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Title: *IN VITRO* ESTRADIOL-INDUCED PROTEIN SYNTHESIS IN THE ENDOMETRIUM OF CYCLIC AND STEROID-TREATED OVARIECTOMIZED EWES

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Abstract approved: ______________________

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The ability of ovine endometrium incubated *in vitro* with estradiol-17β to synthesize a specific induced protein (IP) was studied. Endometrium from cycling, pregnant and ovariectomized ewes was used.

Twelve ewes were allotted in equal numbers to be sacrificed on days 0 (estrus), 3, 6, or 10 of the estrous cycle. In addition uteri from nonpregnant ewes (day 14 or 15, N = 3) were compared with day 15 pregnant ewes (N = 3).

Experiment 2 involved 16 ovariectomized ewes assigned randomly to one of four treatment regimes. The ewes were treated as follows: a) controls, no exogenous steroids, b) estradiol-17β, c) progesterone and d) progesterone plus estradiol. Estradiol was packed into silastic capsules and implanted subcutaneously on day 1 of treatment. Progesterone (15 mg 2x daily) or vehicle (oil) were

injected for 5 consecutive days beginning 48 hr (day 3) after implantation of estradiol. All ewes were sacrificed on the morning of day 8. Blood samples were taken at sacrifice and estradiol and progesterone levels were measured. The ewes treated with estradiol tended to have increased estradiol levels and ewes receiving progesterone had significantly higher serum progesterone levels.

Upon sacrifice of the ewes, the uterus was removed and transported to the laboratory. Three-50 mg endometrial segments were incubated for 1 hr with either 2.5×10^{-8} M estradiol-17β (treated) or ethanol (control), followed by a 2 or 4 hr incubation with labelled amino acids. Treated and control tissues were homogenized together and the resulting supernatant was stored at -20 C until electrophoresed. Following disc gel electrophoresis, the gels were placed into a dye for staining or were fixed in acetic acid. The gels fixed with acetic acid were sliced into sections and then counted in a scintillation spectrometer.

Experiments utilizing immature rat uteri were conducted to verify that IP synthesis in the rat uterus could be detected in our laboratory. Induced protein was detected following estradiol stimulation _in vivo_ and _in vitro_.

Synthesis of an estradiol-induced endometrial protein was not detected during the ovine estrous cycle, on day 15 of pregnancy or in steroid-treated ovariectomized ewes. However, increased

incorporation of both isotopes, running in front of the tracker dye, was detected in two of the four ewes in both the control and estradiol-treated ovariectomized ewes. This increased isotope incorporation was detected in all four ewes receiving progesterone injections and was not present in any of the ewes receiving both estradiol and progesterone. Problems with excess estradiol during the *in vitro* incubation probably prevented any expression of IP during the estrous cycle experiment (experiment 1).

No substantial change in the prealbumin, non-specific protein patterns were observed. Protein profiles of uteri varied from 1 to 3 bands during the estrous cycle and day 15 of pregnancy. The number of uterine prealbumin bands observed in the ovariectomized ewes receiving exogenous steroids varied from 0 to 4 within and among the treatment groups.

In Vitro Estradiol-Induced Protein Synthesis in the Endometrium of Cyclic and Steroid-Treated Ovariectomized Ewes

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IN VITRO ESTRADIOL-INDUCED PROTEIN SYNTHESIS IN THE ENDOMETRIUM OF CYCLIC AND STEROID-TREATED OVARIECTOMIZED EWES

INTRODUCTION

Female reproductive phenomena are regulated by a hypothalmic-pituitary-gonadal feedback mechanism. Under pituitary stimulation, the theca interna cells of the ovarian follicles synthesize and release estrogens. The major and most biologically active estrogen released from the ovary is estradiol-17β. Estradiol-17β (E_2) is converted to estrone by the action of a dehydrogenase enzyme in the liver, and estrone is further metabolized to estriol which is the major estrogen excreted in humans.

Estradiol-17β is found in minute quantities in the blood (picogram levels). It induces behavorial estrus in farm animals and aids in ovulation by stimulating the release of luteinizing hormone from the anterior pituitary. In the uterus, E_2 stimulates proliferation of the endometrium and increased vascularity. It also stimulates development and maintenance of secondary sex characteristics in the female.

The mechanism of how estradiol brings about the above actions is now under intensive investigation. Estradiol, upon entry into the uterine cell, binds with a specific cytoplasmic receptor which transports it into the nucleus. Once in the nucleus, the transformed

estradiol-receptor complex binds to the chromatin where estradiol stimulates RNA synthesis which subsequently results in an increase in protein synthesis in the cytoplasm. Determination of the mechanism by which estradiol initiates protein synthesis and the characterization of the specific proteins produced will allow for a more precise control of various reproductive phenomena.

The present research examined if endometrium from ewes sacrificed on days 0 (estrus), 3, 6, 10, and day 14 or 15 of the estrous cycle had the capacity to synthesize the estradiol induced-protein (IP) first characterized in rat uteri. The effect of pregnancy on the ability of ovine uteri to synthesize IP also was studied. Uteri from ovariectomized ewes receiving exogenous steroids were used to determine if *in vitro* stimulation by estradiol initiates early synthesis of induced proteins. General uterine endometrial protein synthesis also was studied during the estrous cycle and in the ovariectomized ewes receiving hormonal treatment regimes.

REVIEW OF LITERATURE

This review will discuss the early and late uterine response to estrogen with particular emphasis on the synthesis of the induced protein. The early uterine response is defined as occurring within 1 hour (hr) and the late response as that which occurs later than 4 hrs after exposure to estradiol. Previous research has utilized the female rat as the experimental animal. Although our major interest is with large animals, it becomes necessary to rely on results obtained in rats for supportive information.

General Response

The first physiological response of the uterus to estradiol is imbibition of water (Astwood, 1938a). Uterine weight increased by 60% within 6 hrs and a second increase occurred in approximately 12 hrs following a single injection of estradiol into intact immature rats. A more detailed study utilizing intact immature rats demonstrated that water accumulation did not occur until 2 hr after estradiol injection, peaked at 6 hr, then decreased during the following 6 hrs due to dehydration of uterine tissue (Astwood, 1938b). The majority of water accumulation occurred in the endometrial stroma. From histological preparations, the uterine tissue appeared edematous, swollen, translucent and pale in color. An increase in

vascularity of the mesometrium was also noted which tended to parallel the development of edema (Astwood, 1938b).

The secondary increase in uterine weight, occurring between 12 and 13 hr after estradiol injection, was due to increased mitotic activity of the cells, resulting in increased cellular protoplasm (Astwood, 1938a). The increased mitotic activity resulting in increased cell numbers continued for 15 hrs with a gradual return to normal preinjection weight by 48 hr (Astwood, 1938b).

Studies in the adult cycling rat sacrificed during different stages of the estrous cycle indicated that maximal water imbibition, calculated by the difference in wet and dry uterine weight, occurred during pre-estrus (prior to proestrus). Water imbibition remained elevated during proestrus and decreased during estrus. Uterine water content remained low during metestrus and the first day of diestrus (Astwood, 1939). Dry weight measurements of uterine tissue peaked at proestrus with a gradual decrease to minimum levels at diestrus. Uterine distension occurred from proestrus to the middle of estrus at a time when both uterine water content and dry weight were decreasing. Since uterine water content decreased during estrus when estrogen levels are low (Brown-Grant et al., 1970), maintaining estrogen levels should maintain uterine water content. Therefore, adult rats in various stages of the estrous cycle were injected with estradiol and sacrificed 6 hrs later. Injection of estradiol during diestrus and

pre-estrus resulted in increased uterine water content whereas no increase was observed following an injection of estradiol during proestrus or estrus. Therefore, a refractory period or an inhibitory mechanism to estradiol is present during proestrus and estrus (Astwood, 1939). These results were later confirmed by Szego and Roberts (1953).

Previous work in monkeys had demonstrated that water retention by the uterus occurred during the follicular phase when estrogen levels were elevated (Van Dyke and Ch'en, 1936). They also reported cyclic changes in glycogen content of uterine endometrium during the menstrual cycle of the monkey. Maximum glycogen levels were attained during the luteal phase of the cycle when progesterone levels are high. This increase in uterine glycogen coincides with the time of implantation and may act as a nutritive aid for the ovum. For glycogen synthesis to occur, the necessary enzymes, phosphorylase and amylase, must be present in the endometrium. Zondek and Hestrin (1947) found that human endometrium contained phosphorylase and that its activity increased during the secretory phase of the menstrual cycle. Lendrum and Hisaw (1936) also reported increased uterine glycogen content in ovariectomized and immature monkeys receiving progesterone injections.

Contrary to this, Boettiger (1946) found that uterine glycogen content in rats was controlled by estrogen and therefore, must have a

different function than in the primate uterus. Maximal glycogen synthesis occurred during proestrus and remained low from early estrus through diestrus. Water content and glycogen formation increased in ovariectomized rats treated with estrogen but in the continued presence of estradiol, uterine water content decreased while glycogen content remained high. Exogenous progesterone had no effect on uterine glycogen content. In rodents the glycogen content tends to follow the cyclic dry weight change of the uterus as reported by Astwood (1939). The significance of the estrogen-increased glycogen content may be associated with uterine myometrium activity since increased glycogen content and increased muscular activity occurred simultaneously. Walaas and Walaas (1950) observed that rat and rabbit uterine muscle had low levels of adenosine triphosphate and creatine phosphate. Estrogen stimulation did not affect the levels of adenosine triphosphate and creatine phosphate in uterine myometrium. Upon separation of the myometrium and endometrium of the rat uterus, Walaas (1952) noted that the glycogen content of the myometrium doubled while no significant change in the glycogen content of the endometrium occurred following estrogen stimulation. Glycogen content in the myometrium was non-responsive to exogenous progesterone, testosterone or cortisol. During the last stage of pregnancy just prior to parturition, myometrial glycogen content increased. Brody and Westman (1958), utilizing rabbit uteri,

observed that estradiol increased the glycogen content of both the endometrium and myometrium. Progesterone tended to decrease the glycogen content with a greater effect on the endometrium than on the myometrium.

Cole (1950) reported that estradiol injected into ovariectomized rats affected the water and electrolyte balance of the uterine cell. The initial increase in total uterine water content which occurred within 6 to 10 hr following estradiol injection was due to an increase in uterine cell volume and increased extracellular fluid space. The increased cell volume resulted from an increase in the molar concentration of non-diffusible ions without increasing the anionic charge within the cell. This is suggestive of protein catabolism within the cell. The increased extracellular fluid may have been due to increased permeability of the capillaries (Hechter *et al.*, 1941).

The second increase in uterine cell volume was due to an increased electrostatic equivalent (ESE) occurring 18 to 54 hrs after the estradiol injection and the increased water content was due to an increase in intracellular fluid. The observed increase in ESE resulted from an increased diffusion of total nucleoproteins and phosphorus ions. Potassium concentration also increased. An increased ESE and decreased molar concentration of non-diffusable ions is indicative of protein synthesis.

Specific Response

The mechanism of how estradiol brings about its growth-stimulating potential was first suggested by Szego and Roberts (1953) following studies on cellular permeability and the rate of incorporation of radioactive phosphorus (^{32}P) by uterine tissue. They found that ^{32}P incorporation increased 4 hr after estradiol injection and was greatly enhanced by 20 hr. Increased ^{32}P incorporation is indicative of tissue protein synthesis. Estrogen also increased the permeability of the cell membrane, either by a direct binding at its surface or by stimulating the metabolic systems responsible for maintenance of cell membrane permeability. It was hypothesized by these investigators that estradiol exerted its effects by increasing specific protein synthesis which increased cell permeability and allowed for uterine growth.

Mueller *et al*. (1958) and Hamilton *et al*. (1968) observed that the uterine cellular content of ribonucleic acid (RNA) decreased but could be restored upon estradiol stimulation in ovariectomized rats. A time course study revealed that little or no cellular accumulation of protein, RNA or deoxyribonucleic acid (DNA) occurred from 1 to 6 hr (induction phase) post-stimulation. During the RNA accumulation phase (6-24 hr), increases in RNA content and in size of the nucleolus and rough endoplasmic reticulum occurred. Deoxyribonucleic acid

increased 40 to 72 hr after estradiol stimulation; therefore it was thought that estradiol acted as an inducer to remove a specific protein covering the RNA-template thereby allowing for protein synthesis.

Since it had been shown that estradiol increases RNA and protein content in uterine cells, it became necessary to determine if de novo synthesis of RNA occurred. Ui and Mueller (1963) were able to inhibit the early acceleration of phospholipid and protein synthesis in estradiol-treated ovariectomized rats by an intraperitoneal injection of actinomycin D, an agent which blocks the transcription of DNA to messenger ribonucleic acid (mRNA). This demonstrated that estradiol acts by stimulating de novo RNA synthesis. Means and Hamilton (1966) reported a 13% increase in precursor uptake (^{3}H-uridine) by the uterine cell and a 40% increase in ^{3}H-uridine incorporation into nuclear RNA within two minutes of estradiol stimulation in ovariectomized adult rats. The greatest rate of precursor uptake by the uterine cell occurred between the first 5 to 10 min of hormone administration and continued to increase but at a slower rate until 30 min post-stimulation. Peak ^{3}H-uridine incorporation occurred at 20 min with a slight decrease at 30 min which continued until 2 hr following hormone stimulation. The rate of ^{3}H-uridine incorporation into nuclear RNA occurring at 2 hr (50% above control level) was continued to 4 hr post-stimulation. The greatest increase in the incorporation rate occurred between 5 and 10 min following

estradiol stimulation. These results indicate that estradiol initially may stimulate membrane activity and transcription of DNA.

Hamilton et al. (1968) demonstrated that the increased cytoplasmic RNA at 6 to 12 hr post-estradiol administration was due to transport of nuclear RNA to the cytoplasm. The initial transport of RNA could be detected by 80 min after estradiol administration (Hamilton, 1968). The major RNA produced in response to estradiol was rRNA. The nucleoli increased in size and number from 6 to 24 hr after estradiol and were the major site of rRNA synthesis.

In the intact cycling adult rat, RNA synthesis, accumulation and transport were more rapid in the estrous phase than during the diestrous phase (Hamilton et al., 1968; Hamilton, 1968). Hamilton concluded therefore, that the primary action of estradiol in uterine cells was to stimulate synthesis of nuclear RNA, both chromosomal (mRNA) and ribosomal (rRNA).

