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Title: PITUITARY AND UTERINE CONTROL OF OVARIAN FUNCTION IN THE GILT AND EWE

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The role of the pituitary gland and uterus in regulating ovarian function in the estradiol-treated gilt and ewe was investigated in two experiments.

Pituitary gonadotropin content, ovarian follicular development and corpus luteum function were studied following daily intramuscular injection of seven gilts with 7 mg of 17β-estradiol from day 11 through day 16 of the estrous cycle (first day of estrus = day one of the cycle). Seven control gilts were injected with corn oil for the same duration. Control and treated gilts were autopsied on day 17 of the cycle. Exogenous estradiol caused an increase in pituitary FSH and LH activity ($P < .01$, for each gonadotropin) and increased anterior pituitary dry weight ($P < .05$). Treatment of gilts with estradiol inhibited ovarian follicular growth beyond 5 mm in diameter, reduced the number of follicles $\geq$ 3 mm in diameter ($P < .01$) and depressed follicular fluid weight ($P < .01$). Injection of estradiol into gilts was without effect on luteal weight, but increased luteal progesterone content and concentration ($P < .01$, for each characteristic).

Effect of time of hysterectomy on luteal characteristics of estradiol-treated ewes and the effect of estradiol on uterine protein synthesis in these ewes were investigated.

Fifteen ewes were assigned randomly in equal numbers to three treatment groups and were injected intramuscularly with 750 μg of 17β-estradiol on each of days 10 and 11 of the cycle (first day of estrus = day zero of the cycle). Ewes were hysterectomized as follows: group 1, immediately before the first injection (zero hour); group 2, 24 hours following the first injection and prior to the second injection, and group 3, 48 hours following the first injection. In addition, two ewes were injected with vehicle on each of days 10 and 11 of the cycle and hysterectomized 24 and 48 hours following the first injection. All ewes were autopsied on day 15 of the cycle. Following hysterectomy, samples of endometrium from the uterine horn ipsilateral to the ovary containing the corpus luteum from one ewe in each group and the two control ewes were incubated for two hours at 37°C under 95% O_2-5% CO_2 in Eagle's HeLa medium containing 7 μCi of uniformly labelled L-leucine-^{14}C. The tissue was homogenized in 0.05% Na_2 EDTA, centrifuged and the supernatant subjected to polyacrylamide gel electrophoresis, Sephadex column chromatography and dialysis to determine the extent of incorporation of leucine into newly synthesized endometrial protein.

Removal of the uterus at 24 hours following injection of ewes with estradiol blocked corpus luteum regression whereas hysterectomy at 48 hours failed to prevent estradiol-induced luteal regression ($P < .05$). Time of hysterectomy was without effect on luteal progesterone content of estradiol-treated ewes. Luteal progesterone concentration of ewes

hysterectomized 48 hours following the initial injection of estradiol was greater ($P < .05$) than that of ewes hysterectomized at 24 hours, but did not differ from the luteal progesterone concentration of ewes hysterectomized prior to estradiol treatment (zero hour). Luteal progesterone concentration of ewes hysterectomized 24 hours after the initial injection of estradiol did not differ from that of ewes hysterectomized prior to estradiol treatment. Incorporation of labelled leucine into endometrial protein was maximal at 24 hours and was reduced at 48 hours following estradiol injection. Incorporation of leucine into certain endometrial proteins tended to increase progressively with time following estradiol injection, whereas incorporation of this amino acid into some other endometrial proteins tended to decrease with time. Results of Sephadex column chromatography and dialysis indicate that labelled leucine was actually incorporated into uterine protein. Data from the present experiment suggest that there may be a relationship between estrogen-induced uterine protein synthesis and luteal regression in the ewe.

Pituitary and Uterine Control of Ovarian Function in the Gilt and Ewe

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REVIEW OF LITERATURE

The sequence of biological and behavioral events which characterize mammalian female reproduction is dependent to a great extent upon the coordinated function of various endocrine glands. The primary importance of the pituitary gland (hypophysis) in regulating successful female reproduction has long been recognized. Removal of the pituitary results in ovarian atrophy and terminates behavioral estrus in laboratory and domestic animals. At one time it was assumed that the pituitary gland, through the release of various hormones, merely directed the function of other endocrine glands. It is now known, however, that the gonadal hormones as well as hormones from other endocrine glands actually feedback to regulate the function of the pituitary gland. Although a direct action of gonadal hormones on the pituitary is not ruled out, they appear to regulate pituitary function by acting on the higher centers of the brain, particularly the hypothalamus. The hypothalamus in turn controls the release and perhaps the synthesis of various hypophyseal hormones through production of specific releasing or inhibiting factors. Hypothalamic control of pituitary gland function appears to be an essential integrating mechanism for successful reproduction.

Hypothalamo-Hypophyseal Interrelationships

The ability of various external stimuli to alter gonadotropin secretion rate was the earliest indication that higher centers of the brain were involved in the regulation of pituitary function. Anterior

pituitaries of rats kept under constant illumination release more follicle stimulating hormone (Negro-Vilar, Dickerman and Meites, 1968) and contain an increased amount of luteinizing hormone (Bradshaw and Critchlow, 1966). Although the neurohypophysis (posterior pituitary) is innervated to a great extent, the same is not true for the adenohypophysis (anterior pituitary). Harris (1948) described the anterior pituitary as a gland under neural control but lacking an adequate nerve supply. Neural control of the anterior pituitary by humoral agents released from the hypothalamic region of the brain and carried to it by the hypophyseal portal circulation was suggested by Brooks (1938) and Taubenhaus and Soskin (1941). The validity of these early theories was subsequently demonstrated. Hypophysectomy and autotransplantation of pituitaries to a site under the kidney capsule of mature female rats block the occurrence of estrous cycles and cause a significant reduction in ovarian weight (Nikitovitch-Winer and Everett, 1958). Retransplantation of pituitaries to the site under the median eminence of the brain results in the resumption of estrous cycles and increases ovarian weight. Results of these investigations were regarded as strong evidence that the hypothalamus was indeed a regulator of anterior pituitary function. Since these initial experiments a specific hypothalamic releasing factor (RF) has been reported to exist for follicle stimulating hormone (FSH) by Igarashi and McCann (1964) and Mittler and Meites (1964), luteinizing hormone (LH) by McCann (1962), adrenocorticotropic hormone (ACTH) by Royce and Sayers (1958), growth hormone (GH) by Franz et al. (1962) and thyroid stimulating hormone (TSH) by Guillemin et al. (1963).

Inhibition of synthesis and release of prolactin also appears to be regulated by a prolactin inhibiting factor (PIF) of hypothalamic origin (Talwalker, Ratner and Meites, 1963). Acid extracts of the stalk median eminence (SME) of rat or bovine origin causes FSH release in ovariectomized rats in which FSH release has been blocked by administration of estrogen and progesterone (Igarashi and McCann, 1963; Igarashi, Nallar and McCann, 1964). Presence of a hypothalamic FSHRF has also been detected through measurement of FSH released from rat anterior pituitaries incubated in vitro with rat hypothalami (Jutisz and de la Llosa, 1967). There is a possibility that the FSHRF and LHRF in the porcine hypothalamus is one and the same molecule (Schally et al., 1971). In the rat, however, McCann and Dhariwal (1966) have been able to isolate fractions possessing either FSHRF or LHRF activity. Likewise, extracts of sheep hypothalami appear to contain discrete FSH and LH releasing factors (Dhariwal et al., 1965). In addition, evidence for the presence of a specific LHRF in rat SME extract able to induce LH release when injected into rats bearing SME lesions (McCann, 1962) or induce ovulation when injected directly into the rat pituitary (Nikitovitch-Winer, 1962) has been presented. Similar neurohumoral regulation of pituitary LH release appears to be operative in the sheep. Intravenous injections of purified porcine LHRF can cause an increase in the serum LH level of diestrous ewes (Reeves, Arimura and Schally, 1970). In a subsequent experiment injection of porcine LHRF into ewes at various stages of the estrous cycle caused release of pituitary LH but response was maximal during the period immediately following onset of estrus (Reeves, Arimura and Schally, 1971). It

appears that pituitary responsiveness to LHRF is influenced by the stage of the estrous cycle and more specifically by the endogenous levels of progesterone. The circulating levels of estrogen can also affect hypothalamic releasing factors. Treatment of rats with estrogen causes a reduction in pituitary FSH content by inhibiting the synthesis of FSHRF (David, Fraschini and Martini, 1965). This observation indicates that FSHRF may regulate both the synthesis and release of FSH. There is some experimental evidence which suggests the presence of a FSH synthesizing factor in the rat hypothalamus (Corbin and Daniels, 1968). Although conclusive proof of a synthesizing factor is lacking, this factor appears to affect pituitary FSH levels in a manner different from that of FSHRF (Corbin, Milmore and Daniels, 1970).

Involvement of brain catecholamines or monoamines in the regulation of gonadotropin release has been suspected for some time (Friedgood and Bevin, 1938; Markee, Sawyer and Hollinshead, 1948; Markee, Everett and Sawyer, 1952). Mating of sympathectomized female rats with a vasectomized male induced pseudopregnancy indicating a probable existence of another neural pathway (Friedgood and Bevin, 1938). Acetylcholine failed to induce ovulation when injected either systemically or directly into the hypophysis of the rabbit (Markee, Sawyer and Hollinshead, 1948), but epinephrine induced ovulation only when injected directly into the hypophysis. These investigators suggested that the hypothalamic - pituitary pathway involved in ovulation is probably adrenergic in nature. Schneider and McCann (1969) demonstrated that dopamine enhances LH release when added to

rat anterior pituitaries incubated with SME and that this response to dopamine is blocked by the addition of phentolamine, an α-adrenergic blocking agent. Injection of dopamine into the third ventricle of the brain induces release of LHRF into the peripheral circulation of hypophysectomized rats (Schneider and McCann, 1970). Pretreatment of rats with estradiol two hours before dopamine injection, however, completely blocks dopamine stimulated discharge of LHRF. Intraventricular injection of dopamine causes a marked increase in serum LH in the rat, whereas serotonin (5-hydroxytryptamine) decreases serum LH concentrations (Kamberi, Mical and Porter, 1970). These investigators also observed that epinephrine and norepinephrine were effective in causing an increase in the serum LH level when injected in high doses only. Systemic administration of melatonin (N-acetyl-5-hydroxytryptamine), a monoamine from the pineal gland can retard puberty in immature female rats and reduce plasma LH levels when implanted in the median eminence of castrated male rats, but not when implanted in the pituitary gland (Martini, Fraschini and Motta, 1968). Among other indole compounds synthesized by the pineal gland, median eminence implants of 5-hydroxytryptophol can cause a significant reduction of pituitary LH, whereas 5-methoxytryptophol is without effect (Martini et al., 1968). These observations indicate that the brain may contain receptors sensitive to indole compounds which play a role in the regulation of gonadotropin secretion.

