THE EFFECT OF ESTROGENIC ALFALFA CONSUMPTION IN CYCLIC EWES: THE PLASMA GONADOTROPIN PROFILE THROUGHOUT THE ESTROUS CYCLE

by

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ABSTRACT

Phyto-estrogens are known to cause reproductive failures in the ewe by interacting with uterine estrogen receptors. Further studies have demonstrated anomalous ovarian steroid levels which implicate the hypothalamus and pituitary. Estrogen receptors which bind phyto-estrogens have been recently demonstrated in ovine hypothalamic and pituitary tissues. It would then seem logical to assume that estrogen interactions in these tissues will be altered and result in abnormal gonadotropic synthesis or secretion.

This project employed vaginal cytological studies and ovine Luteinizing Hormone radioimmunoassays to characterize the plasma LH profiles and estrous cycles in ewes fed orchard grass hay or phyto-estrogenic alfalfa. Results indicate that the consumption of phyto-estrogenic activity will decrease the pulsatile nature of LH secretion and delay the LH peak further into the estrus period when compared with ewes fed a ration devoid of phyto-estrogenic activity. Vaginal cytological studies also demonstrate phyto-estrogenic activity through elevated karyopyknotic index values.
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INTRODUCTION

A normal and completely integrated hypothalamo-adenohypophysis-gonadal axis is crucial to the occurrence of a regular fertile estrous cycle.

The hypothalamus can control the adenohypophysis when it processes neural sensory inputs into releasing hormone(s) which are carried to the anterior pituitary through a portal vascular system. Extrahypothalamic areas, which include the pineal and limbic structures, also influence the incidence and refinement of these neural impulses.

The Releasing Hormone(s) stimulate the adenohypophysis to release gonadotropins (hence the name Gonadotropin Releasing Hormone GnRH) into the general circulation to induce ovarian follicular development with subsequent steroid biosynthesis, ovulation and corpus luteum formation. The ovarian steroids, in particular the estrogens and progestagens, then exert positive and negative feedback effects on the hypothalamus, higher centres within the CNS and the anterior pituitary. Through these structures ovarian steroids can modify the synthesis and secretion of gonadotropin.
The interplay between GnRH, the gonadotropins, i.e., Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), the estrogens and progesterone is acutely important. Therefore any intake of exogenous hormone which is of sufficient duration and magnitude will disrupt control of the ovarian cycle.

Such is the case when animals ingest "estrogenic" pastures containing principally coumestrol and genistein. These phyto-estrogens are capable of acting as endogenous estrogen agonists and antagonists depending upon the animal's prevailing estrogen blood profile. This is possible because plant estrogens approximate the structure of Estradiol-17β (E2-17β) and contain the critical functional groups. Hence they may bind and react with cytosol receptors in E2-17β target tissues.

Consumption of these compounds is associated with permanent or temporary loss in fertility which has been attributed principally to impaired sperm and ovum transport. However, abnormal folliculogenesis and steroidogenesis have been demonstrated in these animals consequently the hypothalamus and pituitary have been implicated.

The following text will discuss the estrous cycle in depth with the specific intention of identifying the possible sites where phyto-estrogens may affect the
hypothalamo-adenohipophysis-gonadal axis. Special considera-
tions will be developed for the hypothalamus and pituitary as it is hypothesized that gonadotropin output by the adenohipophysis will be modified by phyto-estrogen consumption. Experimental results will be utilized in an attempt to demonstrate this area of concern.
REVIEW OF LITERATURE

1. ESTROGENS AND ESTROGEN RECEPTORS

Naturally occurring estrogens are typically 18-carbon steroids which contain -OH and/or ketone functional groups at carbon atoms 3 and 17. Estrone, estradiol-17β (physiological responses are attributed to E₂-17β) and estriol predominate, however, estriol appears only when synthesized by a functional placenta.

Estradiol-17β is the major estrogen secreted by the theca interna of the ovary (Martin, 1976). However, recent and conclusive results in vitro (Dorrington et al., 1975; Moon et al., 1975; Moon et al., 1978) indicate that the thecal cells synthesize ovarian androgen which is then aromatized to estradiol-17β by the granulosa cells. This will be discussed in greater depth in the following sections.

Estrone and estradiol-17β are biologically inter-convertible but prevailing conditions in the circulatory system favor formation of estrone hence it is one of the major estrogens in blood plasma and along with E₂-17β appears conjugated either to sulfate or glucuronate. The free and conjugated
estrogens are transported to their target tissues by binding with high affinity to sex steroid-binding globulin (SSBG) more commonly known as sex steroid binding plasma protein (SBP), (Martin, 1976; Mercier-Bodard et al., 1977; Funder, 1978). Transportation also occurs when sex steroids bind to plasma albumins which have a low affinity for the steroids but demonstrate many reactive sites which results in a high capacity to bind and carry the circulating steroids.

Imperative to the concept of target tissue, cells must have the ability to recognize and distinguish between the circulating steroid hormones. The recognition mechanisms are achieved because target cells contain receptors that have the properties which facilitate the ability to bind specific hormones and as a consequence initiate a response through the cell's molecular apparatus.

By injecting tritiated estradiol-17β into immature female rats and determining the distribution of radioactivity Jensen et al. (1960, 1962) demonstrated that cells are capable of binding and concentrating specific steroids.

The accepted model for sex steroid-receptor interactions is the uterus. Through this model the mechanism of steroid action has been demonstrated (Gorski et al., 1968; Giannopoulos et al., 1971; Gorski et al., 1976; O'Malley et al., 1976).
Upon entering the uterine cell $E_2^{-17\beta}$ rapidly associates with an 8S binding protein in the cytosol. This hormone-receptor complex undergoes a conformational change and within 1 hour appears within the nuclear compartment (Stumpf, 1969; Giannopoulos et al., 1971; O'Malley et al., 1976). The estrogen now appears bound to a 4-5S component (Gorski et al., 1968) and associates with specific nonhistone proteins of the chromatin effecting transcriptional changes, mRNA formation and subsequent cellular metabolic responses (Means et al., 1972; Gorski et al., 1968; O'Malley and Schrader, 1976; Gorski and Gannon, 1976; Gorski et al., 1977).

Changes within the target cell induced by $E_2^{-17\beta}$ include increased water uptake and retention, increased nitrogen retention, increased RNA polymerase activity and biosynthesis of 'induced protein', an early indication of estrogen action (Katzenellenbogen et al., 1975a).

For a macromolecule to be considered a receptor it must meet several stringent criteria (Munck, 1976):

(i) The binding molecule must be present in the target cells.

(ii) Specificity of binding; the binding protein must have the ability to form complexes with molecules of complementary (and
obligatory) stereochemical configurations and be able to exclude inappropriate structures.

(iii) High affinity; the ability and strength with which a receptor will identify and bind a specific molecule. This principle also counteracts the low concentrations at which hormones are normally present.

(iv) The molecule must demonstrate a high affinity (association) constant ($K_A$) to its specific steroid hormone.

(v) The binding protein must exhibit finite or saturable binding. Non-specific binding represents low affinity, non-saturable binding.

Association of a steroid to its respective target cell receptor involves a combination of several physical properties. The principal interactions are hydrophobic and hydrogen bonding. It is these molecular forces that determine affinity and specificity between steroid-receptor molecules. Strict stereochemical configurations are inherent to the concept of specificity and affinity with the result steroid structural conformation is critical to proper
Table 1 Temporal sequence of metabolic events in uteri of ovariectomized rats after in vivo administration of estrogen.

<table>
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<th>TIME</th>
<th>METABOLIC RESPONSE</th>
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<tr>
<td>36 sec.</td>
<td>9.55 steroid-receptor complex</td>
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<tr>
<td>72 sec.</td>
<td>Translocation of steroid-receptor complex to nuclear compartment</td>
</tr>
<tr>
<td>360 sec.</td>
<td>Induced protein (I.P.) synthesis and nucleoplasmic polymerase II</td>
</tr>
<tr>
<td>1 hour</td>
<td>Nucleolar RNA polymerase I (I.P. synthesis), glucose metabolism</td>
</tr>
<tr>
<td>5 hours</td>
<td>Protein synthesis, water inhibition</td>
</tr>
<tr>
<td>1-020 hours</td>
<td>Histone, DNA, net RNA synthesis</td>
</tr>
<tr>
<td>30 hours</td>
<td>Mitosis</td>
</tr>
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</table>

From Gorski et al. (1969) and Kitts (1976).
Figure 1A: Hypothetical model for estrogen interaction with the uterine cell (Gorski et al. 1968).
Figure 1B: Hypothetical mechanism of gene action by the steroid hormones (O'Malley and Schrader, 1976)
specific binding and the subsequent cellular response (King and Mainwaring, 1974; Munck, 1976).

It appears that the greatest binding affinity is obtained if the natural steroid ring configuration has an unsaturated ring A, a phenolic hydroxyl group on C-3, alcoholic hydroxyl group on C-17 (Hahnel et al., 1973) and substitutions within ring D (Korenman, 1969). Maximum binding affinity between receptor and steroid is achieved with estradiol-17β, the most important feature is the phenolic hydroxyl group on C-3. If this group is missing or substituted the affinity towards the receptor is largely or entirely abolished. Further to this, ring A must be a benzene ring with the result the hydroxyl group must be phenolic and not alcoholic.

The second oxygen function on ring D is important. Its state of oxidation and position will influence the binding activity. Maximum binding occurs when the oxygen function is an alcoholic hydroxyl group on C-17 in the β-configuration (Korenman, 1969; Hahnel et al., 1973). Addition of further oxygen functions to ring D, additional substituents on ring A and/or unsaturation of ring B will decrease the affinity for receptor and prove inhibitory to binding, specifically -OH groups on C-11 or -methyl substitutions on C-2 and C-16.
The two non-steroidal estrogens, hexestrol and diethylstilbestrol (DES), had binding affinities comparable to estradiol-17β with binding considered to occur through the two phenolic hydroxyl groups (Hahnel et al. 1973). However, Korenman (1969) using a rabbit cytosol as opposed to human cytosol (Hahnel et al., 1973) found affinities for DES and 17β-ethynyl-estradiol-17β considerably stronger than E2-17β.

Binding is considered to involve attachment of small molecules (steroids-ligands) to receptors at two points inducing a conformational change in the receptor. The flexible active site of the receptor may consist of two parts, an attractive centre which determines the specificity and orientation of the ligand, and a second centre less specific than the first.

Obligatory to this concept, it has been demonstrated that there is a necessity for the ligand to have two attachment points. The optimum requirements for this two point interaction exist with estradiol-17β where the phenolic -OH group on C-3 and the C-17β -OH group are active binding centres (Hahnel et al., 1973).

Initially there is attachment between the C-3 phenolic hydroxy group and the primary binding centre. This induces a conformational change within the receptor
Figure 2: Structural configurations of natural and synthetic estrogens.
which facilitates the second attachment of the C-17β -OH function and the second less specific binding centre. For this process to occur, an additional requirement must be included. The distance between the two -OH groups on the estradiol-17β molecule is approximately 10.6 Å (Hahnel et al., 1973) and is a prerequisite for optimal binding. Considering the two non-steroidal estrogens, hexestrol is a flexible molecule and its two -OH functional groups can adapt to this distance. DES is a more rigid molecule due to the carbon-carbon double bond and can therefore only achieve a distance of 12.0 Å between functional groups.

2. ANTIESTROGENS

Sankaran and Prasad (1972) and Geynet et al. (1972) define antiestrogens as compounds which antagonize or interfere with any actions of estrogen, principally estradiol-17β. True antiestrogens demonstrate two properties: first, they exhibit competition with estradiol-17β for cytoplasmic receptor sites within target tissues (Geynet et al., 1972; Rochefort et al., 1972; Sankaran and Prasad, 1972; Jordan et al., 1978); secondly,
antiestrogens exhibit varying degrees of estrogenicity specified with reference to target tissue, route of administration, dose and time sequence of action (Sankaran and Prasad, 1972). Terenius and Ljungkvist (1972) demonstrated antiestrogen estrogenicity (i.e., uterotrophic effect) with positive Allen Doisy and mouse uterine growth tests.

Through their biphasic action, antiestrogens may also be considered agonistic to a lesser degree than E₂-17β as well as antagonistic to the uterotrophic effects of estradiol-17β. Depending upon the physiological state and endogenous estrogen content of the animal, antiestrogens at low concentration by themselves are able to cause varying estrogenic effects. However, as a critical concentration is reached they become antagonists and inhibit the uterotrophic response whether by themselves or in direct competition with estrogens (Geynet et al., 1972; Ruh and Ruh, 1974; Capony and Rochefort, 1975).

The exact sites or mechanism of action of the antiestrogens have not been elucidated, however based on work reported, it is logical to assume that the sites will parallel the uterotrophic responses of estradiol-17β (Geynet et al., 1972; Rochefort et al., 1972). These series are demonstrated in Figure 3 as a figure of steps:
1. Competition with estrogen to enter cell
2. Inhibitive binding to cytosol receptor
3. Abnormal translocation of receptor-steroid complex into the nucleus
4. Inhibition of transformation into the nuclear receptor complex
5. Atypical interactions with the nuclear acceptor
6. Incomplete or abnormal receptor recycling or resynthesis, RNA synthesis and subsequent induced protein synthesis.

Figure 3 Proposed sites of antiestrogen action (Rochefort et al. 1972)
Step 1. Competition to enter the cell.
2. Inhibitive binding to cytosol receptor.
3. Abnormal translocation of receptor complex to the nucleus.
4. Inhibition of transformation to nuclear-receptor.
5. Atypical interactions with nuclear acceptor.
6. Incomplete or abnormal RNA synthesis and subsequent induced protein synthesis.

The major non-steroid antiestrogens used to determine the extent and sites of interference are: chlomiphene (cis and trans), nitromophene citrate (CI-628), nafoxidine (U-11,100A), tamoxifen (ICI-46,474), ethamoxypiphetol (MER 25) and to a small extent dimethylstilbestrol (see Figure 4).

Two types of antiestrogens can be distinguished based upon their binding affinities and ability to effect a uterotrophic response (Korenman, 1969; Rochefort et al., 1972).

(1) Compounds which inhibit the uptake of $E_2^{17\beta}$ in uteri and/or binding to its receptor. The relative affinity of these antiestrogens for receptor is much higher than their relative uterotrophic response.
Figure 4: Structural formula of several non-steroidal antiestrogens.
FIGURE 4 (cont’d)

Nitromophene Citrate (CI-628)

Ethamoxytriphetol (MER-25)

Dimethystilbestrol (DMS)
Consequently, the biological action is blocked in at least one of the above steps. Examples of this type of antiestrogen are: nafoxidine, tamoxifen and clomiphene.

(2) Antiestrogens of this type exhibit no affinity for the estrogen cytosol receptor which suggest a different mechanism of action. Examples are progesterone and testosterone.