The enzyme responsible for the transcription of DNA to RNA is RNA polymerase. Gorski (1964) isolated an enzyme from rat uterine tissue with characteristics similar to those of live RNA polymerase. This enzyme increased in activity in response to estradiol, but this response was lost when a high salt concentration was present in the incubation medium. The increased activity of RNA polymerase occurred within 1 hr after estradiol stimulation. Puromycin, which dissociates the developing peptide chain from the ribosome, blocked

the estradiol-induced increase in polymerase activity. This indicates that increased RNA polymerase activity is dependent on prior synthesis of a protein.

Hamilton (1968) and Hamilton et al. (1968) reported the existence of two distinct types of RNA polymerase, depending upon the presence of Mg^{++} or Mn^{++} ions in the media. The Mg^{++}-activated RNA polymerase reaction was found to induce the formation of rRNA while Mn^{++}-activated DNA polymerase synthesized RNA with a DNA-like base (presumably mRNA). The time appearance of each polymerase in response to estrogen also differed. The Mg^{++}-RNA polymerase increased within 1 hr post-estrogen stimulation, peaked at 12 hr, then remained constant until 24 hr. Mn^{++}-RNA polymerase increased at 12 hr and was maintained until 24 hr.

Greenman and Kenney (1964) demonstrated that exogenous estradiol-17β increased the number and capacity of ribosomes in the cytoplasm needed for protein synthesis in ovariectomized rats. Means and Hamilton (1966) found that the estrogen-induced increase in ribosomes occurred within 2 hr.

Induced Protein Synthesis

Since actinomycin D and puromycin can inhibit the estrogenic response of the cell, it has been suggested that estrogen, upon entry into the nucleus, exerts its primary effect by a subsequent stimulation

of the synthesis of specific proteins which in turn bring about the metabolic changes within the cell. Notides and Gorski (1966), using starch gel electrophoresis, were able to separate the soluble uterine proteins from immature rats injected with estradiol into several distinct bands. Isotope incorporation into each protein band was also measured. Increased radioactivity in the estrogen-treated group was detected in protein band A which migrated 6.6-6.8 cm from the origin. A standard rat serum albumin had the same electrophoretic mobility as protein band B which migrated 6.2-6.7 cm from the origin, and an increase in the staining intensity of band B occurred with estradiol administration. Isotope incorporation into proteins of band B did not increase following estrogen stimulation and *in vitro* incubation with labelled amino acids. Therefore band B was determined to be serum albumin. Increased incorporation of labelled amino acids into protein band A was noted one-half hour after *in vivo* estradiol stimulation. By 4 hr, incorporation of labelled amino acids into general proteins was noted. Actinomycin D (4 mg/kg) decreased ^{3}H-leucine incorporation by one-half, whereas cycloheximide (10 μg/ml of medium) blocked ^{3}H-leucine incorporation into all proteins. These results indicated that estradiol induced the synthesis of a specific protein, termed the "Induced Protein" (IP). This protein was found to be specific for the uterus since *in vivo* estradiol did not produce similar isotope incorporation into proteins from liver and ileum tissue.

The isoelectric point of IP as purified on polyacrylamide gels was in the range of 3.5 to 4.0, indicating an acidic protein group (Mayol and Thayer, 1970). Other researchers (Iacobelli, 1973; Sömjen *et al.*, 1973) reported isoelectric points of 4.7 or 4.5 with corresponding molecular weights (MW) of 40,000 and 45,000 daltons, respectively. Katzenellenbogen and Leake (1974) reported two isoelectric points for IP at 4.6 and 5.1 with a MW of 42,000 daltons.

Since IP was not detected unless estradiol was present, indicating *de novo* synthesis (Barnea and Gorski, 1970), it became necessary to determine the role of RNA on its synthesis. Contrary to Notides and Gorski (1966), De Angelo and Gorski (1970) reported that pretreatment with actinomycin D or nogalomycin, which inhibits RNA transcription, blocked IP synthesis. Mayol and Thayer (1970) also reported IP inhibition by the presence of actinomycin D. These results indicated that the synthesis of the estradiol-induced protein may be dependent on prior synthesis of a specific RNA, presumably mRNA which is also estradiol dependent. De Angelo and Gorski (1970) determined that the IP synthesizing capacity (all factors involved in determining the rate of IP synthesis such as mRNA synthesis, transport, stability and translation), which is actinomycin D sensitive, occurred within 15 min of estradiol stimulation and decreased within 30 to 60 min, even though estradiol was still entering the nucleus. Rate of synthesis for the induced protein increased

from 45 to 60 min after estradiol stimulation and possibly was proportional to the amount of accumulated induced protein RNA (IP-RNA). To determine if prior protein synthesis was required for the IP synthesizing capacity step (IP-RNA synthesis), puromycin and cycloheximide were injected intraperitoneally 30 min before an _in vivo_ estradiol injection into ovariectomized or immature rats. Cycloheximide and puromycin under such conditions were unable to block IP synthesis, implying that protein synthesis prior to IP-RNA synthesis (possibly IP-mRNA) is not necessary for IP synthesis (De Angelo and Gorski, 1970).

Following the demonstration that IP synthesis was dependent upon the observed increase of mRNA (actinomycin D sensitive step), a detailed time course study revealed that the concentration of mRNA in the nucleus increased within 5 min and decreased by 30 min after estradiol administration (Gorski _et al._, 1971). The synthesis of IP was not detected until 40 min post-estradiol stimulation, with a significant increase at 60 min and continuing until 2 hr, and a sharp decline at 4 hr. This time sequence indicated an initial lag period of approximately 35 min between the observed accumulation and translation of IP-mRNA into IP. The lag period may allow for the translocation of mRNA from the nucleus to the cytoplasm where IP synthesis occurs. Since the IP-mRNA concentration decreased prior to IP synthesis, a simple negative feedback mechanism is not operative in

the estradiol-induced IP synthesis.

Katzenellenbogen and Gorski (1972) demonstrated that stimulation of IP synthesis occurred using physiological concentrations of estradiol (3.7×10^{-8} M) in an _in vitro_ system. In comparing the _in vitro_ and _in vivo_ systems, the only difference was that the _in vitro_ response was 85% of that observed _in vivo_. It was also noted that maximum synthesis occurred at an estradiol concentration of 2 to 3×10^{-8} M, which is the concentration at which maximal binding to the cytoplasmic receptor occurs, suggesting a relationship between the magnitude of IP synthesis and nuclear binding of estradiol (Katzenellenbogen and Gorski, 1972; Ruh _et al._, 1973). Increased affinity for estrogen by the cytoplasmic receptor resulted in an increased response. Setting the estradiol-17β response at 100%, diethylstilbesterol, estriol and estradiol-17α yielded a 60%, 50%, and 30% increase in IP synthesis, respectively (Katzenellenbogen and Gorski, 1972). High concentrations of estradiol-17β (10^{-7} M) resulted in decreased responsiveness of the uterine tissue.

Incubation with cyclic adenosine 3', 5'-monophosphate (3'5' cAMP), which is implicated in protein hormone mechanism of action, had no effect on IP synthesis (Katzenellenbogen and Gorski, 1972). Prior treatment of immature female rats with propranolol (50 μg/100 g), which blocks the ability of β receptors located on the cell membrane to respond to stimuli, blocked the stimulatory effect of

estrogen on cAMP but did not inhibit IP synthesis (Dupont-Mariesse and Galand, 1975). Studies were also undertaken to determine the effect of cyclic quanosine monophosphate (cGMP) and protein kinase binding activity on IP synthesis (Vokaer *et al.*, 1974). The results were similar to cAMP; thus it was concluded that neither cAMP nor cGMP exert an effect on IP synthesis by the uterus.

Since estrogen elicits a response in both endometrial and myometrial tissue of the uterus, the ability of each tissue to synthesize IP was studied (Katzenellenbogen and Leake, 1974). The endometrium of the immature rat had a greater ability to synthesize IP than the myometrium. The endometrium contained 60-70% of the total IP synthesized. However in the mature uterus, (diestrus with exogenous estradiol) no significant increased IP synthesis occurred in the endometrium compared to the myometrium. This may have resulted from differential estrogen sensitivity between mature and immature rat uteri and/or the ability to synthesize proteins as a function of maturity. These results indicate that the early estrogen response (within 1 hr) is similar for endometrium and myometrium with the divergence occurring in the late response after IP synthesis.

The ability of the uterus of intact cycling rats to produce IP was then explored (Katzenellenbogen, 1975; Iacobelli, 1973). It was observed that the uterus at proestrus had the greatest ability to synthesize IP. During estrus and metestrus, IP synthesis was not

detected, with minimal amounts produced during diestrus. Upon injection of estrogen into the intact rat, maximum IP production occurred during metestrus and diestrus, and only a slight increase was noted during estrus and proestrus. This demonstrated that uterine tissue was refractory to exogenous estradiol during estrus and required a recovery phase which may be related to the number of estrogen nuclear binding sites present. An increase in the availability of cytoplasmic binding sites occurred at proestrus and was correlated with the cyclic variation of plasma estradiol throughout the cycle. Minimal plasma estradiol levels occur at estrus, while peak levels occur at proestrus (Brown-Grant et al., 1970).

The refractory period to estradiol at estrus was reproduced in immature rats by a single subcutaneous (sc) injection of estradiol (5 μg; zero time) followed by a second sc estradiol injection 1 to 42 hr following the initial injection (Katzenellenbogen, 1975). These results indicated that the uterus was unable to respond to a second injection of estradiol within 4 hrs of the initial injection. There was a gradual return of IP synthesis by the uterine tissue 4 to 40 hr after the initial dose of estradiol.

This gradual return to uterine IP synthesis was thought to be correlated with the replenishment of nuclear estradiol receptors. Both maximum IP synthesis and maximum number of nuclear estradiol receptors occurred 1 hr following the initial estradiol injection. By

4 hr, nuclear receptor levels had decreased and remained below control levels up to 42 hr following the initial injection. Cytosol estradiol receptors were found to be in an inverse relationship to nuclear receptors as determined by the cytosol exchange assay. The number of cytosol estradiol receptors began to increase 1 hr after the initial estradiol injection, then returned to control levels by 24 hr and increased above control levels thereafter. The cytosol estradiol receptors are capable of being translocated to the nucleus upon estradiol stimulation. A second estradiol stimulation 24 hr after the first resulted in only a 50% IP response, indicating that a second factor other than replenishment of estrogen receptors may be involved in uterine refractoriness to estradiol.

The actual function of the IP is unknown. It may function as a Key Intermediary Protein (KIP; half life 15-30 min) which increases the metabolic activity of the cell. Baulieu et al. (1972a) proposed a "cascade theory". After estrogen binds to the cytosol receptor and is translocated to the nucleus, it binds to the non-histone chromatin and mRNA, specific for KIP, is synthesized which stimulates KIP formation. The Key Intermediary Protein in turn stimulates the formation of ribosomal ribonucleic acid (rRNA) allowing for protein synthesis, thereby enabling uterine growth to occur. A second possibility is that, after estradiol binds to the chromatin, mRNA is synthesized which directly results in increased rRNA and protein

synthesis. Baulieu *et al.*, (1972b) proposed that IP is the same as KIP or that they are separate proteins which are produced simultaneously by an increase in mRNA and may act to increase the activity of the cell. If IP is KIP it should be localized in the nucleus within 2 hr after estradiol administration to stimulate rRNA synthesis or transport from the nucleolus. A third possibility is that KIP may lead to more RNA polymerase like activity which occurs in the nucleus. However, Katzenellenbogen and Leake (1974) did not observe the presence of the IP (3 hr after estradiol administration) in the nucleoplasm of the endometrium.

General Protein Synthesis

Protein synthesis by rat uterine cell fractions was found to increase 4 hr after estrogen stimulation (Noteboom and Gorski, 1963). The increased protein synthesis was dependent upon RNA synthesis and an increased RNA polymerase activity by 2 hr after estrogen stimulation. Puromycin was capable of blocking the 2 hr increase of RNA and RNA polymerase, indicating that synthesis of a specific protein(s) prior to RNA synthesis must occur to facilitate the 4 hr increase of protein in uterine tissue. Cycloheximide was also found to inhibit the estrogenic acceleration of RNA synthesis and thus the 2 and 4 hr increase in proteins (Gorski and Axman, 1964). Fencl and Villee (1971), using immature rats, injected RNA extracts from rat

uteri previously stimulated with estradiol into one uterine horn while the contralateral horn served as a control. They reported increased protein synthesis similar to an estrogenic response. Maximum incorporation of labelled amino acids into proteins occurred by 24 hr after intraluminal injections of the estradiol-stimulated RNA extracts and returned to control levels by 48 hr. No significant difference was noted in total protein content between treated and control tissue. These results were compared to results obtained from an analogous experiment involving sc estradiol injections into immature rats. Maximal protein synthesis (measured by specific activity) occurred by 24 hr post-estradiol injections; protein synthesis returned to control values by 48 hr post-estradiol injections. The difference between the total protein content of the treated and control uteri disappeared by 72 hr. These results indicate that estradiol increased protein synthesis by increasing RNA and affecting protein turnover rate.

Since estrogen mediates its response in the uterus by increasing protein synthesis, researchers sought to identify and determine the function of uterine fluid proteins throughout the estrous cycle. Macromolecules present around the time of implantation are the most intensely studied group of proteins and may function to control fertility by affecting implantation or embryo development. The majority of research on the proteins of implantation has been conducted utilizing the rabbit.

Implantation in the rabbit normally occurs on day 7 post coitum. Seamark and Lutwak-Mann (1972) determined the levels of progestins from day 5 to day 9 of pregnancy for the free-floating blastocyst, blastocyst fluid and blood plasma in the rabbit. Their results suggest that progestins found in uterine fluid may be of uterine origin and not of blastocyst origin. This is contrary to that which occurs in pig blastocysts which have been shown to synthesize estrogens and progesterone (Perry _et al._, 1973). Prasad _et al._ (1974) reported that incorporation of labelled estradiol ($^3H\text{-}E_2$) into the nuclei of glandular epithelium of the rat uterus occurred when implantation was delayed by ovariectomy on day 3 of pregnancy. Uptake of $^3H\text{-}E_2$ by uterine tissue occurred within 5 min after injection, but they were unable to detect uptake of $^3H\text{-}E_2$ by the blastocyst. However, this does not rule out the possibility that the estrogen binding proteins in cells are in the inner cell mass of the developing embryo or that estrogen exerts an indirect effect on the blastocyst.