Lesions placed in various areas of the hypothalamus can interfere with normal reproductive functions and investigators have used this experimental approach to localize hypothalamic areas controlling the

secretion of pituitary gonadotropins. Hypothalamic lesions cause failure of reproductive functions in the rat (Kennedy and Mitra, 1963), guinea pig (Barry and Mazzuca, 1962) and sheep (Clegg and Ganong, 1960). Lesions in the anterior hypothalamus of immature female rats cause precocious sexual development (Bogdanove and Schoen, 1959). Lesions in similar areas of the hypothalamus of adult female rats cause hypertrophy of the anterior pituitary, ovaries lack corpora lutea and such rats exhibit constant estrus (D'Angelo, 1960; Van Rees, Van der Werff and Wolthius, 1962). It appears that lesions in this hypothalamic area inhibit normal release of LH. Ovulation and luteinization can be induced in such rats by injection of progesterone (Greer, 1953) or by ovariectomy and transplantation of one of the ovaries to the spleen (Flerko and Bardos, 1961). These results indicate that either injection of progesterone or lowering of endogenous levels of estrogen by ovariectomy can cause release of LH and ovulation. Flerko (1962) has suggested that destruction of estrogen-sensitive FSH-inhibitory centers in the hypothalamus results in increased FSH release and release of enough LH to promote estrogen secretion but not sufficient to cause ovulation. Such lesions do not affect the hypothalamic areas controlling LH secretion and so, either injection of progesterone or lowering the estrogen level by ovariectomy can induce sufficient LH release to cause ovulation. Further support for hypothalamic steroid-sensitive sites has been presented by Barraclough, Yrarrazaval and Hatton (1964). Lesions in the suprachiasmatic nuclei of the hypothalamus of the rat causes persistant estrus, but permits ovulation following treatment with progesterone. If in addition to the suprachiasmatic area, the

periventricular portion of the medial preoptic area is also destroyed by lesions, the rats fail to ovulate to progesterone treatment. These experimental data suggest the existence of steroid sensitive hypothalamic site(s) that control LH release. Van Rees and Wolthius (1962) have indicated that testosterone may act to inhibit FSH secretion from anterior pituitaries transplanted under the kidney capsule in the immature rat. Injection of immature rats with estrogen can exert a positive feedback effect upon the pituitary and stimulate both synthesis and release of FSH (Johnson, 1971). Although these experimental observations demonstrate a direct action of gonadal steroids on the pituitary gland, they do not preclude the possibility of the existence of hypothalamic steroid-sensitive areas regulating release of FSH and LH as proposed by Flerko (1962).

Injection or implantation of steroid hormones into the hypothalamo-hypophyseal system can alter pituitary function and reproduction. Ovarian atrophy and atrophy of the reproductive tract occur in rats when estrogen or testosterone is injected into the arcuate or mammillary nuclei of the hypothalamus (Lisk, 1960; 1962). On the other hand, Ramirez and McCann (1964) found that estrogen implanted into the median eminence of the rat can cause synthesis and release of both LH and prolactin. Failure of ovulation and subsequent ovarian atrophy can result in rabbits bearing median eminence estrogen implants, but such implants in the anterior pituitary itself or in other hypothalamic areas are without effect (Davidson and Sawyer, 1961). Synthesis and release of LH occur in rabbits when estrogen is implanted into the anterior pituitary (Kanematsu and Sawyer, 1963). Recent experimental

evidence suggests that the pituitary gonadotropins may regulate their own synthesis by modifying their hypothalamic regulators. Results obtained by Corbin and Cohen (1966, 1967) following LH and FSH implants into the rat median eminence are indicative of the presence of direct internal feedback mechanisms. Anatomical support for such a mechanism has been presented by Szentagothai (1962), who observed that some of the blood reaching the anterior pituitary gland may find its way into the interior plexus of the infundibular stem and is finally drained towards the hypothalamus. Implants of LH in the rat median eminence, but not in the anterior pituitary cause a reduction in pituitary LH content (David, Fraschini and Martini, 1966). Luteinizing hormone implanted into the rat median eminence has the ability to reduce pituitary LH content, but implants of FSH and ACTH are without effect on pituitary levels of this gonadotropin (Corbin and Cohen, 1966). In a similar study, Corbin and Cohen (1967) found that implants of FSH into the rat median eminence significantly lowered pituitary FSH content, whereas implants of LH failed to affect FSH levels in the pituitary.

Control of Luteal Function

In our domestic species, the duration of the estrous cycle of the female is governed by the life span of the corpus luteum. Luteal life span and secretion of progesterone during the estrous cycle is controlled to a great extent by the pituitary gland and the non-gravid uterus. The role of the pituitary and uterus in regulating luteal maintenance and function, however, appears to differ in the various species of animals studied thus far.

Hypophysectomy causes regression of corpora lutea in the pseudo-pregnant mouse (Kovacic, 1965), rat (Evans et al., 1941), hamster (Greenwald, 1967) and rabbit (Kilpatrick, Armstrong and Greep, 1964). Hypophysectomy of the immature ewe immediately following gonadotropin induced ovulation, does not interfere with luteal maintenance up to 12 days post-surgery (Denamur and Mauleon, 1963 a). Secretion of progesterone by the corpus luteum is normal up to nine days following hypophysectomy of mature ewes between days two and five of the estrous cycle, but declines thereafter (Denamur, Martinet and Short, 1966). These experimental observations suggest that continued hypophyseal support is not necessary for the formation and early development of the corpus luteum in the ewe. On the other hand, Kaltenbach et al. (1968 a) have demonstrated that the corpus luteum fails to develop if ewes are hypophysectomized immediately following ovulation. Furthermore, hypophyseal support for normal luteal development is essential for the first five days following ovulation. In contrast to the results of others, these data indicate that in the ewe corpus luteum formation and function is dependent upon the pituitary gland. Evidence has been presented for normal luteal development in the sow when hypophysectomy is performed before or at various times following ovulation (du Mesnil du Buisson and Leglise, 1963; Anderson et al., 1967). Development of corpora lutea in sows hypophysectomized immediately after the onset of estrus is normal up to day seven or eight but luteal regression occurs thereafter (du Mesnil du Buisson and Leglise, 1963). Corpora lutea of hypophysectomized sows weigh less on day ten compared to that of glands of control animals (Anderson et al., 1967). It appears that

in the sow at least, hypophyseal support for the formation and early development of corpora lutea is not necessary.

The term "luteotropin" was coined by Astwood (1941) for the anterior pituitary hormone prolactin because of its ability to stimulate the function and maintenance of corpora lutea in the pseudopregnant mouse and rat. Corpora lutea in the stalk transected rat persist (Nikitovitch-Winer, 1965) indicating that prolactin secretion from the pituitary continues due to the failure of PIF to reach the hypophysis.

Prolactin, however, has not stood the test of being the universal luteotropin. Injection of prolactin fails to prolong luteal life span in the guinea pig (Aldred, Sammelwitz and Nalbandov, 1961), rabbit (Kilpatrick, Armstrong and Greep, 1964), ewe (Denamur and Mauleon, 1963 b), sow (Duncan et al., 1961) and cow (Smith, McShan and Casida, 1957). Extensive investigations have been carried out in various species of animals to characterize the luteotropic hormone(s) of pituitary or non-pituitary origin. Because of its intimate association with ovulation and luteal formation, the role of LH on the maintenance of luteal function has been the object of intensive research.

Injection of LH into rabbits has produced varied results. Corpora lutea are maintained in hypophysectomized pseudopregnant rabbits when LH is injected 12 hours after hypophysectomy, but earlier injection of LH causes luteal regression (Kilpatrick et al., 1964; Spies, Coon and Gier, 1966). These observations indicate that some pituitary factor which is not available beyond a period of 12 hours following hypophysectomy may act in concert with exogenous LH to bring about luteal regression in the rabbit. Intravenous injection of LH into

the intact pseudopregnant rabbit after day five induces luteal regression (Stormshak and Casida, 1965; Spies et al., 1966). Injection of estrogen into the intact pseudopregnant rabbit can maintain corpora lutea (Chu, Lee and You, 1946) and can protect the corpora lutea from the luteolytic action of exogenous LH (Stormshak and Casida, 1965). Destruction of follicles by x-irradiation in the remaining ovary of a unilaterally ovariectomized pregnant rabbit causes abortion within 27 to 60 hours following ovariectomy, but pregnancy is carried to term if the follicles in the remaining ovary are not destroyed (Keyes and Nalbandov, 1967). Abortion can also be prevented by daily injection of estrogen to unilaterally ovariectomized rabbits with one irradiated ovary, whereas injections of LH or ovine pituitary powder are ineffective. It has been suggested that follicular estrogen acts directly to maintain corpora lutea in the rabbit (Keyes and Armstrong, 1968) and if this source of estrogen is removed and not replaced, corpora lutea cannot be maintained by hormones of pituitary or placental origin (Keyes and Nalbandov, 1967; Keyes and Armstrong, 1968). Luteinizing hormone is luteolytic in the pseudopregnant rabbit only in the absence of estrogen (Keyes and Nalbandov, 1967).

Luteinizing hormone appears to be the primary luteotropic hormone in sheep. Hypophysectomy of the ewe at midcycle results in a marked decrease in luteal progesterone secretion within five hours but a single injection of LH or prolactin causes an immediate increase in the ovarian venous progesterone concentration (Hixon and Clegg, 1969). These authors were of the opinion that the functional activity of the ovine corpus luteum is regulated by anterior pituitary hormones.

