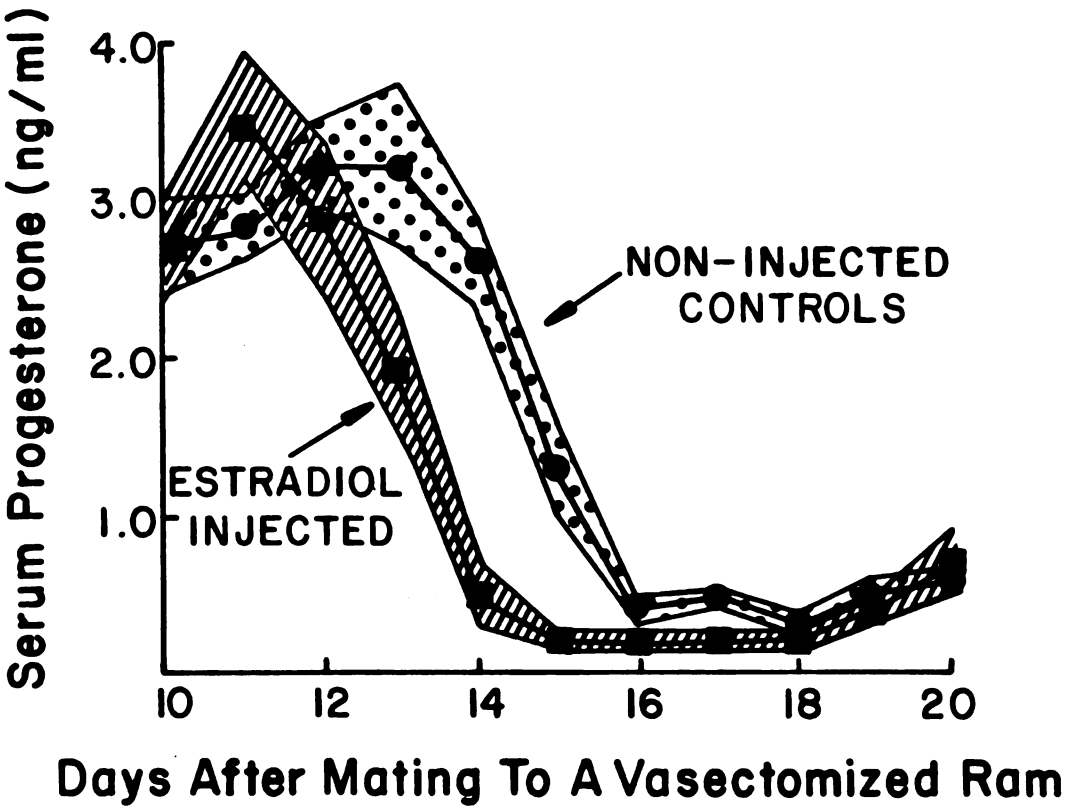

Figure 5.--Serum progesterone in ewes mated to a vasectomized ram and given either 0 or 125 μg 17β-estradiol on days 11 and 12 after mating (Experiment III).

were pregnant at autopsy on day 20 were not different from levels in pregnant ewes which had not received estradiol (Figure 6).

Fourteen ewes mated to the intact rams used in this experiment were all confirmed pregnant on or prior to day 20; these pregnant ewes were either controls in this experiment or involved in a concomitant experiment.

Experiment IV

There was no difference ($P > .05$) in amount of uterine lumenal protein recovered on each day between days 12 and 15 of an estrous cycle (Table 1). Furthermore, pregnancy had no effect on the amount of lumenal protein recovered ($P > .05$).

Table 1.--Uterine lumenal fluid protein recovered from ewes during an estrous cycle or early pregnancy (Experiment IV).

Day	Estrous Cycle		Pregnancy	
	Total Protein (mg)			
12	2.90 ± .67[a]	(3)[b]	3.64 ± .77	(4)
13	3.06 ± .75	(3)	4.29 ± .64	(3)
14	3.36 ± .46	(5)	4.76 ± .99	(4)
15	2.85	(2)	5.89 ± 2.18	(3)

[a]Mean ± S.E.

[b]Number of Observations

Beginning on day 14 of pregnancy (14P), three protein bands were detected in uterine lumenal protein that were not detected in

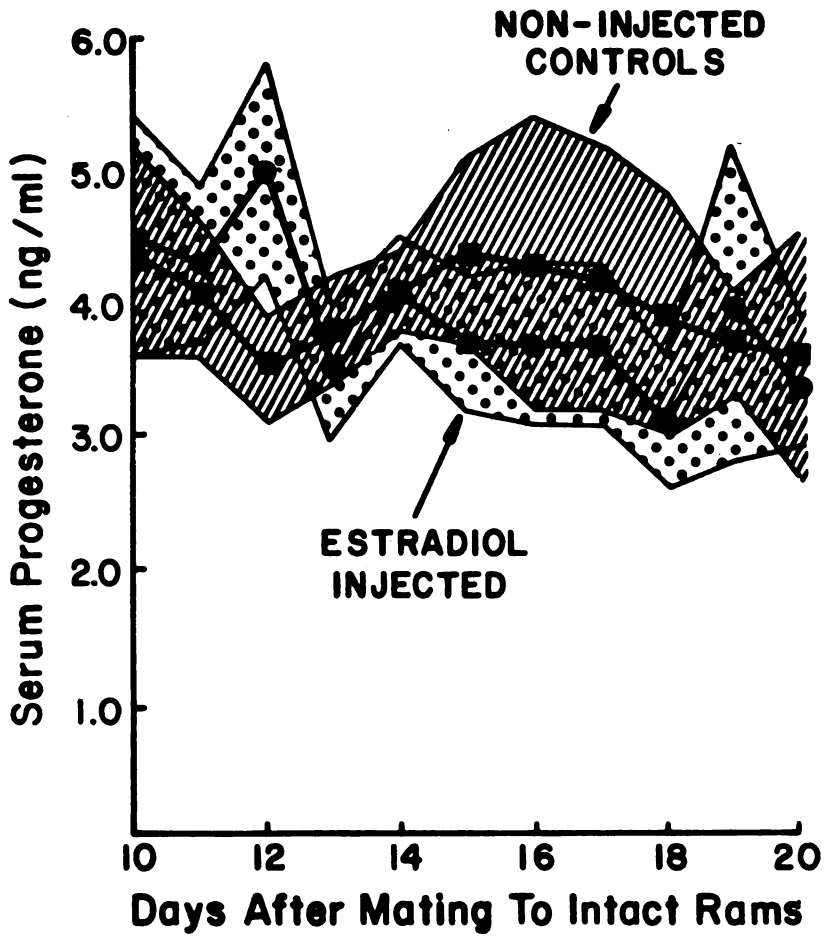

Figure 6.--Serum progesterone in ewes mated to intact rams and given either 0 or 125 μg 17β-estradiol on days 11 and 12 after mating (Experiment III).

serum (Figure 7; S). One of these protein bands (top arrow) migrated slower than albumen (A) and the other two bands (middle and bottom arrows), both of low intensity, migrated faster than albumen. Of the two bands which migrated faster than albumen, the protein band of lesser mobility was not present in lumenal fluid collected on day 14 of an estrous cycle (14C). On day 15 after estrus, a similar difference was detected between pregnant and non-pregnant ewes.

After SDS electrophoresis of uterine lumenal fluid protein collected between days 12 and 15 of an estrous cycle, the only consistent and progressive change in protein profiles was the decrease in stain intensity of a protein below 10,000 MW (Figure 8; 12C, arrow). Lower intensity in that region was observed on days 14 (14C) and 15 (15C) as compared with days 12 (12C) and 13 (13C) of an estrous cycle. Although intensities of several other protein bands varied throughout the period, the differences were related to individual animals rather than day of the cycle.

Comparison of protein profiles obtained from SDS electrophoresis of uterine fluid from pregnant and non-pregnant ewes also emphasized the small molecular weight (< 10,000 MW) protein bands (Figure 8). On day 12, a protein in that region of the gel was more intensely stained during an estrous cycle (12C; arrow) than during pregnancy (12P). Although band intensity did not differ on day 13, differences were again detected in that region on days 14 and 15; on those days, band intensity was greater in profiles of uterine fluid from pregnant ewes (14P and 15P; arrows).

Figure 7.--Electrophoresis in polyacrylamide gel at pH 4.5 of uterine lumenal fluid protein collected on day 14 of an estrous cycle and pregnancy (Experiment IV).

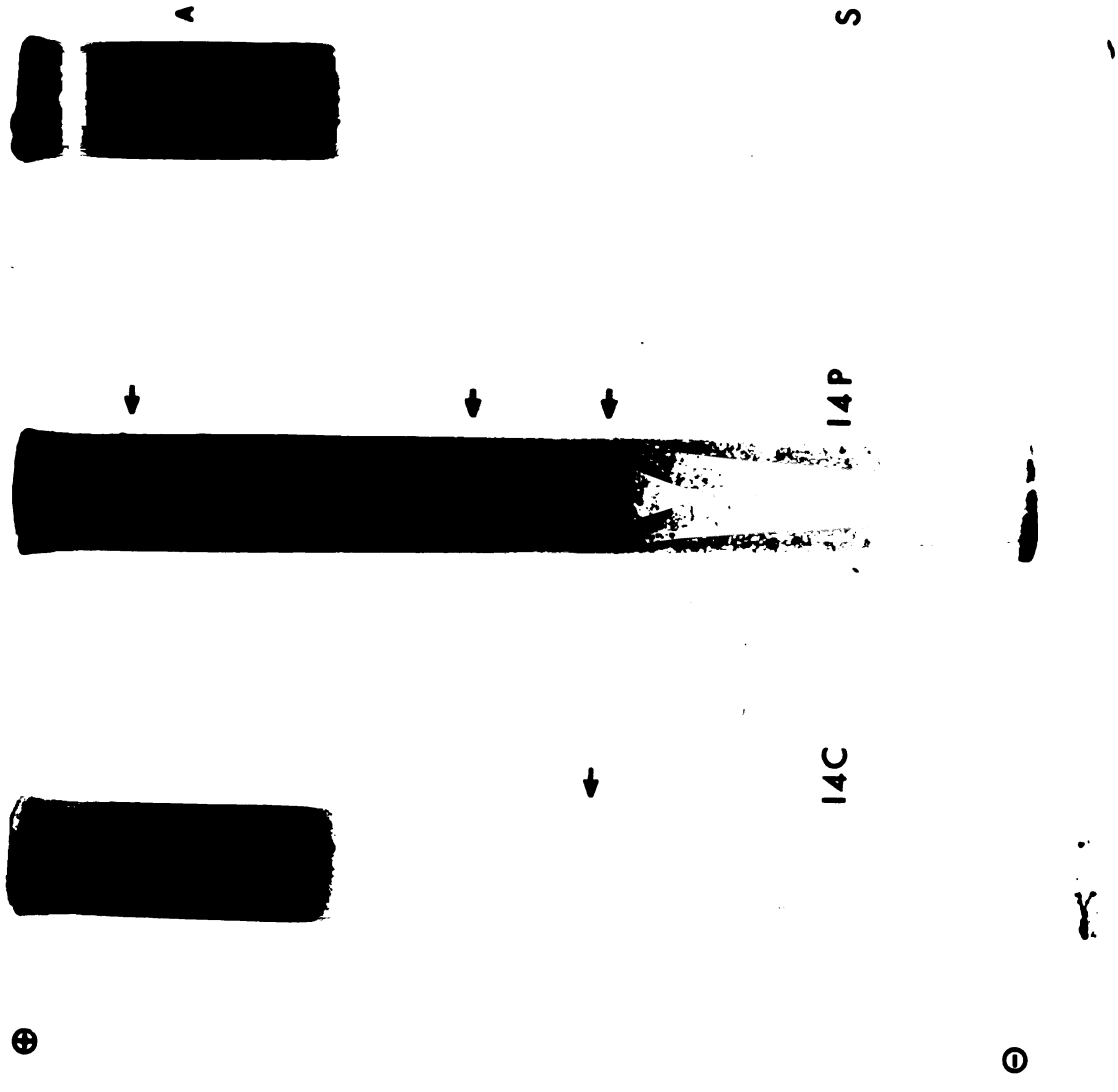
A
S
14P
14C
⊕
⊖

Figure 7.--Electrophoresis in polyacrylamide gel at pH 4.5 of uterine lumenal fluid protein collected on day 14 of an estrous cycle and pregnancy (Experiment IV).

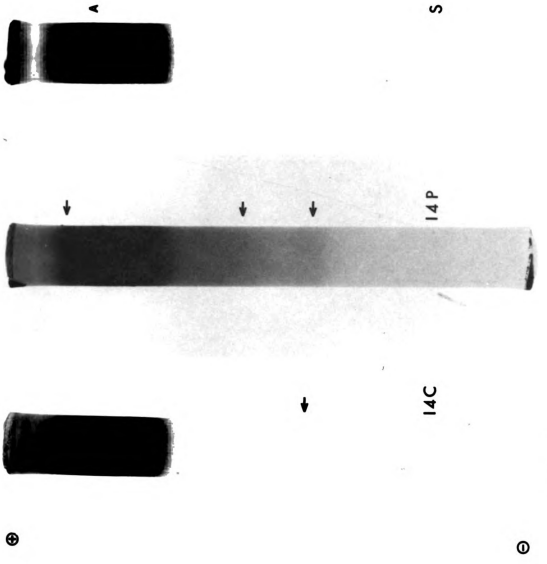
A
S
14P
14C
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Figure 8.--SDS electrophoresis of uterine lumenal fluid protein collected on days 12, 13, 14 and 15 of an estrous cycle and pregnancy (Experiment IV).