Important to this discussion are the antiestrogens described in type (1). These compounds can be further categorized (see Table 2) based on their proposed mechanism of action and time sequence (Terenius and Ljungkvist, 1972; Ruh and Ruh, 1974; Jordan et al., 1978):

Type 1 (a): Long Acting Antiestrogens

The triphenylethylene estrogen derivatives nafoxidine, tamoxifen, clomiphene and CI 628 have been utilized extensively to elucidate the mechanism of action. These compounds occur in this category due to their effect of increasing the nuclear retention time of the steroid-receptor complex. Tamoxifen has been shown to maintain a consistently elevated concentration of estrogen receptor within the nucleus up to 48 hours, whereas the initial rise in nuclear accumulation produced by estrogen returns to control levels within 24 hours.
Another property of these compounds is their prolonged plasma biological half-life \( (t_\frac{1}{2}) \). In the rat, \( E_2-17\beta \) exhibits a biological half-life of a few hours while tamoxifen is maintained for several days (Jordan et al., 1978). Carroll and Cox (1972) have reported the \( t_\frac{1}{2} \) for \( E_2-17\beta \) in the ewe as 18-22 minutes. Even though estrogen receptor is resynthesized and recycled the elevated levels of antiestrogen, due to their prolonged biological half-life, will continually bind and translocate the receptor to the nuclear compartment (Baudendistel et al., 1978). The target tissue then becomes depleted in cytosol receptor and progressively refractory to \( E_2-17\beta \) stimulation.

Type 1 (b): Short Acting Antiestrogens or 'Impeded' Estrogens.

Compounds such as estriol and DMS are termed short acting or 'impeded' estrogens, even though they have relatively high affinity for receptors, because they are rapidly lost from the receptor. Consequently, there is sub-optimal activation of the receptor and an atypical nuclear reaction. The overall effect is an incomplete and diminished estrogenic response.

Not only do the agonistic and antagonistic properties of antiestrogens depend upon the physiological state of the animal due principally to fluctuations in cytosol receptor (Geynet et al., 1972), they also appear to be
associated with specific tissues (Cidlowski and Muldoon, 1976).

Utilizing anterior pituitary, hypothalamus and uterine rat tissues Cidlowski and Muldoon (1976) were able to demonstrate that CI-628 caused extensive depletion of cytoplasmic receptors by translocation to the nucleus in anterior pituitary and uterine tissues but not in the hypothalamus. Results also indicate a complete lack of receptor replenishment. Application of MER 25 was followed by a moderate depletion and a short significant phase of replenishment in cytosol receptor. As with CI 628, MER 25 was effective in the pituitary and uterus but not in the hypothalamus. DMS demonstrated effects intermediate to CI 628 and MER 25 but was a potent inducer of receptor replenishment particularly in the hypothalamus.

3. PHYTO-ESTROGENS

Bennetts et al. (1946) were the first to recognize that infertility in sheep of western Australia was caused by subterranean clover consumption. The substances implicated were of two principal categories; the isoflavones (genistein, formononetin, etc.) and coumestans (coumestrol, etc.). These compounds as well as biochanin A, diadzein,
Table 2 Classification of estrogen agonists and antagonists (a).

<table>
<thead>
<tr>
<th>CLASS</th>
<th>EXAMPLES</th>
<th>NUCLEAR RETENTION</th>
<th>PHARMACOLOGIC CHARACTERISTICS</th>
<th>UTEROTROPHIC PROPERTIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Short acting</td>
<td>estriol, DMS, 16-oxo-estradiol</td>
<td>Short (1-4 hr.)</td>
<td>antagonistic when injected, agonistic when implanted</td>
<td>early responses</td>
</tr>
<tr>
<td>2. Long acting</td>
<td>A. estradiol, DES</td>
<td>Intermediate (6-24 hr.)</td>
<td>Agonistic</td>
<td>early and late responses</td>
</tr>
<tr>
<td></td>
<td>B. triphenylethylene derivatives, e.g. Nafoxidine, Tamoxifen</td>
<td>Long acting, greater than 24-48 hr.</td>
<td>Agonistic - one injection, Antagonistic - multiple injections</td>
<td>early and late responses</td>
</tr>
</tbody>
</table>

Early responses: water imbibition, hyperemia, amino acid and nucleotide uptake, activation of RNA polymerase I and II, stimulation of induced protein.

Late responses: cellular hypertrophy and hyperplasia, sustained RNA polymerase I and II activity.

(a) Table reproduced from Mathieson (1979).
benzofuranocouinarin and psoralidin were subsequently isolated and characterized (Bradbury and White, 1951; Bradbury and White, 1954; Moule et al., 1963; Bickoff et al., 1969; Livingston, 1978).

The isoflavones (genestein) and coumestans (coumestrol) have demonstrated their ability to displace and compete with estradiol-17β for estrogen receptors in target tissues (Shutt, 1967; Foman and Pope, 1966; Folman and Pope, 1969; Lindner, 1976). The phyto-estrogen receptor complex is then able to translocate into the nucleus inducing an estrogenic response (Noteboom and Gorski, 1963; Ostrovsky and Kitts, 1963; Foman and Pope, 1969). The response is typified by water imbibition, increases in RNA, protein, phospholipid and acid soluble uridine triphosphate (UTP) in the uterus of the ovariectomized rat (Noetboom and Gorski, 1963; Kitts, 1976; Kitts et al., 1980).

Since the discovery by Bennetts et al. (1946) more than 50 plant species have been shown to contain estrogenic substances (Bradbury and White, 1954; Kitts et al., 1959; Bieley and Kitts, 1964). According to Lindner (review: 1976) the most important pasture and forage crops shown to contain phyto-estrogen include: *Trifolium subteraneum* L. (clover cultivars; Dwalganup, Mt. Baker, Yarloop, Marrar), *T. pratense* (red clover), *T. fragiferum* (strawberry
clover), *Medicago sativa* (Lucerne, alfalfa) and *Soya hispida* (soya beans).

Variation in coumestrol and genistein content and incidence has been attributed to plant variety, cutting stage of growth, disease and fungal infection (Moule et al., 1963; Hanson et al., 1965; Bickoff et al., 1969; Kitts et al., 1969; Cox and Braden, 1974; Livingston, 1978). When disease and fungal infection are considered, it is unclear whether the plant estrogens are metabolic substances synthesized in response to the invasion or produced by the infective agents. Lindner (1976) demonstrated that coumestrol content increases in alfalfa infected with the fungal agent *Pseudopezia medicagensis* and zearalenone formation occurs in corn during storage by the microbial attack of *Fusarium spp*.

As stated previously coumestrol and genistein are capable of competing with estradiol-17β for receptor sites in target tissue and inducing a uterotrophic response. Whether the response is agonistic (weakly estrogenic) or antagonistic will depend upon the dose ingested and maintained within the blood stream. Folman and Pope (1966) demonstrated that coumestrol, genistein, DMS and norethisterone acetate are agonistic at low doses and antagonistic at higher levels.
The stereochemical configuration of the non-steroidal phyto-estrogen molecule enables competition for estradiol cytoplasmic receptors. This confirms the importance of the functional phenolic hydroxyl groups as binding centre for association with the cytosol receptor (Noeboom and Gorski, 1963; Shemish et al., 1972). Coumestrol has a greater affinity for uterine receptors than do the isoflavones but less than estradiol-17β. Shutt and Cox (1972) estimate the relative potencies of $E_2$-17β: Coumestrol: Genistein to be 1:20:111 respectively while Shemish et al. (1972) estimate the relative potencies to be 1:70:175.

Original studies (Bennetts et al., 1946) attributed infertility to coumestrol and genistein, however, metabolic studies have demonstrated pathways implicating not only coumestrol but equol as the principal infertility agents (see Figure 6). Through action of the rumen microflora biochanin A undergoes O-demethylation to genistein which is further metabolized to inert p-ethylphenol (Braden et al., 1966; Shutt et al., 1970; Cox and Braden, 1974). Formononetin is metabolized through O-demethylation to daidzein which is further transformed to equol and O-desmethylangloensin (Cox and Braden, 1974; Lindner, 1976), again through rumen fermentation processes (Shutt, 1976).
5A Isoflavones:

5B Coumestans:

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Biochanin-A</td>
<td>CH₃</td>
<td>OH</td>
</tr>
<tr>
<td>Daidzein</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Formononetin</td>
<td>CH₃</td>
<td>H</td>
</tr>
</tbody>
</table>

Coumestrol

Figure 5: Structural formula of principle phyto-estrogens.
FIGURE 6A:

Biochanin A \[\xrightarrow{\text{OCH}_3} \] Genistein

p-Ethylphenol

FIGURE 6B

Formononetin \[\xrightarrow{\text{OCH}_3} \] Daidzen

O-Desmethyl angolensin \[\xrightarrow{\text{HO}} \] Equol

FIGURE 6: Structural formula of phyto-estrogen metabolites.
It has been demonstrated that ingesting estrogenic pasture can be associated with such gross physiological changes as dystocia, uterine prolapse, udder development and milk secretion in barren ewes (Noteboom and Gorski, 1963), lamb mortality, retained mummified fetus, metritis, sterility and infertility (Moule et al., 1963).

Pathologically the uterine epithelium, stroma and myometrial layers undergo hypertrophy and proliferation due to increased water imbibition and glycogen synthesis (Ostrovsky and Kitts, 1963). Associated with this abnormal uterine metabolism is cystic glandular hyperplasia of the endometrium with uterine and cervical lesions (Moule et al., 1963). This results in increased fluidity of cervical and uterine mucus leading to impaired ova and sperm mobility with subsequent decreases in fertilization and conception rates (Moule et al., 1963; Lighfoot and Wroth, 1974a; Lightfoot et al., 1974b; Cox and Braden, 1974; Livingston, 1978). Under such abnormal hormonal conditions Ostrovsky and Kitts (1963), Moule et al. (1963), Lightfoot and Wroth (1974), Kelly et al. (1976), and Livingston (1978) doubted that this uterine epithelium could respond to progesterone priming which would therefore result in unsuccessful implantation, another source of infertility.
Lightfoot and Wroth (1974) and Kelly et al. (1976) have demonstrated that ewes on coumestan rich feed exhibit premature regression of the corpus luteum, in fact 58% of the ewes did not have a recently developed corpus luteum. This elucidates two major features of acutely affected ewes; (1) regression of the corpus luteum removes the progesterone required for successful implantation and (2) a complete absence of a corpus luteum indicates an anovulatory estrus. (Firth et al., 1977).

Premature regression of the corpus luteum and anovulatory estrus are two indications of an altered hypothalamic-pituitary-ovarian relationship in which the hypothalamus has been specifically implicated. Noteboom and Gorski (1963) demonstrated an increase in pituitary weights of affected ewes with subsequent hyperactivity (Kelly et al., 1976). In contrast, Cox and Braden (1974) found pituitaries to be histologically normal in clover diseased ewes. Any discrepancies could be a result of ingesting different levels of phyto-estrogen. Wright (1963) fed coumestrol to mice which did not suppress gonadotropin biosynthesis but did inhibit its release. The plant estrogens may act directly upon the pituitary to prevent gonadotropin release (Wright, 1963). However, it is more probable that the hypothalamus and its secretion of GnRH is affected.
(Hernshaw et al., 1972; Findlay et al., 1973; Lindner, 1976, Adams, 1976, Adams et al., 1979). Permanent infertility amongst ewes is characteristic of clover disease with the hypothalamus and higher nerve centres responsible. These neuroendocrine centres which control the estrous cycle become suppressed and permanently desensitized to the positive stimulatory effect of endogenous estrogen (Lindner, 1976; Livingston, 1978; Adams, 1978; Adams et al., 1979).

As a consequence of altered hypothalamo-hypophyseal function one would expect abnormal folliculogenesis. Kelly et al. (1976) found follicular abnormalities indicative of strong estrogenic stimulation whereas Adams (1976) discovered smaller ovaries in clover affected ewes indicating ovarian desensitization to circulating gonadotropins or decreased levels of the same gonadotropins. Adams (1979) found ewes fed subterranean clover showed elevated ovulation rates but no difference in primordial follicles. This indicates an altered pattern of follicular development rather than an increase in the rate of initiation of growth. A further result of atypical folliculogenesis should be anomalous ovarian steroidogenesis which is in fact the case (Obst and Seamark, 1975; Newsome and Kitts, 1977).
4. THE HYPOTHALAMUS IN FEMALE REPRODUCTION

a) **Anatomical Organization**

The hypothalamus provides the major link between endocrine and neural systems that integrate the external and internal environment. It is bilaterally symmetrical with diffuse boundaries and forms the walls and floor of the lower region of the third ventricle. The upper boundary is formed by the hypothalamic sulcus, a structure which defines the boundary between the hypothalamus and thalamus. Lateral boundaries are determined by the cerebral ganglion, subthalamus and optic tracts.

The hypothalamic area also includes the optic chiasma, tuber cinereum, infundibulum and mammillary bodies. The tuber cinereum is a bulging part of the floor of the third ventricle which extends downward towards the infundibulum. The expanded upper infundibulum and lower part of the tuber cinereum are richly supplied with blood vessels and course through to the anterior pituitary defining the Median Eminence (ME) and hypophyseal portal system (Martin, 1976).

Within the hypothalamus is an extensive nerve fibre system which extends throughout other parts of the central nervous system. This neuronal network, which
is responsible for synthesis and secretion of releasing hormones regulating anterior-pituitary function, is referred to as the Perivascular Neurosecretory System (Setalo, 1977). The various neuron cell bodies (perikarya) aggregate in specific areas of the hypothalamus termed nuclei (also islands). Specific nuclei are integral components of the Perivascular Neurosecretory system and are found in the medical basal hypothalamus also known as the hypophy-siotropic area. The nuclei implicated are the suprachiasmatic-preoptic, tuberal-premammillary region, ventromedial periventricular and arcuate (Setalo, 1977; Dyer, 1977; Brawer and Van Houton, 1977).

The fibres from the medial basal hypothalamus form two distinct tracts, (i) the Preoptic Infundibular tract and, (ii) the Tuberoinfundibular tract. These tracts are the endocrine neurones which terminate in the Median Eminence on the capillaries of the hypophyseal portal system. The major concentration of Gonadotropin Releasing Hormone (GnRH) perikarya can be found in the Arcuate Nucleus (Brawer and Van Houten, 1977; Krulich et al., 1977; Zimmerman, 1976; Zimmerman, 1977), the fibers of which constitute a major portion of the Tuberoinfundibular tract. There also appears to be a slight but significant concentration of GnRH neurons in the mediobasal zone of the preoptic
Figure 7: Ventral (A) and Median (B) surfaces of the sheep brain (May, 1964).
Figure 8: Mid sagittal section of the rat diencephalon
(Everett, 1978).

PV Paraventricular Nuc.
ARC Arcuate Nucleus
NAH Anterior Hypothalamic Area
SCH Suprachiasmatic Nuc.
SO Supraoptic Nuc.
DM Dorsomedial Nuc.
VM Ventromedial Nuc.