Continuous infusion of crude LH or FSH and LH can maintain the corpus luteum in the ewe, while FSH, prolactin and estrogen are without effect (Kaltenbach et al., 1968 b). Luteotropic properties of LH have been established in the cow (Simmons and Hansel, 1964; Donaldson, Hansel and Van Vleck, 1965). Oxytocin, the pituitary hormone from the posterior lobe is luteolytic in the cow only when administered during the first seven days of the estrous cycle (Armstrong and Hansel, 1959; Labhsetwar et al., 1964) but not when injected during days 12 to 14 of the cycle (Mares and Casida, 1963). Daily injection of bovine LH can counteract the luteolytic action of oxytocin (Donaldson et al., 1965), whereas equine LH, prolactin and GH are ineffective. A single injection of LH into intact heifers on day 16 of the cycle prolongs the length of the estrous cycle (Donaldson and Hansel, 1965).

Luteinizing hormone by itself is not luteotropic in the sow. Daily injection of LH may prolong the luteal life span in the hypophysectomized sow only in the absence of the uterus (Anderson et al., 1965). In the presence of the uterus, concurrent administration of 5 mg LH and 5 mg 17β -estradiol to hypophysectomized sows beginning on day 12 of the cycle cause corpora lutea to remain functional up to day 20 of the estrous cycle (Anderson et al., 1965). It has been suggested that administration of estrogen may inhibit uterine luteolytic activity in the sow (Denamur, 1968).

Further support for the luteotropic ability of LH has been obtained under in vitro conditions. Luteinizing hormone stimulates progesterone synthesis by luteal slices of the ewe (Kaltenbach et al., 1966), cow (Mason and Savard, 1964; Hansel, 1966) and sow (Cook et al., 1967).

Addition of prolactin or FSH to the incubation medium does not promote progesterone synthesis by porcine corpora lutea (Cook et al., 1967) and a direct stimulatory effect of estrogens on progesterone synthesis has not been demonstrated (Cook et al., 1968).

In some species of animals exogenous estrogen can either maintain or induce regression of the corpus luteum depending on the stage of the estrous cycle during which treatment is begun. Exogenous estrogen is luteotropic in the pregnant or pseudopregnant rabbit (Greep, 1941; Stormshak and Casida, 1965). Although not effective in the hypophysectomized ewe, estrogen injected into the intact animal during the early part of the estrous cycle can maintain the corpus luteum (Denamur and Mauleon, 1963 b; Piper and Foote, 1968). There is evidence to indicate that treatment of ewes with estrogen during the early stages of the cycle brings about release of LH from the anterior pituitary which may be luteotropic (Foote, 1964; Howland et al., 1968 and Akbar, 1971). An increase in serum LH was observed 16 to 20 hours following injection of estradiol into ewes on day four of the cycle (Akbar, 1971). On the other hand, estrogen is luteolytic when injected into ewes beyond mid-cycle (Stormshak, Kelly and Hawk, 1969; Hawk and Bolt, 1970) and Akbar (1971) has reported that estrogen treatment on day 11 of the cycle failed to cause an increase in the serum LH level. Daily injection of estrogen into the gilt beginning on or before day 11 of the estrous cycle causes luteal maintenance (Kidder, Casida and Grummer, 1955; Gardner, First and Casida, 1963). Follicular estrogen, however, does not play any significant role in luteal maintenance of the pregnant sow (Noveroske, 1969). Exogenous estrogen is luteolytic in cattle

(Kaltenbach et al., 1964) and also in the guinea pig when injected during the early luteal phase of the cycle (Choudary and Greenwald, 1968).

Another steroid hormone, progesterone, can affect the luteal life span in some species. Daily injection of progesterone into cows for ten days or into ewes for six days beginning on the day of detected estrus causes a significant reduction in luteal life span, but injection of this hormone into the sow has no effect on the corpora lutea (Woody, First and Pope, 1967). It has been suggested that exogenous progesterone by its negative feedback action on the pituitary may inhibit the release of a luteotropin and as a consequence reduce luteal life span (Nalbandov, 1961). Daily injection of progesterone into ewes for six or twelve days, before or after the day of expected ovulation resulted in significantly smaller corpora lutea on day six of the estrous cycle, whether or not treatment was continued beyond the time of ovulation (Woody, Ginther and Pope, 1967). Luteolysis caused by exogenous progesterone can be prevented by hysterectomy (Woody et al., 1967), indicating that the injected progesterone may act via the uterus.

Investigations in elucidating the control of luteal function in the past have mainly dealt in characterizing the role played by the pituitary gland. It had been assumed that the uterus did not play any significant role in luteal function until Loeb (1923) demonstrated that complete hysterectomy maintained corpora lutea for over 60 days in the guinea pig. Since these initial studies, many investigations have been conducted to study the utero-ovarian relationships. Hysterectomy during the estrous cycle of the mouse, rat, and hamster do not affect the life span of corpora lutea (Anderson, Bowerman and Melampy, 1963).

Luteal maintenance in the pseudopregnant rat (Melampy, Anderson and Kragt, 1964) and hamster (Duby et al., 1969a) is prolonged following hysterectomy but not in the pseudopregnant rabbit (Hunter and Casida, 1967). Hysterectomy causes luteal maintenance and cessation of estrual behavior in the guinea pig (Rowlands, 1961), cow and ewe (Wiltbank and Casida, 1956). Uterine involvement has also been demonstrated in the ewe since hysterectomy abolishes estrogen-induced luteal regression (Stormshak et al., 1969). Luteal involution and regressive changes in ovine luteal cells even when already started, can be arrested as late as day 15 of the cycle by hysterectomy (Moor et al., 1970). Maintenance of corpora lutea in the pig following hysterectomy has been demonstrated by Anderson, Butcher and Melampy (1963).

A local luteolytic effect of the uterus has been demonstrated to exist in the ewe (Moor and Rowson, 1964; Rowson and Moor, 1964) and the pig (du Mesnil du Buisson, 1961). Presence of at least one-fourth of the uterus is required for continuation of estrous cycles in the gilt (Anderson, Butcher and Melampy, 1961). du Mesnil du Buisson (1961) reported that each uterine horn can exert a local luteolytic action on corpora lutea located in the adjacent ovary. In the partially hysterectomized pig, a segment of the uterine horn causes corpora lutea to regress on the adjacent ovary whereas corpora lutea on the opposite ovary are maintained. Maintenance of corpora lutea in the ovary on the ipsilateral side of unilaterally hysterectomized animals and normal luteal regression in the contralateral ovary have been observed in the guinea pig (Bland and Donovan, 1966), ewe (Inskeep and Butcher, 1966) and cow (Anderson, Neal and Melampy, 1962; Ginther et al., 1967). The

amount of uterine tissue necessary for the maintenance of normal estrous cycles appears to vary from species to species. Presence of one uterine horn is necessary for normal estrual activity in the guinea pig (Butcher, Chu and Melampy, 1962), whereas only one-fourth of one uterine horn is sufficient in the pig (Anderson et al., 1961). Half of the estrous cycles were extended over 20 days following removal of one uterine horn in the ewe and one-fourth of the cycles were extended when only the distal half of one uterine horn was removed (Moor and Rowson, 1964; Rowson and Moor, 1964). In the cow, presence of the cervix and the posterior segment of the uterine body is sufficient to maintain nearly normal estrual activity (Wiltbank and Casida, 1956). The local luteolytic effect of the uterus has received further support from ovarian and uterine transplantation studies. Autotransplanation of ovaries to a site under the kidney capsule in the guinea pig (Bland and Donovan, 1968) and the pseudopregnant rat (Anderson, Melampy and Chen, 1966, 1967) prolong the life span of the corpus luteum. On the other hand, an absence of local uterine action in the hamster is indicated since autotransplantation of the ovaries to the cheek pouch did not extend the length of the estrous cycle or pseudopregnancy (Duby et al., 1969b) Homotransplantation of the uterus into the cheek pouch of the hysterectomized pseudogregnant hamster also causes luteal regression (Duby, McDaniel and Black, 1965). These findings suggest that in the hamster, the uterine luteolytic action is mediated systemically. Cyclic estrual behavior can be induced in hysterectomized pigs by uterine endometrial autotransplantation into the abdominal oblique muscle or to the parietal wall of the peritoneum (Anderson et al., 1963; du Mesnil

du Buisson and Rombauts, 1963). An inverse relationship has been demonstrated between the quantity of endometrial glands in the uterine autotransplants and the duration of pseudopregnancy in the rat (Melampy et al., 1964). In the ewe, transplantation of either the entire or a part of the uterus to the omentum, results in similar cycle lengths as in control animals (Niswender, 1968). It thus appears that in the guinea pig, sheep and pig, the species in which a local uterine luteolytic effect is well established, the uterus may also exert this action systemically, possibly by a uterine luteolytic factor(s).

Existence of a uterine luteolytic factor has not been conclusively demonstrated. The only promising results have been obtained using the hysterectomized pseudopregnant hamster as the test animal. The luteolytic activity was found to be contained in the submitochondrial fraction of day six or day seven uterine homogenates of the hamster, whereas, uterine homogenates from days four, five or eight of the pseudopregnancy were without effect (Mazer and Wright, 1968). In all other species of animals investigated so far, evidence for the existence of such a factor has been based primarily on experiments involving hysterectomy indicating the significant role played by the uterus in inducing luteal regression. It also appears that only the endometrium is the site of production of this luteolytic factor. Injection of an irritant or corrosive into the lumen of the remaining uterine horn of a unilaterally hysterectomized pig causes necrosis of the endometrium and maintenance of corpora lutea (Anderson et al., 1961). Butcher, Chu and Melampy (1962) found extended luteal life span following injection of a corrosive into the lumen of the uterine horns of the guinea pig. The subsequent

increase in the luteal life span was proportional to the extent of endometrium destroyed. Many investigators have attempted to identify the elusive uterine luteolytic factor(s) without much success. Crude aqueous extracts of bovine endometrium from day ten to day 13 of the estrous cycle can induce luteal regression in the hysterectomized pseudopregnant hamster and the active factor appears to be a large molecular weight protein or a small protein-bound molecule (Lukaszewaska and Hansel, 1970). Under in vitro conditions, progesterone synthesis by pig luteal slices was enhanced by the addition of endometrial extracts obtained from sows on days 12 and 13 of the estrous cycle, but endometrial extracts obtained on days 16 to 18 of the cycle had an inhibitory effect on steroidogenesis (Duncan et al., 1961). Support for the presence of a bovine uterine luteolytic factor has been presented by Williams et al., (1967) who found that injection of an acetone-ether dried bovine uterine preparation caused luteal regression in pseudopregnant rabbits. On the other hand, administration of aqueous or ether extracts of bovine endometrium from different stages of the cycle failed to cause a reduction in the length of pseudopregnancy in the hysterectomized rat (Malven and Hansel, 1965).