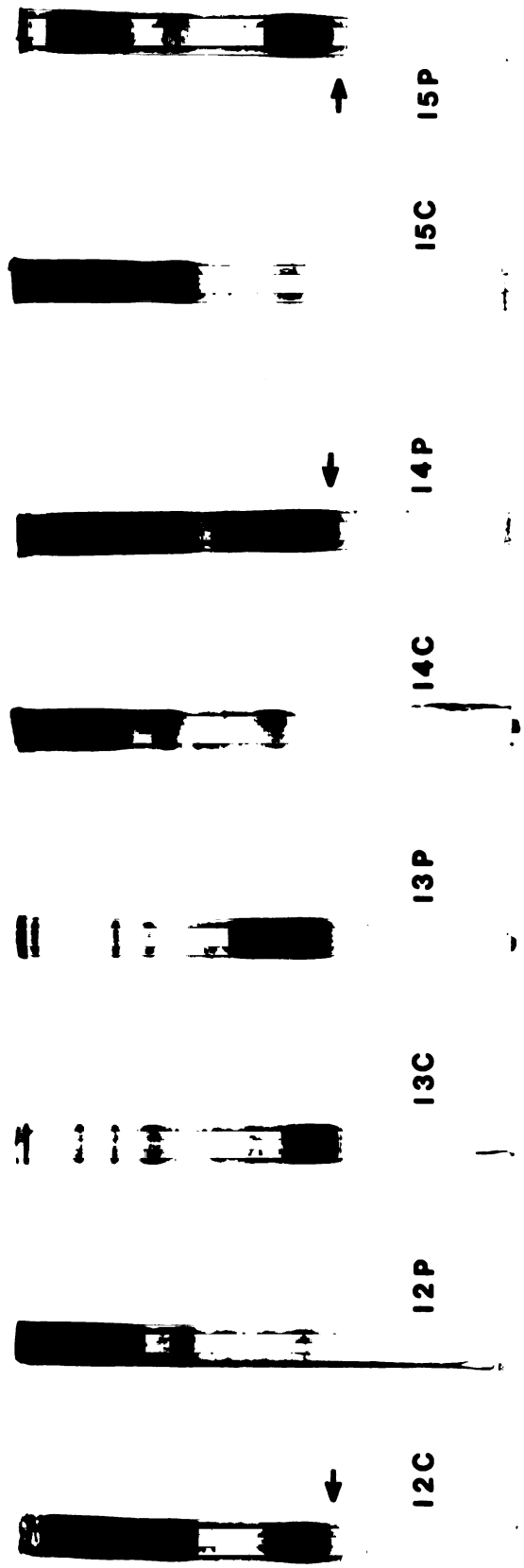
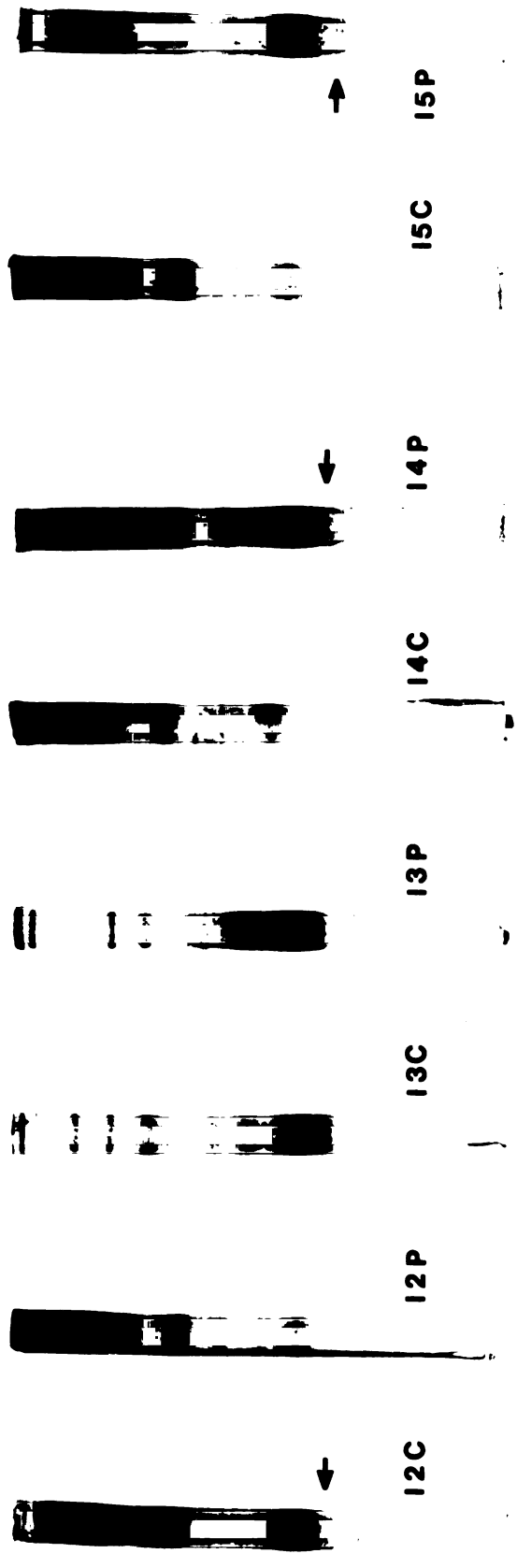

I5P
I5C
I4P
I4C
I3P
I3C
I2P
I2C

Figure 8.--SDS electrophoresis of uterine lumenal fluid protein collected on days 12, 13, 14 and 15 of an estrous cycle and pregnancy (Experiment IV).


15P
15C
14P
14C
13P
13C
12P
12C

The molecular weights of proteins in lumenal fluid ranged from approximately 66,000 to 9,500 (Figure 9). As detected by spectrophotometric scanning, each of the 13 bands of protein observed on day 14 of pregnancy migrated similarly to a comparable protein band detected on day 14 of an estrous cycle. There were few exceptions to this and those that occurred were related to individual ewes rather than reproductive status.

Experiment V

On day 3 of an estrous cycle, 1.84 ± .21 mg total protein was recovered from the uterine lumen (Table 2). Recoverable uterine protein increased to 4.97 ± 1.20 mg on day 9 ($P < .05$) and remained at that level (day 14, 5.1 ± 1.60 mg). Fourteen days of pregnancy did not increase ($P > .05$) the total amount of uterine protein recovered (6.61 ± .76 mg) as compared with day 14 of an estrous cycle.

During the course of isoelectric focusing of uterine lumenal proteins, it was necessary to replenish certain reagents. This change in reagents affected the pH gradient formed in the polyacrylamide gel (Table 3). The pH gradients used for comparison in the present study were averages of all analyses completed with a particular set of reagents.

After isoelectric focusing of uterine protein collected on day 14 (14C) of an estrous cycle, up to 35 bands of protein were detected by spectrophotometric scanning of the gels (Appendix I, Figure 14) and all but two bands (pI 7.2 to 7.6; arrow) of low intensity were also observed in serum (Figure 10; S; the left pH gradient was representative of gels 3C and 14C, the right pH

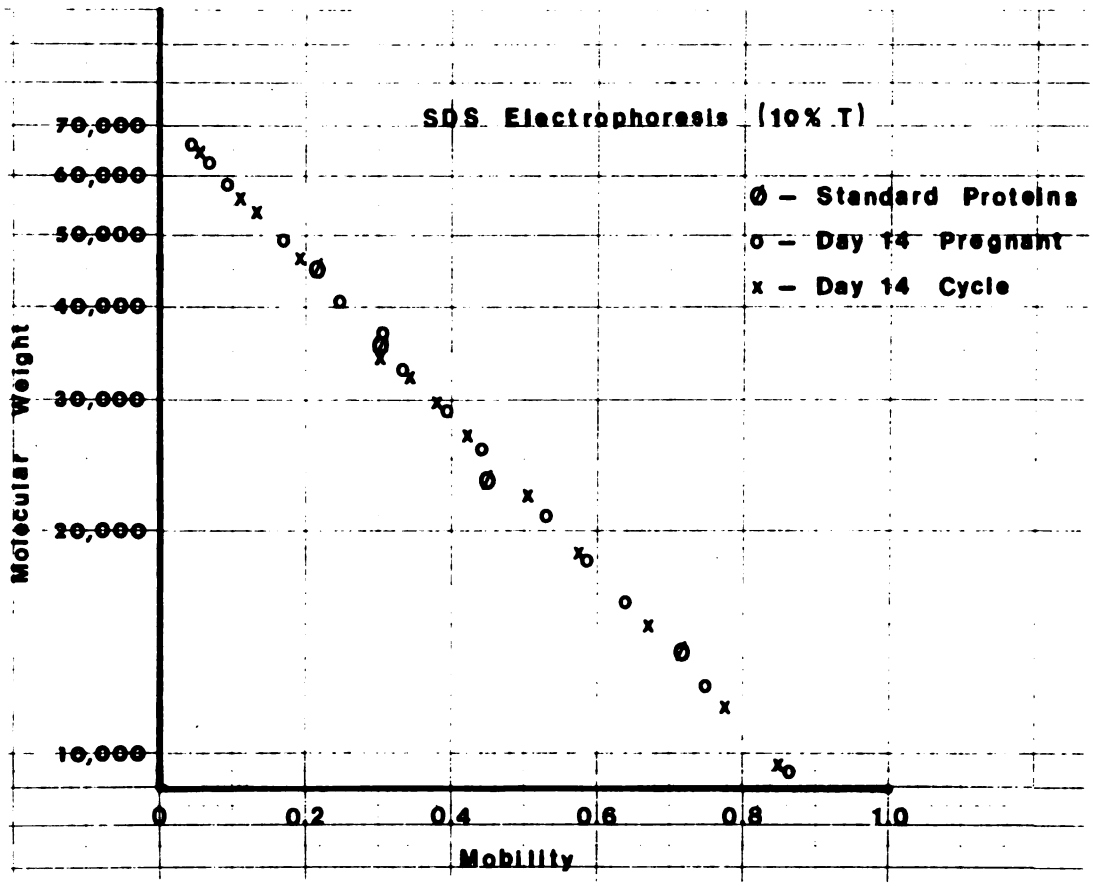


Figure 9.--Determination of molecular weight by SDS electrophoresis of uterine lumenal fluid protein collected on day 14 of an estrous cycle and pregnancy (Experiment IV).

Table 2.--Uterine lumenal fluid recovered from ewes during an estrous cycle or early pregnancy (Experiment V).

	Total Protein -mg-	
Day of Cycle		
3	1.84 ± .21[a]	(5)[b]
9	4.97 ±1.20[c]	(3)
14	5.10 ±1.60[c]	(5)
Day of Pregnancy		
14	6.61 ± .76	(5)

[a]Mean ± S.E.

[b]Number of Observations

[c]Significantly different ($P < .05$) from levels recovered on day 3 of an estrous cycle.

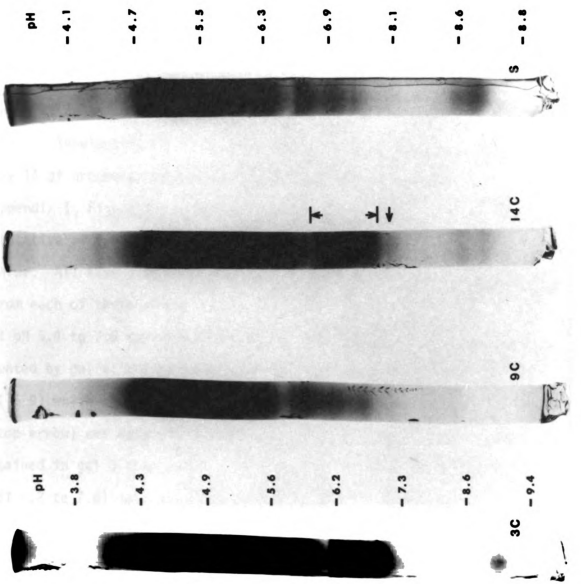
Table 3.--Gradients of pH formed during isoelectric focusing in polyacrylamide gel.