MM Medial Mamillary Nuc.
PM Premamillary Nuc.
LAHY Pars Distalis of Adenohypophysis
LPHY Neural Lobe of Hypophysis
area (Krulich et al., 1977; Brawer and Van Houton, 1977). It is thought that the fibers from this region also contribute to the Tuberoinfundibular tract principally via the arcuate nucleus.

The GnRH endocrine neurons of the medial basal hypothalamus making up the Tuberoinfundibular tract also exhibit branched fibres that extend to other hypothalamic and extrahypothalamic regions (Dyer, 1977). There are also peptide release regulating interneurones which integrate the various impulses arising from the numerous hypothalamic regions. This indicates an integrated control mechanism between other brain centres, the CNS, and the hypothalamus (Dyer, 1977; Kordon et al., 1976).

b) Neurotransmitters and the Hypothalamus

Monoamines are considered to act as neurotransmitters in inputs to the neurosecretory cells of the hypothalamus and in some cases may even act directly on the anterior pituitary. The monoamines consist of two principal groups: (1) the catecholamines; dopamine (DA), adrenalin (A), noradrenalin (NA) and (2) the indolamines; 5-hydroxytryptophan (5-HT), melatonin and histamine (Kalra, 1977).
Dopamine, NA, A and 5-HT pathways originate in the lower brain stem ascending to innervate the thalamus, subcortical and cortical limbic systems, basal ganglia and neocortex. Within the hypothalamus there are a number of intrahypothalamic DA systems. The most prominent is the tuberoinfundibular tract DA system which innervates the lateral and medial palisade zones of the external median eminence (Fuxe et al., 1977). With the involvement of the tuberoinfundibular tract system it has been demonstrated that neurons containing DA are concentrated within the arcuate and anterior periventricular nuclei (Fuxe et al., 1976).

Cell bodies giving rise to the NA neuronal system are localized in the pons and medulla oblongata. Axons form the ventral NA bundle in the mesencephalon and course through the hypothalamic region to terminate in the medial palisade zone and subependymal layer of the median eminence (Fuxe et al., 1976; Fuxe et al., 1977).

Stimulation of the DA system inhibits the release of GnRH from the median eminence while NA will facilitate secretion. The integration of these two systems yields the overall GnRH response and may be a site of estrogen feedback control. Conclusions drawn from indolamines are unclear, however, it is conceded that 5-HT has a dual
effect of inhibiting basal GnRH secretion and controlling the rhythmic GnRH secretion pattern. Melatonin has been shown to inhibit ovulation while histamine will facilitate it (Brownstein, 1977; Fuxe et al., 1976; Fuxe et al., 1977; Kalra, 1977; Kordon, 1978; McCanne, 1977; Sawyer, 1977).

c) Extrahypothalamic Structures in Female Reproduction

Higher neural centres are considered to influence the hypothalamus because external stimuli are known to effect ovulation and sexual behavior. Readily discernible examples of this are: psychological amenorrhea in women and induced ovulators such as the rabbit.

The limbic system is thought to integrate and control circadian, stress, autonomic nervous system and external sensory stimulus responses which influence the reproductive state of the animal. Structures within the limbic system are the medial part of the mesencephalic reticular formation (midbrain limbic system), the septum, cingulate and pyriform cortex, hippocampus and amygdala (Martin, 1977; Hutchinson, 1978).

The amygdala, hippocampus and a number of mesencephalic nuclei all have anatomical access to the GnRH delivery system. The influence on this delivery system
is achieved through connections with the suprachiasmatic-preoptic areas (Brawer and Van Houten, 1977; Ellendorf, 1978). More extensive research has been conducted on the limbic system and it has been demonstrated that sensory inputs are not available to the limbic structures with the exception of the retinohypothalamic connection to the suprachiasmatic nucleus (Wilber et al., 1976). Hutchinson (1978) has illustrated complementary afferent and efferent neural pathways between the limbic structures and hypothalamus indicating a two-way interaction. Sensory inputs are also relayed to the limbic system through the reticular formation of the thalamus and brain stem (Wilber et al., 1976).

Hypothalamic inputs from the hippocampus arise from a massive efferent pathway, the fornix (Everett, 1978). The fornix is the major contribution to the mammillary body and also directs efferent fibers into the arcuate and the ventromedial nuclei of the hypothalamus. Some of these fibers also terminate on tubero-infundibular neurons (Lammers and Lohman, 1974).

The amygdala is perhaps the most studied structure of limbic system. Projections from the corticomedial nuclei of the amygdala extend through the stria terminalis to the septum and external border of the ventromedial
and medial preoptic nuclei of the hypothalamus. From the basomedial amygdala, projections of the amygdalofugal pathway pass to the lateral hypothalamus. The function of this last pathway is however unclear (Ellendorf, 1976).

The amygdala is capable of exerting both a facilitatory and inhibitory influence upon the release of gonadotropin (through the secretion of GnRH). Facilitatory impulses are transmitted through the stria terminalis and mesencephalo-amygdaloid system while inhibitory pulses are implemented through the mesencephalo-hippocampal system. The two systems stated above may be integrated to form an overall inhibitory pathway however the exact inhibitory pathway is unclear (Taleisnile and Beltramino, 1975).

The pineal arises from neuropithelium and is a true endocrine gland because it synthesizes hormonal factors in specific cells, the pinealocytes, and not in neurons as does the neuroendocrine hypothalamus (Kappers, 1976). The pineal in sheep is both indirectly and directly responsive to light (Hutchinson, 1978) which in turn synchronizes reproductive states with season. The pineal then acts as an integrator and intermediary between seasonal photoperiodic changes and the neuroendocrine reproductive axis (Reiter, 1976).
Though the mechanism is unresolved the main function is one of an antigonadotropic nature suppressing gonadotropin activity, again through GnRH secretion. Some secretory products are however released into the systemic blood system. The secretory products are antigonadotropic and are composed of polypeptides and indolamines, principally melatonin (Reiter, 1976).

d) Gonadotropin Releasing Hormone(s)

Gonadotropin Releasing Hormone abbreviated as GnRH (also known as LRF, LH-RH, FSH/LH-RH and Gonadoliberin) is the centre of controversy. The question is constantly raised as to the exact nature of GnRH. Is there just one GnRH responsible for the release of both gonadotropins (FSH and LH) or are there two releasing hormones?

Evidence indicates the existence of one releasing hormone acting upon one gonadotroph to evoke a differential release of either LH or FSH. This differential control of the pituitary by the hypothamus is in turn modified by the prevailing reproductive state and consequently circulating ovarian steroid levels. These prevailing conditions will modify the gonadotroph's responsiveness and secretory mechanisms (Meites, 1969; Fleischer and

GnRH is a decapeptide first isolated and characterized from procine hypothalamic extracts (Schally et al., 1971; Matsuo et al., 1971). As stated previously, GnRH is synthesized within the perikarya of the suprachiasmatic-preoptic area and arcuate nucleus of the medial basal hypothalamus. From these nuclei the releasing hormone is deposited and localized within the median eminence to be subsequently released into the primary capillary plexus of the hypophyseal portal system (McCann, 1977). This neurovascular pathway forms the critical link from nerve cells of the hypothalamus to the endocrine cells of the anterior pituitary.

Daniel and Prichard (1975) determined that specific regions within the pars distalis of the anterior pituitary receive blood from certain areas of the median eminence and neurohypophysis. They also demonstrated that each region of the pars distalis is completely circumscribed. These points may be important because various types of cells of the pars distalis tend to be grouped in particular regions of the lobe (Daniel and Pricard, 1975; Mathieson, 1979).
Once into the hypophyseal portal system the GnRH travels down the pituitary stalk and into sinusoids within the anterior pituitary. Here the GnRH reacts with surface receptors of the gonadotrophs to activate the adenylate cyclase system with subsequent increases in cAMP and protein kinase activity (Justiz, 1971; McCann, 1977; Conn et al., 1979; Labrie et al., 1979; Labrie et al., 1979). As a result of this action membrane proteins are dephosphorylated altering the membrane permeability and enabling extrusion of the secretory granules containing gonadotropin (McCann, 1977). Wagner et al. (1979) demonstrated the existence of a single class of high affinity receptor sites ($D_A 2.33 \pm 3.1 \times 10^{10} M^{-1}$). A complete discussion of peptide hormone-membrane receptor reactions will follow in later sections. It should be noted that a second pathway exists within gonadotrophs involving prostaglandin which may be responsive to GnRH. The PG pathway is thought to be independent of cAMP activation (Conn et al., 1979; Nair et al., 1979).

GnRH also exerts a self-priming effect upon the pituitary which magnifies the adenohypophysis response to GnRH. This property of GnRH is further enhanced by the action of estrogen (Castro-Vazquez and McCann, 1975; McCann, 1977; Pickering and Fink, 1977; Labrie et al.,
However, continual stimulation by GnRH ultimately leads to desensitization of the pituitary towards GnRH (Sandow et al., 1978).

The GnRH is also localized in extra-hypophysiotropic areas and therefore may have other physiological actions within the CNS and brain function (Moss, 1979). GnRH has been found in such regions as the pineal, midbrain, cerebral and cerebellar cortices and brain stem. Acting through these structures GnRH has been demonstrated to affect mating behavior particularly lordosis (Moss and McCann, 1973; Pfaff, 1973; Moss, 1979).

5. THE PITUITARY IN FEMALE REPRODUCTION

The hypophysis develops in the region dorsal to the notochord, arising from two ectodermal primordia: thesaccus infundibularis, a ventral evagination from the diencephalon; and Rathke's pocket, a dorsal outgrowth of the buccal cavity (Everett, 1978). The neural lobe forms the neurohypophysis (posterior lobe) while Rathke's pocket forms the adenohypophysis (anterior lobe). The latter is central to this discussion and will be the only hypophysial lobe considered. The terms anterior and
posterior lobes are misleading because they do not apply to all species and will not be used in subsequent discussions. Anatomically, three zones can be distinguished within the adenohypophysis. The pars distalis consists of glandular cells arranged in irregular columns which are intimately related to a network of sinusoidal capillaries. These capillaries are the extremities of the hypophyseal portal venules which originate from the primary plexus (Rennels and Herbert, 1979).

The glandular cells are composed of chromophobes, acidophils (somatotrophs, lactotrophs), and basophils (thyrotrophs, gonadotroph). The gonadotrophs are responsible for the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in response to GnRH and steroid feedback stimulation. Several workers have identified two distinct gonadotroph cell types and termed them folliculotrophs (which produce FSH) and Luteotrophs (which produce LH) (Holmes and Ball, 1974; Schulster et al., 1976; Purandare et al., 1978). Martin (1976) and Franchimont (1977) however maintain the presence of one type of gonadotroph which can be influenced by extracellular conditions. Evidence for this is based on microscopic immuno-histochemical techniques which demonstrate FSH and LH within the same cells (Nakane, 1975). This dilemma remains unresolved,
however, Dacheux and Dubois (1978) implementing electron microscopic immunocytochemical studies have identified and characterized LH-secreting cells in the ovine pituitary. Both LH and its subunits (LHα and LHβ) were localized on the secretory granules and on the small granules near the Golgi complex. The authors also suggest that these techniques can be used to isolate FSH and as a consequence to determine whether LH and FSH occur in one or two different cell types.

6. GONADOTROPINS

a) General Considerations

Ovine gonadotropins have been isolated and characterized (Papkoff and Ekblad, 1970) while human gonadotropins have been extensively reviewed by Butt (1976).

The gonadotropins are dimer glycoproteins which contain up to thirty per cent carbohydrate. Pituitary FSH has a molecular weight of approximately 35,000, is rich in glutamic acid and cysteine and 10-30 per cent of the molecule appears as an alpha-helical structures. LH has a molecular weight of approximately 33,000 and
is rich in proline which prohibits the formation of the alpha-helical configuration (Butt, 1976).

FSH contains more carbohydrate and sialic acid than LH. The hexose amines which occur are principally 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine) in FSH, N-acetylglucosamine and N-acetylglucosamine (ratio 6:1 respectively) in LH. The hexose amines appear to play a role in receptor binding (Butt, 1976) and will be discussed later.

The FSH and LH individual subunits (alpha and beta) have no intrinsic biological activities; the dimer is required. Hybrid molecules may be obtained, however, the beta subunit determines the nature and characteristics of the hybrid. (Reichert, 1968; Reichert, 1974; Butt, 1976).

b) Gonadotropin Synthesis and Secretion

The gonadotrophs are controlled through the action of GnRH reaching the adenohypophysis via the hypophyseal portal system. This pathway is also influenced by the GnRH itself and prevailing gonadal steroid patterns; these topics will be considered in later sections.
Using phosphodiesterase inhibitors, Jutisz (1971) demonstrated cAMP to be a mediator of GnRH as it is able to release FSH and LH from rat pituitaries in vivo in the presence of Ca\(^{2+}\) ions. By using actinomycin D (RNA synthesis inhibitor) and cycloheximide (inhibits protein biosynthesis) in vitro the releasing activity of GnRH was only partially impaired but over a longer period FSH release did begin to decline. This evidence yielded a two compartment model for pituitary cell gonadotropin synthesis and release (Jutisz, 1971; Yen, 1977).

The model depicted involves a readily releasable pool of FSH and a second compartment responsible for FSH synthesis and storage. Evidence indicates the releasable pool to be under direct influence of the GnRH. The synthesis compartment appears to be controlled by intracellular messengers and feedback mechanisms which may be an indirect result of GnRH action Jutisz, 1971). An example of an intracellular messenger could be the prostaglandin described by Nair et al. (1979) which is independent of the adenylate cyclase-cAMP system. This intracellular PG biosynthesis appears to be under the influence of GnRH as MDA, which is a direct metabolic product of prostaglandin endoperoxide, increases in pituitaries stimulated with GnRH over controls. Prostaglandin may also be responsible
for LH release from gonadotrophs (Nair et al., 1979). In contrast to this concept Labrie et al. (1978) suggest prostaglandins are not involved in GnRH action at the level of the anterior pituitary but stimulate LH release at the hypothalamic level through increased GnRH secretion.