It has been hypothesized that, if the blastocyst is capable of steroid synthesis, these blastogenic steroids alter uterine metabolism to facilitate implantation. A second possibility is that the blastocyst produces a substance, possibly a protein, which initiates implantation. Lau _et al._ (1973), using a day 9 mouse blastocyst, demonstrated that exogenous estrogen increased RNA synthesis in the blastocyst which is necessary for protein synthesis.

Uterine tissue, in response to estrogen, has provided experimental evidence for the existence of an implantation initiating factor (IIF; Mintz, 1970, 1972). The IIF may possibly be a proteolytic enzyme. It is stage specific, affecting the blastocyst but not the morula stage. Pinsker _et al_. (1974) found that proteolytic activity of mouse uterine fluid increased on day 3 of pregnancy and hypothesized that IIF exerts an effect on blastocyst receptors, resulting in an initial attachment or superficial adhesion of the blastocyst to the uterine wall. Further embryo attachment may then be enhanced by proteolytic enzymes produced by the trophoblast. The IIF may also aid in implantation since it could lyse the zona pellicuda. The IIF was also detected in pseudopregnant mice thus supporting data indicating a uterine origin (Pinsker _et al_., 1974).

Research conducted by Beier and Hellwig (1973) on endometrial protein secretions during pregnancy and pseudopregnancy in the rabbit revealed the presence of nine proteins. The predominant protein found in uterine fluid was uteroglobin (blastokinin). This protein was present in the endothelium on the day of coitus. The accumulation of uteroglobin in the endothelial cells peaked between days 3 and 4 post-coitum (Beier, 1974). Uteroglobin has a molecular weight of 15,000 daltons and a sedimentation coefficient of 1.38 S (Beier, 1974). Uteroglobin has been found in the blastocyst fluid but not in the blastocyst tissue (Beier and Maurer, 1975). Uteroglobin synthesis

was stimulated by progesterone, whereas estrogen delayed its synthesis and consequently can delay implantation in the rabbit (Beier and Hellwig, 1973; Beier, 1974). Bullock and Willen (1974) also observed that the presence of estrogen decreased the amount of uteroglobin present in the uterine fluid between days 5 and 10 post-coitum. Therefore, it is thought that estrogen acts by increasing certain proteins which inhibit the synthesis of uteroglobin or that it increases the degradation of uteroglobin.

Uteroglobin is thought to function in the binding and transport of progesterone from the uterine fluid to the blastocyst where the hormone elicits an effect on the blastocyst. Beato and Baier (1975) demonstrated that uteroglobin did bind progesterone in the presence of dithioerythritol and that one molecule of progesterone was bound per molecule of uteroglobin. Uteroglobin was also found to be composed of two subunits.

The proteins of pregnancy may be species specific since uteroglobin had not been detected in human uterine fluid (Beier and Hellwig, 1973; Beier, 1974). The synthesis of these pregnancy proteins is dependent on the ratio of ovarian hormones present during the time of implantation. Estrogens increase the mitotic activity of the blastocyst as well as the concentration of high molecular weight proteins in uterine fluid (Gore-Langton and Surani, 1976). Progesterone stimulates the synthesis of uteroglobin and thus functions to maintain pregnancy.

MATERIALS AND METHODS

Experimental Design

Two separate but related experiments were conducted to determine if ovine uterine endometrium was capable of synthesizing the induced-protein (IP) previously reported in rat uterine tissue after incubation with estradiol-17β (Katzenellenbogen and Gorski, 1975). General uterine protein synthesis was also determined for each experiment. The first experiment was designed to determine if ovine uteri have the capacity to synthesize the IP on various days of the estrous cycle and during pregnancy. This experiment was conducted during the fall and winter of 1975.

The second experiment was conducted during the spring of 1976 to determine if treatment of ovariectomized ewes with estradiol-17β and progesterone would enhance the ability of *in vitro* estradiol-17β to stimulate uterine IP synthesis.

Experiment 1. Intact Cycling Ewes

Eighteen mature (3- to 5-yr old), intact, cycling Suffolk-Hampshire ewes exhibiting normal estrous cycles were assigned in equal numbers to be necropsied on various days of the estrous cycle or pregnancy. The average estrous cycle length for three consecutive cycles prior to animal sacrifice was 16.2 ± .15 days. Ewes were

sacrificed on days 0 (estrus), 3, 6, and 10 and on day 14 or 15 of the estrous cycle and on day 15 of pregnancy. The results from ewes sacrificed on day 14 or 15 of the estrous cycle were compared with the results obtained from day 15 pregnant ewes.

Blood samples were collected on the morning of sacrifice by jugular venipuncture using Vacutainer tubes. The serum samples were analyzed for estradiol and progesterone by specific radioimmunoassays (RIA). Mean (± SE) serum estradiol-17β levels obtained on days 0, 3, 6, and 10 were 2.1 ± 1.3, 5.37 ± 3.47, 13.73 ± 7.13 and 3.37 ± 2.57 pg/ml, respectively. Estradiol levels for the day 15 pregnant and day 14 or 15 nonpregnant ewes were 1.3 ± .06 and 2.05 ± .25 pg/ml, respectively. These values were derived from analysis of three serum samples per group except for the day 0 and day 14 or 15 group where only two serum samples were assayed for estradiol and progesterone. Mean serum progesterone levels for days 0 and 3 were significantly lower (.60 ± .16 ng/ml) than on days 6, 10, 14 or 15 of the estrous cycle (1.25 ± .23 ng/ml) and day 15 of pregnancy (2.61 ± .25 ng/ml), as determined by orthogonal comparisons.

Experiment 2. Ovariectomized Ewes

Sixteen mature (3- to 5-yr old) Suffolk-Hampshire ewes were ovariectomized a minimum of 4 weeks prior to use in the experiment

using a mid-ventral incision under sodium pentobarbitol anesthesia. The ewes were assigned randomly to 1 of 4 treatment groups within a randomized necropsy schedule. The treatment groups consisted of a) a control group (received empty implant plus vehicle injections), b) subcutaneous (sc) estradiol-17β (E_2) implant plus vehicle injections, c) sc progesterone (P_4) injections plus an empty implant and d) estradiol implant and progesterone injections. Silastic capsules (3.35 mm by 10 mm) containing 54 ± 3 mg of estradiol-17β ($\Delta^{1,3,5,(10)}$ Estratrien-3, 17β diol; Sigma Chem. Co.) were placed subcutaneously under the foreleg while control ewes were implanted with an empty capsule. This procedure was first reported by Karsch et al. (1973). The silastic capsules were implanted on day 1 of treatment following pretreatment and removed at the time of sacrifice (day 8). Progesterone (two-15 mg doses at 12 hr intervals; Δ^4-Pregnen-3, 20, -dione, Sigma Chem. Co.) and vehicle (sesame oil) injections began 48 hr following sc implantation of the implant. Progesterone was injected for 5 consecutive days. Ten milliliter blood samples were collected by jugular puncture prior to the implant implacement and preceding each progesterone or vehicle injection. A blood sample also was collected on the morning of day 8, immediately prior to sacrifice.

Serum hormone levels were analyzed statistically by a split-plot analysis of variance with hormone treatments as the main effect.

Further analysis using orthogonal comparisons indicated that ewes receiving an estradiol implant (E_2 and $E_2 + P_4$ groups) tended to have elevated serum estradiol levels (5.92 ± 1.89 pg/ml) in comparison to ewes receiving an empty implant (3.72 ± 0.71 pg/ml). Estradiol levels prior to placement of the implant (2.23 ± 1.05 pg/ml) were less than levels following sc placement of the implant (5.24 ± 3.47 pg/ml; P = .10). Serum progesterone levels for the progesterone-treated ewes (P_4 and $P_4 + E_2$) were significantly higher (55.77 ± 17.12 ng/ml) than for the vehicle-treated ewes (control and E_2; 1.30 ± .10 ng/ml). Mean levels of estradiol and progesterone established during the treatment regime are reported in Tables 1 and 2, respectively.

Table 1. Mean estradiol levels in ovariectomized ewes treated with estradiol-17β and progesterone.[a]

	Day of Treatment						
Treatment	1	3	4	5	6	7	8[c]
Control	1.25[b]	1.68	3.15	11.45	5.20	4.88	5.93
	±.12	.22	1.08	10.72	1.65	2.21	2.15
E_2	2.43	29.85	4.40	4.25	2.25	3.45	3.70
	.76	17.23	1.62	2.14	.56	.71	2.36
P_4	2.33	2.43	3.50	1.52	1.78	3.45	3.48
	.38	.99	1.16	.44	.09	1.59	2.13
$E_2 + P_4$	3.05	4.60	4.10	3.25	9.05	3.53	4.93
	.30	1.47	1.02	1.25	3.35	1.17	1.56

[a]Estradiol was implanted on day 1 with progesterone treatments starting on day 3.

[b]Mean ± SE estradiol (pg/ml) from 4 samples.

[c]Day of sacrifice.

Table 2. Mean progesterone levels in ovariectomized ewes treated with estradiol-17β and progesterone.[a]

Treatment	Day of Treatment						
	1	3	4	5	6	7	8[c]
Control	.20[b]	.18	.39	.29	.34	.45	.38
	±.01	.01	.06	.15	.09	.21	.09
E_2	.40	.32	.45	.42	.19	.30	.19
	.09	.12	.17	.20	.08	.17	.03
P_4	.20	.44	1.24	2.81	45.75	12.82	23.27
	.01	.24	.17	.57	37.12	11.04	15.80
$E_2 + P_4$	.23	.52	13.21	10.47	13.90	23.00	47.34
	.03	.25	7.03	8.18	7.83	20.24	45.00

[a] Estradiol was implanted on day 1 with progesterone treatments starting on day 3.

[b] Mean ± SE progesterone (ng/ml) from 4 samples.

[c] Day of sacrifice.

In Vitro Incubation of Ovine Uterine Tissue

A slightly modified protocol of Katzenellenbogen and Gorski (1975) was followed for the *in vitro* labelling of newly synthesized proteins by ovine uteri.

Each ewe was necropsied at approximately 0700 hr by stunning and exsanquination. The uteri were removed and immediately placed on ice and transported to the lab within 20 min. The uterine horns were slit longitudinally and the endometrium was separated from the myometrium by blunt dissection. The caruncles and fatty tissue were also removed. Only endometrium from the mid portion of the horn

was utilized, and segments were placed in cold 0.9% saline and kept on ice. Two ml of Eagles HeLa TC media (Difco Products) were added to ten ml Erlenmeyer flasks previously coated with Siliclad (Lab Products). The Eagles HeLa TC media was used without modification. A torsion balance was used to weigh three-50 mg segments of endometrium which were placed into each flask. The flasks were kept on ice until the start of the *in vitro* incubation.

The control flask received 10 μl of absolute ethanol whereas 10 μl of estradiol-17β (13.6 ng, $\Delta^{1,3,5,(10)}$ Estratrien 3,17 diol; Schwarz Mann) in absolute ethanol was added to the treated flasks. The flasks were gassed with 95%-O_2 5%-CO_2 and corked with silicon rubber stoppers. After an incubation period of 60 min at 39°C in a Dubnoff tissue incubator (40 cycles/min), the medium was aspirated and the tissue rinsed 3 times with fresh media maintained at room temperature to avoid cold-shocking the tissue. Following the third rinse, 2 ml of Eagles HeLa TC media was added per flask. The control flask received 65 μl (6.5 μCi) of uniformly labelled L-(^{14}C) Leucine (325 mCi/mMole, New England Nuclear [NEN]). The treated flask received 40 μl (40 μCi) of L-(4,5-^{3}H) Leucine (60 mCi/μM, NEN). The flasks were regassed for 10 seconds with 95%-O_2 5%-CO_2 and immediately stoppered. A two hr incubation under conditions similar to the previous incubation was followed. The uteri from the pregnant and nonpregnant ewes were incubated with labelled amino

acids for 4 hr as compared to a 2 hr incubation period for uteri from the cycling ewes. The increased incubation time was established to enhance the incorporation of labelled L-Leucine. Following the 2 or 4 hr incubation period, the tissue from the control and treated flasks were rinsed three times with buffer (Na_2EDTA). The tissue from the control flask was combined with the tissue from the treated flask. The combined tissues were homogenized in 1 ml 0.05% Na_2EDTA (4 C) using a ground glass mortar and pestle attached to a Sargent Cone Drive stirring motor. To provide a more concentrated protein supernatant, the protocol was later modified so that tissue was homogenized in 0.5 ml of 0.05% Na_2EDTA (J. Gorski, personal communication). Following complete homogenization, the homogenate was poured into plastic centrifuge tubes for centrifugation at 27,000 x g at 4 C for 50 min using a refrigerated Sorvall Superspeed RC-2 centrifuge. The supernatant was removed and placed into glass storage vials and stored at -20 C until analyzed by electrophoresis.

Disc Gel Electrophoresis of Ovine Uterine Supernatant

The protein supernatants were thawed and two hundred microliters of the supernatant were withdrawn and placed into a culture tube. The density of the supernatant was increased by adding sucrose crystals (8 mg/100 µl) or Ficoll 400 (13 mg/100 µl; Pharmacia). Three to five microliters of a 0.05% solution of bromphenol blue

(Allied Chemicals) were also added to each supernatant as a tracking dye. The protein preparations were placed into the refrigerator overnight to allow the sucrose or Ficoll to enter into solution.

The following day, a 6% polyacrylamide gel solution (95:5 acrylamide-N, N'-Methylene bisacrylamide; Eastman) was prepared by mixing 5 ml acrylamide-bis, 87 µl 10% ammonium persulfate (Eastman), and 11 µl of N, N, N'N'-tetramethyl ethylenediamine (TEMED; Eastman). Three gels could be poured from the above solution before polymerization occurred.

The solution was dispensed into gel tubes (7.5 x .5 cm) previously coated with Photo Flow 200 (1 part Photo Flow to 200 parts distilled water; Eastman). Gel height was limited to 6.5 cm and polymerization usually occurred within a few min after pouring. The gels were then placed into the upper chamber of a Buchler Instrument disc gel apparatus. The upper and lower chambers were filled with Tris-borate-EDTA buffer (pH 8.6, 0.066 M Tris, 0.02 M Boric acid, 0.003 M Na_2EDTA), and the protein supernatant was layered beneath the buffer of the upper chamber onto the surface of the appropriate gels. A Heathkit Power Supply (Model IP-17) was allowed to warm up before use. Electrophoresis was conducted at room temperature in a water cooled lower chamber at 1 ma/tube for the first hr (until proteins had entered the gel) and then at 2 ma/tube for the remainder of the run. The power was shut off when the tracking dye was within

1 mm of the bottom of the gel. A normal run lasted 3 to 4 hr.