Centimeter of gel length	Reagent Set #1 pH Units	Reagent Set #2 pH Units
1	3.82 ± .06[a]	4.08 ± .06[b]
2	4.32 ± .11	4.69 ± .03
3	4.87 ± .21	5.47 ± .05
4	5.62 ± .05	6.30 ± .11
5	6.15 ± .03	6.91 ± .12
6	7.26 ± .06	8.09 ± .14
7	8.55 ± .07	8.55 ± .04
8	9.35 ± .10	8.84 ± .06

[a]Mean S.E., n = 3

[b]n = 5

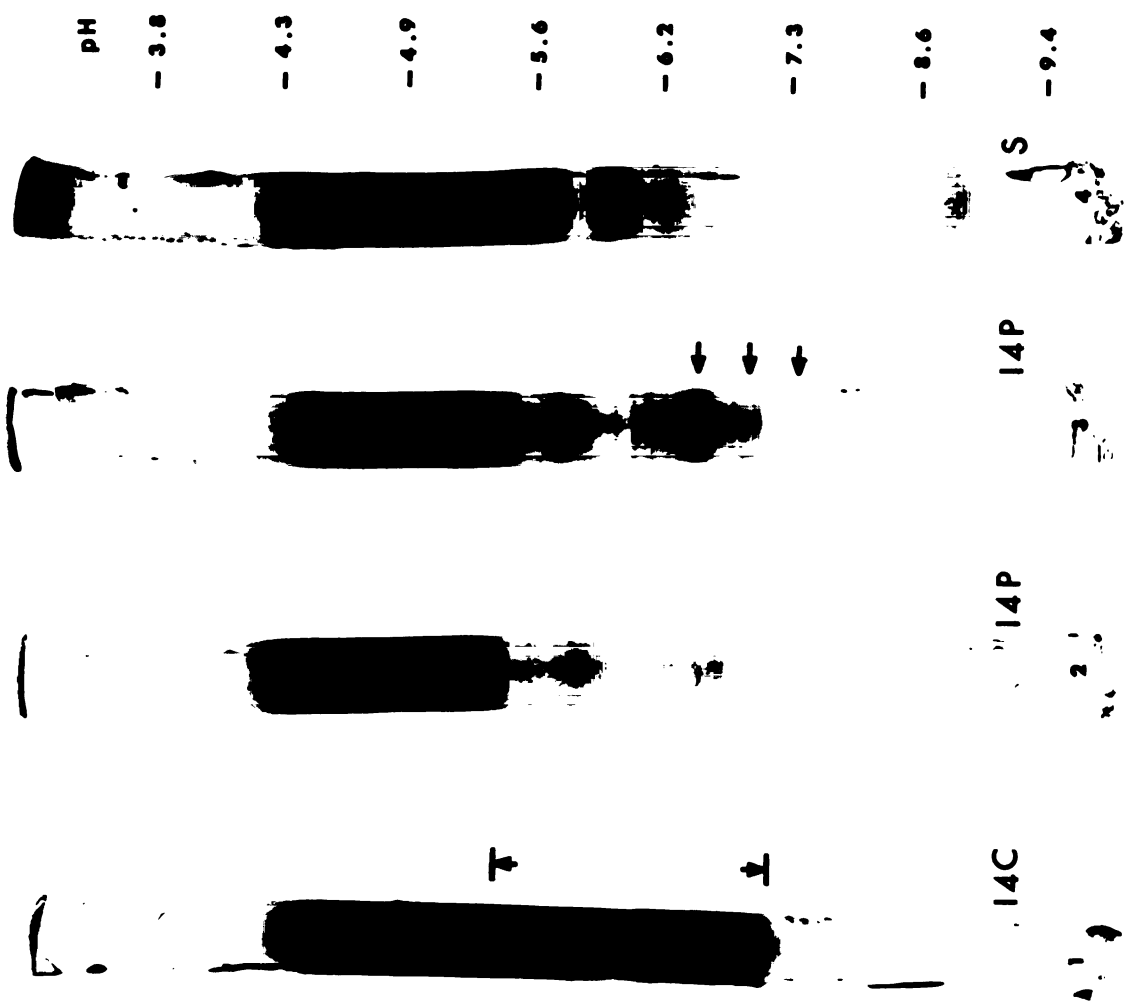
Figure 10.--Isoelectric focusing in polyacrylamide gel of uterine lumenal fluid protein collected on days 3, 9 and 14 of an estrous cycle (Experiment V).


pH
-4.1
-4.7
-5.5
-6.3
-6.9
-8.1
-8.6
-8.8
S
14C
9C
pH
-3.8
-4.3
-4.9
-5.6
-6.2
-7.3
-8.6
-9.4
3C

gradient was representative of gels 9C and S). The number of protein bands in uterine fluid collected on days 3 (3C) and 9 (9C) of an estrous cycle did not differ from day 14 with the exception of the two basic bands (pI 7.2 to 7.6). Band intensity of proteins focused at pH 6.3 to 7.3 was less on day 9 than on day 3. On day 14, protein bands in that region were of the intensity observed on day 3 of an estrous cycle.

Isoelectric focusing of uterine lumenal protein collected on day 14 of pregnancy produced two distinct profiles (Figure 11 and Appendix I, Figure 14). Of the five pregnant ewes, gel 2 was representative of two animals and gel 3 was representative of the other three. All five of these ewes had an embryo of normal size flushed from each of their respective uteri. Stain intensity of bands focused at pH 5.4 to 7.0 was greater in gel 3 than gel 2. In profiles represented by gel 3, one extra protein band was detected (middle arrow; pI 6.9) which was not present in gel 2. A protein focused at pH 6.4 (top arrow) was detected in both gels, but that area was more heavily stained in gel 3 than gel 2. The two uterine-specific protein bands (pI 7.2 to 7.6) detected on day 14 of an estrous cycle were also observed on day 14 of pregnancy in profiles represented by both gels 2 and 3 (bottom arrow), although the bands were much fainter in gel 2. Uterine lumenal proteins collected on day 14 of an estrous cycle (gel 1) differed from those collected on day 14 of pregnancy in stain intensity of proteins focused between pH 5.4 to 7.0. It was not discernible whether the extra band detected in gel 3 (Figure 11; middle arrow) was present on day 14 of an estrous cycle due to lack of

Figure 11.--Isoelectric focusing in polyacrylamide gel of uterine lumenal fluid protein collected on day 14 of an estrous cycle and pregnancy (Experiment V).


pH
3.8
4.3
4.9
5.6
6.2
7.3
8.6
9.4
S
14P
14P
14C

resolution of proteins in that region. If that difference was disregarded, uterine lumenal fluid from pregnant and non-pregnant ewes contained an equal number of detectable proteins.

Uterine lumenal fluid proteins had little gonadotropic activity when assayed with a radioreceptor assay system. Of the uterine protein samples collected on day 14 from five pregnant ewes, gonadotropic activity was undetectable in three and the other two had activity equivalent to 2.5 and .5 ng HCG/mg protein (.0116 I.U. HCG/ng HCG). The homogenates of three of the five embryos collected on day 14 exhibited activity equivalent to .4, 4.1 and 7.1 ng HCG per embryo (3.9 ± 1.9 ng HCG/embryo). The other two embryo homogenates were lost during processing.

Experiment VI

In ovariectomized ewes, a subcutaneous estradiol implant increased serum estradiol from 4.3 ± .6 to 11.0 ± .9 pg/ml ($P < .01$) within three days and maintained increased estradiol throughout the treatment period (Table 4). Serum estradiol after hormone replacement was approximately 75 percent of values observed on the day of estrus (Yuthasastrakosol et al., 1975). A pessary impregnated with progesterone increased ($P < .01$) serum progesterone from .4 ± .05 to 2.2 ± .2 ng/ml (days 0 and 3, respectively). Serum progesterone was maintained above control values for the duration of the treatment. Concentrations of serum progesterone achieved were comparable to those expected during the luteal phase of an estrous cycle (Figure 5; non-injected controls). Similar increases over control values were observed for each hormone in the group of ewes given both

Table 4.--Serum estradiol and progesterone in ovariectomized ewes with estradiol implants and/or progesterone pessaries (Experiment VI).

Treatment	Estradiol (pg/ml) Day 0	Day 3	Day 6	Day 9	Progesterone (ng/ml) Day 0	Day 3	Day 6	Day 9
Control[a]	5.7 ± .4[b]	4.7 ± .6	4.4 ± .7	4.7 ± .7	.3 ± .01	.3 ± .05	.4 ± .01	.4 ± .05
Estradiol[a]	4.3 ± .6	11.0 ± .9	8.1 ± .7	7.3 ± .7	.3 ± .05	.3 ± .05	.3 ± .01	.4 ± .01
Progesterone[a]	4.3 ± .7	4.9 ± .2	4.6 ± .6	5.7 ± .6	.4 ± .05	2.2 ± .2	1.8 ± .3	1.6 ± .2
Estradiol and Progesterone[c]	4.0 ± .6	8.8 ±1.8	8.9 ±1.5	8.0 ±1.9	.3 ± .05	1.5 ± .4	2.0 ± .4	2.1 ± .3

[a] n = five ewes

[b] Mean ± S.E.

[c] n = four ewes

estradiol implant and progesterone pessary (Table 4). One ewe in the estradiol-progesterone treatment group was excluded from the experiment because of expulsion of a pessary.

In ovariectomized ewes, replacement of progesterone and estradiol increased ($P < .05$) total recoverable uterine lumenal protein from $1.20 \pm .25$ to 3.48 ± 1.20 and 4.98 ± 2.37 mg, respectively (Table 5). A combination of the two hormones produced neither an additive or synergistic protein increase (6.02 ± 1.62 mg). One ewe in the estradiol treatment group produced 14 mg of lumenal protein, nearly six-fold more than the other four ewes in the group ($2.62 \pm .36$ mg).

After isoelectric focusing, uterine lumenal protein from control ovariectomized ewes (C) focused to a greater extent in the region less than pH 4.7 when compared to serum (Figure 12; S). After ten days of estradiol replacement (E), the profile of proteins in uterine lumenal fluid did not differ from profiles in control ewes. Progesterone replacement (P) decreased the proportion of proteins focused at less than pH 4.7. Of the 21 protein bands detected after isoelectric focusing of lumenal protein collected after progesterone treatment, all but one band of low intensity (pI 7.9) were present in serum (S). Upon replacement of both progesterone and estradiol (PE), the majority of proteins in lumenal fluid focused between pH 4.7 and 5.8.

Experiment VII

The amount of uterine lumenal fluid protein recovered on day 14 was not affected ($P > .05$) by administration of 125 µg estradiol per day on days 11 and 12 in ewes mated to a vasectomized ram (Table 6).

Table 5.--Uterine lumenal fluid protein recovered from ovariectomized ewes with estradiol implants and/or progesterone pessaries (Experiment VI).

Treatment	Total Protein - mg -	
Control	1.20 ± .26[a]	(5)[b]
Progesterone	3.48 ±1.20[c]	(5)
Estradiol	4.98 ±2.37[c]	(5)
	(2.62 ± .36)	(4)
Progesterone and Estradiol	6.02 ±1.62[c]	(4)

[a]Mean ± S.E.

[b]Number of observations

[c]Significantly different ($P < .05$) from levels in control ewes.

Table 6.--Uterine lumenal fluid protein recovered on day 14 from ewes mated to intact or vasectomized rams and given either 0 or 125 µg 17β-estradiol on days 11 and 12 after mating (Experiment VII).

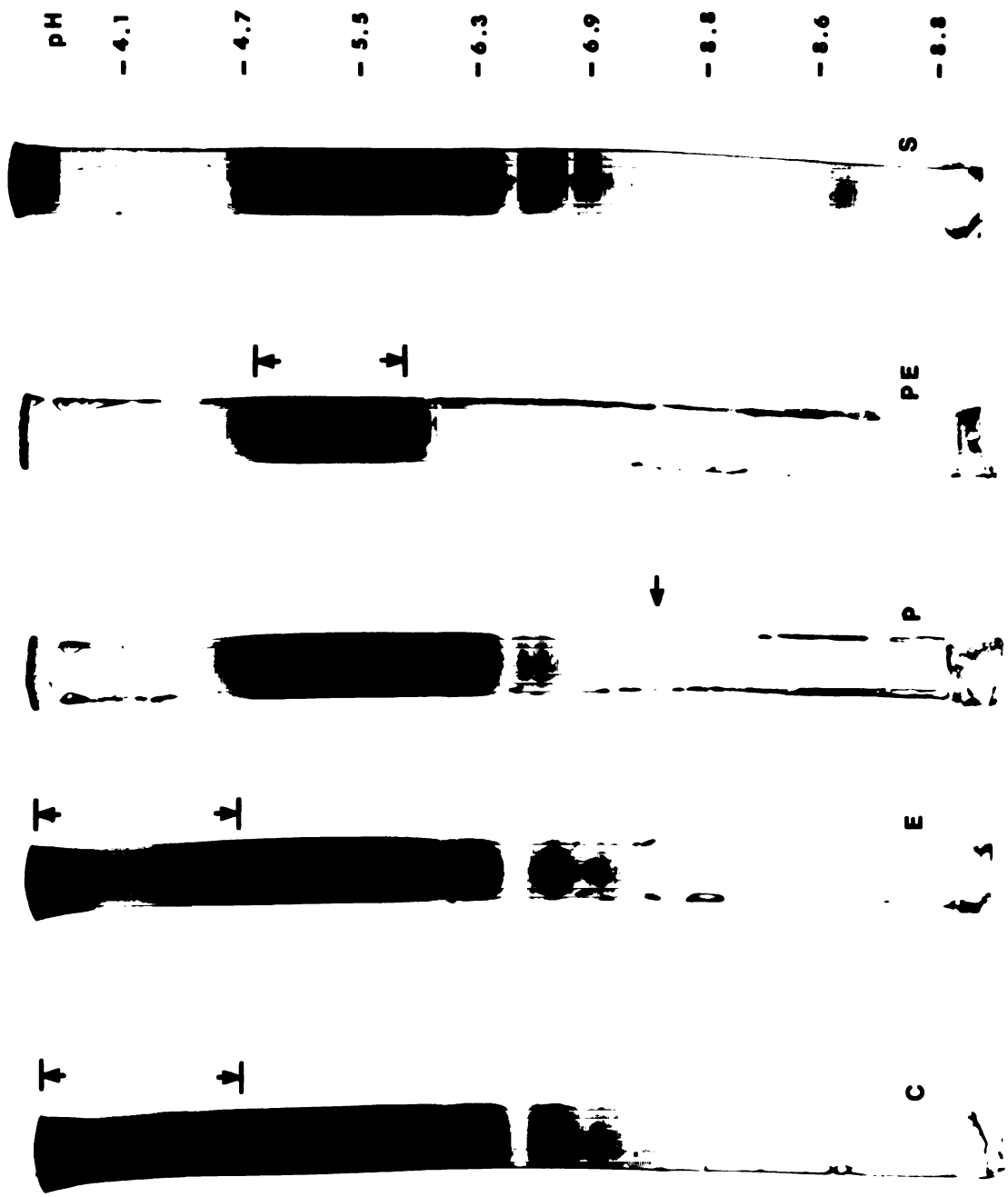
Treatment	Total Protein - mg -	
Mated to Vasectomized Ram		
Control	5.10 ± 1.60[a]	(5)[b]
Estradiol	4.32 ± .31	(5)
Mated to Intact Rams		
Control	6.61 ± .76	(5)
Estradiol (conceptus present)	5.84 ± .50	(7)
Estradiol (no conceptus)	4.19 ± .56[c]	(3)

[a]Mean ± S.E.