The feedback mechanisms modulating the two pools of gonadotropin may involve FSH and LH themselves (Jutisz, 1971), the self-priming effect of GnRH (Castro-Vazquez and McCann, 1971; McCann, 1977; Pickering and Fink, 1977; Yen, 1977; Foster, 1978; Hoff et al., 1979) and ovarian estrogen (Yen, 1977). Through the early mid-follicular phase the second pool of synthesized and stored LH parallels the estradiol-17β pattern and is preferentially augmented. Enlargement of the releasable pool is not apparent until the late follicular phase which represents a shifting of LH between the two compartments. Estrogen appears to promote acceleration of gonadotropin synthesis within the second pool and transfer to the first pool but appears to impede the GnRH mediated LH release. During the mid-luteal phase progesterone and estrogen levels appear to maintain a large second pool while the first pool is much smaller. This environment is also associated with extremely low GnRH release (Yen, 1977). Hoff et al. (1979) also demonstrated
Figure 9A: Proposed mechanism of action for GnRH (McCann, 1977 and Labrie et al. 1978).
Figure 9B: Interrelationship between estrogen and GnRH in the proposed 2 pool hypothesis for gonadotropin (Jutisz, 1971 and Yen, 1977)
Figure 10: Prostaglandin biosynthesis (Nair et al., 1979).
that large elevations of GnRH favor gonadotropin release whereas small increments promote priming. This is most evident in the presence of high estradiol levels.

c. Gonadotropin Receptors and Activation

Polypeptide hormone receptors obey the stringent criteria described for steroid receptors; however, hormone specificity resides in the structure of plasma membrane receptors, the enzyme profile of the target cell and the ability to change membrane permeability. This is in contrast to intracellular steroid receptors which ultimately affect gene expression (Blake, 1978).

The gonads are capable of binding 400-500 times more gonadotropin than necessary to evoke maximum steroidogenesis. Only 10-15 per cent of the total sites need be saturated to achieve the maximum response (Saxena and Rathnam, 1976). Consequently two orders of binding sites have been described (Saxena and Rathnam, 1976):

(i) high affinity-low capacity receptors,
(ii) low affinity-high capacity, representing non-specific binding.

In contrast, Catt and Dufau (1977) have indeed demonstrated "spare receptors" and occupation of any 1 percent of the
total Leydig cell membrane receptors will produce complete activation of LH induced steroidogenesis. This indicates equivalent saturation binding and affinity between all LH receptors. Erickson et al. (1979) have determined the LH-Granulosa cell receptor dissociation constant ($K_D$) to be $1.4 \times 10^{-11}$ M. Utilizing human FSH and seminiferous tubule homogenates, Bhalla and Reichert (1974) have demonstrated the $K_D$ for FSH-receptor interactions to be $6.7 \times 10^{-10}$ M.

For optimal hormone-receptor interactions and maximum cell response several critical factors must be considered. Carbohydrate moieties are essential for receptor integrity and when FSH is considered, sialic acid and mannose residues are required for optimal binding (Saxena and Rathnam, 1976; Catt and Dufau, 1977). Phospholipids, principally phosphatidylethanolamine, phosphatidylserine and lecithin are vital requirements for functional membrane receptors (Saxena and Rathnam, 1976) and disulfide groups within the LH receptor appear to be implicated for the biologically active conformation (Catt and Dufau, 1977).

The polypeptide hormone-receptor interaction involves hydrophobic (consequently hydrogen bonding) and electrostatic forces which are facilitated by specific
amino acid residues within the hormone and receptor site (Catt and Dufau, 1977; Blake, 1978). Results obtained with insulin and glucagon indicate that peptide hormones and receptor proteins are capable of hydrogen bonding within themselves exposing hydrophobic regions. The complementary hydrophobic regions between the hormone and receptor then associate in what is termed the "zipper concept" (Blake, 1978). Binding occurs by the interaction of a single amino acid region within the hormone with the appropriate subsite on the receptor. The hormone reorganizes its conformation with the result other specific amino acid residues interact in succession with appropriate receptor subsites until the hormone and receptor are optimally aligned (Blake, 1978).

It is accepted that cAMP is the secondary messenger within FSH and LH target cells (Saxena and Rathnam, 1976; Catt and Dufau, 1977; Hay and Moor, 1978; Blake, 1978; Richards et al., 1979), however, the exact mechanism of cAMP activation has only been recently described.

The original view that hormone receptors were structurally coupled to adenylate cyclase (EC 4.6.1.1) and served as regulatory subunits during the enzymatic conversion of ATP → cAMP has since been replaced by other models. This is a consequence of demonstrations that several hormones can influence dispersed fat cells through
the same adenylate cyclase system and yet still bind to separate specific receptors (Cuatrecasas et al., 1975). A model is also required which is more consistent with the fluid nature of plasma membranes. The model proposed is "The Mobile Receptor Hypothesis" which is based upon the assumption that receptor and enzyme molecules are normally discrete and separate structures (Cuatrecasas, 1974; Cuatrecasas et al., 1975; Catt and Dufau, 1977; Blake, 1978).

Occupancy of the receptor site by hormone increases the affinity of the receptor for other membrane structures such as adenylate cyclase (Cuatrecasas, 1974; Blake, 1978). Although the receptor sites appear to be physically distinct from adenylate cyclase (Catt and Dufau, 1977; Levitzki and Helmreich, 1979) they obviously possess intimate functional connections.

The adenylate cyclase system consists of three components the hormone receptor, the GTP regulatory unit (GTP Binding Protein) and the adenylate cyclase catalytic unit (Catt and Dufau, 1977; Levitzki and Helmreich, 1979). Activation of the membrane bound enzyme requires simultaneous binding of hormonal agonist and guanyl nucleotide to their respective sites. Within the GTP unit is the enzyme GTPase which promotes GTP $\rightarrow$ GDP + Pi. When the hormone reacts
Figure 11: The hormone mobile receptor adenylate cyclase interaction (Blake, 1978; Levitzki and Helmreich, 1979).
with the receptor it facilitates and maintains an exchange of GDP with GTP which is a vital requirement in adenylate cyclase activation (Levitzki and Helmreich, 1979).

Once activated adenylate cyclase, through its catalytic site, reacts with ATP chelated to magnesium to produce cAMP. The cAMP then induces the observed target cell response by activating protein kinase enzymes either alone or in combination with other intracellular messengers and effects membrane permeability depending upon target cell function.

d) The Ovarian Response to Gonadotropins

Ovarian follicular development involves steroid and gonadotropin regulation of granulosa-theca cell proliferation, differentiation, morphology and function. These specific changes in response appear to be related, in part, to hormone specific regulation of hormone receptors. Mammalian ovaries are considered to contain a non-proliferating pool of germ cells which are eventually surrounded by a layer of cells and through the process of maturation form three cell types; granulosa cells, basement membrane, thecal layers external to the basement membrane and stromal connective tissue (Saxena and Rathnam, 1976; Richards et al., 1978).
Follicle growth is presumed to begin as a random initiating event which, once started, proceeds until ovulation or atresia occurs. The process involves the formation of a pool of committed growing follicles which subsequently become large pre-antral follicles. If this stage of maturation and pre-ovulatory surge of gonadotropin are achieved further folliculogenesis will occur simultaneously and culminate with ovulation during the next reproductive cycle. Atresia occurs when the gonadotropin surge and pre-antral stage are not synchronized (Richards et al., 1978).

Folliculogenesis and consequently steroidogenesis are believed to involve a "Two Cell Theory". During follicle maturation to the pre-antral stage FSH is bound solely by the granulosa cells and LH by the thecal cells (Saxena and Rathnam, 1976; Lindner et al., 1977; Richards et al., 1979). Synthesis of ovarian estrogen requires an intimate interaction between thecal cells, the sole source of androgen, and granulosa cells which are necessary for aromatization (Dorrington et al., 1975; Moon et al., 1975, 1978; Lindner et al., 1977; Hay and Moor, 1978; Richards et al., 1978).

LH facilitates the conversion of cholesterol to pregnenolone with the ensuing formation of androgens, principally dehydroepiandrosterone (DHA), androstenedione
and testosterone. This conversion and pathway occurs via the \( \Delta^5 \)-3\( \beta \)-hydroxysteroid pathway within the theca cells. The granulosa cells are also capable of androstenedione and testosterone biosynthesis through the \( \Delta^4 \)-3-ketosteroid pathway with such intermediates as progesterone and 17-hydroxyprogesterone (Fotherby, 1975; Gower and Fotherby, 1975; Gower, 1975a; Marsh et al., 1976). FSH then stimulates aromatization of androstenedione and testosterone within the granulosa cells to produce principally estradiol-17\( \beta \) (Moon et al., 1975; Dorrington et al., 1975; Richards et al., 1978). Gower and Fotherby (1975) contradict this concept by maintaining that theca cells are responsible for aromatization and estrogen production during the first half of the menstrual cycle.

As estradiol-17\( \beta \) is synthesized and secreted it in turn exerts a positive feedback upon the ovary to increase the follicular response to gonadotropins. As the follicle matures FSH increases its own number of receptors while \( E_2 \)-17\( \beta \) increases the responsiveness of the FSH adenylate cyclase system to FSH. With the approach of the Graafian Follicle stage FSH and estradiol work synergistically to induce granulosa cell differentiation and promote increases in the number of granulosa cell LH receptors (Saxena and Rathnam, 1976; Richards et al.,
Figure 12: Pathways of steroid biosynthesis in human ovarian tissue (Marsh et al. 1976)
1976, 1978, 1979; Erickson et al., 1979). This discussion demonstrates three methods of hormone specific regulation of hormone receptors which occur with the mammalian ovary (Richards et al., 1976);

(i) Autoregulation: hormone affects only its own receptors.

(ii) Coordinated regulation: steroid-protein hormone interaction.

(iii) Heteroregulation: one hormone affects the content of receptor for an entirely different hormone.

With the appearance of LH receptors on granulosa cells LH binding alters the steroidogenic function and morphology of theca and granulosa cells initiating ovulation and spontaneous luteinization (Saxena and Rathnam, 1976; Hay and Moor, 1978). With the onset of LH stimulation there is a preliminary increase in estrogen synthesis which is then rapidly terminated. This is followed by a series of sequential but transient peaks of testosterone, 3β-17α-dihydroxypregn-5-en-20-one and pregnenolone all of which reflect a progressive loss of steroidogenic activity in the thecal component. As luteinization of the granulosa
cells occurs, the cells exhibit enhanced synthetic activity producing mainly progesterone with some 17-hydroxyprogesterone, 20α-hydroxypregn-4-3n-3-one and 20α-dihydroprogesterone (Gower and Fotherby, 1975; Hay and Moor, 1978). LH induced steroidogenesis in the human corpus luteum involves the Δ^4 pathway where LH facilitates the synthesis of cholesterol from acetate and the subsequent 20α-hydroxylation step whereby cholesterol is converted to pregnenolone (Gower, 1975b; Moon et al., 1975; Marsh et al., 1976).

LH and prolactin constitute the ovine luteotrophic complex (Denamur et al., 1973) and both are required for the establishment and maintenance of the ovine corpus luteum (Denamur et al., 1973). Associated with LH-induced granulosa cell luteinization is a decrease in the number of receptors for LH and FSH with subsequent increases in prolactin receptor. Prolactin in turn raises the number of luteal cell receptors for LH. Prolactin and estradiol are also required to stimulate and maintain the steroidogenesis within the corpus luteum induced by the LH surge (Denamur et al., 1973; Crosignani et al., 1976; Martin, 1976; Richards et al., 1978; Poindexter et al., 1979). The concentration of circulating prolactin, if sufficiently elevated will in contrast, inhibit human ovarian luteal formation and function in response to LH (Rolland et al., 1976; Friesen and Shiu, 1977).
In addition to activation of the adenylate cyclase-cAMP system, LH and prolactin appear to incorporate prostaglandins as intracellular messengers in the luteinization and steroidogenic processes during the ovine estrous cycle. There is conflicting evidence that prostaglandins, principally PGE$_2$, may act as an obligatory intermediate between the protein hormone-adenylate cyclase system and/or mediators in other intracellular processes (Kuehl et al., 1973, 1976; Weiss et al., 1976; Marsh and LeMaire, 1976; Marsh et al., 1976; Lindner et al., 1977; Hay and Moor, 1978; Behrman, 1979). Gower (1975b) again offers a contradictory discussion by stating that the prostaglandin-adenylate cyclase relationship holds in vitro but is not applicable in vivo as there can be a demonstrable reduction of plasma progesterone and 20α-hydroxyprogesterone levels. Gower (1975b) does not specify the type of prostaglandin, the reproductive states of the various species or in fact the species in question, all of which are critical to his conclusions.

7. THE HYPOTHALAMIC-HYPOPHYSEAL-OVARIAN AXIS

a) The Ovine Estrous Cycle

The mean estrous cycle length of the ewe is determined to be 16.5-17.5 days (Fraser, 1971; Terrill,
1974; Robertson, 1977) and can be partitioned into four distinct phases depending upon the stage of follicular maturation (Terrill, 1971; McDonald, 1975). Proestrus lasts approximately two days and is characterized by follicular growth with increasing estrogen production. Estrus represents the period of sexual receptivity which lasts 26-40 hours with ovulation usually occurring 24-27 hours after the onset of estrus. The luteal phase commences with metestrus, a period which lasts two days characterized by the formation of a corpus luteum and progesterone secretion. Diestrus follows representing the period of a functional corpus luteum with large amounts of progesterone secretion. Diestrus lasts approximately 12 days.

The endocrine events associated with these four phases are essential for control of the ovarian cycle. Serum concentrations of estradiol begin to rise 12-14 hours prior to, and peak with, the onset of estrus (Baird et al., 1976; Pant et al., 1977; Scaramuzzi and Land, 1978). Closely correlated with this secretion of ovarian estradiol is androstenedione which indicates a common pathway or site of synthesis (Baird et al., 1976). Serum LH remains low during the luteal phase (Pant et al., 1977), rises to peak levels 0-16 hours after the onset of estrus and then rapidly declines to low concentrations during the
Figure 13: Schematic reproduction of the hormonal changes during the ovine estrous cycle (Robertson, 1977).
ensuing luteal phase (Cunningham *et al.*, 1975; Pant *et al.*, 1977; Baird *et al.*, 1978). FSH can exhibit two peaks, the first coincides with the LH surge, the second occurs 24 hours later (Cunningham *et al.*, 1975; Pant *et al.*, 1977).

Progesterone levels vary in a cyclic manner, the highest values are attained during the mid-luteal phase and remain elevated until 35 hours prior to the onset of estrus. By this time the uterine luteolytic factor PGF$_2\alpha$ (Goding, 1974) has facilitated regression of the corpus luteum resulting in a rapid significant decrease in serum progesterone levels which remain low until days 2-4 of the following cycle (Baird *et al.*, 1976; Pant *et al.*, 1977).

b) **The Role of Ovarian Steroids**

It is apparent from the previous discussion concerned with the ovine estrous cycle that there exist distinct relationships between circulating hormone levels and the integration of various endocrine events, all of which collectively provide accurate control of the reproductive cycle.
The major steroids secreted by the ovary, as stated previously, are: progesterone, 20α-dihydroprogesterone, estrone, 17α-dihydroprogesterone, androstenedione and estradiol-17β (Scaramuzzi et al., 1974; Baird, 1976, 1977). The steroids relevant to this discussion are principally progesterone, androstenedione and estradiol-17β.

Estrogenic effects can be genital or non-genital in nature (Hebert, 1977). In the ewe genital effects are characterized by stimulation in growth and function of the ovaries, fallopian tubes, uterus, cervix, vagina, external genitalia and mammary glands. This is achieved through increased receptor, RNA and protein metabolism (Miller, 1976; Miller et al., 1977). The non-genital effects of estrogens are summarized by: the development and maintenance of secondary sex characteristics, anabolic effects, and critical to the following discussion, feedback effects on cyclic gonadotropin secretion and action within the ovary.