The object of recent investigations involving the identification of the uterine luteolytic factor has been the prostaglandins, a group of related 20-carbon chain hydroxy fatty acids widely distributed in mammalian tissues. It has been demonstrated that prostaglandin $F_{2\alpha}$ can cause luteal regression in the pseudopregnant rat (Pharris and Wyngarden, 1969), guinea pig (Blatchley and Donovan, 1969), rabbit (Gutknecht, Cornette and Pharris, 1969) and sheep (McCraken, Glew and

Scaramuzzi, 1970). Further support for prostaglandin $F_{2\alpha}$ being a uterine luteolytic factor has been provided by the investigations of Blatchley et al. (1971). These investigators detected the presence of substantial amounts of prostaglandin $F_{2\alpha}$ in the utero-ovarian venous blood when intact guinea pigs were subcutaneously injected with estradiol benzoate on days four through six of the estrous cycle, but not when animals were hysterectomized on day four of the cycle. Bioassays indicated the presence of more prostaglandin $F_{2\alpha}$ in the estrogen treated animals compared to untreated controls.

Estrogen Action on the Uterus

Administration of estrogen to an ovariectomized female brings about a series of anabolic changes in the uterus which have been most intensively studied in the rat. A single injection of estrogen into the rat causes generalized hyperemia of uterine tissue, uterine fluid imbibition, enlargement of cell nuclei and expansion of the endoplasmic reticulum, respectively (Mueller, Herranen and Jervell, 1958). Following injection of rats with estrogen, one of the earliest uterine changes detected is a rise in phospholipids which occurs simultaneously with the imbibition of uterine fluid (Aizawa and Mueller, 1961). Increased glucose utilization by the rat uterus occurs as early as four hours following estrogen administration (Szego and Roberts, 1953). On the other hand, incorporation of glucose-^{14}C into protein and ribonucleic acid (RNA) of the rat uterus is markedly increased within two hours after estrogen treatment (Nicolette and Gorski, 1964). Estrogen treatment also causes an increase in uterine glycogen content in the rat (Wallaas, 1952) and rabbit (Brody and Westman, 1958).

Ability of exogenous estrogen to induce a rapid increase in uterine protein synthesis has received considerable attention in recent years. Pretreatment of rats with physiological doses of estradiol greatly increases incorporation of radioactive glycine and tryptophan into protein by uterine tissue under *in vitro* conditions (Mueller, 1953). Availability of increased amounts of certain amino acid-activating enzymes may be necessary as a first step in estrogen-stimulated synthesis of new proteins. Treatment of rats with estrogen causes a rapid increase in the activities of seven uterine amino acid-activating enzymes during the ensuing 24-hour period (McCorquodale and Mueller, 1958). The series of events that culminate in an increase in protein synthesis by the estrogen-stimulated uterus have not yet been completely elaborated. Jensen and Jacobson (1962) demonstrated that physiological doses of estrogen administered to immature rats were selectively taken up by the uterus whereas this specificity was lost when higher doses were used. It is possible that higher doses of hormone may result in a saturation of uterine estrogen binding sites or receptors. Using an *in vitro* technique, Lee and Jacobson (1971) have recently estimated that the total receptor content of the rat uterus increases from 940 to 1760 femtomoles between 21 and 45 days of age. These investigators also observed cyclic fluctuations in the receptor content during the estrous cycle, indicating that the uterine content of available estrogen receptors may be under hormonal control. Similar cyclic variations in the estrogen receptor content of the rat uterus have been observed by Feherty *et al.* (1970) who reported that the uterine receptor concentration was considerably higher in immature compared to adult rats.

One of the early effects of estrogen is to promote an increase in uterine protein synthesis between two and four hours following treatment of rats (Noteboom and Gorski, 1963). Early estrogenic acceleration of RNA and phospholipid synthesis, as well as estrogen-induced uterine imbibition of water can be prevented by puromycin, an inhibitor of protein synthesis (Mueller, Gorski and Aizawa, 1961). The anabolic changes occuring as a result of estrogen stimulation may be dependent on prior protein synthesis. Noteboom and Gorski (1963) found that estrogen-induced increases in rat uterine RNA synthesis and RNA polymerase activity could be blocked by puromycin. These observations indicate that estrogen-induced uterine protein synthesis is dependent on prior RNA synthesis. Estrogen effects on uterine glucose metabolism also appear to be dependent on prior synthesis of both protein and RNA (Nicolette and Gorski, 1964).

Further evidence that estrogen-stimulated uterine protein synthesis is mediated by RNA has been presented by Fencl and Villee (1971). Ribonucleic acid extracted from uteri of estrogen treated immature rats and injected into the lumen of one uterine horn of immature rats caused an increased incorporation of amino acids into protein compared to that of the control horn. The difference in incorporation of amino acids between experimental and control horns was maximal at 24 hours and disappeared 48 hours following administration of RNA. Protein synthesis was not stimulated by RNA preparations from the kidney, adrenals, lungs or skeletal muscle, but RNA extracted from the liver of estrogen treated rats caused stimulation of uterine protein synthesis similar to that of uterine RNA. These investigators claimed that the effect of uterine

RNA on protein synthesis is organ specific even though liver RNA also stimulated uterine protein synthesis. It is possible that the liver may also contain specific estrogen receptors since Kidson and Kirby (1964) detected an increase in liver m RNA following estrogen treatment of female rats. Gorski (1964) has demonstrated that in vitro incubation of immature rat uteri in high ionic strength medium evokes polymerase activity in controls to a level equal to that of the estrogen treated animals. This observation suggests that the estrogen response may not be due to an increase in the amount of the enzyme, but to increased enzymic activity.

Use of actinomycin D, a potent inhibitor of DNA-dependent synthesis of RNA has greatly clarified the role of estrogen in uterine protein synthesis. Although actinomycin D prevented in vivo acceleration of protein synthesis by estrogen stimulated rat uteri, it did not affect basic protein synthesis in control uteri, suggesting that part of the information (m RNA) was already available for protein synthesis and could be activated by hormone treatment (Ui and Mueller, 1963). Greenman and Kenney (1964) found that ovariectomy of rats resulted in a drastic reduction in the yield of uterine ribosomes which could be restored by short-term treatment with estrogen, although the fall in ribosomal activity as measured by their ability to incorporate amino acid was only transient. These observations indicate that both the number and the capacity of uterine ribosomes for protein synthesis were estrogen dependent.

Recent investigations by Barnea and Gorski (1970) and DeAngelo and Gorski (1970) have considerably increased the understanding of

early estrogenic action on protein synthesis by rat uteri. A single injection of 17β-estradiol into immature or mature ovariectomized rats induces *in vivo* protein synthesis detectable after a lag period of only 40 minutes (Barnea and Gorski, 1970). DeAngelo and Gorski (1970) were the first to demonstrate that synthesis of this induced protein by estrogen could not be blocked by the protein synthesis inhibitors puromycin or cyclohexamide. Significant accumulation of protein band RNA was observed in the rat uteri within 15 minutes following estrogen treatment. This actinomycin D - sensitive step which cannot be inhibited by puromycin appears to be the earliest synthetic event to occur.

Based on these findings, Gorski, Shyamala and Toft (1970) have proposed that immediately following administration, estrogen is bound to the receptor in the cytoplasm and this complex then moves into the cell nucleus. Following its entry into the nucleus, changes in RNA synthesis occur and the movement of this newly synthesized RNA into the cytoplasm causes increased protein synthesis followed by the anabolic changes observed as a result of uterine stimulation by estrogen. This hypothesis would serve to explain the observed delay in gross metabolic changes of the uterus in the estrogen-treated rat.

STATEMENT OF THE PROBLEM

The necessity of endogenous pituitary gonadotropins for the development and maturation of ovarian follicles and ovulation is well established. On the other hand, the endocrine factors which regulate luteal maintenance and regression in most species remain unknown although the gonadotropins have been implicated in both. Experimental evidence accumulated in recent years suggests that the uterus is also involved in regulating the life span of the corpus luteum in our domestic species. It appears that luteal life span is determined by the interplay of two endocrine systems, one luteotropic and the other luteolytic. A "luteotropin" presumably controls the growth and function of the corpus luteum. A uterine "luteolysin" may be the factor which causes demise of the corpus luteum at the end of the estrous cycle.

Exogenous ovarian hormones have been used extensively to induce alteration of ovarian follicular development as well as to cause prolonged maintenance or early regression of the corpus luteum. Exogenous estrogen has been found to either prolong or shorten the life span of the corpus luteum depending upon the species. In the gilt estrogen prolongs the luteal life span whereas in the intact ewe estrogen causes early luteal regression. In the hysterectomized ewe, however, estrogen fails to cause regression of the corpus luteum.

The present studies were conducted to investigate the role of the pituitary gland on follicular development and luteal function in the estradiol-treated gilt and to elucidate the role of the uterus on luteal regression in the estradiol-treated ewe.