[b]Number of observations

[c]Significantly different ($P < .05$) than values recovered from control ewes mated to intact rams.

Figure 12.--Isoelectric focusing in polyacrylamide gel of uterine lumenal fluid protein collected from ovariectomized ewes after replacement of estradiol and/or progesterone (Experiment VI).

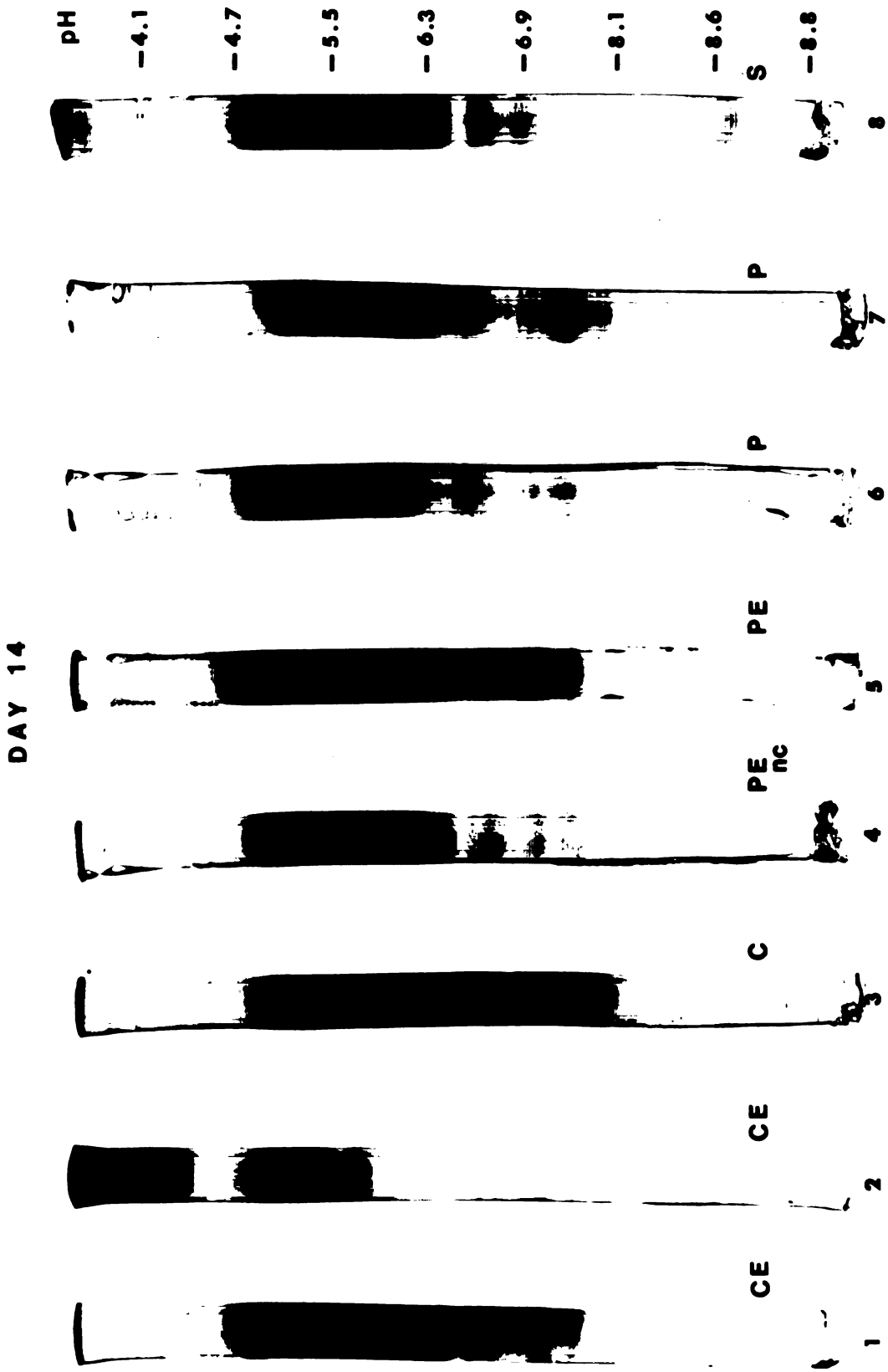

pH
4.1
4.7
5.5
6.3
6.9
8.8
8.6
8.8
S
PE
P
E
C

Similar lack of response was observed in estradiol treated ewes mated to intact rams provided a conceptus was present on day 14. Less uterine protein ($P < .05$) was recovered in the absence of an embryo compared with control ewes mated to intact rams ($4.19 \pm .56$ vs $6.61 \pm .76$ mg).

After estradiol administration to ewes mated to a vasectomized ram, two distinctly different protein profiles were obtained from isoelectric focusing of lumenal fluid protein collected on day 14 (Figure 13; gel 1, CE and gel 2, CE). Gel 2 (representative of three ewes) differed from gel 1 (representative of two ewes) by increased band intensity of proteins focused at less than pH 4.7 and decreased intensity of bands focused between pH 5.2 and 6.9. The two uterine-specific protein bands (pI 7.2 to 7.6) detected on day 14 of an estrous cycle (gel 3, C) were not observed after estradiol administration.

The presence of a conceptus was associated with the fact that profiles of uterine lumenal proteins collected from ewes mated to intact rams and given estradiol were divided into two distinct types. If the conceptus was present on day 14, the protein profile (gel 5, PE) was similar to one of the two representative profiles obtained on day 14 of pregnancy (gel 7, P) with the exception that proteins focused between pH 5.4 and 6.3 were in higher proportion in the estrogen treated ewes. The absence of a conceptus (gel 4, PE-nc) resulted in profiles similar to uterine protein from ewes mated to a vasectomized ram and given estradiol (gel 1, CE). (In Figure 13, the pH gradient presented was not representative of gels 3, 6 and 7, consult Figure 11 for proper pH gradient.)

Figure 13.--Isoelectric focusing in polyacrylamide gel of uterine lumenal fluid protein collected on day 14 from ewes mated to intact or vasectomized rams and given 125 μg 17β-estradiol on days 11 and 12 after mating (Experiment VII).


DAY 14
pH
4.1
4.7
5.5
6.3
6.9
8.1
8.6
8.8
S
P
P
PE
PE NC
C
CE
CE
8
7
6
5
4
3
2
1

Discussion

In the first three experiments of the present study, all ewes mated to vasectomized rams had decreased serum progesterone within two days after daily injection of estradiol (125 or 250 μg/day) on days 11 and 12 or days 12 and 13 of an estrous cycle. These data support the evidence that exogenous estradiol causes premature regression of the corpus luteum in the ewe (Stormshak et al., 1969; Hawk and Bolt, 1970).

In the ewe, $PGF_{2\alpha}$ is apparently the naturally occurring uterine luteolysin (Goding, 1974) and it increases in association with elevated endogenous estradiol (Barcikowski et al., 1974; Baird et al., 1976). Premature luteolysis after estrogen is probably mediated through increased uterine $PGF_{2\alpha}$ secretion (Ford et al., 1975).

If normal regression of the corpus luteum in the ewe is mediated through estradiol and if during pregnancy the conceptus prevents the normal regression of the corpus luteum (Moor and Rowson, 1964; 1966c; 1966d), then the conceptus should be capable of blocking estradiol-induced luteolysis. However, the ability of the conceptus to prevent estradiol-induced luteolysis had not been investigated. The first three experiments were designed to assess the antiluteolytic action of the conceptus when pregnant ewes were given doses of estradiol capable of causing premature regression of the corpus luteum in cycling ewes.

In Experiment I, serum progesterone decreased in only six of 12 ewes mated to intact rams and given 250 μg estradiol on days 11

and 12 or days 12 and 13 after mating (Figure 2). These data suggested that pregnancy prevented the luteolytic effect of estrogen in some ewes, however, pregnancy was not diagnosed in these animals.

To confirm that the conceptus had antiluteolytic capacity, a second experiment was performed in which six of 16 ewes mated to intact rams of known fertility and given 125 or 250 μg estradiol on days 11 and 12 were pregnant on day 16. Pregnant ewes had higher serum progesterone than non-pregnant ewes on days 13, 14, 15 and 16 (Figure 3). However, serum progesterone in pregnant ewes declined from 3.3 ng/ml on day 13 to .9 ng/ml on day 16, suggesting the luteolytic effect of estrogen was not fully overcome. Data from this experiment contradicted the results obtained from Experiment I and inferred the presence of the conceptus in the uterus of the ewe on days 11 and 12 after mating only delayed estradiol-induced luteolysis.

It was therefore necessary that a third experiment be performed to resolve the question of whether pregnancy prevents estradiol-induced luteolysis or just delays its completion. In Experiment III, five of 17 ewes mated to intact fertile rams and given estradiol (125 μg/day) on days 11 and 12 after mating were pregnant on day 20. As in the previous experiment, pregnant ewes had higher serum progesterone on day 14 than non-pregnant ewes (Figure 4). Serum progesterone in estradiol-injected pregnant ewes did not differ from levels in non-injected pregnant ewes (Figure 6). These data supported the evidence obtained in Experiment I, that pregnancy prevented the luteolytic effect of estradiol.

Thus, from these three experiments, it can be concluded that the conceptus has a definite antiluteolytic capacity and exerts that capacity on days 11 to 13 of pregnancy.

Previous reports have provided contradictory evidence as to whether the ovine conceptus could maintain the corpus luteum by suppression of $PGF_{2\alpha}$ secretion from the uterus or blockage of its luteolytic effect at the ovary. Although no difference was detected in mean uterine venous PGF levels from pregnant and non-pregnant ewes on day 15 (Pexton et al., 1975) or days 11 to 17 (Nett et al., 1976) after estrus, fewer peaks of $PGF_{2\alpha}$ were detected in uterine venous drainage from pregnant ewes (Nett et al., 1976). During pregnancy, Barcikowski et al. (1974) observed suppression of the peaks of $PGF_{2\alpha}$ normally observed on days 15 and 16 of an estrous cycle. Alternatively, the uterine luteolysin may be inhibited at the ovary. Luteolysis occurred in non-pregnant but not in pregnant ewes after $PGF_{2\alpha}$ was injected on day 12 after estrus into a follicle on the same ovary as the corpus luteum (Inskeep et al., 1975). Infusion of $PGF_{2\alpha}$ into the ovarian artery on day 13 after estrus caused luteolysis in ewes hysterectomized after mating but not in pregnant ewes (Mapletoft et al., 1976a). The activity of uterine luteolysin was blocked at the ovary by a blood-borne factor secreted from the gravid horn of the uterus into the uterine venous drainage (Mapletoft et al., 1975) and transferred by a venoarterial pathway into the ovarian artery (Mapletoft et al., 1976b).

The antiluteolytic capacity of the conceptus may be derived from the production or stimulated secretion of a gonadotropin,

since exogenous gonadotropins can block both naturally occurring (Karsch et al., 1971) and experimentally induced luteolysis (Bolt et al., 1971; Bolt, 1973). However, failure to detect gonadotropic activity in the conceptus or uterine lumenal fluid protein in the present study (Experiment V) inferred that the above effect of gonadotropins as inhibitors of luteolysis was pharmacological. Also, during normal pregnancy there was no serum progesterone increase indicative of gonadotropin stimulation (Table 10).