The dual and reciprocal action between gonadal steroids and gonadotropin release illustrates the presence of integrated control mechanisms. Castration results in pituitary hypertrophy and associated increases in gonadotropin secretion (Dorner, 1976; Hutchinson and Sharp, 1977). Administration of estrogen to these castrates
results in a subsequent reduction in gonadotropin secretion (Lisk et al., 1972). Pant et al. (1978) and Rawlings et al. (1978) actively immunized ewes against E₂-17β. As a consequence the ewes exhibited complete absence of estrous behavior, the ovaries contained large Graafian follicles and plasma LH levels paralleled increases in serum antibody titer. Injection with high doses of stilbestrol dipropionate reduced the LH concentrations.

The concept presented thus far represents an overall negative feedback for estrogen on gonadotropin secretion which maintains gonadotropin levels within physiological limits (Dorner, 1976). Estrogen also exerts a positive feedback upon FSH and LH release. The most noticeable is the LH surge which induces ovulation and luteal formation (Hutchinson, 1978). What then, are the primary roles for gonadal steroids in the control of ovulation?

During early proestrus the corpus luteum regresses, removing the progesterone block which has inhibited sufficient gonadotropin release for maximal follicular growth and ovulation (Robertson, 1977). With removal of progesterone, intrinsic hypothalamic and ovarian rythyms facilitate initial release of FSH and estradiol respectively. The ovarian steroids, in this instance E₂-17β, are capable of interacting with neuroendocrine tissue
(Stumpf, 1970, 1971a,b; Stumpf et al., 1975; Stumpf and Sar, 1976; Challis et al., 1976; McEwen, 1976; Kato, 1977a,b; Stumpf and Sar, 1977) to modify the synthesis and secretion of GnRH neurons.

The positive feedback effect of estrogen appears to be facilitated within the preoptic-suprachiasmatic area (DePaolo and Barraclough, 1979; Fink, 1979). The inhibitory action of estrogen upon GnRH release occurs in part within the arcuate nucleus of the medial basal hypothalamus (McCann, 1977) and the tuberoinfundibular tract dopamine neurons in the lateral palisade zone of the median eminence. Stimulation of these dopamine neurons with associated desensitization of norepinephrine neurons results in reduced GnRH secretion (Fuxe et al., 1976, 1977).

This estrogen mediated release of GnRH facilitates GnRH release from the median eminence into the hypophyseal portal system to stimulate the adenohypophysis gonadotrophs, initially and through its self-priming affect, to secrete FSH. Estradiol-17β is also capable of increasing the pituitary response to GnRH (Fink et al., 1977; Labrie et al., 1977; Foster, 1978; Labrie, 1978; Fink, 1979). This promotes follicular maturation with associated increases in E2-17β, androstenedione and progesterone.
Together FSH and E₂-17β induce the formation of granulosa cell LH receptors during late proestrus and early estrus in preparation for ovulation (Richards, 1976, 1978, 1979). Estrogen peaks with the onset of estrus and triggers the preovulatory surge of LH inducing ovulation (Baird and Scaramuzzi, 1976; Franchimont et al., 1976; Yen, 1976; Karsch et al., 1977; Fink, 1979). Associated with the pre-ovulatory surge of gonadotropin is a progressively increasing GnRH turnover. Justiz et al. (1973) reported that the highest level of GnRH immunoreactivity occurs during the preovulatory surge of LH and FSH and is not detectable outside the estrus period. Gonadotropin-releasing hormone exhibits pulsatile release patterns (Crighton et al., 1973) with detectable peaks increasing in frequency and magnitude immediately prior to and during the pre-ovulatory LH surge (Crighton et al., 1974; Foster et al., 1976; Foster, 1978).

In addition to the pre-ovulatory surge in gonadotropin, estrogen is also capable of facilitating prolactin release from pituitary lactotrophs (Meites, 1969; Fuxe et al., 1977; Wuttke, 1977; Ferland et al., 1979; Padmanabhan and Convey, 1979; Shupnik et al., 1979; Vician et al., 1979). This aspect of estrogen metabolism is important as prolactin and LH form the luteotrophic complex in the
ewes (Denamur, 1973). Dopamine is a prolactin inhibiting factor. Estrogen is anti-dopaminergic within the medial palisade zone of the median eminence and can indirectly facilitate prolactin release from lactotrophs (Ferland et al., 1979). Prolactin mediates its own secretion via a short loop feedback mechanism involving dopamine turnover in the medial palisade zone. Prolactin also increases dopamine turnover in the lateral palisade zone which indirectly inhibits GnRH release from the median eminence (Fuxe et al., 1976, 1977; Wuttke, 1977; Rudd et al., 1979).

Under the influence of LH and prolactin, a functional corpus luteum is formed which secretes progesterone during diestrus. Progesterone now appears to be the primary controller of tonic gonadotropin secretion in the ewe (Karsch et al., 1977). Progesterone alters the frequency of pulsatile GnRH release prohibiting LH release allowing pituitary LH reserves to build up (Pelletier and Thiomoner, 1975). Progesterone priming is also necessary for the positive estrogen feedback on gonadotropin release (Baird and Scaramuzzi, 1976; Fink et al., 1977; Karsch et al., 1977, 1979). In the ewe, Robertson (1977) describes an intrinsic follicular and ovulatory cycle of 4-5 days in absence of a functional corpus luteum and a 4-5 day follicular cycle without
ovulation in the presence of a corpus luteum. The follicular cycle consists of 3-4 discrete waves of graafian follicular development followed by atresia during the normal estrous cycle. Ovulation in all but one follicle is suppressed by progesterone secretion from a functional corpus luteum (Karsch et al., 1977; Robertson, 1977). The wave of follicle development and subsequent atresia is associated with slight elevations in E$_2$-17$\beta$ and LH (due to the positive estrogen effect); however a full preovulatory surge is inhibited due to the increasing progesterone secretion (Baird and Scaramuzzi, 1976; Robertson, 1977).

The other major ovarian steroid, androstenedione, parallels the secretion of estrogen and as an androgen may be implicated in ovarian follicular atresia (Lindner et al., 1977; Hay and Moor, 1978). The ultimate fate of follicles may depend on the balance between androgen and estrogen at critical stages of follicular development. An imbalance in favour of androgen may affect the ovarian response to gonadotropin or gonadotropin secretion. In the ewe Baird (1976) neutralized the biological activity of androstenedione by active immunization which resulted in increased LH secretion.
Figure 14: Schematogram of the endocrine reproductive systems in females.
c) **Exfoliative Vaginal Cytology during the Estrous Cycle**

Throughout the estrous cycle uterine and vaginal epithelium undergo cyclic changes which parallel the ovarian steroid profile prevailing in the blood circulation (Papanicolaou et al., 1948; Sanger et al., 1958; Wachtel, 1969; McDonald, 1975; Sorensen, 1979). The vaginal wall consists of a fibrous coat, muscular coat and internal mucosa lining covered with stratified squamous epithelium. Within this epithelium of a mature female 4 distinct zones can be recognized (Figure 15). The proliferation and dominance of any one zone will depend upon the gonadal steroid hormone profile (Wachtel, 1969).

The basal cells lie adjacent to the basement membrane and separate the overlying epithelium from the underlying stroma. These cells are cuboidal in shape and form a single layer firmly attached to the basement membrane. As a result basal cells do not exfoliate and are absent from vaginal smears. Basal cells are the undifferentiated cells from which regeneration of epithelium is maintained.

Superficial to the basal layer are the Parabasal cells. This zone consists of several rows of polyhedral cells with comparatively large nuclei and glycogen processes
Figure 15: The vaginal mucosa (Wachtel 1969).

A: Basal layer  B: Parabasal layer
C: Intermediate layer  D: Superficial layer
extending from one cell to another. These cells may exfoliate, losing the intercellular bridges and appear in smears as round or oval shaped cells.

The intermediate layer is composed of several rows of slightly larger flatter cells which also possess intercellular bridges. Their nuclei are vesicular and appear smaller in relation to cell size than those of parabasal cells. When exfoliated these cells appear larger and less round than parabasal cells.

The superficial zone consists of several layers of large flat cells with pyknotic nuclei. In vaginal smears the cells are large and polyhedral with a clear transparent cytoplasm and pyknotic nucleus. The nucleus stains uniformly dark while the cytoplasm stains pink (eosinophilic).

These four zones which make up the vaginal epithelium are very sensitive to stimulation by the sex hormones to which they respond. The response characteristically involves alteration of epithelium height (number of cell layers) and thickness (number of cell rows per layer).

During the follicular phase estrogen has a proliferative and maturing affect on the vaginal epithelium which promotes growth and differentiation and results
in a well developed superficial zone. The level of estrogen and duration of its stimulation is depicted by the eventual proliferation and maturation of the epithelial cells. Estrogen also has a clearing effect on cervical mucus which becomes transparent, consequently vaginal smears obtained with a high level of estrogenic stimulation have a clear background. During the luteal phase progesterone has a regressive influence on the mature estrogen primed epithelium. Consequently with the onset of a functional corpus luteum the superficial layer is sloughed off. Once this occurs progesterone will maintain the epithelium within the parabasal and intermediate squamous zones. The cells often appear oval in shape with thickened cell borders, clear blue staining cytoplasm and an oval eccen­tric vesicular nucleus. These cells are termed "navicular cells" as they often appear boat shaped. Cells of this type have a tendancy to crowd together, fold and curl their edges forming the boat shape. Parabasal and intermediate cells take up the cyanophilic stain therefore the overall staining reaction produces bluish-green smears. Under the influence of progesterone cervical mucus is opaque, exhibits little elasticity and contains many leukocytes.
8. A POTENTIAL ROLE FOR PHYTO-ESTROGENS IN FEMALE REPRODUCTION

Hauger et al. (1977) state the presence or absence of various hormones which may act synergistically with, or antagonistically to, estrogen must be considered in interpreting the overall sequence of events. With this statement in mind it becomes obvious that phyto-estrogens will affect the reproductive physiological responses within the animal in question.

The possible sites for coumestan and isoflavone action parallel those for endogenous estrogen. Negative and positive feedback action at the hypothalamic and pituitary levels to inhibit or stimulate GnRH release becomes an important consideration. Phyto-estrogens are capable of binding to and altering hypothalamic and pituitary estradiol cytosol (Tang and Adams, 1978; Mathieson, 1980). The potential of plant estrogens to modify prolactin secretion may also play a role as elevated prolactin levels are commonly associated with ovarian dysfunction (Besser, 1976; Crosignani et al., 1976; Rolland et al., 1976). Working with females during the puerperium, Crosignani et al. (1976) and Besser (1976) have demonstrated a refractoriness of gonadotrophs to GnRH, delayed gonadotroph
recovery and ovarian insensitivity to gonadotropin stimulation resulting in hypogonadism and reduced steroidogenesis.

Phyto-estrogen activity may also demonstrate itself within the ovary. It may compound the estrogen-FSH effect inducing greater gonadotropin receptor formation and increases the ovarian response to circulating gonadotropin. The opposite also holds true if plant estrogens prove antagonistic to the action of endogenous estrogen at the ovarian level. Groom and Griffiths (1976) utilized the antiestrogen tamoxifen in pre-menopausal women to demonstrate little difference in LH, FSH and progesterone secretion; however, a two to eight-fold increase in estradiol level and a significant decrease in prolactin was found.

The antiestrogen CI 628 also demonstrates the ability to inhibit the induction of progestin receptors by estradiol in the preoptic-hypothalamus area and pituitary (Roy et al., 1979). This capability of antiestrogen is of importance also as it is now known that progesterone appears to be the primary organizer of the ovine estrous cycle (Karsch et al., 1977, 1979). The ability of plant estrogens to modify this aspect of the cycle could also affect sexual behavior and hormonal sequences during the ensuing ovulatory cycle. The intent of the research
reported here is to determine the effect, if any, of estrogenic alfalfa consumption upon the endogenous gonadotropin profile in the cycling ewe and to deduce the possible pathways involved.
EXPERIMENTAL PROCEDURE

I. INTRODUCTION

The objectives of this study are to determine the effect of estrogenic alfalfa consumption on reproductive performance in the ewe. The parameters observed were the response to estrus synchronization, exfoliative vaginal cytology and blood plasma gonadotropin levels.

To accomplish these objectives, ten maiden Dorset Horn ewes exhibiting normal estrous cycles, as determined through actions of a vasectomized marker ram, were randomly assigned into experimental groups. Five ewes in Group I each received a ration of Orchard Grass Hay (Dactylus glomerata; 1.0 kg/day) with a Whole Barley: Beef Concentrate Supplement (Buckerfields; 0.30 kg/day). The remaining five ewes in group II each received a ration of Creston Alfalfa Cubes (Medicago sativa; 1.2 kg/day), produced and purchased from Kootney Dehydrators, Creston, B.C.

Supplement was included with the Group I ration in order to balance protein and energy content of the orchard grass hay with the alfalfa cubes.

Plant, barley and beef concentrate extracts were obtained by a modified method of Francis and
Millington (1965) as described by Newsome and Kitts (1977). The extracts were then assayed for phyto-estrogen content using the Competitive Binding Assay technique of Korenman (1968) and Shutt (1969). Orchard grass hay, barley and concentrate were shown to contain lower amounts of phyto-estrogenic activity than alfalfa cubes (Table 3). As a consequence group I animals, which received the orchard grass hay - supplement, formed experimental controls and the group II animals received an experimental test ration of alfalfa cubes, high in phyto-estrogenic activity.

Animals were synchronized for estrus using progesterone impregnated intravaginal pessaries then placed on their respective rations one week prior to the first scheduled bleeding. Vaginal cytological studies were conducted in conjunction with the use of a vasectomized marker ram to characterize the stage of the estrous cycle and determine the onset of behavioral estrus.

Jugular venous blood was withdrawn into heparinized syringes, centrifuged and aliquots of the plasma frozen until assay. During metestrus, diestrus and early-mid proestrus blood samples were obtained on alternate days during late proestrus and estrus blood was withdrawn at eight hour intervals. The object of this sampling schedule was to provide an accurate representation of the ovine gonadotropin profile throughout the estrous cycle.
Table 3. Estrogenic content of experimental rations (i)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>START OF EXPERIMENT</th>
<th>COMPLETION OF EXPERIMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>OG</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>16.9±2.9</td>
</tr>
<tr>
<td>II</td>
<td>118±12.3</td>
<td>-</td>
</tr>
</tbody>
</table>

(i) Results expressed as 'ppm±S.E.M.' Genistein equivalents  
(ii) A = Alfalfa cubes, OG = Orchard grass, B = Whole barley  
     BC = Beef concentrate
II. MATERIALS

A. Intravaginal Pessaries

Polyurethane foam (0.036 g/cc) cut into the shape of small plugs 3.5 cm (dia) x 2.54 cm; synthetic thread 46 cm in length; distilled ethanol; progesterone (pregn-4-3n3-3,20-dione, sigma).