At the completion of the run, the gel tubes were removed rapidly from the chambers and the gels removed by ringing with a 25 gauge needle while submerged in cold water. Care was taken to avoid cutting the gel with the needle as the gel was being removed. Upon removal of the gel, the length of the gel and the distance the tracker dye had migrated from the origin was recorded. The top of the gel was notched to distinguish it from the bottom. The gels were then placed in 7.5% acetic acid overnight or in a staining solution for a minimum of 2 hr.

The polyacrylamide gels were stained with one of the following dyes; Amido Black 10B (1%, Merck Ag· Darmstacht), Aniline Blue (1%, CI 42755; Allied Chem.), Buffalo Black NBR (1%, CI 20470; Allied Chem.) or Coomassie Brilliant Blue G (2%, Sigma). Amido Black, Aniline Blue, and Buffalo Black were prepared in 7% glacial acetic acid and Coomassie Brilliant Blue G was prepared in methanol:acetic acid:distilled water (45:10:45). The sensitivity of the dyes to stain proteins was determined by comparing prealbumin protein patterns observed for each dye using duplicate uterine supernatant samples. Coomassie Brilliant Blue G was more sensitive and yielded sharper bands compared to the other dyes. For example, Coomassie Brilliant Blue G stained 5 prealbumin bands, while only 2 bands were stained with Buffalo Black NBR (Figure 1, gels D and E respectively).

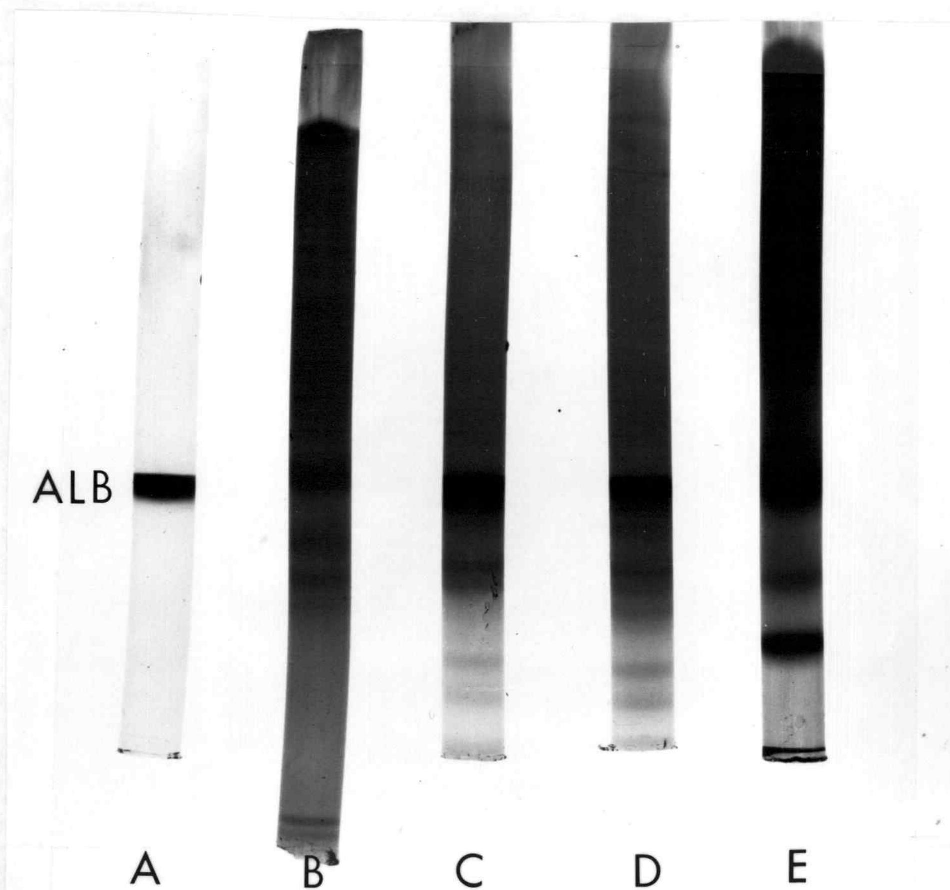

Figure 1. Polyacrylamide electrophoric patterns of soluble uterine proteins.

a. Bovine serum albumin (25 μg) stained with Coomassie Brilliant Blue G.

b. Rat uteri incubated in vivo with estradiol-17β, stained with Amido Black 10 B (trial 2).

c. Ovariectomized ewe treated with estradiol implant, incubated in vitro with estradiol-17β and stained with Coomassie Brilliant Blue G.

d. Ovariectomized ewe treated with estradiol implant and progesterone injections; incubated in vitro with estradiol-17β and stained with Coomassie Brilliant Blue G.

e. Same as D except stained with Buffalo Black NBR.

Buffalo Black NBR and Amido Black 10B gave similar staining patterns although Buffalo Black NBR yielded slightly sharper bands. Aniline Blue tended to be less sensitive and had a reduced resolving ability due to less destaining of background when compared with the other dyes.

After a 2 hr staining period, the gels were placed into a beaker containing 7.5% acetic acid to remove excess stain. To facilitate the destaining procedure, the acetic acid was changed frequently until distinct protein bands could be distinguished. Upon completion of destaining, gel length, the distance the protein bands had migrated from the origin and the position of the albumin band were recorded for each gel. The stained gels were then stored in 7.5% acetic acid.

After remaining in 7.5% acetic acid overnight, the unstained gels were placed into the upper chamber of the electrophoretic apparatus to remove the labelled free amino acids by electrophoresis. The upper and lower chambers were filled with 7.5% acetic acid and electrophoresis was run at 5 ma/tube for 1 hr. At the termination of the run, the gels were removed from the destaining tubes and placed in 7.5% acetic acid until they were sliced.

To keep the gel straight during freezing, gels were positioned in an aluminum angle bar noting which end was the top of the gel, and frozen over dry ice. When frozen the gel was removed from the bar and placed on plexi-glas (13 x 13 cm). The frozen gel lengths were

recorded since gel expansion occurred during freezing. The gel was sliced by applying even pressure on a plexi-glas (5 x 11 cm) placed over the gel cutter. The gel cutter was constructed by spacing double edged razor blades with washers and bolted together to provide slices 1.9 mm in thickness. Each gel yielded about 36 slices; each slice was placed into a scintillation vial containing 0.8 ml of 1% sodium dodecyl sulfate (SDS; Bio Rad) in distilled water.

The gel slices were incubated in the SDS for a minimum of 12 hr at 37 C in an oven. Ten milliliters of 10% BBS-3 (Beckman) toluene scintillation fluid (.3 gms dimethyl POPOP, 5 gms PPO/ liter) were added to each vial. The vials were counted in a Packard Liquid Scintillation Spectrometer (Model 2425) for 10 min or 10,000 counts. For dual label counting, the upper and lower windows were set at 250-30 and 1000-90 for the ^{3}H and ^{14}C channels, respectively. The gain settings for the ^{3}H and ^{14}C channels were 100% and 19.5%, respectively. Under these conditions counting efficiency was 35% for ^{3}H and 71% for ^{14}C. It was determined that 7% of the ^{14}C spilled over into the ^{3}H channel.

Determination of Ovine IP Synthesis and General Protein Synthesis

The ability of estradiol to stimulate IP synthesis *in vitro* was determined by noting the incorporation of labelled

L-(4, 5, -^{3}H)-Leucine in the estradiol-treated tissue as compared to the incorporation of L-(^{14}C)-Leucine in the control tissue. Since the tissues (control and treated) were homogenized together, the relative incorporation of each label could be calculated for each gel slice. Therefore, the presence of the IP was indicated by an increase in the $^{3}H/^{14}C$ ratio (^{3}H cpm/^{14}C cpm) occurring in the prealbumin region of the gel.

To calculate this ratio, cpm of the ^{3}H channel were adjusted since 7% of the ^{14}C activity was also detected in the ^{3}H channel by the spectrometer. For the ^{14}C channel, cpm were converted to dpm (disintegrations per min) by dividing the ^{14}C cpm by 0.713 (^{14}C counting efficiency). The resulting ^{14}C-dpm were multiplied by 0.07 since the ^{3}H channel detected ^{14}C with 7% efficiency. This correction factor yielded the cpm of ^{14}C detected in the ^{3}H channel and therefore was subtracted from the ^{3}H cpm, leaving the actual ^{3}H cpm. The $^{3}H/^{14}C$ ratio was determined for each gel slice by dividing the calculated ^{3}H cpm (treated tissue) by the ^{14}C cpm (control tissue). The advantage of monitoring the incorporation of different isotopic labels in stimulated and non-stimulated tissue is that it is more sensitive relative to single isotopic labelling in detecting newly synthesized proteins.

Differences in non-specific protein synthesis in experiments 1 and 2 were determined by recording the number of protein bands

occurring between the albumin band and tracker dye front (prealbumin region) in each of the stained gels. These markers (albumin and dye front) were used due to their prominant staining ability and electrophoretic mobility and is the area in which the induced-protein (IP) occurs (Notides and Gorski, 1966).

IP Synthesis by Rat Uterine Tissue as Detected in Our Laboratory

Since it has been established that rat uterine tissue stimulated *in vivo* or *in vitro* by estradiol is capable of synthesizing IP, similar experiments using immature rat uteri were conducted to verify that IP synthesis in the rat uterus could be detected in our laboratory.

A. Materials and Methods

Twelve immature (22-day-old, 50-60 gm) female Long Evan's rats (Simonson's Lab) were decapitated, the uteri were excised and three uteri were placed randomly into each of four flasks (trial 1). A 1 hr incubation with estradiol-17β (E_2, 13.6 ng/10 μl) or absolute ethanol (10 μl/flask) preceded the 2 hr incubation with either L-(4,5-^{3}H)-Leucine (6.68 x 10^{-4} μM, 60 mCi/μM) or uniformly labelled L-(^{14}C)-Leucine (2 x 10^{-2} μM, 325 mCi/mM), respectively. Following incubation, estradiol-treated uteri were combined with control uteri and homogenized in 1.2 ml 0.05% Na_2EDTA. Following centrifugation, the supernatant was subjected to polyacrylamide gel

electrophoresis, allowing for separation of soluble uterine proteins. Electrophoresis was carried out as stated previously.

The second trial utilized twelve immature Long Evan's female rats which were assigned randomly to two groups for an in vivo and in vitro estradiol stimulation study. The six rats within the in vivo study were divided equally and randomly into a control group, receiving an intraperitoneal injection (ip) of ethanol-saline (0.5 cc), and a treated group, receiving an ip injection of E_2 (5 µg in 0.5 cc ethanol). One hr later, the rats were decapitated, uteri were removed and the three uteri from each group were placed into separate flasks containing 2 ml of Eagles HeLa TC media (3 uteri/flask). Incubation with radioactive isotopes was conducted for 2 hr, as in trial 1, prior to homogenization of the tissue. The six rats in the in vitro study were divided into two groups and treated as in trial 1.

B. Rat Uterine Response

Trial 1 failed to repeat the results of Katzenellenbogen and Gorski (1972). The inability to detect the presence of IP, as shown by an absence of change in the $^{3}H/^{14}C$ ratio (Figure 2), was later determined to be caused by a 100,000-fold excess in the concentration of estradiol used during the incubation. This resulted in exposure of uterine tissue to an estradiol concentration of 2.5×10^{-3}M. Katzenellenbogen and Gorski (1972) have reported the optimal

estradiol concentration to be 2 to 3 x 10^{-8} M, with decreased rat uterine IP responsiveness occurring with increased estradiol concentrations. Estradiol at 2 x 10^{-5} M resulted in 68% of the maximal level of IP induction.

Trial 2 was conducted using an estradiol concentration (2 x 10^{-8} M) similar to that of Katzenellenbogen and Gorski (1972). In the in vivo experiment, increased isotopic incorporation in gel slice 23, together with an increased $^{3}H/^{14}C$ ratio, indicates that IP synthesis occurred (Figure 4). The relative rate of IP synthesis was calculated by determining the ratio of cpm in the IP band (3 peak slices) to the cpm in 2 gel slices above and below the IP band for both ^{3}H and ^{14}C (Figure 4b). The ratio for the ^{3}H (E_2) was divided by the ^{14}C (control) ratio; the value obtained--1.26--is an estimate of IP synthesis. Induced protein was also detected in the in vitro experiment (Figure 3). The increased radioactive incorporation and $^{3}H/^{14}C$ ratio occurred in gel slice 24. The relative rate of IP synthesis obtained from the in vitro study was calculated to be 1.27. Therefore, the IP response was similar for both the in vivo and in vitro estradiol incubation. This is different than the observation of Katzenellenbogen and Gorski (1972), who found that the in vitro estradiol stimulation was 85% of the in vivo response.

Staining the gels allowed for the detection of protein bands with an electrophoretic mobility greater than albumin and less than the

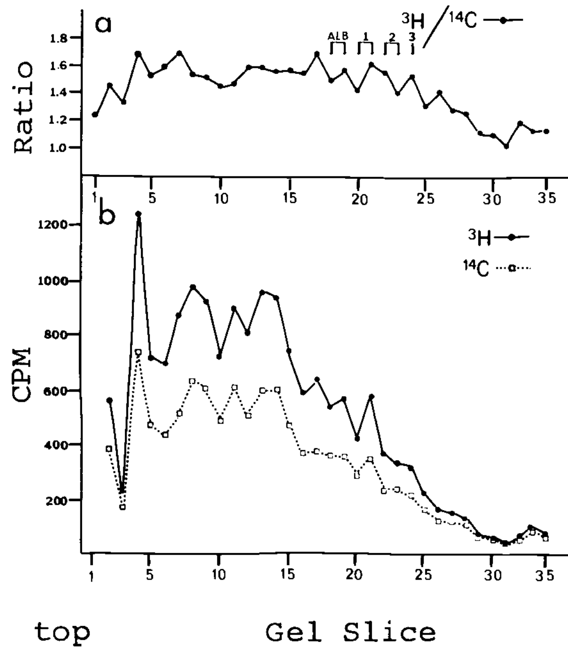

Figure 2. Incorporation of ^3H L-Leucine (estradiol-treated [E_2]) and ^{14}C L-Leucine (control) into immature rat uterine proteins following E_2 stimulation (2.5 x 10^{-3} M) in vitro (trial 1). Uteri were homogenized together and the supernatant subjected to electrophoresis. Albumin (ALB) and pre-albumin bands (1, 2, 3) are identified.

a. Ratio of ^3H and ^{14}C incorporation.
b. Total ^3H and ^{14}C incorporation.