It is conceivable that the conceptus exerts its effect through a combination of luteotropic and antiluteolytic actions and in the present study, the additional luteolytic effect of exogenous estradiol may have been beyond the corpus luteum prolonging capacity of the conceptuses, since estrogen treatment resulted in luteolysis in a significant percentage of ewes mated to fertile rams (Experiment I, six of 12 ewes; Experiment II, 11 of 16 ewes; Experiment III, 12 of 17 ewes). This suggests there may be a delicate balance between maintenance and regression of the corpus luteum especially since Bolt (1973) found that the size of luteolytic challenge determined whether exogenous gonadotropin was capable of blocking induced luteal regression.

In Experiment II, serum progesterone in pregnant ewes averaged 1.6 ± .4 ng/ml on day 13 of pregnancy and decreased to .9 ± .4 ng/ml by day 16, suggesting the luteolytic effect of estrogen was not fully blocked even though a conceptus was present in the uterus on day 16. Insufficient levels of progesterone may have been responsible for the retarded development of two of the six embryos recovered in

Experiment II. Recovery of a higher percentage of conceptuses on day 14 (Experiment VII) than on day 16 or 20 (Experiments II and III, respectively) provides evidence for a reduced ability of the embryo to survive the effects of the estradiol treatment. Lack of progestational support from the mother may have caused embryonic mortality.

Another aspect of the present study was to investigate the route by which the conceptus was capable of preventing luteolysis. Evidence had indicated the embryo produced a protein (Rowson and Moor, 1967) which was active prior to day 13 (Moor and Rowson, 1964; 1966c; 1966d). It was assumed that this protein must pass through the lumenal fluid to leave the uterine lumen since placental attachment does not begin until day 15 in the sheep (Boshier, 1969). Since preliminary information on uterine lumenal fluid protein in the ewe was inadequate, Experiments IV and V were performed to determine if proteins in the uterine lumenal fluid changed qualitatively or quantitatively during the estrous cycle and to determine the contribution of the conceptus to uterine lumenal fluid protein.

Initially, lumenal protein was subjected to electrophoresis at pH 4.5 but this technique was discontinued due to poor resolution of protein bands. Thereafter, SDS electrophoresis and isoelectric focusing were implemented to characterize the proteins in uterine lumenal fluid.

After uterine lumenal protein collected during an estrous cycle was subjected to SDS electrophoresis, the only consistent and progressive change detected was the decrease in staining intensity

after day 13 (13C) of a protein less than 10,000 MW (Figure 8). Although SDS electrophoresis revealed other changes in band intensity throughout an estrous cycle, these differences were associated with individual animal variation.

After isoelectric focusing of protein collected on day 14 of an estrous cycle (Figure 10; 14C) only two of 35 protein bands detected in uterine fluid were not also present in serum. These two bands which focused between pH 7.2 and 7.6 were not present on days 3 and 9 and constituted the only difference in number of detectable proteins observed on various days of an estrous cycle. Utilizing cellulose acetate electrophoresis, Wales (1973) detected no change in relative mobilities of the various protein fractions of uterine fluid collected during estrus, diestrous and anestrous periods in the ewe.

Decreased stain intensity of proteins focused between pH 6.3 and 7.3 was observed on day 9 of an estrous cycle. By day 14, intensity of the bands in this region had returned to the intensity observed on day 3. Whether changes in band intensity (pI 6.3 to 7.3) observed after isoelectric focusing of uterine fluid collected on days 3, 9 and 14 of an estrous cycle were related to changes observed with SDS electrophoresis was not investigated due to lack of sample material.

Uterine lumenal proteins collected during an estrous cycle and pregnancy differed as follows: (1) after day 14 of pregnancy, a protein appeared that migrated toward the cathode at pH 4.5 (Figure 7; 14P, middle arrow); (2) after day 13, staining intensity

of a protein less than 10,000 MW was increased during pregnancy (Figure 8; 14P and 15P, arrows); and, (3) increased band intensity of proteins focused at pH 5.4 to 7.0 occurred on day 14 of an estrous cycle (Figure 11; 14C, arrows). Possible interrelationship of these three changes in uterine lumenal protein was not investigated due to lack of sample material. Band intensity of a protein less than 10,000 MW increased after day 12 of pregnancy and had a mobility similar to the protein that decreased in intensity during an estrous cycle. However, comparable migration on SDS electrophoresis may simply indicate similar molecular weight rather than identity. The role of these protein changes in the ewe occur later than the period of blastulation in the sheep and suggest these changes do not act analogous to blastokinin which has been reported to influence blastocyst formation in the rabbit (Krishnan and Daniel, 1967). Changes in uterine lumenal protein may be involved in embryonic development since days 12 to 14 of gestation is a period of rapid elongation of the trophoblast (Rowson and Moor, 1966). Another possibility is that these protein changes reflect the mechanism by which the presence of an embryo in the uterus of ewes 12 and 13 days after estrus results in maintenance rather than regression of the corpus luteum (Moor and Rowson, 1964; 1966c; 1966d). Although the changes in lumenal protein may be a product of the embryo, this was not likely since isoelectric focusing revealed no protein band that was present only during pregnancy; differences were in staining intensity only. However, the presence of the embryo may have affected existing secretion.

Uterine-specific proteins are minor and subtle components of lumenal fluid collected during an estrous cycle and pregnancy in the ewe. The majority of proteins in uterine lumenal fluid of the ewe have similar electrophoretic properties and isoelectric points as serum proteins. Although most of the proteins in uterine lumenal fluid had physical identity with serum proteins, their proportional part of the total sample differed from serum (Figure 11). Wales (1973) observed albumen to be higher and β-globulin to be lower in uterine fluid than in serum of ewes during estrus, diestrous and anestrous periods. The data of the present study suggest that presence of serum proteins in lumenal fluid is not the product of passive diffusion or transudation of serum into the lumen of the uterus. In the cow, which has a syndesmochorial morphological type of placenta, similar to the ewe, Roberts and Parker (1974) observed all but two of 35 proteins detected in uterine lumenal fluid were also present in serum and these two uterine-specific proteins made up a small proportion of the total lumenal protein. However, uterine-specific blastokinin comprises up to 40 percent of the total uterine protein on day 5 of pregnancy in the rabbit (Arthur and Daniel, 1972). And after day 12 of an estrous cycle, seven uterine-specific proteins were detected in the pig (Murray et al., 1972; Squire et al., 1972); one of the seven, a purple-colored protein makes up 15 percent of the total uterine protein on day 15 (Chen et al., 1973). Less well characterized uterine-specific proteins have been described in the baboon (Peplow et al., 1973; 1974), ferret (Daniel, 1970), hamster

(Noske and Daniel, 1974), human (Bernstein et al., 1971; Shirai et al., 1972; Daniel, 1973) and northern fur seal (Daniel, 1971; 1972).

After preliminary description of the uterine lumenal fluid proteins, Experiment VI was performed to determine how ovarian steroid hormones influence their secretion. In ovariectomized ewes, ten days of progesterone replacement (P) had both inhibitory and stimulatory effects on protein in the uterine lumen (Figure 12). Progesterone reduced the intensity of proteins focused at less than pH 4.7 although the total amount of protein in the uterine lumen increased (Table 5). The simulatory effect of progesterone was also evidenced by the subtle appearance of a protein band of low intensity focused at pH 7.9. Estradiol replacement (E) had no effect on the proportion of proteins in the uterine lumenal fluid but it did increase total protein. In ovariectomized animals, uterine-specific proteins of the pig (Knight et al., 1973) and rabbit (Urzua et al., 1970; Arthur and Daniel, 1972) were demonstrated after progesterone replacement. Uterine lumenal fluid protein in the ewe is not influenced by progesterone alone, since up to 14 of 35 bands observed on day 14 of an estrous cycle (Figure 10; 14C) were not detected in lumenal fluid collected from ovariectomized ewes after ten days of progesterone replacement (Figure 12; P). The possible requirement for estradiol in addition to progesterone was not supported, since replacement of both hormones (PE) did not result in secretion of all 35 bands of diestrus. However, estradiol replacement in Experiment VII resulted in serum concentrations (Table 4) approximately two-fold greater than levels observed during the luteal phase of an estrous cycle.

Since the administration of estradiol on days 11 and 12 of an estrous cycle in Experiments I, II and III resulted in premature luteal regression by day 14 and the presence of the conceptus prevented luteal regression, Experiment VII was performed to determine if lumenal fluid protein was affected by the estradiol treatment.

Administration of estradiol during an estrous cycle produced two types of profiles (Figure 13); one (gel 2) similar to that obtained from an ovariectomized ewe, and the other profile (gel 1) similar to that obtained on day 14 of an estrous cycle (gel 3) except that the uterine-specific proteins focused at pH 7.2 to 7.6 were not detected and band intensity between pH 5.4 and 7.0 was greater in estradiol treated ewes. This difference may reflect the endocrine environment of the respective ewes. In the ewe represented by gel 2, estradiol-induced luteolysis had probably prematurely decreased progesterone secretion. However, in ewes represented by gel 1, even though estradiol-induced luteolysis had probably decreased serum progesterone, the animals may have not been without progesterone long enough to affect uterine secretory products. Administration of estradiol during pregnancy did not affect the uterine protein profile if the conceptus was present on day 14 (gel 5); in the absence of the conceptus on day 14 (gel 4), the resultant protein profile was similar to that obtained from ewes given estradiol during an estrous cycle (gel 1). In ewes mated to intact rams and given estradiol but having no conceptus on day 14, it is probable that insufficient antiluteolytic capacity allowed luteal regression and decreased progesterone secretion resulted in altered uterine protein

profiles and embryonic mortality. And it is likely that alterations of uterine proteins during an estrous cycle by estradiol administration were also an effect of decreased progesterone rather than a direct influence of estrogen on protein secretion.

SUMMARY AND CONCLUSIONS

In a series of three experiments to assess the antiluteolytic capacity of the conceptus, comparison of luteal function in ewes mated to either intact or vasectomized rams and given estradiol revealed distinct differences in corpus luteum maintenance. In ewes mated to a vasectomized ram (Experiment I), two consecutive days of estradiol injection (250 μg/day) on days 11 and 12 or 12 and 13 decreased serum progesterone as compared to non-injected controls. However, when ewes were mated to intact rams, serum progesterone decreased in only six of 12 ewes. In Experiment II, pregnancy was confirmed on day 16 in four of eight and one of eight ewes mated to intact fertile rams and given either 125 or 250 μg estradiol, respectively, on days 11 and 12. Serum progesterone was higher on day 15 after mating in pregnant than in non-pregnant ewes. On day 20 of Experiment III, pregnancy was confirmed in five of 17 ewes mated to intact fertile rams and given 125 μg estradiol on days 11 and 12. After day 14, serum progesterone in estrogen-treated ewes mated to intact rams was lower in non-pregnant ewes than pregnant ewes. Concentrations of serum progesterone in estrogen-treated pregnant ewes did not differ from control pregnant ewes.

The present study demonstrates that a conceptus in the uterus on days 11 to 13 after mating prevented the luteolytic effect

of exogenous estradiol in some ewes. However, the combination of endogenous and experimentally induced luteolytic factors may have been beyond the corpus luteum prolonging capacity of many of the conceptuses since estradiol administration resulted in luteolysis in 50 to 70 percent of ewes mated to fertile rams. The involvement of estradiol in stimulation of uterine $PGF_{2\alpha}$ secretion suggests the conceptus maintains the corpus luteum by suppression of $PGF_{2\alpha}$ secretion from the uterus or blockage of its luteolytic effect at the level of the ovary. However, the alternative of luteotropin stimulation by the conceptus cannot be ruled out.

To investigate the means by which the conceptus exerted its antiluteolytic capacity, uterine lumenal fluid protein was subjected to examination. The intent of this investigation was to determine if proteins in uterine lumenal fluid changed qualitatively or quantitatively during the estrous cycle and early pregnancy. Recoverable uterine lumenal protein increased from $1.84 \pm .21$ mg on day 3 to 4.97 ± 1.20 mg on day 9 of an estrous cycle and remained at that level until day 15. Pregnancy did not increase total uterine lumenal protein recovered 14 days after mating compared to that recovered on day 14 of an estrous cycle. Of the 35 protein bands detected after isoelectric focusing of uterine lumenal protein collected on day 14 of an estrous cycle, only two (pI 7.2 to 7.6) were not present in serum also and could be considered uterine-specific. The two bands focused at pH 7.2 to 7.6 were absent in uterine fluid collected on days 3 and 9 of an estrous cycle, but with that exception, the number of protein bands detected on those days did not differ from

that on day 14. However, band intensity of proteins focused between pH 6.3 and 7.3 was less on day 9 than on days 3 and 14. After SDS electrophoresis, the only consistent change observed in uterine lumenal protein collected during an estrous cycle was a decrease after day 13 in staining intensity of a protein of less than 10,000 MW. During pregnancy uterine lumenal protein differed from that collected during an estrous cycle as follows: (1) after day 14 of pregnancy, a protein appeared that migrated toward the cathode at pH 4.5; (2) after day 13 of pregnancy, there was greater staining intensity of a protein less than 10,000 MW; and (3) decreased intensity of proteins focused between pH 5.4 and 7.0 occurred during pregnancy. Gonadotropic activity was undetectable in three of five samples of uterine lumenal protein collected on day 14 of pregnancy and was equivalent to 3.9 ± 1.9 ng HCG per day 14 embryo homogenate.