B. Exfoliative Vaginal Cytology

EA-65 (Papanicalaou Stain, Fisher); hematoxylin; OG-6 stain; syringe with adapter for a teflon pipette; speculum.

C. Ovine Luteinizing Hormone (oLH) RIA

Anti-oLH serum (#15; G.D. Niswender); anti-rabbit gamma globulin (Anti-RGG, ICN Biochemicals); normal rabbit serum; oLH (DNW-9-109-5, 2.43 x NIH-LH-S18, D.N. Ward).

Assay Buffer systems; phosphate buffered saline (PBS) containing 0.14 M NaCl and 0.01 M sodium phosphate, pH 7.0; PBS made 0.1% gelatin, pH 7.0; PBS-EDTA, .05 M EDTA in PBS, pH 7.0.
D. Protein Radioiodination

Chloramine-T (N-chloro-p-toluene-sulfonomide sodium; Matheson Coleman and Bell); Hamilton micro-syringe (Pierce); iodine-125 (1 mCi, Amersham); sephadex G-100 (Pharmacia); sodium metabisulphite (Na$_2$S$_2$O$_5$, Fisher).

Buffer systems; 0.05 M phosphate, pH 7.5; 0.05 M phosphate made 0.2% gelatin, pH 7.5; 0.2 M phosphate, pH 7.5.

E. Preparation of Gonadotropin Free Plasma

Beckman model L5-65 ultra centrifuge with T-65 fixed angle rotor (Bekman); 2-mercaptoethanol (Eastman).

Assay buffer system; 0.10 M tris-HCl, pH 7.4 containing 5.0 mM MgCl$_2$, 0.10 M sucrose.

Homogenization buffer system; same as above with the addition of 1.0 mM 2-mercaptoethanol.

III. METHODS

A. Progesterone Impregnated Intravaginal Pessaries

For the purposes of an efficient bleeding schedule and ability to correlate the gonadotropin profile with
the various stages of the estrous cycle, it was imperative that all experimental animals be synchronized for estrus. This requirement can be easily achieved with the use of intravaginal sponges and a suitable progestagen.

Pessaries were prepared according to the method of Wishart (1967) and Robinson et al. (1967). Two 46 cm lengths of synthetic thread were passed through the polyurethane sponges 1.5 cm apart and knotted twice, once immediately on the surface of the pessary and again approximately 2.5 cm from the lower surface opposite the original knot. This left approximately 30 cm of double strand which protrudes from the vulva and provides a means to remove the pessary from the animal.

A stock solution of progesterone in ethanol equivalent to 800 mg/15 ml was prepared and stored under refrigeration until use. The constructed pessaries were suspended from a horizontal support and the progesterone solution applied in two 7.5 ml aliquots allowing the sponge to air dry completely between applications. Sufficient volume of carrier solvent should be used to ensure good dispersion of progestagen throughout the sponge (Haresign, 1978). However, the pipette method of Robinson et al. (1967) proved inadequate for this volume and amount of progesterone. As an alternative the progesterone was
injected slowly throughout the sponge utilizing a syringe and then permitted to air dry.

Insertion was achieved by first positioning the pessary in the tip of the speculum and placing it in the vagina such that the pessary can be deposited as far as possible into the anterior vagina adjacent to the cervix. Several researchers have encountered problems with this level of progesterone. Moore and Holst (1967) found that estrus and ovulation were not controlled as there appears to be a limit to the amount of progesterone that can be absorbed through the vagina. Moore and Robinson (1967) also demonstrated that progesterone in excess of 500 mg causes the sponge to lose much of its elastic and soft qualities producing an irritant effect. Upon pessary withdrawal the author did observe blood residues throughout many of the sponges; however, no deleterious effects were observed.

Pessaries were withdrawn after eighteen days at which time all ewes were immediately run with a vasectomy marker ram to determine the onset of behavioral estrus.
B. Exfoliative Vaginal Cytology

Throughout the ovarian cycle karyopyknotic index (KI) values provide useful and reliable information on endogenous estrogen effects. In this experiment, KI values will be used as criteria to assess a phyto-estrogenic effect and perhaps by inference an abnormal or modified corpus luteum and associated progesterone secretion.

Vaginal smears were obtained by inserting a teflon pipette through a speculum and aspirating the cervical region of the vagina. Smears taken in this fashion were obtained on alternate days throughout the course of the experiment and stained according to Papanicalaou (1954) and Wachtell (1969).

The smears were examined microscopically to determine the KI values. According to Wachtell (1969) the KI value establishes the percentage of 200 cells counted with pyknotic nuclei, omitting parabasal cells from the count. The KI value varies from day to day showing a continuous rise throughout the follicular phase reaching a peak at ovulation. After ovulation progesterone is secreted and the KI falls sharply.
C. Development of the oLH RIA

To measure gonadotropin quantitatively in ovine blood plasma a sensitive and reliable oLH RIA is required. The components of such a RIA will include: purified oLH for use as standard and labelled tracer, ovine plasma cleared of gonadotropin which serves as a vehicle to carry oLH standards in the development of a standard curve, an immuno-precipitation reaction and finally an anti-oLH sera of high specificity, titer and affinity.

The oLH used in this RIA was prepared and supplied by Dr. D.N. Ward. The purified oLH (DNW-9-109-5) has been assayed by Dr. Ward according to the procedure of Moyle and Ramachandran (1973) which involves testosterone production by isolated Leydig cells. Potency estimates were (Ward, personal communication, 1979):

\begin{align*}
2.15 \times \text{NIH-LH-S18} & \quad (95\% \text{ CL } 1.33-3.81; \lambda = 0.16) \\
2.72 \times \text{NIH-LH-S18} & \quad (95\% \text{ CL } 1.72-4.54; \lambda = 0.15) \\
\text{Mean potency estimate} & \quad 2.43 \times \text{NIH-LH-S18}
\end{align*}

Anti-oLH sera #15 was provided by Dr. G.D. Niswender; its preparation and characterization have been described
elsewhere (Niswender et al., 1968, 1969). According to the instruction of Dr. G.D. Niswender (personal communication, 1979) the #15 anti-oLH sera was used in the RIA at a final dilution of 1:40,000.

The RIA described below is a modification of the method outlined by Niswender et al. (1969). Disposable culture tubes (12x75 mm) were used for the RIA. Varying amounts of sample, 200 μl in this instance, or standard in 200 μl cleared plasma were placed in each tube and PBS-0.1% gelatin was added to bring the volume to 500 μl.

The lyophilized aliquot of #15 anti-oLH serum was reconstituted with 10 ml distilled water which then equalled a 1:400 dilution of antibody in 0.05 M EDTA-PBS. This was further diluted to 1:40,000 using 1:400 normal rabbit serum which had also been diluted in EDTA-PBS. The presence of EDTA in the reaction mixture will perform two functions; as a chelating agent it will minimize differences in complement and will also increase the total buffering capacity of the incubate. Immuno-precipitation is dependent to some extent on concentration of antigen (equivalent to the first antibody with its bound hormone). Since this concentration is extremely low, non-immune globulin of the same species as the first antibody (rabbit) must be added to ensure optimal immunoprecipitation (Midgley et al., 1969). This may be done at different times. Some
investigators have added carrier globulin with the second antibody, others add it as part of the diluent for the sample (Midgley et al., 1969); however, in this instance the non-immune rabbit globulin is added as part of the diluent for the first antibody (Niswender et al., 1969).

Two hundred microliters of the 1:40,000 anti-ovine LH serum were added to each assay tube, the contents mixed and incubated at 4°C for 24 hours. $^{125}$I-oLH, 50,000 cpm/100 μl, was then added to each tube. The LH-anti-LH complexes were soluble at the concentrations employed here therefore an immunoprecipitation step was required. This step utilized goat-anti-rabbit gamma globulin (anti-RGG) at a dilution which optimally precipitated the RGG. 200 μl of the anti-RGG stock solution was added to each tube and after 72 hours of further incubation 2.0 ml of cold PBS was added to dilute the unbound hormone radioactivity. This addition of cold PBS negates any wash step required (Midgley et al., 1969).

The antibody bound hormone was separated from the free hormone by centrifugation in the cold (0-2°C) at 1000 g for 30 minutes. The supernatant was poured off, the rim of the tube blotted and the precipitate counted.

Three critical areas in this oLH RIA, specifically protein iodination, anti-RGG titer and the use of gonadotropin free plasma will be discussed separately below.
D. Protein Iodination

The main objective in protein iodination is to achieve a high specific activity with minimal loss in immunological activity. When biological activity is considered the conditions of iodination become even more rigorous (Sonada and Schlamowitz, 1970; Leidenberger and Reichert, 1972; Reichert and Bhalla, 1974).

The iodination method employed for oLH and oFSH is a modification of the chloramine-T procedure described by Greenwood et al. (1963) for human growth hormone and is outlined in the appendix. FSH was iodinated according to McNeilly and Hagen (1974), LH according to Niswender et al. (1969). Both pituitary preparations iodinated in this fashion and subsequently purified by gel filtration through sephadex G-100 were shown to be suitable for use in the appropriate RIA without decreasing assay specificity or sensitivity (Niswender et al., 1969; McNeilly and Hagen, 1974).

E. Anti-RGG Titer Analysis

As stated previously, the oLH-anti-oLH complexes are soluble in the RIA system employed. Consequently, a method must be devised which will quantitate these
complexes. Several procedures for this purpose are described (Ransom, 1976); however, the system recommended by Niswender et al. (1969) for use in this particular oLH RIA employs an immunoprecipitation step involving anti-RGG. What remains to be determined is the optimal anti-RGG dilution in the RIA which will maximize the immunoprecipitation reaction.

$^{125}$I-oLH (50,000 cpm/100 µl) was added to a series of assay tubes containing 500 µl PBS-0.1% gelatin. This was followed with a 200 µl aliquot of the #15 anti-oLH serum (1:40,000) and a 24 hour incubation period at 4°C. 200 µl aliquots from serial anti-RGG dilutions were then added and the assay tubes again incubated 72 hours at 4°C. Serial dilutions ranged from stock to 1:1000 in 0.05 M EDTA-PBS, pH 7.0.

F. Preparation of Gonadotropin Free Plasma

To obtain a valid standard curve for use in the RIA described it is necessary to utilize ovine plasma, as a vehicle to carry standards, which has been cleared of endogenous gonadotropin. The use of cleared plasma will ensure that endogenous gonadotropin in the carrier plasma does not compound the effect of added oLH standards.
The use of plasma from hypophysectomized ewes has been demonstrated (McNeilly et al., 1976); however, the source and availability of this plasma proved an inconvenience. Rapid and readily accessible methods incorporate tissue membrane receptor preparations specific for gonadotropin and when incubated with plasma will bind and subsequently remove any endogenous gonadotropin.

The method described here is a modification of the procedure outlined by Haour and Saxena (1974) which incorporates differential and sucrose density centrifugation to purify gonadotropin receptor from bovine corpus luteum. The purified membrane receptor preparation was subsequently used according to Saxena et al. (1974); Saito and Saxena (1976).

Male rats of body weight 350-400 grams were sacrificed by CO₂ asphyxiation and the testes removed. The rat testes homogenate was prepared as described by Leidenberger and Reichert (1972), Reichert et al. (1973). The tunica albuginea was removed, the testes weighed and homogenized briefly on ice in assay buffer (1 g testes/2 ml buffer). The resultant homogenate was filtered through a double layer of cheese cloth to remove the seminiferous tubule fraction and the turbid, pink filtrate obtained is the RTH.
The RTH was subsequently re-homogenized and centrifuged 500xg for 20 minutes at 2-4°C. The supernatant was again re-centrifuged 15,000xg for 60 minutes at 4°C, the supernatant discarded, the pellet resuspended in 2.0 ml assay buffer and subjected to sucrose density centrifugation.

Of this pellet suspension 0.2 ml was layered onto the surface of a discontinuous sucrose gradient which covered a density range of 0.4-1.6 M sucrose in assay buffer. Ultra centrifugation proceeded at 55,000xg for 2.0 hours at 2-4°C. At the termination of this centrifugation 0.75 ml fractions were drawn off the bottom of the polyallomer centrifuge tube, each fraction diluted x5 with Tris-HCL Buffer and centrifuged 3000 rpm for 15 minutes at 4-8°C. The pellet was resuspended in the original volume of Tris-HCL and the wash step repeated three times. A 200 μl aliquot of each fraction was subsequently incubated with ^{125}I-oLH (20,000 cpm/100 μl) and 200 μl Tris-HCl containing 0.1% BSA at 37°C for 45 minutes.

The pooled membrane receptor preparation was analyzed for protein content according to the trinitrobenzenesulfonic method of Snyder and sobocinski (1975, see appendix). Aliquots equivalent to 0.0, 0.5, 1.0, 1.5, 2.5, 5.0, 10.0 and 50.0 mg protein were centrifuged
and the pellet incubated with 1.0 ml ovine plasma as outlined by Haour and Saxena (1974), Saito and Saxena (1976). After a 30-minute incubation period at 37°C the plasma-receptor mixture was centrifuged for 15 minutes at 2500 rpm and 4°C. The plasma was withdrawn and assayed for LH content with the oLH RIA of Niswender et al. (1969) previously described.
EXPERIMENTAL RESULTS AND DISCUSSION

1. PROCEDURAL DEVELOPMENT

A. Protein Iodination  (Refer to Appendix II)

Upon completion of the iodination reaction the next objective is purification of the labelled hormone. This is accomplished with filtration through sephadex-G100 which separates the iodination mixture into labelled hormone, damaged protein and free iodine fractions. A typical elution pattern for iodinated gonadotropin is demonstrated in Figure 16.

For each hormone two peaks are readily discernible. The first represents $^{125}$I-gonadotropin while the second indicates free $^{125}$I. The system implemented in this study involved a 1x10 cm sephadex column with a flow rate of 40 ml/hr. which characterized oFSH and oLH with elution volumes ($V_e/V_o$) of 1.53 and 1.68 respectively.

These values differ from Reichert et al. (1968), who in their study employed a system which consisted of a 2x90 cm column, 8.0 ml/hr. flow rate and produced elution volumes for hFSH and hLH of 1.65 and 1.78 respectively.
Figure 16: Elution profiles of 125-oLH (○-○) and 125 I-oFSH (●-●).
B. Anti-RGG Titer Analysis

The anti-RGG dilution which will yield the maximum $^{125}\text{I-OLH}$-anti-OLH bound dpm represents the optimal immunoprecipitation reaction in the OLH RIA previously described. The titer analysis curve is presented in Figure 17 and from it one can determine the anti-RGG dilution for the immunoprecipitation step. Maximum $^{125}\text{I-OLH}$ dpm bound and precipitated is achieved when 200 μl stock (undiluted) anti-RGG are added to each RIA tube.