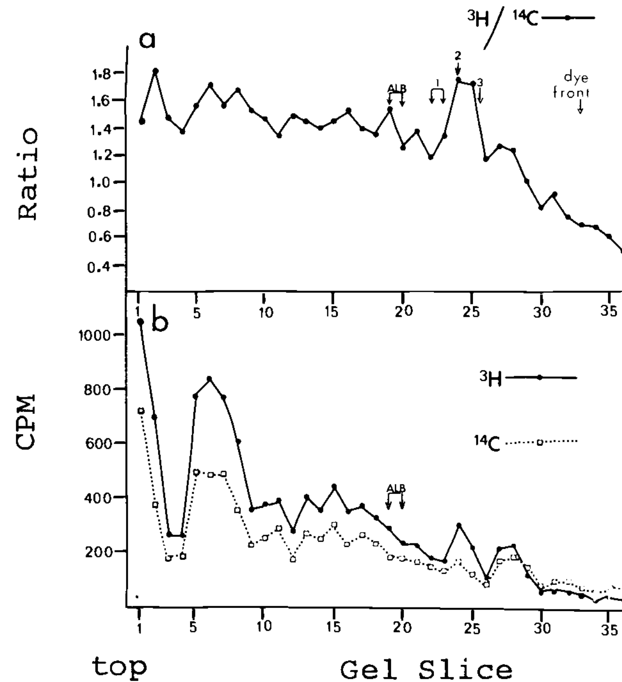

Figure 3. Incorporation of ^3H L-Lucine (estradiol-treated [E_2]) and ^{14}C L-Leucine (control) into immature rat uterine proteins following E_2 stimulation (2.5 x 10^{-8} M) in vitro (trial 2). Uteri were homogenized together and the supernatant subjected to electrophoresis. Albumin (ALB) and pre-albumin bands (1, 2, 3) are identified.

a. Ratio of ^3H and ^{14}C incorporation.
b. Total ^3H and ^{14}C incorporation.

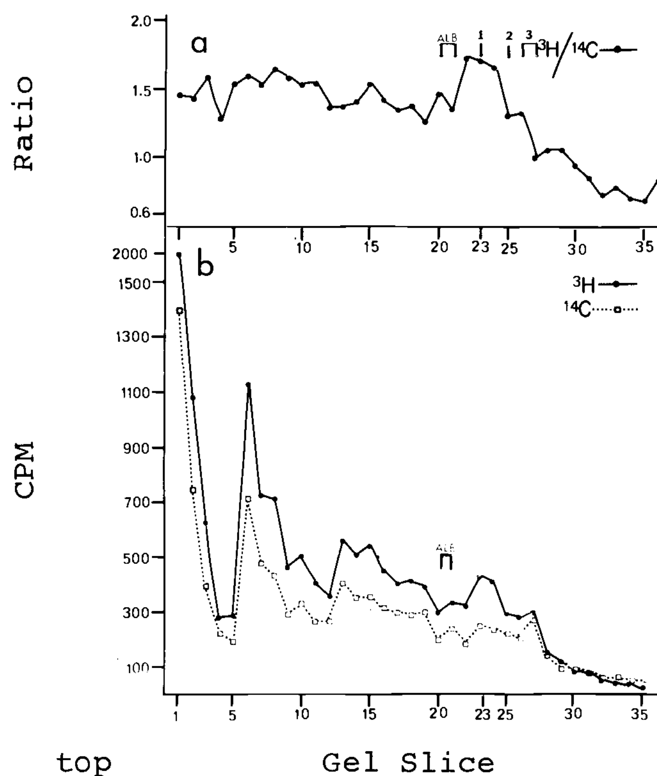

Figure 4. Incorporation of ^3H L-Leucine (estradiol-treated [E_2]) and ^{14}C L-Leucine (control) into immature rat uterine proteins following E_2 stimulation (2.5×10^{-8} M) <u>in vivo</u> (trial 2). Uteri were homogenized together and the supernatant and prealbumin bands (1,2,3) are identified.

a. Ratio of ^3H and ^{14}C incorporation.
b. Total ^3H and ^{14}C incorporation.

tracker dye (prealbumin region). The variability occurring between electrophoric runs reduced the consistency of the Rf determined for each protein during different electrophoretic runs and therefore was not used. The results were simply recorded as number of bands stained in this region. The bovine serum albumin (BSA) standard (25 μg) migrated 4.25 ± .10 cm (N = 15) from the origin (Figure 1a). The serum albumin in the rat protein supernatant migrated 4.36 ± .14 cm (N = 10) from the origin (Figure 1b). The presence of the three characteristic prealbumin protein bands (Katzenellenbogen and Gorski, 1975) was detected in trial 1 and 2 (Figure 1b). The relationship between the stained bands and isotope incorporation is indicated in Figures 2, 3 and 4 for each rat experiment. Barnea and Gorski (1970) have reported that the middle prealbumin band contains the IP. This relationship was only observed for uterine proteins after *in vivo* estradiol in trial 2 (Figure 4). Therefore, the presence of three prealbumin protein bands alone is not a reliable indicator of IP synthesis.

Radioimmunoassay for Estradiol-17β and Progesterone

Serum levels of estradiol and progesterone were quantified by means of specific radioimmunoassays (RIA) validated in our laboratory.

The progesterone RIA was modified from the protocol of Koligian and Stormshak (1976). The addition of 200 μl of 0.1% gelatin in phosphate buffered saline following overnight incubation was deleted. Utilization of a new antibody purchased from Dr. G. D. Niswender (Colorado State Univ.), prepared against progesterone-11α-succinyl BSA, resulted in a need for revalidation of the assay. The specificity of the progesterone antibody (1:1000 in 0.1% gelatin) was determined by a cross-reactivity check against specific steroids (Table 3). No significant cross-reactivity of the progesterone antibody with these steroids occurred within physiological concentrations. The sensitivity of the assay was 10 pg progesterone/tube. This value differed significantly from the 0 pg standard (paired t-test; N = 10). Serum pooled from ovariectomized ewes, when assayed in 5 different assays, had a mean progesterone level of 0.25 ± 0.04 ng/ml. The inter-assay precision and accuracy was measured by the addition of known quantities of progesterone (0.25, 0.50, 1, 2 and 10 ng/ml) to pooled serum from ovariectomized ewes. The inter-assay coefficient of variation was 12.4%. Following subtraction of endogenous progesterone, an average of 98% of the added progesterone was recovered (Table 4). The intra-assay variability was determined from replicates (N = 10) of a serum pool from luteal phase ewes. The resulting value was 1.83 ± 0.03 ng/ml (mean ± SE) with a coefficient of variation of 4.6%.

Table 3. Specificity: Cross-reaction of selected steroids with progesterone antiserum.

Steroid[a]	% Cross-Reaction
Progesterone	100.0
Pregnenolone	3.7
20α-hydroxpregn-4-en-3-one	1.5
20β-hydroxpregn-4-en-3-one	1.0
Testosterone	0.8
Corticosterone	1.8
Estradiol-17β	36.0

[a]Each steroid was included in the assay at concentrations of 20 ng/ml with the exception of estradiol-17β, which was assayed at a concentration of 10 ng/ml. 100 μl of the sample was extracted and analyzed.

Table 4. Recovery of known quantities of progesterone (ng/ml) added to pooled serum from ovariectomized ewes.

Quantity Progesterone Added (ng)	Progesterone Detected[a] (ng)	% Recovery[b]
0.25	0.25 ± .02	100
0.50	0.44 ± .02	88
1.00	0.98 ± .06	98
2.00	2.02 ± .07	101
5.00	5.12 ± .14	102
10.00	10.13 ± .35	101

[a]Endogenous progesterone levels of the pooled serum were subtracted.

[b]Data based on five assays.

Modification of the procedure of Wu and Lundy (1971) allowed for a more sensitive measurement of ovine estradiol-17β. To determine procedural losses, 3,000 dpm of 2,4,6,7-^{3}H estradiol-17β (114 Ci/mmole, New England Nuclear) was added to each tube and then dried under filtered air. Two ml of the serum sample were added, vortexed and then extracted twice with three volumes of double distilled benzene. The solvent phase following each extraction and centrifugation at 500 x g for 10 min was aspirated to a second tube. The combined extracts for each sample were dired and washed twice with distilled water to remove water contaminants. The aqueous-solvent phase was separated by centrifugation and the solvent phase was recovered and dried.

The estrogen extracts were chromatographed on a prewashed and preconditioned Sephadex LH 20 column (2 ml disposable glass syringe packed with .4 gm Sephadex < H_2O) as described by Wu and Lundy (1971). The 1 ml eluate fraction containing estradiol-17β was separated into .9 ml and .1 ml aliquots. The mean extraction efficiency was 71% (N = 4). The specificity of the assay was determined by the addition of estrone, progesterone, testosterone or corticosterone to serum (Table 5). Although estrone cross reacted with estradiol by 15%, Moore et al. (1969) reported that estrone comprises only 12.5% of the serum estrogen levels in the ewe, indicating that estrone levels are below the sensitivity of the assay. Two sets of

standards (0, 2, 5, 10, 20, 30, 50, 75, 100, 150 pg/tube) were incorporated into each assay. The antibody was prepared against estradiol-17β-succinyl-BSA in sheep and was provided through the courtesy of Dr. B.V. Caldwell (Yale Univ.). The antibody was used at a dilution of 1:16,000 in 0.1% gelatin-PBS buffer. To the tubes containing the dried extracts from the Sephadex columns, 100 μl of antibody and 100 μl of 2 4,6,7,-^{3}H estradiol-17β (114 Ci/mM, NEN; 10,000 dpm) were added and incubated for 2 hr at 4 C. Free and bound estradiol were separated by adding 1 ml dextran-coated charcoal (.25 g Dextran T-70 and 2.5 g Neutralized Norit/l PBS). A portion of the supernatant (.7 ml) was counted in 7 ml Triton scintillation fluid (7 g PPO/liter of toluene and Triton X 100 [2:1]) at 45% efficiency.

Table 5. Specificity: Cross-reaction of selected steroids with antiserum for estradiol-17β.

Steroid[a]	% Cross-Reaction
Estrone	15.00
Progesterone	0.14
Testosterone	0.48
Corticosterone	0.14

[a]All selected steroids were included in the assay at approximate peak systemic serum concentrations.

The inter-assay precision and accuracy as determined by the addition of 2.5, 5.0, 10.0, 15.0, 25 and 75 pg/ml to a serum pool of an anestrous ewe, resulted in the recoveries indicated in Table 6.

Table 6. Recovery of known quantities of estradiol (pg/ml) added to pooled serum from ovariectomized ewes.

Quantity Estradiol-17β Added (pg)	Estradiol-17β Detected[a,b] (pg)	% Recovery[b]
2.5	3.0 ± 0.3	120
5.0	4.6 ± 0.2	92
10.0	8.7 ± 1.2	87
15.0	14.4 ± 1.2	96
25.0	26.2 ± 2.4	105
75.0	71.4 ± 0.6	95

[a] Endogenous estradiol levels of the pooled serum was subtracted.

[b] Data based on 4 assays.

The assay sensitivity was determined to be 2 pg/ml since a significant difference (N = 10) between the 2 and 0 pg standard was observed using a paired t-test. Analysis of an anestrous ewe serum pool compared to a serum pool plus 2.5 pg/ml of estradiol showed that the assay could detect a difference of 2.5 pg/ml between samples ($P < 0.05$, $N = 4$). Also, solvent blanks (benzene and benzene: methanol) from 4 assays averaged 0.8 ± 0.1 pg/tube.

RESULTS AND DISCUSSION

Synthesis of the estradiol-induced protein (IP) following incubation of endometrium with estradiol-17β was not detected on days 0 (estrus), 3, 6, or 10 of the ovine estrous cycle. This is depicted by the lack of any substantial change in the $^{3}H/^{14}C$ ratio occurring in the prealbumin region for days 0, 3, 6, or 10 (Figures 5 through 8, respectively). The albumin band was detected in gel slices 22 and 23. Each figure represents 1 of the 3 ewes sacrificed on each of the selected days. Furthermore, using the same criteria as above, uteri from day 14 or 15 nonpregnant and day 15 pregnant ewes did not elicit IP synthesis upon incubation with estradiol-17β (Figure 9 and 10, respectively). Albumin was detected in gel slice 23 (day 14 or 15 nonpregnant, Figure 9) and 21 (day 15 pregnant, Figure 10).

The inability to induce IP synthesis in ovine endometrial tissue on days 0, 3, 6, or 10 of the estrous cycle and day 15 of pregnancy was likely caused by the 100,000-fold excess concentration of estradiol used during the _in vitro_ incubation. The endometrial sections (three 50 mg pieces) were exposed to an estradiol concentration of 2.5×10^{-3} M. This probably resulted in "refractoriness" of the ovine uterine tissue to estradiol. This "refractoriness" was also observed in the experiment utilizing the rat (trial 1) which substantiates the findings of Katzenellenbogen and Gorski (1972) who found the

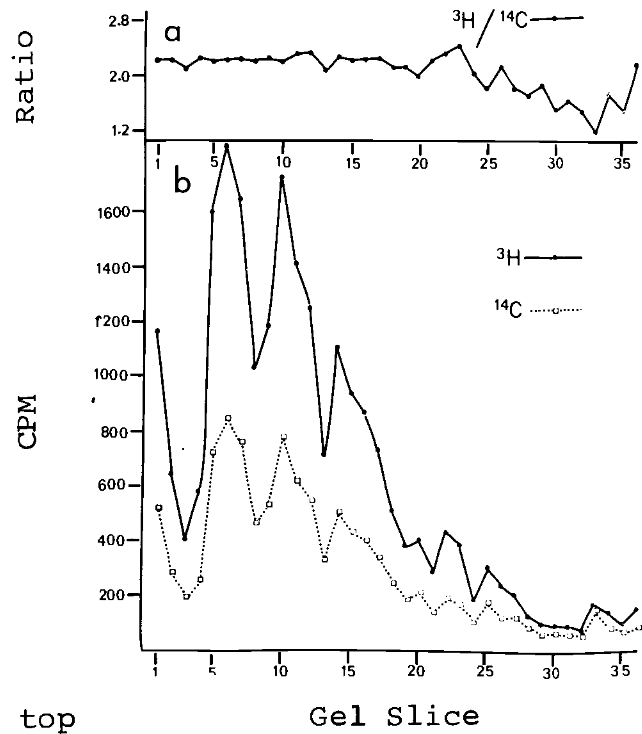

Figure 5. Incorporation of ^{3}H L-Leucine (estradiol [E_2]-treated in vitro, 2.5 x 10^{-3} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of an estrus (day 0) ewe. Treated and control uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^{3}H-^{14}C incorporation.
b. Total ^{3}H and ^{14}C incorporation.