EXPERIMENT I

Daily injection of estrogen into the gilt, beginning on or before day 11 of the estrous cycle, causes luteal maintenance (Kidder et al., 1955; Gardner et al., 1963), however, the mechanism(s) by which exogenous estrogen maintains corpora lutea is not clear. Exogenous estrogen fails to prolong the life span of corpora lutea in hypophysectomized (du Mesnil du Buisson, 1966) or hypophyseal stalk transected sows (Anderson et al., 1967), suggesting an involvement of pituitary gonadotropins in luteal maintenance. Concurrent administration of luteinizing hormone (LH) and estrogen to hypophysectomized sows beginning on day 12 of the cycle causes corpora lutea to remain functional to day 20 of the estrous cycle. Treatment of hypophysectomized sows with LH alone, however, does not maintain corpora lutea unless the animal is hysterectomized on day 12 of the cycle (Anderson et al., 1965).

The present experiment was conducted to study the effect of short-term daily injections of 17β-estradiol on pituitary gonadotropins and various ovarian characteristics of the gilt during the late stages of the estrous cycle.

Materials and Methods

Fourteen crossbred gilts weighing 105 to 130 kg were assigned randomly in equal numbers to either a control or a treatment group. All gilts were checked for estrus twice daily using a vasectomized boar. A gilt was considered to be in estrus when she stood for mounting by the boar. All gilts were allowed to complete at least one estrous cycle of normal duration before being assigned to the experimental groups.

Treatment consisted of a daily intramuscular injection of 7 mg of 17β-estradiol in 2 ml of corn oil from day 11 through day 16 of the estrous cycle (first day of estrus = day 1 of the cycle). This dose level was chosen since 5 mg of estradiol has been reported to be an effective minimum daily dose for luteal maintenance in the gilt (Gardner et al., 1963). Gilts in the control group received 2 ml of corn oil daily for the same duration. All gilts were autopsied on day 17 of the estrous cycle.

At autopsy, the ovaries were removed, weighed, and the number and diameter of follicles in each ovary were recorded. The corpora lutea were removed from the ovarian stroma and their number and weights recorded. A minced representative sample of 500 mg luteal tissue from each ovary was stored in 95% ethanol under refrigeration until analyzed for progesterone. The ovarian stroma was minced, blotted, weighed, and the follicular fluid weight determined as described by Short et al. (1968). The anterior pituitary gland from each animal was weighed and stored at -20°C. These were later homogenized, lyophilized, weighed and kept in a desiccator until assayed for FSH and LH. The stalk median eminence from each animal was also removed at autopsy, weighed and stored in 0.1 ml of 0.1 N HCl at -20°C for possible assay of FSH releasing factor (FSHRF).

Pituitary FSH activity was determined by use of the rat ovarian weight augmentation method of Steelman and Pohley (1953). Twenty-two day old immature female Holtzman rats (Holtzman Company, Madison, Wisconsin) weighing between 50 and 55g were used in the bioassays. Along with the dose of pituitary powder, dissolved in physiological

saline, each rat received a total of 22.5 I.U. of human chorionic gonadotropin (HCG) as a subcutaneous injection. The rats were injected twice daily for three days and autopsied 96 hr following the first injection. Preliminary assay for pituitary FSH activity indicated that a dose of 1.5 and 3.0 mg but not 6.0 mg of pituitary powder per rat would fall on the linear portion of the dose response curve. Accordingly, doses of 1.5 and 3.0 mg of pituitary powder from each gilt were used with two rats per dose. Rat ovarian weights were analyzed statistically by use of analysis of variance.

The method of Parlow (1961) was used to estimate pituitary LH activity. Twenty-six day old Holtzman female rats were subcutaneously injected with 50 I.U. of pregnant mare serum (PMS) and with 25 I.U. of HCG 56 hours later. Seven days later, the rats were injected via tail vein under mild ether anaesthesia with doses of 0.1, 0.2 and 0.4 mg of pituitary powder from each gilt dissolved in 1 ml of physiological saline using one rat per dose. Four hours following injection of rats both ovaries were removed, weighed, and analyzed for ascorbic acid by the method of Mindlin and Butler (1938). Test rat ovarian ascorbic acid content was used as an estimate of pituitary LH potency. Ovarian ascorbic acid is inversely related to LH activity. The ascorbic acid content was adjusted for ovarian weight by analysis of covariance (Sakiz and Guillemin, 1963). Validity of the bioassays was established using the methods described by Howland et al. (1966).

An attempt was made to determine whether FSHRF activity of porcine SME could be estimated using the method described by Mittler and Meites (1966). It was anticipated that this method might be used to analyze

the SME from gilts of the present experiment for FSHRF activity. The SME used in preliminary trials were collected from a local slaughter house and represented gilts at various stages of the estrous cycle. Mature male Wistar rats (Simonsen Laboratory Inc., California) weighing between 280 and 300 g served as pituitary donors. The only modification of the procedure was the use of Kreb's-Ringer-Bicarbonate medium (pH-7.2) containing 80 mg % glucose, in place of Difco medium 199. The SME were homogenized in 0.1 N HCl, centrifuged at 12,000xg for 40 minutes at 4°C and the supernatant boiled for 15 minutes in a water bath to deactivate any contaminating FSH. The supernatant was allowed to cool to room temperature and diluted with 0.1 N HCl to yield the various doses used in the experiment. Gilt cerebral cortex extract was similarly prepared to serve as a control. A dose range of 1/64 to 1/4 porcine SME extract per incubated pituitary was used in these trials.

The donor rats were decapitated, anterior pituitaries separated from the posterior lobe, hemisected and eight separate pituitary halves were placed into 20 ml beakers containing 2 ml of Kreb's-Ringer-Bicarbonate-Glucose medium. Incubations were carried out in a Dubnoff metabolic shaker (60 cycles/min) under constant gassing with 95% O_2-5% CO_2 at 37°C. Following a preincubation period of 30 minutes, the media in the beakers was replaced with 2 ml of fresh medium and graded doses of SME or cortex in 1 ml of acidic extract neutralized to pH 7.2-7.4 with 1 N NaOH were added to each flask. Incubation was continued as before and terminated 4 hours later. The medium from each flask was stored at -20°C until assayed for FSH activity using the method of Steelman and Pohley (1953).

Luteal tissue was analyzed for progesterone using the procedure of Stormshak et al. (1970). Progesterone -4-^{14}C was added to each sample to correct for procedural losses. Luteal tissue was homogenized in 95% ethanol and filtered. The filtrate was dried under vacuum and further purified by column chromatography as described by Stormshak et al. (1963). Final purification of samples was made by use of thin layer chromatography (E. Merck, silica gel F-254, preparative, with inorganic fluorescent indicator). The chromatogram was washed in a chromatography tank containing chloroform:methanol (50:50 V/V) for one hour and then dried for 30 minutes at 80-90°C, before spotting the sample on the plate. One half of each sample was spotted on the chromatogram and was developed in one dimension for one hour in a solvent system of chloroform:acetone (85:15 V/V). The chromatography tank was lined with filter paper and the solvents added on the same day the chromatograms were to be developed. Authentic progesterone was chromatographed along with the samples to aid in the visual detection of the sample steroids under ultraviolet light. The sample steroid and appropriate blank areas of silica gel were removed from the plates by vacuum and eluted with 10 ml of chloroform:methanol (2:1 V/V). Progesterone was quantified by measuring sample absorption at 230 and 240 mμ in a Beckman DU spectrophotometer as described by Reinke (1956). The mean progesterone recovery was 87.2 ± 2.1%.

Luteal weights were adjusted by covariance for the number of corpora lutea. Loy et al. (1958) reported that total progesterone in the corpora lutea of a gilt is dependent upon the amount of luteal tissue. Since treatment had no effect on luteal weight, progesterone content and

concentration were analyzed statistically by use of covariance using luteal weight as the independent variable.

Results and Discussion

Treatment of gilts with estradiol increased pituitary FSH and LH activity relative to that of control animals ($P < .01$, for each gonadotropin) and increased anterior pituitary dry weight ($P < .05$, Table 1). The LH assay met all criteria for validity as indicated by the lack of any statistically significant quadratic regressions. There were no significant treatment interactions with dose in either the FSH or LH assay. Injection of estradiol into gilts did not affect the weight of the stalk median eminence.

Exogenous estradiol caused a reduction in the number of ovarian follicles ($\geq$ 3 mm diameter) and follicular fluid weight ($P < .01$, for each characteristic; Table 2). Moreover, gilts treated with estradiol had no follicles $\geq$ 5 mm in diameter, although there were 5.9 follicles ($\geq$ 5 mm diameter) per control animal. As shown in Table 2, treatment failed to affect the weight of corpora lutea but increased luteal progesterone content and concentration ($P < .01$, for each characteristic). Prolonged treatment of gilts with estrone or estradiol has been demonstrated to reduce corpus luteum weight without apparently altering luteal progesterone content (Gardner et al., 1963).

The data from the present experiment indicate that short-term treatment of gilts with estradiol during the latter half of the estrous cycle can increase pituitary FSH content. Lack of follicular development, significant reduction in the amount of follicular fluid and

TABLE 1. Mean hypophyseal characteristics of gilts treated with 17β-estradiol

Treatment	No. of gilts	Anterior pituitary dry wt. (mg)	Stalk median eminence wt. (mg)	Test rat[a]	
				Ovarian wt. (mg)	OAA content (µg)
None	7	39.7±1.0	34.9±3.3	144.8	111.9
17β-estradiol[b]	7	44.2±1.8*	30.3±2.4	227.2**	96.9**

[a] Test rat ovarian ascorbic acid content was used as an estimate of pituitary LH activity. Ovarian ascorbic acid is inversely related to LH activity. Test rat ovarian weight was used as a measure of pituitary FSH activity.

[b] 7 mg of 17β-estradiol daily from day 11 through 16 followed by autopsy on day 17 of the estrous cycle.

* $P < .05$

** $P < .01$

TABLE 2. Mean ovarian characteristics of gilts treated with 17β-estradiol[a]

Treatment	No. of gilts	No. of follicles (≥ 3 mm)	Follicular fluid wt. (gm)	No. of CL	Total CL wt. (gm)	Avg. CL wt. (mg)	Luteal progesterone content (µg)	Luteal progesterone concentration (µg/gm)
None	7	44.4±5.8	4.0±0.4	13.0±0.5	3.8±0.4	279.8±25.8	142.5±12.1	35.9±4.4
17β-estradiol[b]	7	8.9±2.3**	2.1±0.1**	13.7±1.4	3.2±0.4	244.6±25.8	249.6±12.1**	73.0±4.4**

[a] Luteal weight was adjusted for number of corpora lutea by covariance. Progesterone content and concentration were adjusted for luteal weight by covariance.