C. Preparation of Gonadotropin Free Plasma

The first step in this procedure employs discontinuous sucrose centrifugation to purify LH receptors from the rat testes homogenate. Fractions were drawn from the bottom of the centrifuge vial in volumes which facilitated collection of an interface into each fraction. This approach was used since cellular material concentrates at the interface between each sucrose layer in discontinuous sucrose gradient centrifugation.

Figure 18 depicts the sucrose density profile of these purified fractions for binding $^{125}\text{I-OLH}$. Fractions 6-8 inclusive demonstrated maximum $^{125}\text{I-OLH}$ bound and were used in all subsequent steps of this procedure.
Figure 17: Titer analysis to determine the optimal anti-RGG dilution for use in the oLH RIA.
Figure 18: The sucrose gradient elution profile for 125-I-oLH.
Aliquots containing increasing quantity of purified membrane receptor were then incubated with 1.0 ml of ram plasma to determine that amount of receptor which effectively binds all detectable endogenous LH (see Figure 19). Maximum $^{125}$I-oLH bound to immunoprecipitated antibody, which represents minimum competition with endogenous LH, is achieved with approximately 10 mg protein receptor preparation. Additional aliquots of ram plasma were incubated with purified membrane receptor at a concentration of 10 mg protein/1.0 ml plasma. The resultant standard curves which compare normal and cleared ram plasma appear in Figure 20.

In evaluating standard curves regression analysis according to the method of Bliss (1952; 1967) requires the use of the term lambda ($\lambda$). Lambda is defined as $S_{y \cdot x}/b$ hence $\lambda$ approaching zero will indicate either a decreasing standard error of the estimate ($S_{y \cdot x}$), an increase in the slope ($b$) or a combination of the two. $\lambda$ provides a mechanism for comparisons between assays by evaluating the parameters of precision and sensitivity in a given system. Regression analysis performed in this manner (Bliss, 1972, 1967) yields $\lambda$ values for normal and cleared ram plasma of 0.1168 and 0.1072 respectively.
Figure 19: 125-I-oLH bound to receptor plotted against the quantity of receptor used to adsorb oLH per ml of plasma.
Performing the Logit transformation (Feldman and Rodbard, 1971) where

\[ \text{Logit } B/B_0 = \ln \left( \frac{(B/B_0)/(1-B/B_0)} \right) \]

will produce the curves illustrated in Figure 21. Parallel line analysis on the transformed data demonstrate equal slopes for each regression line \((F_{0.05(1)}, 1, 4.71; \text{ probability } = 0.971)\). Further tests for equality of elevation show that the regression lines are derived from separate and distinct data sets \((F_{0.05(1)}, 1, 5.61; \text{ probability } = 0.014)\).
Figure 20: The comparison between normal (○) and gonadotropin cleared (●) plasma.
Figure 21: Equality of slope between normal (o) and cleared (●) plasma.
2. TREATMENT EFFECTS

A. Estrus Synchronization

If one accepts the argument that intrinsic mechanisms are responsible for the initial secretion of FSH and estrogen to initiate the ovarian cycle then prevailing endocrinological conditions upon pessary removal should have a critical effect upon the ensuing return to estrus with ovulation. One factor which could contribute to the overall endocrinological pattern is the occurrence of circulating phyto-estrogens and/or metabolites from estrogenic forages consumed.

In both the control and experimental groups all ewes generally exhibited behavioral estrus 2-4 days after sponge withdrawal. This result is in close agreement with literature values (Robinson et al., 1967). Ewes which consumed estrogenic alfalfa deviated somewhat from the control ewes. When compared to the control ewes, these ewes demonstrated an onset of behavioral estrus which occurred over a less stringent time period. Of the 5 ewes fed alfalfa one exhibited behavioral estrus 8 hours after sponge withdrawal while another failed to show behavioral estrus through the 5-day period after pessary removal.
B. Exfoliative Vaginal Cytology

The characteristic cell patterns described in the human female (Papanicolaou, 1954) have been applied to the estrous cycle of the ewe (Sanger et al., 1958 a,b). This study again attempted to characterize the estrous cycle of the ewe with the methodology of Papanicalaou (1954) and Sanger et al. (1958) but with the addition of KI values and their interpretation as outlined by Wachtel (1969).

KI values were obtained from 4 ewes which consumed orchard grass hay and 4 which consumed alfalfa cubes (Figure 22). In each case all ewes exhibited an initial increase and plateau in the KI values within a period 3-10 days prior to estrus. This region is followed with a period of rapid increase in the KI to peak levels which corresponded to estrus. The KI values subsequently drop to low levels with ovulation and formation of a functional corpus luteum.

Upon closer examination one can see that KI values from ewes which consumed the alfalfa used in this study demonstrate a slower rate of descent 0-3 days post ovulation. This decreased rate of descent becomes more
Figure 22: The karyopyknotic index obtained from ewes which consumed (A) orchard grass hay and (B) alfalfa.
pronounced during the period 3-5 days from estrus. This result would seem to indicate that greater estrogenic stimulation has occurred in ewes fed estrogenic alfalfa and the effect is maintained through a period of low endogenous estrogen levels. Such a period is encountered between ovulation and the establishment of a functional corpus luteum 0-6 days after estrus (Robertson, 1977). Sanger et al. (1958b), Folman and Pope (1966) and Newsome and Kitts (1980) have shown phyto-estrogens to demonstrate estrogenic activity when endogenous estrogen levels are minimal.

A critical feature of the normal ovarian cycle is the abrupt change from the proliferative to secretory phase which indicates ovulation and subsequent formation of a functional corpus luteum. This transition leads to the characteristic cell patterns described by Papanicolaou (1954), Sanger et al. (1958) and Wachtel (1969). These cell patterns were observed during this study and are described below.

Smears collected during the estrus period are typically clear and contain individual discrete squamous cells with pyknotic nuclei (Figure 23). The cytoplasm of these cells is transparent with a slight pale blue hue. Vaginal mucus observed during this period appears thin, copious and transparent.
Figure 23: Photograph of exfoliated cells characteristic of the estrus period (x 400).
With the onset of metestrus there is a transition from transparent pyknotic cells to keratinized eosinophilic squamous cells. These cells are large flat squames which appear folded and often appear in dense cell masses. The cells observed here are the type (ii) cornified eosinophilic squames described by Sanger et al. (1958). As metestrus progresses the vaginal mucus-cellular material collected for the smear becomes thicker and opaque-white in colour. The cells are now cyanophilic and indicate the onset of diestrus.

Diestrus is associated with the secretory phase of the estrous cycle consequently the cell pattern is indicative of progesterone secretion. The smears are characteristically heavy and composed of cyanophilic intermediate squamous cells with thickened cell borders and folded edges. The cytoplasm lacks transparency and the cells form thick dense masses (Figure 24). Many neutrophiles are also discernible during this period.

The ovarian cycle is completed with proestrus, a period which indicates a cessation of the secretory phase and start of the proliferative phase. Smears taken during proestrus are characterized by decreasing cell numbers and thin clearer mucus, a result of increasing estrogen secretion.
Figure 24: Photograph of exfoliated cells characteristic of the diestrus period (x 400).
C. LH Plasma Profiles

In this study peak LH levels were determined to be within the range 23.5-46.5 ng/ml and 50.4-83.2 ng/ml for ewes fed orchard grass hay or alfalfa cube respectively (Figures 25 and 26). Luteinizing Hormone levels were undetectable through the secretory phase which indicates plasma LH levels less than 0.5 ng/ml, the lower limit for sensitivity in the RIA employed.

These values are in close agreement to the oLH levels previously demonstrated. The oLH concentrations during the cycle have been reported as: 2-3 ng/ml (Geschwind and Dewey, 1968), less than .5-2 ng/ml (Niswender et al., 1968), 2.9±.9 ng/ml (Goding et al., 1969), .5-2.2 ng/ml (Niswender et al., 1969) and 2.6 ng/ml (Pant et al., 1977). Peak levels of oLH have been reported as: 80 ng/ml (Geschwind and Dewey, 1968), 25-73 ng/ml with a mean of 47 ng/ml (Niswender et al., 1968), mean of 26 ng/ml (Niswender et al., 1969) and 75.3±7.4 ng/ml (Pant et al., 1977).

Discrepancies between results in this study and those cited from the literature can be attributed primarily to samples which are obtained from different regions of the LH peak. For this reason mean peak LH levels were not obtained for comparison between treatments.
Figure 25: Plasma LH levels in ewes fed orchard grass hay with supplement. (arrow indicates onset of estrus).
Figure 26: Plasma LH levels in ewes fed alfalfa cubes (arrow indicates onset of estrus). The time from onset of estrus to LH peak is significantly longer than the control group ($F_{cal} > F_{05(1,8)}$).
The results illustrated in Figures 25 and 26 indicate that plasma LH levels peak with the onset of estrus in ewes fed orchard grass hay, however, ewes fed estrogenic alfalfa demonstrated plasma LH peaks which occurred 0-24 hours into the estrus period. Pre- and post-LH peak levels were also elevated and fluctuating in ewes fed orchard grass hay when compared to corresponding periods in ewes fed alfalfa.

One must be critical of this comparison as it appears the LH peak is out of synchronization with behavioral estrus in ewes which consumed alfalfa. As a consequence the bleeding schedule may have in fact missed the pulsatile release of LH prior to the LH peak in these animals.

Niswender et al. (1968), Goding et al. (1969) and Pant et al. (1977) have reported that the LH peak occurs within a 0-16 hour period from the onset of estrus and generally has a 12-hour duration.
GENERAL DISCUSSION AND CONCLUDING REMARKS

The work of Tang and Adams (1978), Rodgers (1979) and Mathieson (1979; 1980) has demonstrated on $E_2$-17$\beta$ receptor-coumestrol or genistein interaction in both the hypothalamus and pituitary. These results implicate the hypothalamic-hypophyseal axis as a possible site where phyto-estrogens can exert an effect upon control of the ovarian cycle and consequently upon fertility in temporary and permanent infertile 'clover infected' ewes.

Rodgers et al. (1980) found permanent clover-infertile ewes to demonstrate significantly higher plasma LH levels than fertile ewes during anestrus and breeding seasons. Clover fertile ewes exhibited LH secretion intermediate to that of control and clover-infertile ewes. This relationship prevailed even after the ewes had been removed from estrogenic pasture for several years (Rodgers et al., 1980). Further results reported by Rodgers et al. (1980) indicate no significant difference in plasma FSH levels between fertile and infertile ewes during anestrus or the breeding season.

Results from this study were obtained from ewes fed alfalfa of low-moderate phyto-estrogenic activity (Table 3) which did not facilitate the occurrence of
infertility. Mathieson (1979), however, demonstrated that a phyto-estrogenic content of this level had the capability of competing with $\text{E}_2$-$17\beta$ at the tissue receptor level. The results here do in fact demonstrate aberrations in the estrous cycle and associated LH secretory pattern between control and experimental groups.

Mean peak plasma LH levels of ewes fed alfalfa appear elevated when compared with control ewes which consumed orchard grass hay ($66.4\pm16.8$ ng/ml versus $40.1\pm5.55$ ng/ml respectively). This would agree with Rodgers et al. (1980); however, this difference between mean peak LH levels may be attributed to sampling different regions of the plasma LH peak. Rodgers et al. (1980) attribute the elevation in LH levels to estrogenic clover interference with the negative feedback of $\text{E}_2$-$17\beta$ upon LH secretion.

Perhaps of greater importance is the demonstration of a delayed LH peak (Figures 25 and 26). This indicates a tendency toward abnormal LH synthesis and/or secretion. Using the two pool hypothesis for LH synthesis and release (Figure 9B) described by Justiz (1971) and Yen (1977), the results may become clearer.

During periods of low $\text{E}_2$-$17\beta$ levels coumestrol, genistein and their metabolites may actually augment the action of $\text{E}_2$-$17\beta$ in its induction of LH synthesis in pool
2 and transfer to pool 1. As endogenous $E_2-17\beta$ levels increase through the follicular phase of the estrous cycle, the phyto-estrogens may gradually become antagonistic to the action of $E_2-17\beta$ which could result in decreasing LH production and transfer into the releasible pool.

As the follicular phase progresses the plant estrogens may also impede the action of GnRH which is augmented and facilitated by $E_2-17\beta$ at both the hypothalamic and pituitary level. The eventual outcome is a detectable LH peak which occurs later in the estrus period. The discussion presented above could also account for the apparent absence of, or reduced, fluctuations in plasma LH levels prior to the LH surge and afterwards in ewes which consume alfalfa. This aspect would indicate an "all or none" response required to overcome the antagonistic effects of phyto-estrogens. Rodgers et al. (1980) also describe a period where LH secretory patterns are not well defined during the breeding season of clover infertile ewes.

If the apparent increase in LH of 'clover' infected ewes is accepted, the LH autoregulation cycle in the rat as outlined by Barraclough et al. (1979) would also explain the reduced fluctuations in LH levels after the LH peak. The mechanism implicated involves an LH negative feedback
loop from the pituitary to the catecholamine pathways controlling the MPOA-hypothalamic activity and GnRH release.

Barraclough et al. (1979) and Turgeon (1979) used the rat to demonstrate a critical aspect of the E2-17β-LH interrelationship. They have shown that a rapid drop in E2-17β levels during late proestrus influences the secretory pattern and magnitude of the LH surge. If this decrease in peripheral E2-17β is prevented the magnitude of the LH peak is decreased. It was anticipated in this study that the phytoestrogen concentration in the ewe would be sufficient to mask any rapid decrease in endogenous E2-17β resulting in a relationship similar to that described by Turgeon (1979). Results from Rodgers et al. (1980) indicate this is not the case and while the results of this study support this view the levels of phyto-estrogen in the alfalfa ration consumed were not of sufficient magnitude to induce a response which would prove or disprove this concept.

The KI values demonstrate an estrogenic effect of phytoestrogens once ovulation has occurred and endogenous E2-17β levels are low. The KI values of the experimental group fall to the base levels of control ewes 6-7 days from the morning of estrus which also indicates normal luteal formation and function.
In summary, permanent infertility can be characterized by impaired ova and sperm transport and elevated LH levels which are an indication of hormonal imbalances which have desensitized the hypothalamus to estrogenic control mechanisms (Hearnshaw et al., 1972; Findlay et al., 1973; Rodgers et al., 1980).