In the present study, only a very small proportion of uterine lumenal fluid protein was uterine-specific during an estrous cycle and pregnancy. Although a pregnancy-specific protein was observed after electrophoresis at pH 4.5, pregnancy-specific protein bands were not associated with profiles obtained by SDS electrophoresis and isoelectric focusing. After these techniques, samples collected during an estrous cycle and pregnancy differed in staining intensity of several proteins that were also present in serum.

The observed changes in uterine lumenal proteins during an estrous cycle suggested their secretion into the uterine lumen may be under the influence of ovarian steroid hormones. To investigate this, ovariectomized ewes were subjected to ovarian hormone

replacement. More uterine lumenal protein from control ovariectomized ewes focused at less than pH 4.7 compared to serum protein. Progesterone replacement for ten days decreased the proportion of proteins that focused at less than pH 4.7 although it increased the amount of protein recovered from the uterine lumen. Of the 21 protein bands detected after progesterone, one band focused at pH 7.9 was uterine-specific. Estradiol replacement did not change the protein profiles of uterine protein, although the amount of protein recovered increased. After replacement of both progesterone and estradiol, the majority of proteins in lumenal fluid focused between pH 4.7 and 5.8.

Uterine lumenal protein does not appear to be under the exclusive control of progesterone, since only 21 of 35 protein bands observed on day 14 of an estrous cycle were detected after progesterone replacement. The requirement of progesterone and estradiol to achieve the secretion observed on day 14 of an estrous cycle was not supported by the replacement of both hormones. Uterine protein secretion may be sensitive to the progesterone-estradiol ratio and the ratio of hormones replaced in the present study may not have been adequate.

Since estradiol administration on days 11 and 12 of an estrous cycle was luteolytic and the conceptus had the capacity to prevent estradiol-induced luteal regression, the objective of the final experiment was to determine if luteolytic doses of estradiol affect uterine lumenal fluid protein. Two different protein profiles were obtained after estradiol administration during an estrous cycle. One profile was similar to that observed on day 14 of an estrous

cycle; the other had a higher proportion of protein focused at less than pH 4.7 and less protein focused between pH 5.2 and 6.9. After estradiol administration, the two uterine-specific proteins (pI 7.2 to 7.6) detected on day 14 of an estrous cycle were not observed. Uterine lumenal proteins collected from ewes mated to intact rams and given estradiol produced two different protein profiles. On day 14, if a conceptus was present the profile resembled that obtained on day 14 of a normal pregnancy except that proteins focused between pH 5.4 and 6.3 were in higher proportion in estradiol-treated ewes. Absence of a conceptus was associated with a protein profile similar to ewes mated to a vasectomized ram and given estradiol.

The altered profiles of uterine lumenal fluid protein collected during an estrous cycle after luteolytic doses of estradiol were probably the effect of the decrease in progesterone (luteolytic effect) rather than a direct estrogen effect on protein secretion.

The antiluteolytic capacity of the embryos observed was limited. The combination of endogenous and experimentally induced luteolysis was in excess of the capacity of several embryos. Further investigation is required to adequately assess the capacity of the embryo to an experimental luteolytic challenge. And there is a need to determine if the antiluteolytic capacity is realized from suppression of secretion of luteolysin from the uterus or blockage of the luteolytic effect at the level of the ovary. Contradictory reports on the site of action of the antiluteolytic capacity need to be either confirmed or disproven.

The minor contribution of the uterus to proteins found in the uterine lumenal fluid may be characteristic to the morphological type of placenta found in the ewe since a similar situation has been described in the cow. Changes in proportion of the individual serum proteins in uterine lumenal fluid need to be associated with the physiological events that these changes control or facilitate. And the function of the uterine-specific proteins requires elucidation. Although physical identities were established between uterine lumenal and serum proteins in the ewe, it remains that identities be observed by immunological techniques.

It is the opinion of the author that an antiluteolytic factor is produced by or in response to the embryo in the uterus of the pregnant ewe. This factor is secreted into the uterine venous drainage, transferred via a venoarterial pathway to the ovarian artery and has an antiluteolytic effect at the level of the ovary. The suppression of luteolysis may be either direct blockage of the lytic effect or overriding the effect by increased gonadotropic support of the corpus luteum. In view of the data of the present study and the proposed avenue of transport to the ovary, it is likely that the factor is not a protein and has no gonadotropic activity.

Alternatively, although uterine-specific proteins are minor and subtle components of lumenal fluid during early pregnancy in the ewe, their activity may suffice to serve as a premonition of pregnancy. While not detected in the present study, this possibility of a protein specific to pregnancy can not be discarded. Regardless of whether uterine lumenal protein is involved in the antiluteolytic

capacity of the embryo, it can be concluded that an ovine embryo can accomplish the various developmental phenomena without requirements of large amounts of uterine-specific proteins. This development either requires no control or facilitation from a detectable protein, or this need is fulfilled by the changing proportions of serum proteins in uterine lumenal fluid.

Further study is warranted to gain more complete information concerning maternal recognition of pregnancy and resolve the function(s) of proteins in the uterine lumenal fluid of the ewe.

APPENDICES

APPENDIX A

EXTRACTION AND RADIOIMMUNOASSAY OF PROGESTERONE

APPENDIX A

EXTRACTION AND RADIOIMMUNOASSAY OF PROGESTERONE

(Louis et al., 1973. Proc. Soc. Exp. Biol. Med. 143:152)

Extraction

1. Duplicate aliquants of serum (100 µl) were pipetted into disposable culture tubes (16 X 100 mm).

2. To account for procedural loss, a third aliquant from a representative number of unknowns (eight to 12 per assay) was added to a culture tube (16 X 100 mm) which contained 3000 dpm ^{3}H-1, 2,6,7-progesterone (New England Nuclear; 80 to 100 Ci/mM; repurified by column chromatography).

3. Tubes containing serum and ^{3}H-progesterone were vortexed (10 seconds); labeled and endogenous progesterone were allowed to equilibrate for 30 minutes.

4. Serum was extracted with 2 ml benzene:hexane (1:2) by vortexing by 30 seconds and was then stored at -20 C for at least one hour to freeze the aqueous phase.

   Solvent extracts destined for assay were decanted into disposable culture tubes (12 X 75 mm).

   Extracts from aliquants for procedural loss were decanted into scintillation vials. Recovered radioactivity was averaged to determine a single correction factor to account for procedural losses in all samples.

Radioimmunoassay

1. Progesterone (Sigma Chemical Company) for standards (0, .025, .05, .1, .25, .50, .75 and 1.0 ng) was pipetted into disposable culture tubes (12 X 75 mm) from a stock solution of 10 ng/ml in absolute ethanol and included in each assay.

2. Standards and serum extracts were air-dried.

3. Antibody[1] (200 µl), diluted 1:4500 in .01 M phosphate buffered saline pH 7.4 containing a .1 percent gelatin (Knox Gelatin, Inc., Johnstown, N.Y.), was added to each tube, vortexed and allowed to incubate for 30 minutes at room temperature.

4. Then, 200 µl of .1 percent gelatin in .01 M phosphate buffered saline pH 7.4 containing 45,000 dpm ^{3}H-1,2,6,7-progesterone was added to each tube. The contents of each tube was vortexed and incubated for 12 to 18 hours at 4 C.

5. One ml of .5 percent dextran T70 (Pharmicia, Uppsala, Sweden) and .25 percent carbon decolorizing neutral norit (Fisher Science Company) in distilled H_2O was added to each tube. Contents of each tube was mixed, incubated for ten minutes in an ice bath and then centrifuged at 2500 g for ten minutes at 4 C.

6. A .5 ml aliquant of supernatant was diluted with scintillation fluid (3a70B Preblend; Research Products International Corp., Elk Grove Village, Illinois) for quantification of radioactivity in a liquid scintillation spectrophotometer.

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[1]Anti-progesterone, generously supplied by Dr. G.D. Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins. The rabbit antiserum was prepared against 6β-succinyl-progesterone conjugated to bovine serum albumen.

APPENDIX B

DISC GEL ELECTROPHORESIS pH 4.5

APPENDIX B

DISC GEL ELECTROPHORESIS pH 4.5

(Reisfeld et al., 1962. Nature 195:281)

Gel Preparation

1. Reagent stock solutions:

(a)	1 N KOH	48.0 ml
	acetic acid (glacial)	17.2 ml
	tetramethylethylenediamine (TEMED)	4.0 ml
	H_2O to bring volume to	100.0 ml
(b)	1 N KOH	48.0 ml
	acetic acid (glacial)	2.9 ml
	TEMED	.5 ml
	H_2O to bring volume to	100.0 ml
(c)	acrylamide/5% methylene-bis-acrylamide premix (Cyanogum 41 Gelling Agent; Fisher Sci.)	30.0 g
	H_2O to bring volume to	100.0 ml
(d)	acrylamide/5% methylene-bis-acrylamide premix (Cyanogum 41)	10.0 g
	H_2O to bring volume to	100.0 ml
(e)	riboflavin-5'-phosphate	4.0 mg
	H_2O to bring volume to	100.0 ml

2. Running gel solution (7.5%T, 5%C; pH 4.3) was prepared from stock solutions a and b, and H_2O in a ratio of 1:2:1 (v), respectively. An equal volume of freshly prepared .28% ammonium persulphate was added to the solution.

3. After thorough mixing, the acrylamide solution was dispensed into vertically supported glass tubes (6 mm I.D.) to a depth of 5 cm and approximately .1 ml H_2O was layered over the acrylamide solution.

4. After polymerization, the H_2O layer was removed.

5. Stacking gel solution (2.5%T, 5%C; pH 6.8) was prepared from stock solutions b,d and e, and H_2O in a ratio of 1:2:1:4 (v), respectively.

6. After mixing, the stacking gel solution was dispensed into tubes on top of the polymerized running gel to a depth of 1 cm and approximately .1 ml H_2O was layered over the acrylamide solution.

7. Tubes were supported vertically and irradiated under fluorescent light for at least one hour to polymerize the gel.

Electrophoresis

During electrophoresis, buffer temperature in the lower reservoir was controlled by circulation of H_2O (15 C) through the cooling jacket of the electrophoresis cell.

1. The lower reservoir was filled with alanine-acetate buffer pH 4.5 (31.2 g β-alanine and 8.0 ml glacial acetic acid per 1).

2. After the H_2O overlayer was removed, the gel columns were placed in the electrophoresis cell so the entire length of gel was submersed in the lower buffer.

3. The upper reservoir was filled with alanine-acetate buffer pH 4.5.

4. Samples (200 μg in alanine-acetate buffer pH 4.5) were applied to the gel columns as 10% sucrose.

5. A dirrect current (DC) potential of 90 volts with 4.0 mA/gel with the cathode (-) in the lower reservoir was applied for five minutes; then, 6.0 mA/gel was applied for 115 minutes.

Staining and Destaining Gels

1. Gels were removed from glass tubes by rimming the gel with a 22 ga needle under water and applying pressure with a pipette bulb.

2. Gels were placed in .1% Amido black in 10% acetic acid for three hours.

3. Gels were destained by diffusion in 10% acetic acid.

4. The profile of stained protein bands was displayed on a recorder as a gel was scanned (540 nm) by a spectrophotometer (Gilford 2400-S) with a linear transport attachment).

APPENDIX C

TOTAL PROTEIN DETERMINATION

APPENDIX C

TOTAL PROTEIN DETERMINATION

(Miller, 1959. Anal Chem. 31:964)

1. Duplicate aliquants of samples were pipetted into disposable culture tubes (12 X 75 mm) and volume adjusted with H_2O to .5 ml.

2. Bovine serum albumen (Sigma Chemical Company) for standards (0, 30, 60, 90, 120 and 150 µg) was pipetted into disposable culture tubes (12 X 75 mm) from a stock solution of 300 µg/ml in H_2O and the volume of each tube was adjusted with H_2O to .5 ml.

3. Alkaline copper reagent (.5 ml) was added; then, contents of each tube was vortexed and incubated for ten minutes at room temperature.

   Alkaline copper reagent was prepared from one volume .5 percent $CuSO_4$ in 1 percent Na-K-tartrate and ten volumes 10 percent Na_2CO_3 in .5 N NaOH. Reagents were kept as stock solutions of 1.0 percent $CuSO_4$ and 2 percent Na-K-tartrate and mixed 1:1 (v:v) prior to addition of 10 percent Na_2CO_3 in .5 N NaOH.

4. Phenol reagent (1.5 ml) was added to each tube, its contents vortexed and incubated for ten minutes at 50 C.

   Phenol reagent was prepared from 2N phenol (Folin and Ciocalteu) reagent (Harleco, Philadelphia, Pennsylvania) diluted 1:10 (v:v) with H_2O just prior to use.