[b] 7 mg of 17β-estradiol daily from day 11 through day 16 followed by autopsy on day 17 of the estrous cycle.

**P < .01.

increased pituitary FSH are suggestive of estradiol-inhibited release of FSH rather than increased synthesis with unimpared release of this gonadotropin. Foote et al. (1958) and Wagner and Veenhuizen (1965) have also reported inhibition of follicular development in the gilt following treatment with estrogen. Treatment of gilts with estradiol stimulated an increase in pituitary LH content. Whether this estradiol-induced increase in pituitary LH causes a greater or lesser amount of this gonadotropin to be released, needs to be determined. The increase in luteal progesterone content and concentration of estradiol-treated gilts may, however, be a reflection of an increase in the circulating level of LH. Increased in vitro progesterone synthesis by porcine luteal tissue occurs following addition of LH to the incubation medium, whereas addition of FSH or prolactin are ineffective in stimulating steroidogenesis (Cook et al., 1967). The experimental results of Kidder et al. (1955) suggest that estrogen may be luteotropic in the gilt by facilitating release of pituitary LH in the presence of an inhibitory level of endogenous progesterone, but such a release has not been detected (Foote et al., 1958). Corpora lutea of hypophysectomized gilts can be maintained by daily injections of LH only if the uterus is removed or if accompanied by concurrent injections of estrogen (Anderson et al., 1965). A direct stimulatory effect of estrogens on porcine luteal progesterone synthesis in vitro has not been demonstrated (Cook et al., 1968).

The results of the preliminary assay for FSHRF are presented in Table 3. No linear dose-response relationship could be established over a dose range of 1/64 to 1/4th of porcine SME extract per incubated

rat pituitary. Moreover, porcine cerebral cortex extract also appeared to cause release of pituitary FSH into the incubation medium, indicating that the assay lacked specificity. Due to the inability to establish a dose-response relationship for porcine stalk median eminence FSHRF and the lack of specificity of the assay, no attempt was made to assay the SME of gilts of the present experiment for FSHRF.

TABLE 3. Follicle stimulating hormone releasing activity of porcine SME extract

Treatment	Dose/incubated pituitary	Test Rat[a] Mean ovarian wt.(mg)
Trial I		
Cortex[b]	1/4	147.7
SME	1/4	141.1
SME	1/8	178.6
SME	1/16	156.7
Trial II		
Cortex[b]	1/16	263.4
SME	1/16	196.2
SME	1/32	200.6
SME	1/64	190.7

[a]Test rat ovarian weight was used as a measure of SME activity

[b]Cortex equivalent in weight to 1/4 or 1/16 of one porcine SME

EXPERIMENT II

Injection of estradiol into intact ewes anytime between days nine and 12 of the estrous cycle induces premature luteal regression (Stormshak et al., 1969; Hawk and Bolt, 1970). Estradiol injected into hysterectomized ewes on each of days 11 and 12 of the cycle, however, fails to cause luteal regression (Stormshak et al., 1969), suggesting an involvement of the uterus. Moor et al. (1970) have reported that luteal involution and regressive changes in luteal cells in the ewe, can be arrested by hysterectomy as late as day 15 of the cycle.

Injection of estrogen into immature or mature ovariectomized rats results in an increased incorporation of amino acids into uterine proteins (DeAngelo and Gorski, 1970; Barnea and Gorski, 1970). Injection of estrogen initially causes an increased synthesis of RNA and Fencl and Villee (1971) have suggested that the RNA mediated uterine protein synthesis reaches a peak at 24 hours and may be organ specific.

The present experiments were conducted to investigate the effect of uterine removal at different time intervals following estradiol treatment on luteal characteristics of ewes and also to determine the effect of estradiol on uterine protein synthesis of these ewes.

Materials and Methods

Mature crossbred ewes used in this experiment were checked for estrus twice daily using vasectomized rams. A ewe was considered to be in estrus when she stood for mounting by rams. Ewes were allowed

to complete at least one estrous cycle of normal duration before being assigned to an experimental group. The first day of estrus was designated day zero of the cycle. Fifteen ewes were assigned randomly in equal numbers to three treatment groups. Ewes in all three groups were injected intramuscularly with 750 μg of 17β-estradiol dissolved in corn oil on each of days 10 and 11 of the estrous cycle and hysterectomized as follows: group 1, immediately before the first injection (zero hour); group 2, 24 hours following the first injection and immediately prior to the second injection; and group 3, 48 hours after the first injection. Two additional ewes were injected with corn oil only on each of days 10 and 11 of the cycle. One ewe was hysterectomized at 24 and the other at 48 hours following the first injection of corn oil to provide control uterine endometria. The ovaries and uterus of each ewe were exteriorized through a mid-ventral incision at the designated time. The number of corpora lutea in each ovary was recorded and the uterus including the uterotubal junctions and the proximal half of the cervix was excised. The uterus was immediately placed in an ice bucket, covered with crushed ice and transported to the laboratory for processing.

All animals were autopsied on day 15 of the same estrous cycle. At autopsy, corpora lutea were enucleated from the ovarian stroma and weighed. A small section from each corpus luteum was stored in Bouin's fluid for histological study. The remainder of each corpus luteum was weighed and stored in 95% ethanol until analyzed for progesterone. Luteal tissue from each ewe was analyzed for progesterone using the procedure described by Stormshak et al. (1970) with the following

modifications: one half of each sample was spotted on a thin layer chromatogram and developed in one dimension for two hours in a solvent system of chloroform:methanol (99:1 V/V). Sample steroids were eluted from the chromatogram with 10 ml of chloroform:methanol (2:1 V/V). Mean progesterone recovery was 92.1 $\pm$ 3.7%.

Luteal characteristics were analyzed statistically by use of analysis of covariance using number of corpora lutea as the independent variable. Differences between group treatment means were tested for statistical significance by use of Duncan's multiple range test.

Portions of corpora lutea preserved in Bouin's fluid were washed in a saturated solution of lithium carbonate in 50% ethanol, dehydrated in dioxane, embedded in paraffin and cut into five micron sections using a hand operated microtome. The sections were mounted on slides and stained with hematoxylin and eosin.

Endometria from one animal in each treatment group and the two control ewes were incubated according to the method described by DeAngelo and Gorski (1970). All incubations were carried out within one hour of the time of removal of the uterus. The uterine horn adjacent to the ovary containing the corpus luteum was cut open, the caruncles removed and the endometrium sliced using a hand slicer (Harvard Tissue Slicer #140) pre-rinsed with ice-cold saline. If corpora lutea were present in both ovaries, the uterine horn to be used as a source of endometrium was chosen randomly. Care was taken not to cut the underlying muscle layers. Endometrial slices were immediately placed on filter paper moistened with cold saline and placed over ice. Approximately 200 mg of endometrium was placed in 20 ml beakers containing 2.85 ml of Eagle's

HeLa Medium (Difco Co., Detroit) and 7.0 μCi of uniformly labelled L-leucine-^{14}C in 0.15 ml of 0.01 N HCl. Endometrium added to beakers containing an equivalent amount of medium and 0.15 ml of 0.01 N HCl served as incubated controls. All incubations were carried out in a Dubnoff metabolic shaker (60 cycles/minute) at 37°C for two hours under an atmosphere of 95% O_2-5% CO_2. Whenever possible, duplicate samples of endometrium were incubated. Following incubation all beakers were immediately placed in an ice bath to stop any further synthesis. The medium from each beaker was decanted and stored in vials at -20°C. The tissues were rinsed with 20 ml of ice-cold 0.05% disodium ethylenediaminetetracetate (Na_2EDTA) and homogenized separately in 1 ml of Na_2EDTA solution. The homogenates were centrifuged at 4°C for 30 minutes at 15,000 x g and the supernatant fractions stored frozen.

Endometrial supernatant was subjected to vertical polyacrylamide gel electrophoresis using the procedure described by Taylor (1970). Spacer gel buffer (0.062 M Tris-HCl, pH 6.7) was prepared by dissolving 22.5 g of Tris and 12 ml of concentrated HCl in water to a final volume of 3 litres. Three litres of running gel buffer (0.38 M Tris-HCl, pH 9.0) were prepared by dissolving 138 g of Tris and 12 ml of concentrated HCl in water. The electrode buffer (0.0165 M Tris - 0.0390 M glycine, pH 8.75) was prepared by dissolving 11.6 g of glycine and 8 g of Tris in four litres of water. Final pH adjustments were made with 1N HCl or NaOH. Plug and running gel solutions were prepared by dissolving 10 g and 7 g of Cyanogum-41 (95% acrylamide and 5% N, N' methlenebisacrylamide) in running gel buffer to make 100 ml of 10% and 7% concentrations, respectively. Spacer gel solution was made by

dissolving 4 g of Cyanogum-41 in 100 ml of spacer gel buffer. One-tenth ml of N, N, N', N'-tetramethylethylenediamine (TEMED) and 0.02 ml of Tween 80, a wetting agent, were added to each of the three 100 ml solutions and the solutions were filtered using Whatman #12 paper. Since these solutions are unstable they were prepared immediately prior to use. To effect polymerization of the gel solutions, 0.1 g of ammonium persulphate was added.