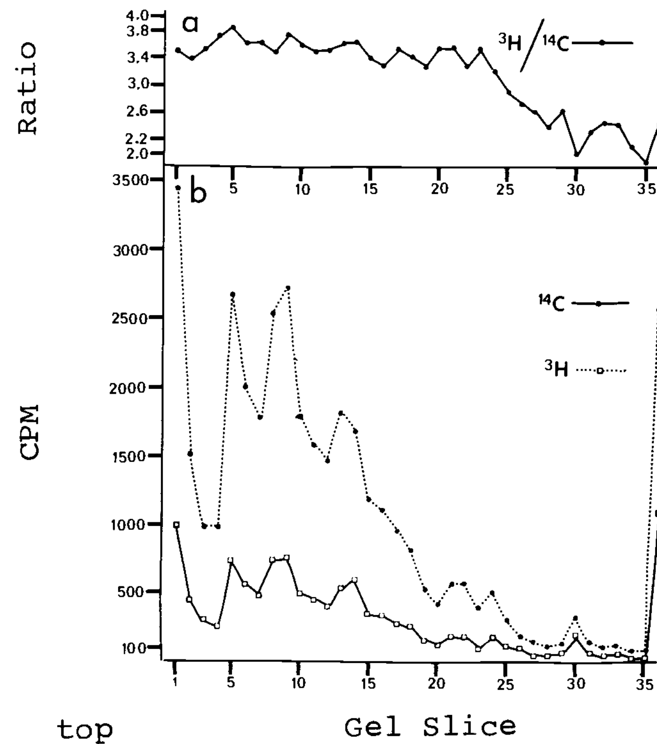

Figure 6. Incorporation of ^{3}H L-Leucine (estradiol [E_2]-treated in vitro; 2.5 x 10^{-3} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of a day 3 ewe. Uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^{3}H-^{14}C incorporation.
b. Total ^{3}H and ^{14}C incorporation.

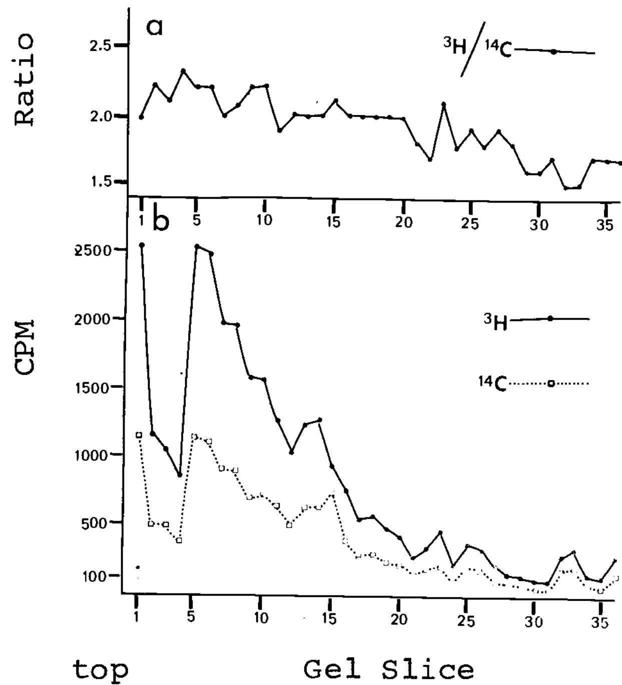

Figure 7. Incorporation of ^3H L-Leucine (estradiol [E_2]-treated in vitro; 2.5 x 10^{-3} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of a day 6 ewe. Treated and control uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^3H-^{14}C incorporation.
b. Total ^3H and ^{14}C incorporation.

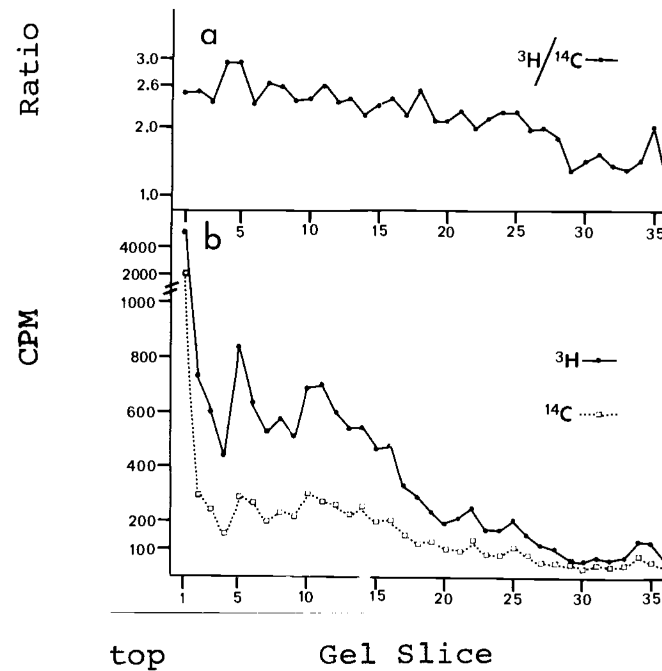

Figure 8. Incorporation of ^3H L-Leucine (estradiol [E_2]-treated in vitro; 2.5 x 10^{-3} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of a day 10 ewe. Treated and control uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^3H-^{14}C incorporation.
b. Total ^3H and ^{14}C incorporation.

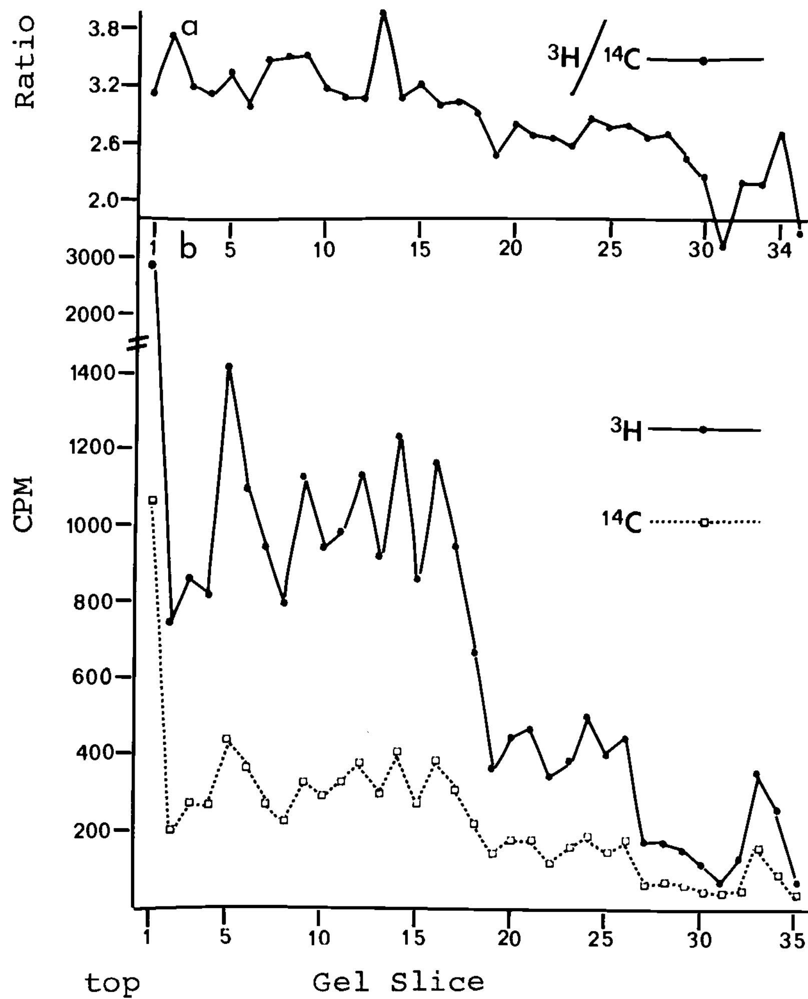

Figure 9. Incorporation of ^3H L-Leucine (estradiol [E_2]-treated *in vitro*; 2.5 x 10^{-8} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins from a day 15 nonpregnant ewe. Treated and control uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^3H-^{14}C incorporation.
b. Total ^3H and ^{14}C incorporation.

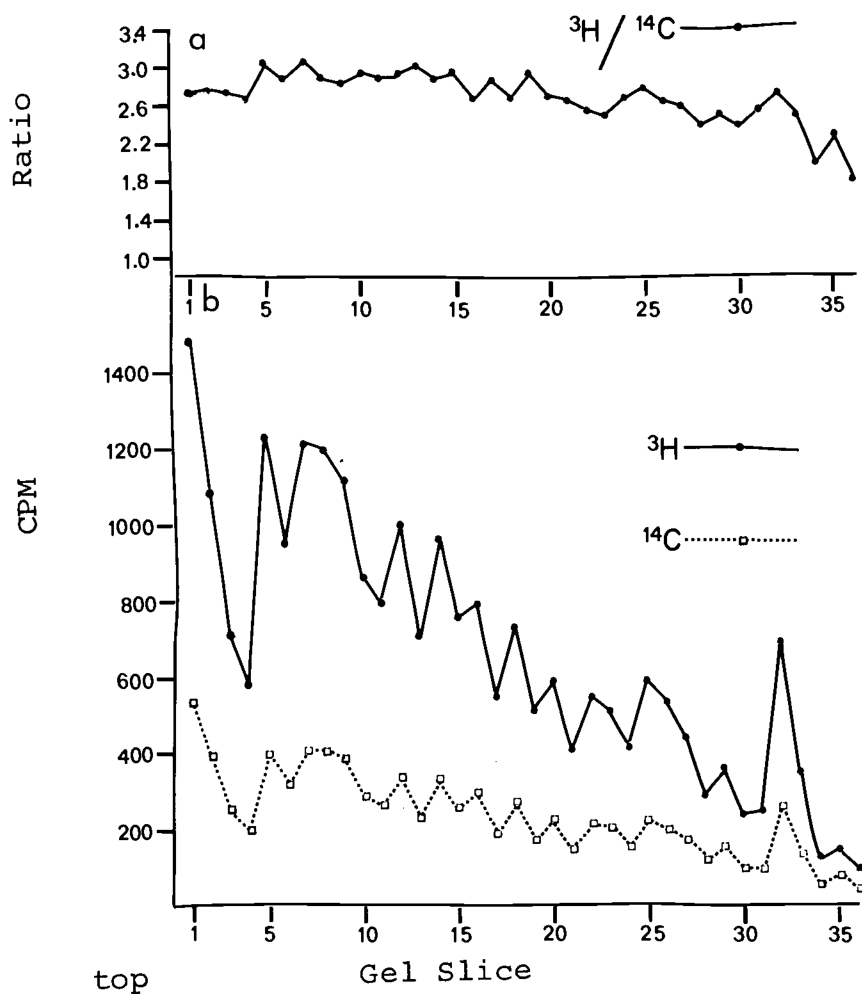

Figure 10. Incorporation of ^{3}H L-Leucine (estradiol [E_2]-treated <u>in vitro</u>; 2.5 x 10^{-8} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins from a day 15 pregnant ewe. Treated and control uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^{3}H-^{14}C incorporation.

b. Total ^{3}H and ^{14}C incorporation.

optimal in vitro estradiol concentration to be 2 to 3 x 10^{-8} M. They observed a decrease in rat uterine responsiveness when the estradiol concentration was increased (2.5 x 10^{-5} M gave 68% of the maximal response. An adjustment in the estradiol concentration resulted in incubation of endometrium with 2.5 x 10^{-8} M (13.6 ng/2 ml Eagles HeLa). However, endometrial segments from a day 15 nonpregnant ewe (Figure 10) did not elicit IP synthesis when incubated with the corrected concentration of estradiol (2.5 x 10^{-8} M).

Similar to the experiment involving cycling ewes, induced protein was not detected in uteri of 16 ovariectomized ewes receiving exogenous steroids and then incubated in vitro with estradiol-17β (2.5 x 10^{-8} M). No prealbumin change in the $^{3}H/^{14}C$ ratio was observed for uterine proteins in the control, estradiol, progesterone or the estradiol plus progesterone ewes (Figure 11 through 14, respectively). However, increased incorporation of both isotopes, occurring in front of the tracker dye, was detected in two of four ewes in the control (Figure 11) and estradiol-treated ewes (Figure 12). This increased isotope incorporation also was detected in all ewes receiving progesterone (Figure 13) but was not present in ewes receiving both estradiol and progesterone (Figure 14). The increased isotope incorporation is illustrated in part b of Figures 11, 12 and 13 by the clearly defined peak occurring between gel slices 30 to 35. However, in vitro estradiol stimulation of ovine uteri did not affect

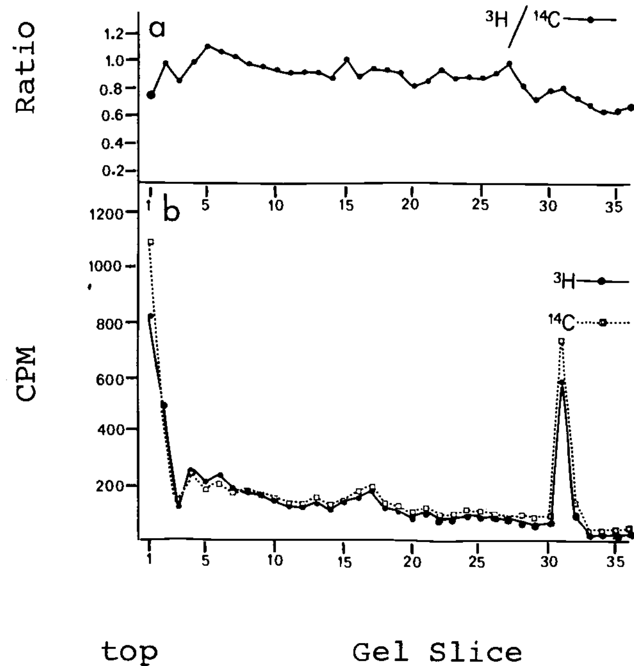

Figure 11. Incorporation of ^{3}H L-Leucine (estradiol [E_2]-treated in vitro; 2.5 x 10^{-8} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of an ovariectomized ewe receiving no exogenous steroids (control). Uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^{3}H-^{14}C incorporation.
b. Total ^{3}H and ^{14}C incorporation.