5. Reaction mixtures were cooled to room temperature and a spectrophotometer was used to determine absorbance at 540 nm.

APPENDIX D

SDS ELECTROPHORESIS

APPENDIX D

SDS ELECTROPHORESIS

(Weber and Osborn, 1969. J. Biol. Chem. 244:4406)

Gel Preparation

1. Reagent stock solutions:

   (a) gel buffer

NaH_2PO_4-H_2O	7.8 g
$NaHPO_4$-$7H_2O$	38.6 g
sodium dodecyl sulphate	2.0 g
H_2O to bring volume to	1.0 l

   (b) acrylamide solution

acrylamide	22.2 g
methylene-bis-acrylamide	.6 g
H_2O to bring volume to	100.0 ml

2. Gel buffer (15 ml) and 13.5 ml acrylamide solution were mixed and deaerated. To this mixture, 1.5 ml of freshly prepared ammonium persulphate solution (15 mg/ml) and 50 µl tetramethylethylenediamine (TEMED) were added. After thorough mixing, the solution was dispensed into vertically supported glass tubes (6 mm I.D.) to a depth of 7 cm and approximately .1 ml H_2O was layered over the acrylamide solution.

Sample Preparation

1. Samples (300 µg) and standards (20 µg) were dissolved in gel buffer diluted 1:1 with H_2O (v:v), mixed with one drop of mercaptoethanol and heated in a boiling water bath for two minutes.

   Proteins used for standards were: ovalbumen, 45,000 MW; pepsin, 35,000 MW; trypsin, 23,300 MW; and, ribonuclease, 13,700 MW.

2. Twenty µl of diluted gel buffer saturated with sucrose and containing .0025% bromphenol blue was added to each sample and standard.

Electrophoresis

1. After the H_2O layer was removed, the gel columns were placed in the electrophoresis cell so the entire length of gel would be below the surface of the buffer in the lower reservoir.

2. Both the lower and upper reservoirs were filled with gel buffer diluted 1:1 with H_2O.

3. Samples and standards were applied to the gel columns.

4. A current of 3 mA/gel with the anode (+) in the lower reservoir was applied for 30 minutes; current was then increased to 7 mA/gel and maintained for 5½hours.

Staining and Destaining Gels

1. Gels were removed from glass tubes by rimming the gel with a 22 ga needle under water and applying pressure with a pipette bulb.

2. Gel length and distance of dye migration were measured.

3. Gels were placed in .05% Coomasie blue in acetic acid: methanol: H_2O (10:50:40) for at least ten hours.

4. Gels were destained by diffusion in acetic acid:methanol:H_2O (10:5:85).

5. A profile of stained protein bands was displayed on a recorder as gel was scanned (540 nm) by a spectrophotometer (Gilford 2400-S) with a linear transport attachment.

Molecular Weight Estimations

1. From the spectrophotometric scan, gel length after destaining and distance of protein migration were recorded.

2. Mobility of a protein was computed as:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

3. Mobilities of standards were plotted against their respective molecular weights expressed on a semi-logarithmic scale.

APPENDIX E

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS

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ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS

(Bio-Rad Lab. Tech. Bul. 1030, 1975)

Gel Preparation

1. The following reagents in their respective order were added to a 25 ml volumetric flask:

   (a) 6.25 ml acrylamide monomer stock solution (29.25 g acrylamide and .75 g methylene-bis-acrylamide in 100 ml H_2O);
   (b) 1.25 g glycerol (reagent grade);
   (c) approximately 10 ml H_2O;
   (d) 1.25 ml ampholyte solution (Bio-Lyte 3/10, 40% solution as packaged, Bio-Rad Lab.);
   (e) after mixing the previously added reagents, .5 ml riboflavin solution (20 mg riboflavin-5'-phosphate in 100 ml H_2O);
   (f) enough H_2O to bring the volume to 25 ml.

2. After thorough mixing, the acrylamide solution was dispensed into glass tubes (6 mm I.D.) to a depth of 8.5 cm.

3. Approximately .1 ml H_2O was layered over the acrylamide solution.

4. Gel columns were supported vertically and irradiated under fluorescent light overnight before use.

Isoelectric Focusing

During the following procedure, electrolyte temperature in the lower reservoir was controlled by circulation of H_2O (4 C) through the cooling jacket of the electrophoresis cell. Also the lower reservoir electrolyte was continuously stirred with a magnetic stirring bar.

1. The lower reservoir was filled with .02 N $Ca(OH)_2$ and .04 N NaOH.

2. After the H_2O layer was removed, the gel columns were placed in the apparatus so the entire length of gel was submersed in the lower electrolyte.

3, Samples (500 µg in .01 M phosphate buffered saline, pH 7.4) were applied as 25% sucrose.

4. Two layers (100 µl) of 20% and 10% sucrose, respectively, were placed over the sample and the remainder of the tube filled with upper reservoir electrolyte (.06 N H_2SO_4).

5. The upper reservoir was filled with electrolyte (.06 N H_2SO_4).

6. A direct current (DC) potential equal to 20 volts per cm of gel length was applied for 16 hours with the anode (+) in the upper reservoir.

Monitoring pH

1. Gels were removed from glass tubes by rimming the gel with a 22 ga needle under water and forcing the gel from the tube with pressure applied by a pipette bulb.

2. The pH gradient formed was monitored along the length of the gel at 1 cm intervals with a minature electrode (Bio-Rad. Lab., Cat. No. 163-2011).

Staining and Destaining Gels
(Minor and Heston, 1972.
Anal. Biochem. 50:313)

1. After removal from glass tubes, gels were placed in 12% trichloro-acetic acid (TCA) for at least 12 hours.

2. Gels were then placed in .05% Coomasie brillant blue in acetic acid:ethanol:H_2O (5:50:45) for at least 12 hours.

3. Gels were destained by diffusion in acetic acid:ethanol:H_2O (10:25:65).

4. The profile of stained protein bands was displayed on a recorder as a gel was scanned (540 nm) by a spectrophotometer (Gilford #2400-S) with a linear transport attachment.

APPENDIX F

RADIORECEPTOR ASSAY OF GONADOTROPIN ACTIVITY

APPENDIX F

RADIORECEPTOR ASSAY OF GONADOTROPIN ACTIVITY

(Saxena et al., 1975. Science 184:793)

Isolation of Plasma Membranes

1. The ovary containing the corpus luteum was obtained at slaughter from pregnant cows before day 60 of gestation. Ovaries were placed in cold (4 C) .01 M tris-HCl buffer pH 7.8, containing 1 mM $MgCl_2$ and 1 mM mercaptoethanol.

2. After transport to the laboratory, corpora lutea were dissected from the ovaries and minced into 1 to 2 mm^3 pieces with a scalpel. The minced tissue was washed in .01 M tris-HCl containing $MgCl_2$ and mercaptoethanol, and the wash decanted.

3. The minced tissue was suspended in .01 M tris-HCl with $MgCl_2$ and mercaptoethanol and homogenized (Servall Omni Mixer; Ivan Sorvall, Inc., Norwalk, CN) for five second intervals six times with precautions taken to prevent heating of the homogenate.

4. Homogenate was subjected to six strokes of a Dounce tissue grinder using the pestle with large clearance and then stirred for two minutes.

5. Homogenate was centrifuged at 550 g for ten minutes and the supernatant was decanted.

6. The supernatant was then centrifuged at 10,000 g for ten minutes. The resulting supernatant was then decanted and discarded, and the pellet resuspended in 50 ml .01 M tris-HCl buffer with $MgCl_2$ and mercaptoethanol.

7. An equal volume of 62% sucrose in .01 M tris-HCl buffer was added to the resuspended pellet solution.

8. Then, 25 ml of 31% sucrose-pellet solution was layered over 20 ml of 45% sucrose contained in each of four 50 ml centrifuge tubes and was then centrifuged at 10,000 g for 20 minutes.

9. The top layer was aspirated off and diluted (1:3; v:v) with .01 M tris-HCl with $MgCl_2$ and mercaptoethanol, and centrifuged at 27,000 g for 20 minutes. The supernatant was decanted and discarded.

10. The pellet was resuspended in .01 M tris-HCl pH 7.2 containing .1% bovine serum albumen, 1 mM $CaCl_2$ and 200 I.U. Trasylol (FAB Pharm.) per ml.

11. Protein concentration of the resuspended pellet was estimated by the method described by Miller (1959; Appendix D). Protein destined for assay was solubilized by heating .1 ml of protein solution with .3 ml 1 N NaOH in a boiling water bath for 30 minutes (a marble was placed on top of the heated tube to reduce evaporation loss).

    A sample of .01 M tris-HCl with .1% bovine serum albumen was assayed to assess the contribution of albumen to the total protein.

12. Volume of the resuspended pellet solution was adjusted to obtain 200 µg plasma membrane protein per 100 µl of buffer.

Radioiodination of Human Chorionic Gonadotropin

1. Human chorionic gonadotropin[1] (HCG) was dissolved in .1 M sodium acetate buffer pH 6.0 at the concentration of 1 µg/µl and 25 µl of this solution was placed in a 1 ml reaction vial.

2. Iodine^{125} (2 mCi; Amersham/Searl Corp., Arlington Heights, Illinois) was added to the reaction vial in 20 µl NaOH solution (pH 8 to 11, as packaged).

3. Then, 50 ng lactoperoxidase (Sigma Chemical Company) in 20 µl of .1 M sodium acetate buffer pH 6.0 and 200 ng H_2O_2 in 10 µl H_2O were added and the solution was mixed by gentle swirling (two minutes) of the vial.

4. Three 100 ng additions of H_2O_2 (10 µl) were made at five minute intervals and the contents of the reaction vial was mixed for two minutes after each addition.

5. Twenty minutes after the first H_2O_2 addition .5 ml of .15 M NaCl with 1% bovine serum albumen pH 7.0 was added to stop the reaction.

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[1]Highly purified human chorionic gonadotropin (HCG-CR 119) was generously supplied by the Center for Population Research of the

6. Specific activity of the protein was estimated by trichloroacetic acid (12%) precipitation of the protein in 10 µl of the reaction mixture and centrifugation at 2500 g for 15 minutes. Specific activity was determined to be 16.5 µCi/µg HCG.

7. Labeled hormone was separated from free iodine by gel filtration (1 X 15 cm column) utilizing Sephadex G-100 (Pharmacia, Uppsula, Sweden) equilibrated with .15 M NaCl with 1% bovine serum albumen, pH 7.0.

Radioreceptor Assay

1. All dilutions were made in .01 M tris-HCl buffer with .1% bovine serum albumen, 1 mM $CaCl_2$ and 200 I.U./ml Trasylol. Uterine lumenal protein (200 µg) or embryo homogenate protein (¼ embryo equivalent) was pipetted into disposable culture tubes (12 X 75 mm) and total volume brought up to 100 µl.

2. Highly purified human chorionic gonadotropin for standards (0, .3, .6, 1.2, 2.5, 5.0 and 10.0 ng) was pipetted into disposable culture tubes; total volume was brought up to 100 µl and the standards were included in each assay.

3. Approximately 1.5 ng ^{125}I-human chorionic gonadotropin (25,000 cpm) and 200 µg luteal cell plasma membrane protein, each dispensed in 100 µl of buffer, were added to each tube. Tube contents was vortexed after each addition.

4. Standards and unknowns were incubated for 20 minutes at 37 C.

5. Tubes were then placed in an ice bath and 2 ml of cold (4 C) .01 M tris-HCl buffer with .1% serum albumen was added, the contents vortexed and centrifuged at 2500 g for 15 minutes.

6. Supernatant was aspirated off and the radioactivity of the pellet quantified in an automatic gamma counter.

Validation

1. Definitions:

   (a) total binding--percent of total amount of ^{125}I-HCG bound to 200 µg of plasma membrane protein in the absence of added HCG;

National Institute of Child Health and Human Development of the National Institutes of Health. Biological potency was determined to be 11,600 I.U./mg.

(b) non-specific binding--percent of total amount of ^{125}I-HCG bound to 200 µg plasma membrane protein which could not be displaced by the addition of 1000 ng HCG;

(c) depression of binding--proportion of total amount of ^{125}I-HCG bound to 200 µg plasma membrane protein in the presence of standard hormone or unknown, when the amount bound in total binding tubes was considered 100%;

(d) cross-reaction--percent derived from amount of HCG equivalent activity of added amount of suspect reactant divided by the amount of suspect reactant added.

2. Values:

(a) total binding 14.0%

(b) non-specific binding 3.2%

(c) depression of binding

ng HCG added	percent bound	ng ovine LH added	percent bound
0	100.0	0	100.0
.3	91.8	.3	97.6
.6	89.0	.6	93.6
1.2	75.0	1.2	90.0
2.5	60.3	2.5	76.6
5.0	43.5	5.0	64.4
10.0	40.0	10.0	56.2
1000.0	23.0	1000.0	25.3

(d) cross-reaction with:

ovine luteinizing hormone, NIH-LH-S11, 31.0% at 10 ng;
ovine follicle-stimulating hormone, NIH-FSH-S9, .20% at 100 ng;
ovine thyroid-stimulating hormone, NIH-TSH-S2, 3.60% at 100 ng;
bovine prolactin, NIH-PRL-B3, .20% at 100 ng;
bovine growth hormone, NIH-GH-B11, .12% at 100 ng.