The electrophoresis cell was assembled with liberal amounts of 0.5% Araganose on the rubber gaskets. The plug gel was poured and allowed to polymerize before the running gel was added. After all the gels were polymerized, the cell was connected to a circulating water bath at 0°C, cold electrode buffer was placed in the cell and the slot former was removed. A supernatant fraction of 150 µl (with 10% sucrose and bromothymol blue) was added to each slot. Voltage (200 volts) was then applied to the cell for 45-60 minutes or until the dye front reached the running gel. The voltage was then raised to 400 volts and the run continued for about two and a half hours. Proteins in the supernatant incubated in the absence of labelled leucine were stained by placing the gel strip in a solution of 0.25% Amido Black (in methanol:water:acetic acid-5:5:1 V/V) for 30 minutes and then in destaining solution for three days. The corresponding gel strip containing the supernatant of tissue incubated in the presence of leucine-^{14}C was cut into 2 mm slices. Each slice was placed in a counting vial and dried at 75°C for two hours. One ml of 30% hydrogen peroxide (H_2O_2) was added to each vial, caps were placed on the vials and the vials placed in the oven at 75°C for four to six hours to

dissolve the gel. The caps were then removed from the vials and the vials were placed back in the oven for two hours until all the H_2O_2 was evaporated. Three drops of distilled water were added to each vial to dissolve the residue followed by the addition of 1 ml of NCS solubilizer (Nuclear Chicago Corp.) and 10 ml of scintillation fluid (0.03% dimethyl POPOP and 0.5% PPO in toluene). The vials were then cooled and counted in a liquid scintillation counter. Counting efficiency was 76%.

All photographs of strips were taken at f/6.5 with 1 second exposure, using a red filter and P/N 55 film.

In order to ascertain that the L-leucine-^{14}C was actually incorporated into newly synthesized proteins, 200 µl of supernatant from each of the treated ewes was fractionated using a column (9 mm x 30 cm) packed with Sephadex G-15. Eight grams of Sephadex G-15 was weighed out and soaked overnight in distilled water prior to packing. The void volume of this column was 7 ml. Thirty five 2 ml fractions were collected (equal to 10 void volumes). Fractions containing protein were determined by measuring their absorbancy at 280 mµ using a spectrophotometer. Fractions were dried and counted as described above.

As a further test of the incorporation of leucine into protein, the supernatant fraction and the incubation medium from the ewe hysterectomized 48 hours following the first estradiol injection were subjected to dialysis. This consisted of adding 200 µl of supernatant or medium diluted with 10 ml of distilled water to dialysis tubing which permitted the passage of substances having a molecular weight

of less than 10,000. The dialysis tubing containing the sample was placed into a 100 ml beaker containing 50 ml of distilled water and was kept at 4°C under continuous stirring using a magnetic stirrer. The stirring facilitated the passage of dialyzable substances from inside the tubing and prevented mold growth. The water in the beaker (dialysate) was replaced every 12 hours over a period of 48 hours. At the end of dialysis, an aliquot of each of the dialysate fractions and the content inside the dialysis tubing (protein) were counted.

Results and Discussion

Effect of time of hysterectomy on mean luteal characteristics of estradiol-treated ewes is presented in Table 4. Corpora lutea of ewes hysterectomized at 48 hours (group 3) were smaller in size ($P < .05$) than glands of ewes hysterectomized at zero (group 1) or 24 hours (group 2) following the first estradiol injection. Removal of the uterus either at zero, 24 or 48 hours failed to affect luteal progesterone content. Luteal progesterone concentration was greater ($P < .05$) in ewes hysterectomized at 48 hours as compared to ewes hysterectomized at 24 hours post-injection, but did not differ from the luteal progesterone concentration of ewes hysterectomized prior to estradiol treatment (zero hour). Luteal progesterone concentration of ewes hysterectomized 24 hours after the initial injection of estradiol did not differ from that of ewes hysterectomized prior to estradiol treatment.

Injection of estradiol into intact ewes beyond midcycle causes premature luteal regression (Stormshak et al., 1969; Hawk and Bolt, 1970),

TABLE 4. Effect of time of hysterectomy on mean luteal characteristics of estradiol treated ewes

Groups	Time of* hysterectomy	Luteal weight (mg)	Progesterone content (µg)	Progesterone concentration (µg/g)
1	0 hr	508.3±20.6[a]	20.9±4.2[a]	34.0±3.9[a,b]
2	24 hr	480.1±21.5[a]	15.6±4.4[a]	24.2±4.0[b]
3	48 hr	400.6±21.0[b]	23.2±4.3[a]	41.2±4.0[a]

* Time of hysterectomy relative to first estradiol injection. Ewes were injected with 750 µg of 17β-estradiol on each of days 10 and 11 of the estrous cycle.

[a,b]Means of each luteal characteristic not having an identical superscript are significantly different ($P < .05$).

and reduces luteal progesterone content and concentration (Stormshak et al., 1969). Estradiol injected into hysterectomized ewes on each of days 11 and 12 of the estrous cycle did not cause luteal regression (Stormshak et al., 1969). Results of the present experiment are in agreement with these findings. Removal of the uterus from ewes prior to estradiol treatment (group 1) or 24 hours following estradiol injection (group 2), failed to cause luteal regression indicating the significant role played by the uterus in estradiol-induced ovine luteal regression. Results of the present experiment suggest, that in order to bring about any significant reduction in luteal weight, the estradiol-stimulated uterus needs to be present for more than 24 hours. These observations are also in agreement with the findings of Moor et al. (1970), who reported that removal of the uterus as late as day 15 of the ovine estrous cycle can arrest further luteal involution of functional glands. Failure of luteal progesterone content of ewes to differ at the three stages following estradiol treatment suggests that the mechanism(s) controlling luteal weight may be different from that controlling luteal progesterone content. The significance of higher luteal progesterone concentration ($P < .05$) of ewes of group 3 compared to that of group 2 animals is not fully understood. It is, however, possible that lower luteal weight and higher progesterone content of ewes in group 3 compared to that of ewes of group 2, may have resulted in a statistically significant difference in the present study. Histological study of corpora lutea of ewes of different groups did not reveal any marked histological differences. Moor et al. (1970) reported that regressive changes in

luteal cells can be arrested by hysterectomy.

The effect of estradiol stimulation of the uterus on the in vitro incorporation of L-leucine-^{14}C into endometrial protein is presented in Table 5. Endometrial protein synthesis was maximum 24 hours following estrogen injection and was reduced to approximately control levels at 48 hours. Total leucine incorporation into protein of control endometrium at the three different stages of the estrous cycle studied remained relatively constant. Mueller (1953) reported that maximum incorporation of labelled glycine into uterine protein of estradiol-treated ovariectomized rats occurs at 18 to 20 hours. Using RNA extracted from estrogen-stimulated endometrium of immature rats to induce protein synthesis, Fencl and Villee (1971) found that incorporation of labelled amino acids into uterine protein was maximum at 24 hours and the difference between control and treated uterine horns disappeared 48 hours following the administration of RNA. These results are similar to those of the present study. Incorporation of labelled amino acid into protein by control endometrium indicates that the uterus can synthesize proteins without exogenous estrogen treatment. This is supported by the findings of Ui and Mueller (1963) who found that actinomycin D does not affect basic protein synthesis in control uteri, suggesting the ability of stored uterine mRNA to continue protein synthesis. The data from the present investigations suggest that there may be a relationship between estradiol-induced luteal regression and uterine protein synthesis in the ewe. In the present study significant luteal regression was obtained (Table 4) only after maximal uterine protein synthesis at 24 hours (Table 5). It is

TABLE 5. *In vitro* incorporation of L-leucine-^{14}C into endometrial protein

Groups	Time of[a] hysterectomy	Total dpm	
		Control	Treated
1	0 hr	-	17,100
2	24 hr	21,800	46,500
3	48 hr	19,400	25,000

[a]Time of hysterectomy relative to first estradiol injection. Ewes were injected with 750 μg of 17β-estradiol on each of days 10 and 11 of the estrous cycle.

possible that estrogen may activate the synthesis of a uterine luteolytic factor which in turn brings about ovine luteal regression. Lukaszewaska and Hansel (1970) suggested that this uterine factor in the bovine may be a large molecular weight protein or a small protein-bound molecule.

A representative electrophoretogram of the proteins present in the endometrium is shown in Figure 1. Induction of new protein due to estradiol treatment was not detected. The gel strips containing protein were divided into six sections to determine the effect of estradiol on incorporation of leucine into proteins in the different sections. Table 6 presents percent in vitro incorporation of labelled leucine into endometrial protein within the different sections of the electrophoretogram. Percent incorporation of leucine into protein of control endometrium at the different stages of the estrous cycle studied remained relatively constant over all the sections, whereas there was increased incorporation in sections I, III, and VI and decreased incorporation in section IV with time following estradiol treatment. Increased incorporation in section I suggests synthesis of large molecular weight proteins that did not migrate from the origin.

Results of the trials conducted to determine whether labelled leucine was actually incorporated into protein are presented in Figure 2 and Table 7. It can be seen in Figure 2 that fractionation of the labelled endometrial protein by use of Sephadex column chromatography resulted in the label being concentrated only in one peak associated with the majority of protein (fractions six and seven). No other protein peak had any radioactivity associated with it. Results of