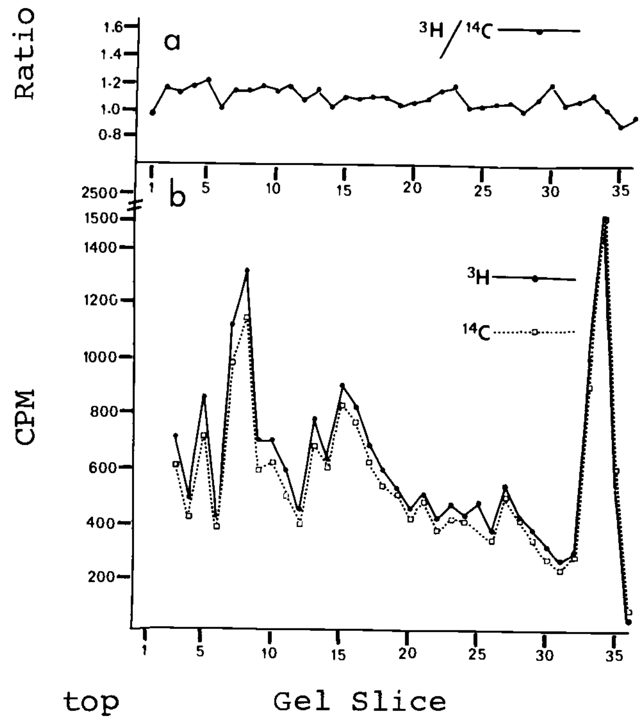

Figure 12. Incorporation of ^{3}H L-Leucine (estradiol [E_2]-treated in vitro; 2.5 x 10^{-8} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of an ovariectomized ewe receiving an estradiol implant and vehicle injections. Uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^{3}H-^{14}C incorporation.
b. Total ^{3}H and ^{14}C incorporation.

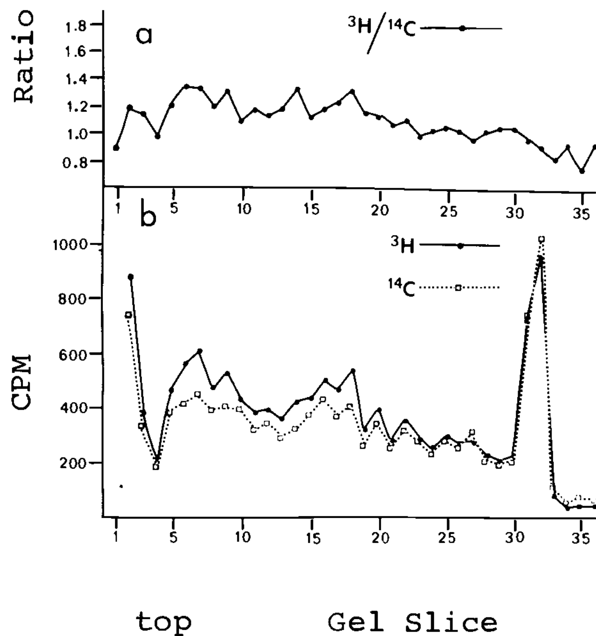

Figure 13. Incorporation of ^{3}H L-Leucine (estradiol [E_2]-treated in vitro; 2.5 x 10^{-8} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of an ovariectomized ewe receiving progesterone injections. Treated and control uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^{3}H-^{14}C incorporation.
b. Total ^{3}H and ^{14}C incorporation.

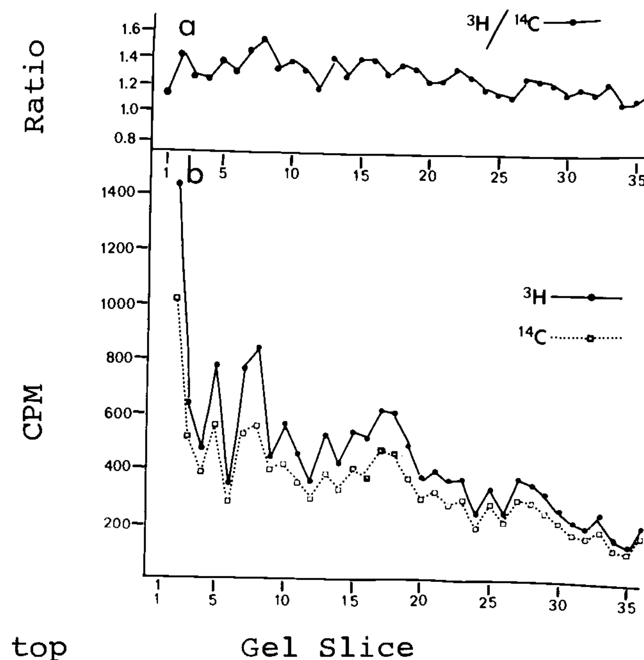

Figure 14. Incorporation of ^{3}H L-Leucine (estradiol [E_2]-treated in vitro; 2.5 x 10^{-8} M, 1 hr) and ^{14}C L-Leucine (control) into uterine proteins of an ovariectomized ewe receiving an estradiol implant plus progesterone injections. Uteri were homogenized together and the supernatant subjected to disc gel electrophoresis.

a. Ratio of ^{3}H-^{14}C incorporation.
b. Total ^{3}H and ^{14}C incorporation.

the incorporation of ^{3}H-L-Leucine in the pre-tracker dye region of the gel as the ratio was unchanged. The $^3H/^{14}C$ ratios obtained for individual intact and ovariectomized ewes are recorded in the Appendix (Table A and B, respectively).

The presence of IP in the rat is detectable during the stage of the estrous cycle when estrogen levels are elevated. This occurs at proestrus; estradiol levels decrease at estrus (Brown-Grant et al., 1970). Induced-protein synthesis by rat uteri was maximal at proestrus, non-detectable during estrus and metestrus with increased IP synthesized during diestrus (Katzenellenbogen and Gorski, 1975). Uteri from ovariectomized rats also have the capacity to synthesize IP when stimulated in vivo with estradiol-17β (Katzenellenbogen and Gorski, 1975).

Therefore, IP synthesis by ovine endometrium would be expected to correlated with the fluctuating levels of estradiol during the ovine estrous cycle. Peak ovine estrogen levels (13.3 ± 0.7 pg/ml) have been reported to occur on the day of estrus (day 0; Yuthasastrakosol et al., 1975). As expected, peak ovarian vein estradiol levels occurred 20 to 30 hr prior to the onset of estrus and rapidly decreased, reaching non-detectable levels 24 hr following the onset of estrus (Moore et al., 1969). This indicates that IP synthesis should occur during proestrus and estrus.

Ovine serum estradiol-17β levels (2.1 ± 1.3 pg/ml), determined on the day of estrus by our lab, were lower than the peripheral serum levels reported by Yuthasastrakosol et al. (1975) for unknown reasons. Estradiol levels in ovariectomized ewes receiving a sc estradiol implant were 5.92 ± 1.89 pg/ml compared to 3.72 ± 0.71 pg/ml in ewes receiving an empty implant; both are substantially lower than those reported by Yuthasastrakosol et al. (1975).

Ovine serum progesterone levels from the cycling ewes on days 0 and 10 were .76 ± .40 ng/ml and 1.52 ± 0.09 ng/ml, respectively. Yuthasastrakosol et al. (1975) reported serum progesterone levels of .45 ± .09 ng/ml and 5.67 ± .94 ng/ml on days 0 and 12 of the estrous cycle, respectively. Serum progesterone levels in the ovariectomized ewes receiving progesterone injections (two-15 mg doses at 12 hr intervals) were 55.77 ± 17.12 ng/ml compared to 1.30 ± .10 ng/ml in the vehicle-treated ewes.

No substantial change in the prealbumin non-specific protein patterns was observed for ovine uterine supernatants on days 0, 3, 6, or 10 of the estrous cycle. Staining patterns (Amido Black 10B) from day 3 and day 10 ewes revealed 1 and 2 prealbumin bands, respectively, while prealbumin protein profiles of uteri from ewes on day 0 and day 6 showed 1 or 2 bands (Table 7). Furthermore, the prealbumin protein profiles did not differ between the day 15 pregnant and day 14 or day 15 nonpregnant ewes; staining with Amido Black 10B

revealed 2 bands for both (Table 7).

Table 7. Prealbumin protein patterns of uterine supernatant from cycling and pregnant ewes stained with different dyes following disc gel electrophoresis.

Treatment Group	Ewe No.	Protein Dye		
		Buffalo Black	Amido Black 10B	Coomassie Blue G
		- - - number of bands detected - - -		
Day 0	1	-[a]	2	-
	2	-	2	-
	3	-	1	2
Day 3	4	1	1	-
	5	-	1	-
	6	1	1	4
Day 6	7	-	2	-
	8	1	1	-
	9	-	2	-
Day 10	10	1	2	-
	11	-	2	-
	12	-	2	-
Day 14 and 15 non-pregnant	13	-	2	5
	14	-	2	7
	15	-	2	-
Day 15 pregnant	16	-	2	-
	17	-	-	3
	18	-	2	3

[a]Electrophoretic gels were not stained with these dyes.

The number of prealbumin bands observed for uteri of the ovariectomized ewes receiving exogenous steroids (control, estradiol, progesterone or estradiol plus progesterone) varied from 0 to 4 within treatment groups (Table 8). Difficulty in interpreting the

Table 8. Prealbumin protein patterns of uterine supernatant from the ovariectomized ewes stained with various dyes following disc gel electrophoresis.

Treatment Group	Ewe No.	Protein Dye: Buffalo Black NBR	Amido Black 10B	Coomassie Brilliant Blue G	Aniline Blue
		- - - - number of bands detected - - - -			
Control	1	-[a]	1	-	0
	2	0	0	-	1
	3	0	0	-	1
	4	0	-	3	2
E_2[b]	5	0	2	-	1
	6	0	2	-	1
	7	0	-	5	0
	8	1	-	-	0
P_4[c]	9	0	-	-	0
	10	1	-	-	0
	11	0	1	-	-
	12	4	2	-	0
$E_2 + P_4$	13	0	1	-	1
	14	0	0	-	1
	15	1	1	5	1
	16	1	-	5	1

[a]Electrophoretic gels were not stained with these dyes.

[b]Estradiol-17β.

[c]Progesterone.

results occurred since the number of stained bands varied within and between replicate electrophoresis of the same uterine supernatant. Variability in the number of bands detected also was affected by the different dyes used. The low repeatability also may be due to the different degrees (heavy, medium or faint) in which the protein bands stained, resulting in non-detection of the faint staining bands in some instances. Likewise, the degree of background staining may have interfered with consistent detection of protein bands.

Contrary to Katzenellenbogen and Gorski (1975), results from the staining procedures with various dyes showed that Coomassie Brilliant Blue G was the most sensitive, as noted by the greatest number of protein bands detected for both the intact and ovariectomized ewe experiments (Table 7 and 8, respectively). The sensitivity also was enhanced by a reduced background staining of the gel when compared with Amido Black 10B, Buffalo Black NBR or Aniline Blue. Unfortunately, only a few gels were stained with Coomassie Brialliant Blue G before the supernatants were depleted. However, Coomassie Brilliant Blue G would appear to be the preferred dye.

The possibility exists that the mechanism of action of estradiol relating to early protein synthesis is species specific. This would imply that the induced-protein, as characterized in the rat, is not synthesized by the ovine uteri. Species specific proteins in response to various hormones have been isolated in rabbits and chickens. The

pregnant rabbit endometrium synthesizes uteroglobin (Beier and Hellwig, 1973) in response to progesterone. Uteroglobin has not been detected in human uterine fluid (Beier, 1974). Avidin synthesis is specific for the chicken oviduct stimulated with progesterone following pretreatment with estradiol (Korenman and O'Malley, 1968). Ovalbumin is a specific protein also synthesized in the estrogen-stimulated chick oviduct (Chan _et al._, 1973).

However, studies in our laboratory indicated that the results we achieved in the rat (trial 2, Figures 3 and 4) were not as pronounced as those obtained by Katzenellenbogen and Gorski (1972). In their investigations, the $^{3}H/^{14}C$ ratio obtained from rat uteri stimulated with _in vitro_ estradiol was approximately 6.0 for the IP peak, whereas adjacent ratios were between 2.8 and 3.3. The IP peak ratio ($^{3}H/^{14}C$) was 1.74 as calculated from our study, with adjacent ratios between 1.2 and 1.52 (trial 2, Figure 4). Thus, our procedure (adapted from Katzenellenbogen and Gorski, 1975) to either induce and/or detect IP synthesis in rat uteri was not as sensitive as procedures of their laboratories. This decreased sensitivity would affect the ability of the procedure to determine if the ovine endometrium was capable of producing the estradiol-induced protein. While Katzenellenbogen and Gorski (1975) used larger electrophoretic gels (9 x .7 cm) than we (7.5 x .5 cm), Mayol and Thayer (1970) indicated that staining intensity decreased as gel length increased

from 6 to 12 cm.

Several problems were encountered with the procedure throughout the course of the two experiments and the attempts to modify them are discussed.

The procedure was modified to allow for a more concentrated protein supernatant during the homogenization step of ovine uteri. This was accomplished by reducing the volume of 0.05% Na_2EDTA in which the uteri were homogenized from 1 ml to 0.5 ml (J. Gorski, Univ. of Wisconsin; personal communication).

Diffusion of the tracker dye front (5 µl of 0.05% Bromphenol blue) during electrophoresis resulted in a poor estimation of the termination of the run. The tracker dye front was sometimes diffused over an area of 0.47 cm ± 0.05 (N = 9) to 0.90 ± 0.05 cm (N = 10). Variability also was sometimes noted in the running time between various gels within a run and between separate electrophoretic runs. This may have resulted from slight variation in the pore size among gels which is affected by the proportion of acrylamide and bisacrylamide (Brackenridge and Bachelard, 1969).

The solutions of acrylamide and bis were found to have a shorter refrigeration life than the several months as reported by Davis (1964). Our acrylamide-bis solution was prepared and used for approximately one month. Clearer gels were obtained with freshly prepared stock solutions when compared with gels from refrigerated

solutions. Therefore, it was suggested that the acrylamide-bis solution be prepared prior to each electrophoresis or stored no longer than 1 week (M. Montgomery, Oregon State Univ.; personal communication). To reduce the number of stock solutions and to increase consistency within gel matrix, the premix, Cyanogum is recommended.