Temporary infertility involves subtle aberrations within the hypothalamo-pituitary axis which may prove cumulative in nature and result in variable fertility problems. Using alfalfa with a modest phyto-estrogen concentration results from this study demonstrate apparent elevated plasma LH levels which coincide with clover-fertile ewes described by Rodgers et al. (1980). Results presented in this study also indicate phyto-estrogen consumption will prolong the estrus period by delaying the LH surge further into estrus. This characteristic indicates that normal estrus, primarily between onset and ovulation, and the LH surge are not synchronized. This may ultimately increase the incidence of prolonged estrus, abnormal estrus, estrus without ovulation, atypical folliculogenesis, impaired luteal function and consequently anomalous hormonal profiles which further disturb the delicate control mechanisms within the hypothalamo-hypophyseal-ovarian axis.
A CRITIQUE

Through the course of this study considerable effort was required to overcome two important aspects of the RIA employed. The areas of concern were development of the double antibody-immunoprecipitation step and development of LH-free plasma as a carrier for the LH standards. With reference to the immunoprecipitation step the quality, availability and cost of commercially prepared immunoglobulins necessitates development of a procedure to provide precipitating antibody.

To conduct further studies of this nature several conditions should be modified. Individual animals should serve as their own controls. This can be accomplished with the animals maintained on their control diet and changed to the experimental ration. The bleeding schedule should be rigorous, a 15-minute interval between sampling would be appropriate. In addition to this, blood should be collected from the juglar vein and ovarian artery and assayed for both estrogen and LH levels. This approach would accurately establish the LH peak and its relation to ovarian estrogen.


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APPENDIX I

SPECIFICITY OF #15 ANTI-oLH SERUM

By using ovine pituitary preparations containing various levels of TSH, FSH, LH, prolactin and growth hormone, Niswender et al. (1969) demonstrated that the radioimmunoassay estimates of LH potency were consistent with bioassay estimates [ovarian ascorbic acid depletion tests (OAAD)]. In all preparations tested the indices of discrimination (OAAD LH/RIA LH) approached unity, this suggests that FSH, TSH, growth hormone and prolactin do not affect the estimation of LH potency by the radioimmunoassay employed in this study (see Appendix Table 1).

a As presented in Niswender et al. (1969).
<table>
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<th>PREPARATION</th>
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<th>FSH U/mg</th>
<th>LH OAAD U/mg</th>
<th>LH RIA U/mg</th>
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* From Niswender et al. (1969).
APPENDIX II

PROTEIN IODINATION

- 1 mCi Na-\(^{125}\)I-iodide
- 25 \(\mu\)l of 0.2 M phosphate buffer
- 5.0 \(\mu\)g oLH/25 \(\mu\)l 0.05 M phosphate buffer
- 40 \(\mu\)g chloramine-T/25 \(\mu\)l 0.05 M phosphate buffer

Agitate 2.0 min (finger tap)

- 240 \(\mu\)g sodium metabisulfite 30 \(\mu\)l/0.05 M phosphate buffer
- 1 \(\mu\)g potassium iodide/100 \(\mu\)l 0.05 M phosphate buffer made 16% sucrose

This entire reaction mixture was layered onto a 1 cc x 10 cc column of sephadex-G 100 equilibrated in 0.05 M phosphate buffer and pre-saturated with 1.0 ml of 50 mg BSA/ml 0.05 M phosphate buffer followed by a 20 ml wash. Elution was conducted with 0.05 M phosphate buffer. One ml fractions were collected into 1.0 ml of 0.05 M phosphate containing 0.2% gelatin. Aliquots of each fraction
were counted and the $^{125}$I-protein peak diluted to 50,000 cpm/100 μl PBS containing 0.1% gelatin.

Protein iodination requires ionization of iodine ($I_2$) to higher oxidative states of 0 or +1 to produce active $H_2OI^+$ molecules which react with protein residues. Reactions with protein are generally irreversible involving interactions between $H_2OI^+$ and the tyrosine residues, some histidine residues and sulfhydryl groups of the protein. Reactions with -SH groups occur much more readily but do not result in stable bonding of iodine. Consequently in any labelling experiment the first iodine consumed, equivalent to -SH content, must be considered lost as depicted by the following equation (Hughes, 1957):

$$H_2OI^+ + R-SH + RSI + H^+ + H_2O$$

$$RSI + R-SH + RS-SR + H + I \text{ (Unusable iodide)}$$

As a consequence the basis of all tagging experiments with iodine involve substitution into the tyrosine residues as depicted below (Hughes, 1957; 1966).
Chloramine-T is an oxidizing agent which facilitates the conversion of iodide (I\(^-\)) to iodine (I\(_2\)) and subsequently to H\(_2\)OI\(^+\), presumably by the following overall equation (Hughes, 1966):

\[
\text{CH}_3\text{SO}_2\text{NHCL} + 2\text{I}^- \rightarrow \text{CH}_3\text{SO}_2\text{NH}^+ + \text{Cl}^- + \text{I}_2
\]

Chloramine-T is also capable of regenerating iodine from the iodide produced when iodine disassociates in solution according to the following equations (Hughes, 1957; 1966):

\[
\text{I}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{OI}^+ + \text{I}^-
\]

\[
\text{I}_2 + \text{OH}^- \rightarrow \text{IOH}^- + \text{I}^-
\]

\[
\text{HOI} \rightarrow \text{H}^+ + \text{OI}^-
\]

\[
3\text{I}^- \rightarrow \text{IO}_3^- + 2\text{I}^-
\]
As it is an oxidizing agent, the use of chloramine-T does create certain disadvantages. Changes within the protein will occur since some of the iodine formed may cycle between the reagent and protein alternately decreasing the reagent and oxidizing the protein. The protein molecule is also susceptible to attack directly by chloramine-T.

As a consequence, the oxidative nature of chloramine-T will impose strict conditions on the actual iodination reaction. This aspect is particularly evident when LH and FSH are to be iodinated for use as a labelled ligand in tissue receptor assays where biological (rather than immunological activity) is critical for optimal competitive binding to receptor. The requirements involve the protein: Chloramine-T ratio, reaction temperature and reaction time prior to addition of sodium metabisulfite (Leidenberger and Reichert, 1972; Reichert and Bhalla, 1974). Excess protein oxidation and $^{125}$I substitution may result in greater specific activity of labelled protein however % nonspecific binding increases, % specific binding decreases and % biological activity retained after iodination drastically decreases (Reichert and Bhalla, 1974).

Sonada and Schlamowitz (1970) conducted detailed studies of parameters which effect trace iodination of immunoglobulin G with iodine-125 and chloramine-T. Conditions
of oxidant, iodide and protein concentration as well as pH, ionic strength, temperature, exposure time and protein purity all affect the overall iodination and subsequent protein integrity. Sonada and Schlamowitz (1970) determined a pH 7.0-7.5 and 2°C temperature optimal for formation of active iodine \( \text{H}_2\text{OI}^+ \) and its incorporation in protein. Oddly enough, Leidenberger and Reichert (1972) as well as Reichert and Bhalla (1974) utilized iodination procedures incorporating pH 7.5 and 2-3°C temperature to maintain the biological activity of the protein in question.

Several alternative procedures are available for protein iodination; McFarlane's hydrogen peroxide or monochloride methods (1956). The Bolton and Hunter reagent which utilizes an \( ^{125}\text{I} \)-labelled acylating agent (1973) and the enzymatic radiiodination of gonadotropins by a system composed of lactoperoxidase and hydrogen peroxide as described by Miyachi et al. (1972) and Pinto et al. (1977). These methods provide a milder iodination reaction which does not predispose the protein molecule to damage by oxidation. The chloramine-T method in contrast, is rapid, combines high efficiency with simplicity and consequently is the most widely used.
In the event of protein damage proper measures must be taken to ensure a pure homogenous iodinated hormone preparation is used in assays. Purification of the iodinated protein, which also separates free unbound iodine-125, can be achieved through several methods. A common procedure involves gel filtration through sephadex-G100 (Reichert and Bhalla, 1974; McNeilly et al., 1976), through Bio-Gel P60 columns (Niswender et al., 1969) or cellulose Whatman CF 11 ion exchange resins (McNeilly et al., 1976).

Assessment of protein damage, which would indicate further purification or reiodination of fresh protein material, can be achieved through several methods. Hunter (1969) describes three such procedures; the first involves the separation pattern of labelled hormone, damaged labelled hormone and free iodine-125 on Whatman 3MC chromatography paper, a modification consists of using a small column of cellulose (Whatman CF 11) to absorb intact iodinated gonadotropin (specifically LH) and so separate it from damaged $^{125}\text{I}}$-LH, $^{125}\text{I}}$-iodide and other reactants. Third and finally the use of polyacrylamide gel electrophoresis to determine the homogeneity of $^{125}\text{I}}$-FSH.

According to Sonada and Scholamowitz (1970) iodinated proteins vary widely in their ability to retain native state and function in vivo and in vitro depending
on the number of atoms of $^{125}$I introduced, side effects due to reacting with oxidant and radiation damage by $^{125}$I. The iodination method implemented for oLH and oFSH are modification of the chloramine-T procedure described by Greenwood et al. (1963) for human growth hormone. FSH was iodinated according to McNeilly and Hagen (1974), LH according to Niswender et al. (1969). Both pituitary preparations iodinated in this fashion and subsequently purified by gel filtration through sephadex-G100 were shown to be suitable for use in appropriate radioimmunoassays without decreasing specificity or sensitivity.
APPENDIX III

TRINITROBENZENESULFONIC ACID (TNBS) METHOD FOR DETERMINING AMINES

Materials

Buffer  0.10 M sodium tetraborate (Na₂B₄O₇·10H₂O), pH 9.3

Reagent  TNBS [2,4,6-trinitrobenzenesulfonic acid, (NO₂)₃C₆H₂SO₃H]

Procedure

Twenty-five mg of TNBS was dissolved in 10 ml of 0.10 M sodium tetraborate buffer, pH 9.3. Twenty microlitres of this reagent was then added to a known aliquot of unknown in 200 μl of buffer. The vials were mixed (finger tapped), held at room temperature for 45 minutes and the analyzed for colour development (A₄₅₀).

a From Snyder and Sobocinski (1975).
APPENDIX IV

ESTRUS SYNCHRONIZATION: BACKGROUND INFORMATION

The classical approach for estrus synchronization simulates prolongation of the luteal phase through the use of a suitable progestagen (progesterone or one of its analogues). The use of progestational agents has resulted from three critical results; Dutt and Casida (1948) found that daily progesterone injections given to cyclic ewes for 14 days inhibited both estrus and ovulation, removal of the progestagen resulted in a return to estrus approximately 3 days later. Dutt (1952) and Robinson (1952) next administered progesterone followed by a PMSG injection to seasonally anestrous ewes which induced coincident estrus and ovulation. Finally, it was demonstrated that progesterone may be taken up from the vagina (Shelton and Moore, 1967; Shelton and Robinson, 1967a).

Development of a technique which involved a progestagen created four specific problems: type of progestagen, route of administration, dosage and endocrinological (physiological) response. Progesterone and several analogues have been evaluated on their ability to inhibit estrus, and most important their ability to release this
suppressive effect upon progestin removal to permit estrus coincident with ovulation (Shelton and Robinson, 1967b). Progesterone, Provera (17α-acetoxy-6α-methylpregn-4-ene-3,20-dione; MAP) and SC-9880 (17α-acetoxy-9α-Fluoro-11α-hydroxypregn-4-ene-3,20-dione; Fluorogestone Acetate; cronolone) have proven the most effective (Robinson and Moore, 1967; Robinson et al., 1967; Haresign, 1978). SC-9880 appears indistinguishable from progesterone in all criteria with a biological activity 20-25 times as potent. This in conjunction with earlier estrus following SC-9880 versus MAP has proven SC-9880 the progestational agent of choice and is the active ingredient in the commercially prepared synchro-mate pessaries (Robinson and Moore, 1967; Robinson et al., 1967; Shelton and Moore, 1967).

Oral, intramuscular injection, implants and intravaginal pessaries are the primary routes of administration (Gordon, 1975), however, individual animals are not guaranteed an adequate daily intake of progestin when given orally and injection requires daily manipulation of the animals. As a consequence intravaginal sponges have become the method of choice when synchronizing ewes (Robinson, 1965, Robinson and Moore, 1967; Robinson and Smith, 1967; Wishart, 1967).
Dose and method of progestagen administration are critical factors when release of estrus, ovulation and ensuing fertility are considered (Haresign, 1978). Low doses of SC-9880 (10 mg/sponge) resulted in poor estrus synchronization when pessaries are removed. This is related to the pattern of progestin absorption, most is absorbed during the first few days after insertion, consequently, the daily rate of absorption in the final days is insufficient to exert a full negative feedback. With low dose pessaries during the final days of insertion there may also be a sub-optimal amount of steroid required for maximum expression of subsequent estrus and fertility (Morgan et al., 1967; Haresign, 1978). Morgan et al. (1967) state on the average sixteen per cent of steroid present at any time will be absorbed over a twenty-four hour period.

Increasing the level of synthetic progestin over a defined limit (40 mg SC-9880) soon has an overall deleterious effect (Shelton and Moore, 1967). Release to estrus with ovulation is impaired as is sperm survival and transport, all of which decrease ewe fertility. These effects are attributed to circulating endocrine abnormalities which result primarily in LH release significantly earlier than onset of estrus and increased cervical mucus (Rexroad
and Barlo, 1977; Haresign, 1978). The optimal and most efficient dose of SC-9880 appears to be within the range 20-40 mg/pessary and inserted fifteen to eighteen days (Robinson et al., 1967; Robinson and Moore, 1967; Robinson and Smith, 1967a). Haresign (1978) found conception rate and proliferacy increases by eight per cent and twelve per cent respectively as the SC-9880 dose increases from 30 mg up to 40 mg.

PMSG treatment (750 I.U. in 5.0 ml saline, subcutaneously) zero-twenty-four hours after pessary removal has demonstrated the ability to increase the incidence of estrus, estrus with ovulation, conception and proliferacy (Robinson and Smith, 1967; Gordon, 1975; Haresign, 1978). With a PMSG injection, it appears a lower level of SC-9880; 10-20 mg/pessary, can be used without adverse affects (Moore and Holst, 1967; Robinson and Smith 1967b; Christenson, 1976).

The prevailing endocrinological conditions upon pessary removal have a critical effect upon the ensuing return to estrus, ovulation with estrus and conception rate as stated previously. Time till onset of estrus after SC-9880 pessary withdrawal occurs in a does dependent manner. The greatest incidence of estrus associated
with ten, twenty and forty mg SC-9880 occurred twenty-nine, thirty-four - forty-eight and forty-eight - fifty-three hours respectively after pessary removal (Robinson and Smith, 1967 a). Non-returns to service also increased from 18.4% to 41.7% as the dose of SC-9880 increased from 20 mg to 40 mg respectively (Robinson and Smith, 1967 a). Wishart (1967) demonstrated 95% of all ewes treated with SC-9880 impregnated pessaries (20 mg, 30 mg, 40 mg & PMSG) demonstrated estrus within the first 5 days, Robinson et al. (1967) found 85% of all SC-9880 treated ewes (20-40 mg) exhibited estrus between days 2 and 4 after pessary removal while Shelton and Robinson (1967) found intramuscular injections of progesterone and SC-9880 induced ewes to show estrus 1-4 days after the last injection.