40 µl ovine serum collected on day 10 of an estrous cycle was equivalent to 1.87 ng HCG.

APPENDIX G

EXTRACTION AND RADIOIMMUNOASSAY OF ESTRADIOL

APPENDIX G

EXTRACTION AND RADIOIMMUNOASSAY OF ESTRADIOL

(Britt et al., 1974. J. Anim. Sci. 39:915)

Extraction

1. Triplicate aliquants of serum (.5 ml) were pipetted into disposable culture tubes (16 X 125 mm).

2. To account for procedural loss, a fourth aliquant from a representative number of unknowns (8 to 12/assay) was added to a culture tube (16 X 125 mm) which contained 3000 dpm ^{3}H-2,4,6,7-estradiol (New England Nuclear; 100 Ci/mM; repurified by column chromatography).

3. Tubes containing serum and ^{3}H-estradiol were vortexed (ten seconds); and labeled and endogenous hormone were allowed to equilibrate for 30 minutes.

4. Serum was extracted with benzene by vortexing for 60 seconds.

5. Samples were centrifuged (2500 g; five minutes) to separate solvent and extracted plasma.

6. Extracted serum was then frozen over dry ice in methanol and solvent extract decanted into a culture tube (12 X 75 mm) for radioimmunoassay.

7. Solvent extracts from aliquants for procedural losses were decanted into scintillation vials. Radioactivity recovered in these extracts was averaged to determine a single correction factor to allow for procedural losses in all serum samples.

Radioimmunoassay

1. Estradiol-17β (Sigma Chemical Company) for standards (0, .5, 1.0, 2.0, 4.0, 7.0, 10.0, 20.0, 50.0 and 100.0 pg) was pipetted into 12 X 75 mm culture tubes from a stock solution of 100 pg/ml in absolute ethanol and incorporated into each assay.

2. Standards and serum extracts were dried under reduced atmospheric pressure (-29 lb/in^2) at 50 C. Tube walls were rinsed with minimal amounts of benzene and dried again.

3. Antibody[1] (200 µl), diluted 1:20,000 in .01 M phosphate buffered saline pH 7.4 containing .1% gelatin (Knox Gelatin, Inc., Johnstown, N.Y.), was added to each tube, the contents vortexed and allowed to incubate for 30 minutes at room temperature.

4. Then, 200 µl of .1% gelatin in .01 M phosphate buffered saline pH 7.4 containing 15,000 dmp ^{3}H-2,4,6,7-estradiol was added to each tube. The contents of the tubes were vortexed and incubated for three hours at 4 C.

5. Five hundred µl of 1% Dextran T70 (Pharmacia, Uppsula, Sweden) and .5% carbon decolorizing neutral norit (Fisher Sci. Co.) in distilled H_2O was added to each tube. Contents were mixed, incubated for ten minutes in an ice bath and then centrifuged at 2500 g for ten minutes at 4 C.

6. A .5 ml aliquant of supernatant was diluted with scintillation fluid (3a70B Preblend; Research Products International Corp., Elk Grove Village, Illinois) for quantification of radioactivity in a liquid scintillation spectrometer.

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[1]Anti-estradiol, generously supplied by Dr. G.D. Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins. The rabbit antiserum (#825) was prepared against 6-oxime-estradiol conjugated to bovine serum albumen.

APPENDIX H

SERUM PROGESTERONE IN EWES GIVEN ESTRADIOL DURING THE ESTROUS CYCLE OR EARLY PREGNANCY

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SERUM PROGESTERONE IN EWES GIVEN ESTRADIOL DURING THE ESTROUS CYCLE OR EARLY PREGNANCY

Table 7.--Serum progesterone (ng/ml) in ewes mated to a vasectomized ram and given 250 μg 17β-estradiol on days 11 and 12 or days 12 and 13 and in non-injected control ewes mated to a vasectomized ram (Experiment I).

Days After Mating	Days Estradiol Injected			Non-Injected Controls (n=4)
	11 & 12 (n=5)	12 & 13 (n=6)	Average (n=11)	
11	3.3 ± .4[a]	2.9 ± .5	3.0 ± .3	4.6 ± 1.9
12	2.5 ± .1	2.5 ± .4	2.5 ± .2	3.2 ± .6
13	1.5 ± .3	2.7 ± .8	2.1 ± .5	3.8 ± .9
14	.4 ± .1	.5 ± .2	.4 ± .1[b]	1.9 ± .6
15	.2 ± .1	.2 ± .1	.2 ± .05	.6 ± .2
16	.2 ± .1	.2 ± .05	.2 ± .01	.1 ± .05
17	.2 ± .05	.2 ± .05	.2 ± .01	.2 ± .05
18	.2 ± .1	.2 ± .01	.2 ± .05	.3 ± .1

[a]Mean ± S.E.

[b]Significantly different ($P < .01$) from control ewes on the respective day.

Table 8.--Serum progesterone (ng/ml) in ewes mated to intact rams and given 250 μg 17β-estradiol on days 11 and 12 or 12 and 13 (Experiment I).

Days After Mating	Progesterone Maintained[a,b] (n=6)	Progesterone Declined[b] (n=6)
11	4.0 ± .9[c]	3.0 ± .6
12	3.1 ± .8	2.4 ± .3
13	3.1 ± .4	2.2 ± .6
14	2.7 ± .7[d]	.8 ± .2
15	3.2 ± .5[d]	.4 ± .1
16	2.7 ± .5[d]	.2 ± .01
17	2.9 ± .7[d]	.3 ± .05
18	2.3 ± .6[d]	.3 ± .1

[a]Includes only ewes which had > 2.0 ng/ml serum progesterone on day 14 after mating.

[b]Data were pooled from ewes treated on days 11 and 12 or 12 and 13.

[c]Mean ± S.E.

[d]Significantly different ($P < .01$) from ewes which progesterone levels declined on respective days.

Table 9.--Serum progesterone (ng/ml) in ewes mated to intact or vasectomized rams and given 125 or 250 μg 17β-estradiol on days 11 and 12 after mating (Experiment II).

Days After Mating	Mated to Intact Rams[a] Pregnant (n=5)	Non-Pregnant (n=11)	Mated to Vasectomized Ram[a] (n=8)
10	2.4 ± .5[b]	2.6 ± .2	2.4 ± .5
11	3.1 ± .4	2.5 ± .4	2.8 ± .5
12	2.8 ± .6	2.4 ± .2	2.6 ± .5
13	3.3 ±1.0[c]	1.6 ± .1	1.7 ± .5
14	1.6 ± .4[c]	.8 ± .2	1.1 ± .4
15	1.6 ± .7[c]	.3 ± .1	.3 ± .1
16	.9 ± .4[d]	.2 ± .01	.2 ± .05

[a]Data were pooled from ewes given 125 or 250 μg 17β-estradiol.

[b]Mean ± S.E.

[c]Significantly different ($P < .01$) from levels in non-pregnant ewes on respective days.

[d]Significantly different ($P < .05$) from levels in non-pregnant ewes on respective days.

Table 10.--Serum progesterone (ng/ml) in ewes mated to intact or vasectomized rams and given either 0 or 125 μg 17β-estradiol on days 11 and 12 after mating (Experiment III).

Days After Mating	Mated to Intact Rams			Mated to Vasectomized Ram	
	Pregnant Non-Injected (n=5)	Pregnant Estradiol (n=5)	Non-Pregnant Estradiol (n=12)	Non-Injected (n=5)	Estradiol Injected (n=5)
10	4.4 ± .8[a]	4.5 ± .9	3.6 ± .2	2.7 ± .3	2.7 ± .3
11	4.1 ± .5	4.3 ± .6	4.1 ± .3	2.8 ± .2	3.5 ± .4
12	3.5 ± .4	5.0 ± .8	3.9 ± .4	3.2 ± .3	2.9 ± .5
13	3.8 ± .4	3.5 ± .5	2.9 ± .4	3.2 ± .5	1.9 ± .4[b]
14	4.1 ± .7	4.1 ± .4	1.4 ± .3[c]	2.6 ± .3	0.5 ± .2[b]
15	4.4 ± .7	3.7 ± .5	.9 ± .3[c]	1.3 ± .3	.2 ± .05[b]
16	4.3 ± 1.1	3.7 ± .6	.5 ± .2[c]	.4 ± .1	.2 ± .05
17	4.2 ± 1.0	3.7 ± .6	.3 ± .01[c]	.5 ± .05	.2 ± .1
18	3.9 ± .9	3.1 ± .5	.3 ± .01[c]	.3 ± .05	.2 ± .1
19	3.7 ± .4	4.0 ± 1.2	.4 ± .1[c]	.5 ± .1	.4 ± .1
20	3.6 ± .9	3.4 ± .5	.6 ± .1[c]	.6 ± .1	.7 ± .2

[a]Mean ± S.E.

[b]Significantly different ($P < .05$) from levels in non-injected ewes mated to a vasectomized ram on respective days.

[c]Significantly different ($P < .01$) from levels in pregnant, non-injected ewes.

APPENDIX I

REPRESENTATIVE SPECTROPHOTOMETRIC SCANS OF POLYACRYLAMIDE GELS AFTER ISOELECTRIC FOCUSING OF UTERINE LUMENAL FLUID PROTEIN

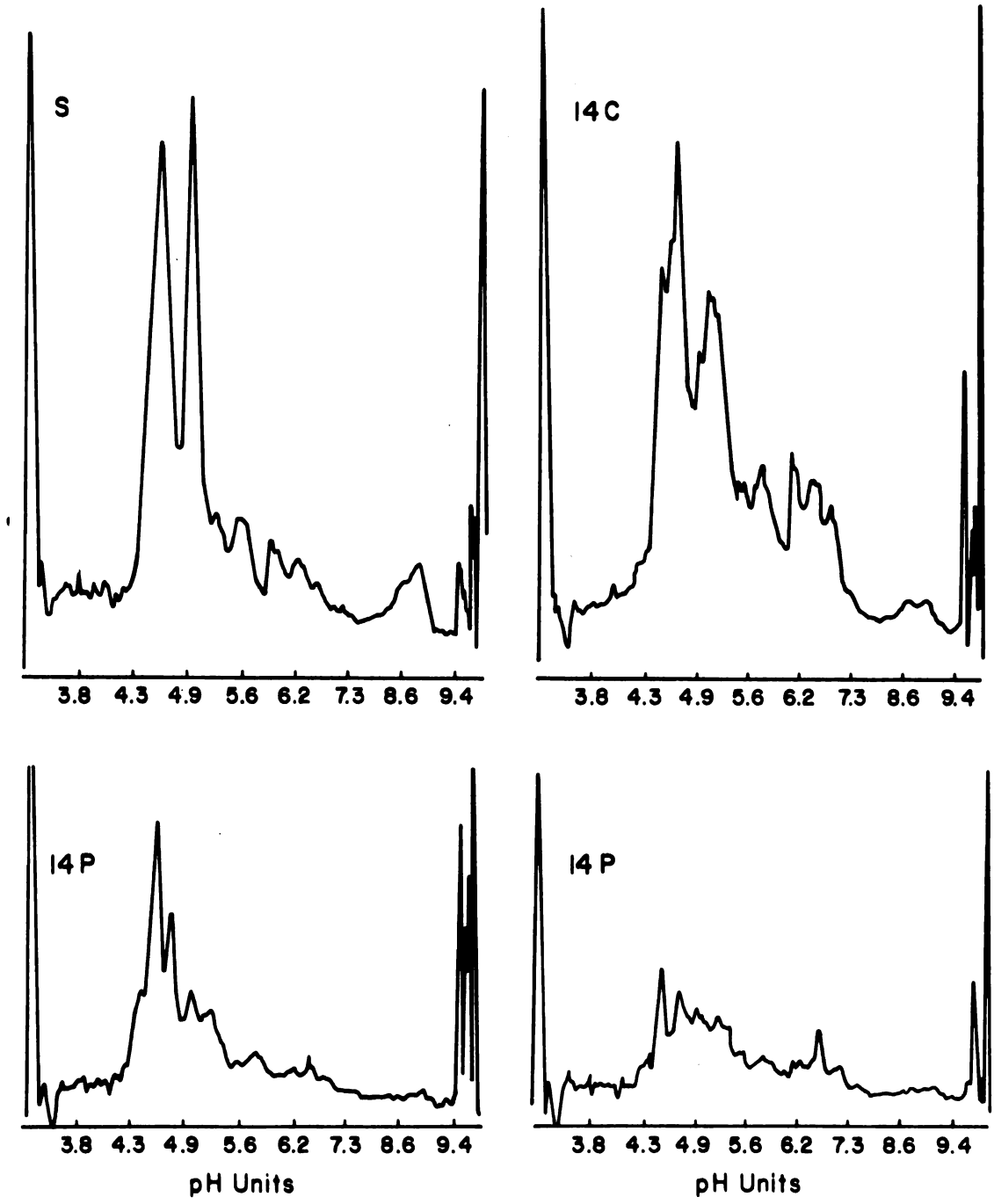

Figure 14.--Spectrophotometric scans of polyacrylamide gels after isoelectric focusing of uterine lumenal fluid protein collected on day 14 of an estrous cycle and pregnancy (Experiment V).

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