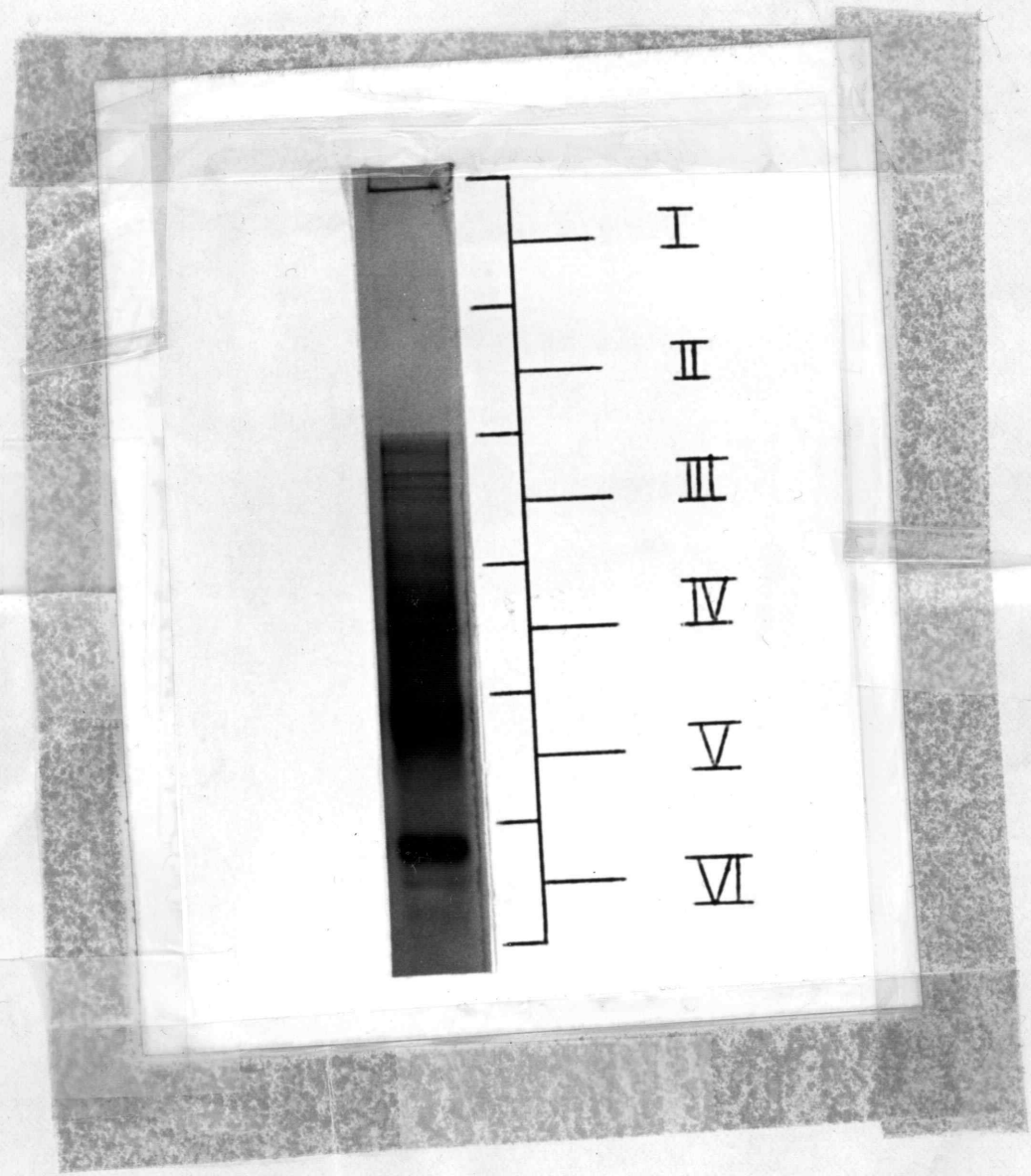

FIGURE 1. Polyacrylamide gel electrophoretic separation of endometrial proteins of a ewe hysterectomized 24 hours after the first injection of 750 µg of 17β-estradiol on day 10 of the cycle. The electrophoretogram has been divided into six sections. The origin is at the top.

TABLE 6. Percent *in vitro* incorporation of L-leucine-^{14}C into endometrial protein

Groups	Time of[a] hysterectomy		Section I	II	III	IV	V	VI
1	0 hr	Control	3.4	1.8	12.3	58.3	4.8	19.2
		Treated						
2	24 hr	Control	3.8	4.0	15.3	56.6	4.2	18.9
		Treated	13.1	1.9	17.9	39.2	7.3	20.6
3	48 hr	Control	2.6	1.0	13.4	64.4	3.4	15.3
		Treated	16.6	4.0	24.3	28.1	6.5	20.5

[a]Time of hysterectomy relative to first estradiol injection. Ewes were injected with 750 µg of 17β-estradiol on each of days 10 and 11 of the estrous cycle.

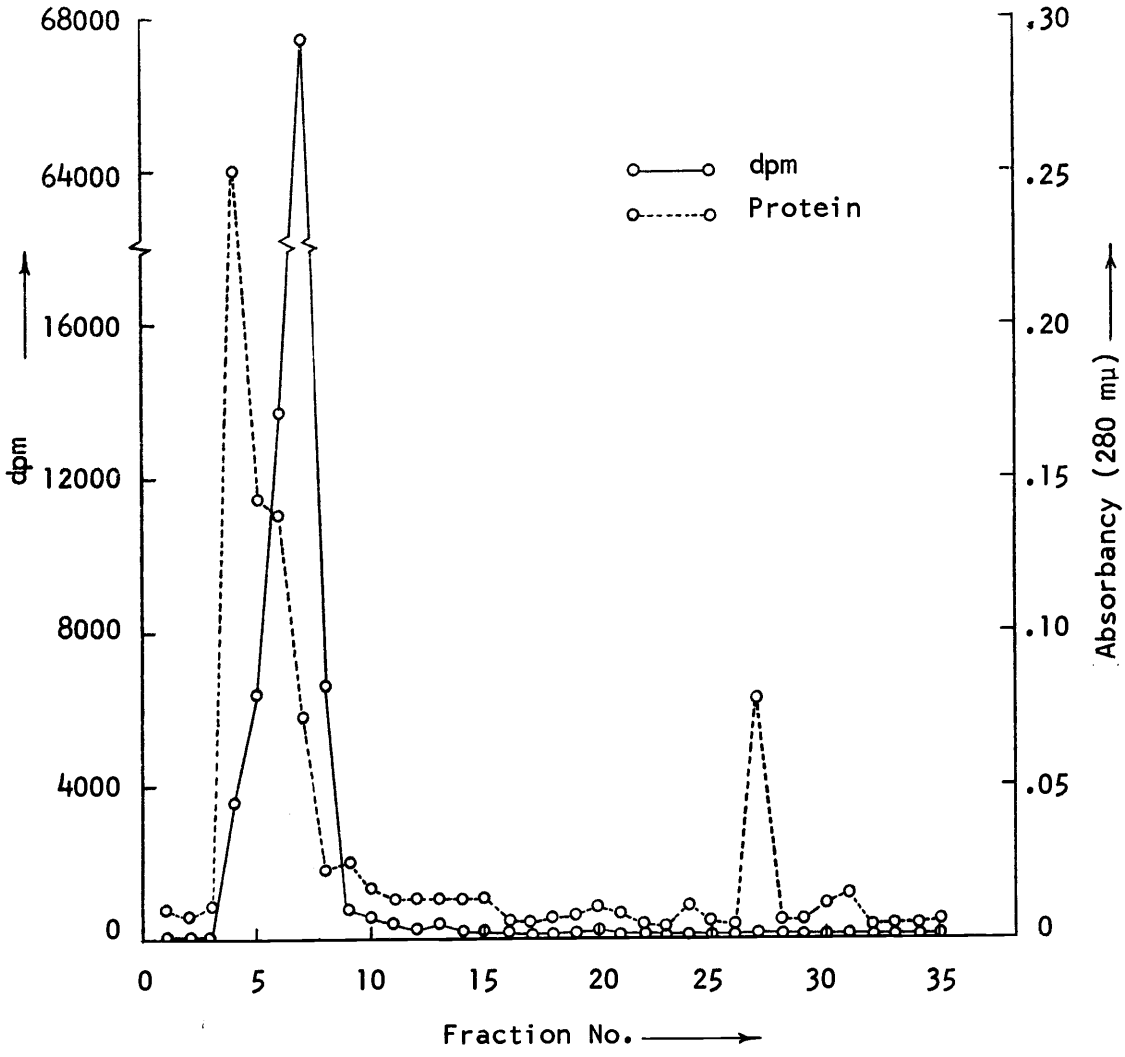

FIGURE 2. Sephadex column (G-15) fractionation of ^{14}C-labelled endometrial proteins of a ewe hysterectomized 24 hours after the first injection of 750 μg of 17β-estradiol on day 10 of the cycle. Endometrium was incubated in Eagle's HeLa medium containing 7 μCi of L-leucine-^{14}C

TABLE 7. Dialysis of incubation medium and endometrial supernatant

Fractions		Percent radioactivity: Incubation media	Percent radioactivity: Endometrial supernatant
Dialysate	1[a]	78.8	49.6
	2	15.1	10.7
	3	3.4	3.1
	4	1.1	1.1
Protein		1.6	35.5

[a]Dialysate fractions collected every 12 hours over a period of 48 hours.

dialysis (Table 7) conclusively demonstrated that free labelled leucine from the medium and the supernatant can be separated. Following dialysis over a period of 48 hours only 1.6% of the total radioactivity was associated with the protein fraction in the medium, whereas 35.5% of the total radioactivity in the supernatant was associated with proteins. The small amount of radioactivity found in the medium was possibly due to the presence of small amount of labelled protein that may have leached out into the medium during incubation.

GENERAL DISCUSSION

Short-term treatment of gilts with 17β-estradiol during the late stages of the estrous cycle caused an increase in pituitary FSH and LH activity. Lack of follicular development, reduction in follicular fluid and increased pituitary FSH content suggest that treatment resulted in an inhibition of FSH release from the pituitary rather than increased synthesis of this gonadotropin. The increase in pituitary LH of estradiol-injected gilts may be due to an inhibition of release or stimulation of synthesis of this gonadotropin, or both. The relationship of increased pituitary LH in treated gilts to changes in ovarian function of these animals was not apparent. The data indicate that short-term estradiol treatment of gilts during the estrous cycle promotes increased luteal function as detected by an increase in luteal progesterone content and concentration. Luteinizing hormone may be luteotropic in the gilt, however, data of the present study do not indicate if the increase in pituitary LH results in a concomitant increase in the circulating levels of this hormone. Knowledge of the circulating levels of LH following estradiol treatment of gilts is needed for a better understanding of the relationship of this gonadotropin to corpus luteum function.

Estradiol-induced luteal regression in the ewe during the estrous cycle appears to be mediated via the uterus. In order to obtain any significant reduction in luteal weight, the estradiol stimulated uterus needs to be present for a period of more than 24 hours. Preliminary data indicate that uterine endometrial protein synthesis reached a maximum at 24 hours and was reduced at 48 hours following an injection

of estradiol. A second injection of estradiol into ewes 24 hours after the first injection of estradiol failed to cause a further increase in uterine endometrial protein synthesis. This lack of additional protein synthesis may indicate a uterine refractory period during which the uterus is unable to respond to further stimulation by estradiol. In the present study, significant luteal regression was obtained only after maximal uterine protein synthesis occured. This is suggestive of a functional relationship between estradiol-induced luteal regression and uterine protein synthesis in the ewe. Further research needs to be conducted on uterine protein synthesis and its possible luteolytic role in the ewe.

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