Therefore, further investigation needs to be conducted to determine for certain if ovine endometrium does or does not synthesize an induced protein as an early response to estradiol.

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APPENDIX

Table A. Treated/control ratios ($^{3}H/^{14}C$) per gel slice for the intact cycling and pregnant ewes.

Treatment		Gel Slice											
		1	2	3	4	5	6	7	8	9	10	11	12
Day 0	#1	0.29	0.28	0.26	0.24	0.24	0.25	0.26	0.24	0.23	0.26	0.26	0.26
	2	3.35	3.71	3.82	4.16	4.12	4.18	3.94	3.68	3.97	3.72	3.82	3.88
	3	2.46	2.43	2.32	2.45	2.46	2.41	2.37	2.41	2.46	2.46	2.52	2.49
Day 3	#4	0.61	0.47	0.42	0.40	0.22	0.21	0.25	0.21	0.21	0.18	0.20	0.25
	5	2.87	2.84	3.02	3.05	3.00	2.86	2.70	2.89	2.92	3.80	2.81	2.88
	6	3.81	3.92	3.81	4.02	4.14	3.95	3.96	3.86	4.07	3.92	3.79	3.85
Day 6	#7	0.46	0.40	0.36	0.33	0.29	0.25	0.31	0.29	0.27	0.26	0.24	0.17
	8	0.41	0.36	0.33	0.29	0.25	0.25	0.26	0.18	0.23	0.24	0.27	0.19
	9	2.22	2.46	2.35	2.48	2.40	2.44	2.25	2.31	2.44	2.42	2.14	2.22
Day 10	#10	0.33	0.29	0.29	0.29	0.28	0.31	0.31	0.32	0.31	0.31	0.28	0.30
	11	3.34	3.77	3.79	4.83	4.09	4.00	3.72	4.07	3.84	3.76	3.80	3.91
	12	3.12	2.96	3.04	3.16	3.09	3.28	3.09	3.01	3.01	3.22	2.99	3.05
Day 15	#13	2.51	2.82	2.78	2.80	3.12	2.97	2.94	2.94	2.90	3.01	2.90	2.85
Non	14	0.70	3.01	3.19	2.68	3.47	3.37	3.40	3.26	3.28	3.22	3.32	3.40
Pg[a]	15	3.14	3 68	3.16	3.10	3.28	3.01	3.45	3.52	3.46	3 21	3.06	3.05
Day 15	#16	1.22	1.05	0.90		1.18	1.73	1.71	1.63	1.23	1.36	1.52	1.69
Pg	17	1.93	2.08	2.15	2.29	2.52	2.22	2.24	2.23	2.48	2.22	2.31	2.18
	18	2.70	2.80	2.70	2.70	3.00	2.90	3.04	2.89	2.84	2.92	2.91	2.91

Table A. Continued.

Treatment		Gel Slice											
		13	14	15	16	17	18	19	20	21	22	23	24
Day 0	#1	0.24	0.28	0.27	0.31	0.30	0.29	0.26	0.29	0.31	0.31	0.27	0.49
	2	3.46	3.86	3.66	3.63	3.44	3.40	3.47	3.34	3.35	3.37	3.27	2.97
	3	2.36	2.46	2.44	2.40	2.39	2.33	2.31	2.18	2.45	2.53	2.66	2.17
Day 3	#4	0.27	0.26	0.22	0.23	0.23	0.29	0.27	0.36	0.36	0.42	0.29	0.33
	5	2.98	3.42	2.67	2.82	2.57	2.61	2.65	2.84	2.49	2.48	2.68	2.31
	6	3.97	3.92	3.77	3.67	3.80	3.76	3.67	3.85	3.78	3.61	3.82	3.48
Day 6	#7	0.14	0.16	0.13	0.14	0.17	0.20	0.20	0.22	0.21	0.38	0.34	0.29
	8	0.17	0.21	0.17	0.19	0.18	0.24	0.24	0.30	0.23	0.26	0.22	0.29
	9	2.18	2.21	2.35	2.17	2.18	2.24	2.17	2.08	1.98	1.89	2.35	2.00
Day 10	#10	0.32	0.32	0.31	0.30	0.33	0.33	0.34	0.34	0.34	0.33	0.39	0.34
	11	3.86	4.19	3.70	3.80	3.69	3.77	3.71	3.62	3.70	3.91	3.50	3.34
	12	3.22	3.09	3.00	2.98	2.82	3.28	2.89	2.85	2.74	3.02	2.59	2.51
Day 15	#13	3.00	3.00	3.00	2.99	2.89	2.83	2.68	2.76	2.75	2.56	2.52	2.24
Non	14	3.31	3.21	3.51	3.32	3.05	3.10	3.00	2.90	2.63	2.49	2.50	2.53
Pg[a]	15	4.02	3.14	3.20	3.05	3.04	2.98	2.54	2.81	2.72	2.67	2.59	2.86
Day 15	#16	2.06	1.97	1.96	2.13	1.90	2.13	2.21	2.12	1.99	2.66	2.42	2.42
Pg	17	2.19	2.25	2.05	1.98	2.11	1.82	1.86	1.62	1.78	1.85	1.74	1.70
	18	2.97	2.88	2.92	2.74	2.76	2.74	2.89	2.69	2.65	2.59	2.54	2.65

Table A. Continued.

Treatment		Gel Slice											
		25	26	27	28	29	30	31	32	33	34	35	36
Day 0	#1	0.45	0.45	0.53	0.54	0.47	0.46	0.59	0.67	0.57	0.63	0.53	0.35
	2	2.82	3.34	3.00	2.40	2.64	2.70	2.59	2.59	2.33	3.35	2.39	2.32
	3	2.03	2.32	1.97	1.88	2.00	1.62	1.07	1.63	1.35	1.80	1.57	2.15
Day 3	#4	0.28	0.31	0.54	0.53	0.67	0.67	0.67	0.38	0.72	0.60	0.63	0.63
	5	2.02	1.62	1.59	1.48	1.30	1.37	1.37	1.58	1.57	1.38	2.27	1.87
	6	3.20	2.98	2.85	2.67	2.51	2.16	2.57	2.57	2.64	2.34	2.11	2.60
Day 6	#7	0.23	0.17	0.31	0.38	0.44	0.51	0.43	0.37	0.59	0.51	0.56	0.60
	8	0.24	0.32	0.35	0.45	0.49	0.63	0.53	0.38				0.90
	9	2.07	1.98	2.06	1.93	1.78	1.71	1.83	1.63	1.65	1.88	1.90	1.84
Day 10	#10	0.35	0.36	0.38	0.44	0.38	0.20	0.42	0.47	0.51	0.46	0.42	0.59
	11	3.38	3.22	3.04	2.99	3.04	2.86	2.63	2.46	2.34	2.46	2.92	
	12	2.70	2.76	2.59	2.37	2.53	2.23	2.12	2.05	1.96	2.04	1.96	2.21
Day 15	#13	2.49	2.43	2.53	2.58	2.31	2.14	2.08	1.88	2.08	1.98	1.36	1.76
Non	14	2.60	2.54	2.60	2.62	2.43	1.98	1.90	1.95	2.47	1.89	2.03	1.75
Pg[a]	15	2.79	2.77	2.69	2.73	2.45	2.33	1.58	2.21	2.17	2.73	1.73	
Day 15	#16	2.99	2.78	2.52	2.64	2.67	2.58	2.50	2.45	3.12	2.46	2.51	2.67
Pg	17	1.82	1.42	1.50	1.41	1.82	1.42	1.21	1.00	0.83	0.86		1.10
	18	2.77	2.65	2.58	2.41	2.45	2.39	2.56	2.73	2.52	2.05	2.24	1.86

[a]Pregnant.

Table B. Treated/control ratios ($^3H/^{14}C$) per gel slice for the steroid treated-ovariectomized ewes.

Treatment		1	2	3	4	5	6	7	8	9	10	11	12
Control	#1	4.60	4.60	5.00	4.90	7.50	4.90	4.70	4.90	4.80	5.00	5.00	4.80
	2	0.72	0.80	0.81	0.74	0.85	0.75	0.78	0.84	0.85	0.84	0.83	0.83
	3	1.08	1.04	1.08	0.87	0.99	0.99	1.11	1.14	1.03	1.09	1.08	1.02
	4	0.74	0.97	0.84	0.97	1.09	1.05	1.02	0.96	0.95	0.92	0.90	0.91
E_2 [a]	#5	1.82	1.80	1.86	1.76	1.97	1.97	1.82	1.91	1.87	1.79	1.88	1.57
	6	0.79	0.72	0.72	0.71	0.73	0.76	0.73	0.72	0.74	0.68	0.72	0.73
	7	0.85	0.88	1.01	0.99	0.92	0.97	1.31	1.05	0.99	0.98	0.87	0.97
	8	0.98	1.15	1.13	1.16	1.20	1.02	1.13	1.13	1.17	1.15	1.17	1.09
P_4 [b]	# 9	1.17	1.13	1.06	1.14	1.21	1.16	1.16	1.06	1.19	1.11	1.07	1.19
	10	1.35	1.32	1.27	1.46	1.28	1.43	1.42	1.38	1.59	1.31	1.25	1.35
	11	0.88	1.18	1.14	0.98	1.21	1.35	1.33	1.19	1.30	1.11	1.17	1.13
	12	0.82	0.97	1.12	1.18	1.17	1.16	1.09	1.07	1.13	1.11	1.17	1.18
$E_2 + P_4$ [c]	#13	0.92	0.99	0.87	0.92	1.05	1.01	0.92	0.98	0.95	0.85	1.22	1.51
	14	1.20	1.47	1.40	1.37	1.49	1.30	1.41	1.34	1.40	1.44	1.25	1.35
	15	1.10	1.14	1.12	1.16	1.09	1.12	1.10	1.09	1.11	1.06	1.03	1.08
	16	1.12	1.40	1.25	1.24	1.37	1.28	1.44	1.53	1.31	1.37	1.31	1.17

Table B. Continued.

Treatment		Gel Slices											
		13	14	15	16	17	18	19	20	21	22	23	24
Control	#1	5.00	4.70	4.50	4.60	4.50	4.50	4.40	4.40	4.40	4.30	4.50	4.40
	2	0.81	0.84	0.84	0.83	0.82	0.88	0.83	0.84	0.67	0.81	0.72	0.72
	3	1.04	1.09	1.05	1.00	1.05	1.02	1.09	0.98	1.05	0.97	1.06	1.09
	4	0.90	0.87	1.00	0.87	0.94	0.92	0.90	0.80	0.84	0.92	0.86	0.87
E_2 [a]	#5	1.74	1.83	1.60	1.58	1.59	1.49	1.51	1.63	1.21	1.27	1.13	1.18
	6	0.78	0.72	0.75	0.67	0.67	0.77	0.82	0.75	0.76	0.76	0.62	0.70
	7	0.91	0.99	0.91	0.94	0.94	1.02	0.87	0.96	0.92	1.00	0.88	0.92
	8	1.14	1.03	1.09	1.08	1.09	1.09	1.05	1.06	1.09	1.14	1.16	1.03
P_4 [b]	# 9	1.03	1.09	1.00	1.07	0.99	1.11	1.11	1.06	1.04	0.97	1.02	1.11
	10	1.42	1.38	1.36	1.31	1.22	1.28	1.25	1.36	1.25	1.31	1.32	1.23
	11	1.18	1.32	1.13	1.17	1.24	1.31	1.17	1.13	1.08	1.09	1.01	1.02
	12	1.07	1.13	1.10	1.07	1.10	1.16	0.95	1.06	1.07	1.01	0.99	1.11
$E_2 + P_4$ [c]	#13	0.97	0.86	0.84	0.93	0.93	0.79	0.86	0.73	0.82	0.82	0.71	0.71
	14	1.35	1.25	1.24	1.27	1.18	1.27	1.19	1.08	1.07	1.19	1.07	1.05
	15	1.01	1.07	1.07	1.05	1.04	1.04	1.06	1.04	1.00	1.05	0.97	1.01
	16	1.38	1.27	1.38	1.39	1.28	1.34	1.31	1.22	1.24	1.32	1.27	1.19

Table B. Continued.

Treatment		Gel Slices											
		25	26	27	28	29	30	31	32	33	34	35	36
Control	#1	4.30	4.00	3.60	3.90	3.80	3.90	3.70	3.50	3.50	3.40	3.40	3.50
	2	0.70	0.67	0.64	0.73	0.80	0.86	0.92	0.93	0.88	0.91	0.85	0.95
	3	1.02	0.98	1.02	1.07	0.92	0.83	0.92	0.99	0.90	0.92	0.90	0.85
	4	0.86	0.89	0.97	0.80	0.71	0.77	0.79	0.71	0.67	0.62	0.62	0.66
E_2[a]	#5	1.13	1.25	1.34	1.11	1.26	1.17	1.19	1.00	1.22	0.90	1.25	1.18
	6	0.74	0.78	0.61	0.65	0.82	0.80	0.89	0.91	0.74	0.85	0.74	
	7	0.83	0.93	0.91	0.83	0.89	0.79	0.79	0.85	0.82	0.90	0.80	0.78
	8	1.03	1.04	1.05	1.01	1.08	1.18	1.06	1.07	1.11	1.02	0.92	0.94
P_4[b]	# 9	1.11	1.16	1.10	1.19	1.06	1.03	1.00	1.10	0.88	1.00	0.95	0.83
	10	1.22	1.39	1.24	1.22	1.20	1.17	1.09	1.30	1.06	1.37	1.14	1.07
	11	1.05	1.04	0.96	1.02	1.05	1.04	0.98	0.92	0.84	0.91	0.77	0.96
	12	1.08	1.05	1.09	1.04	1.03	1.02	1.04	0.82	0.81	0.89	0.77	0.95
$E_2 + P_4$[c]	#13	0.69	0.68	0.71	0.68	0.71	0.93	0.81	0.90	0.86	0.85	0.86	0.81
	14	1.03	0.89	1.00	1.02	0.92	0.95	1.06	1.17	1.06	1.01	1.10	1.14
	15	0.90	0.88	0.98	0.95	0.96	1.05	1.03	1.04	0.92	0.98	1.02	1.05
	16	1.15	1.14	1.27	1.25	1.23	1.16	1.19	1.17	1.24	1.10	1.11	1.17

[a]Estradiol-17β.

[b]Progesterone.

[c]Estradiol-17 β plus progesterone.