ESTROGEN AND PHYTO-ESTROGEN BINDING IN EWE PITUITARY, HYPOTHALAMUS, AND OTHER STRUCTURES

by

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We accept this thesis as conforming to the required standard

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Phyto-estrogens are known to bind to estrogen receptors of the uterus and they can initiate the early events of estrogen stimulation including water imbibition and synthesis of induced protein as well as late events such as uterine growth. There is indirect evidence that these compounds affect the functioning of the hypothalamus and pituitary with respect to the release of gonadotropins. The purpose of this study was to determine if the phyto-estrogen compounds, genistein and coumestrol, could interact with the estrogen receptor molecules in the cytoplasm of pituitary and hypothalamus tissue from ewes. Estrogen binding characteristics were also examined. Cytosol preparations from the various experimental tissues were incubated fifteen minutes at thirty degrees C. with $^3$H-estradiol; separation of bound from free label was carried out on Sephadex LH-20 columns. Estrogen binding parameters were determined by double reciprocal plots. Competitions with $^3$H-estradiol in the presence of either coumestrol or genistein were carried out in a similar manner. Apparent inhibition constants ($K_i$) were determined from Dixon plots.

The apparent dissociation constants ($K_D$) for estradiol in ewe pituitary cytosol was determined to be $0.26\pm0.12$ nM. The apparent $K_i$ for coumestrol in the ewe pituitary cytosol was determined to be 59-61 nM and the apparent $K_i$ for genistein was determined to be 130-210 nM. These compounds were shown to displace estradiol from receptors in ewe hypothalamus and amygdala cytosols. Results of preliminary binding experiments with ewe pineal and uterus cytosols are also presented.

These results suggest that phyto-estrogens can interfere with normal estrogen feedback mechanisms with respect to gonadotropin release in the ewe.
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INTRODUCTION

The female reproductive system with its many hormonal interrelationships has been the subject of study for many years. The elucidation of the molecular action of hormones has led to intensive research on the complex interplay and metabolic effects brought about by these endogenous messengers. A series of delicate checks and balances operate to coordinate the reproductive system and modulate hormonal effects. The hormonal relationship between ovary and anterior pituitary has long been recognized (Moore and Price, 1932). Studies of many workers have demonstrated a further complexity of the gonadal-pituitary relationship in that feedback mechanisms are mediated through the brain, in particular, the hypothalamic area which processes impulses from sensory centres. The hypothalamus exerts control over the adenohypophysis by means of releasing hormones with the essential link being a portal vascular system. Therefore the hypothalamus provides a major link between the endocrine system and neural stimuli from the external and internal environments and has been termed a neuro-endocrine transducer. An intricate system relying on chemical compounds to convey feedback messages is vulnerable to interference by compounds which mimic the effects of endogenous hormones. Such might be the case of compounds termed phyto-estrogens which are found in plants and which can cause estrogen effects when ingested by animals. This is possible because they contain certain chemical groupings similar to steroid estrogens which are recognized by intracellular estrogen receptors. If these compounds bind to the estrogen receptors of pituitary and hypothalamus they could act either as estrogen antagonists against endogenous estrogen or as estrogens when endogenous estrogen levels are low.

It was the purpose of this study to determine if these compounds do bind to estrogen receptors in ewe hypothalamus and pituitary. This problem
has been approached using competitive protein binding techniques and standard methods of binding analysis. Other tissues were examined for comparative purposes and as a check on the assay system and analysis used.

REVIEW OF LITERATURE

A. THE PITUITARY IN FEMALE REPRODUCTION

The pituitary gland is extremely complex both in structure and function. Through its hormones, the pituitary has direct effects on growth, metabolism, conservation of water, reproduction and lactation as well as an indirect influence on body metabolism through hormone effects on the adrenal cortex and thyroid (Holmes and Ball, 1974). Considerable confusion has arisen regarding nomenclature for the various subdivisions of the pituitary due to the great species variation involved. The terms anterior and posterior pituitary, commonly used in the past, are not satisfactory for all species and tend to be misleading.

A generally accepted division is on the basis of embryonic origin of the various tissues composing the pituitary gland. The neurohypophysis which forms the neural component of the gland is derived from neural ectoderm and consists of the pars nervosa or infundibular process, and the infundibular stem and median eminence which together form the hypophyseal stalk. The glandular portion of the pituitary is the adenohypophysis which arises from buccal ectoderm. It is composed of the pars distalis, the pars tuberalis, and the pars intermedia, which is present only in some species. The pars distalis forms the major portion of the adenohypophysis and constitutes eighty percent or more of the total pituitary gland weight (Martin, 1976). This area has no direct arterial blood supply and receives blood via a portal
system which arises from capillaries in the median eminence of the neural component of the pituitary (Daniel and Prichard, 1975; Page and Bergland, 1977). The pars distalis secretes a number of hormones, two of which are directly involved in the reproductive process. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) together form the gonadotropins, so named for their stimulating effect on the gonads. The gonadotropic hormones control the maturation and release of ova directly and indirectly control the development of secondary sex characteristics, reproductive cycles, and fertility, by stimulating the secretion of steroid sex hormones.

There are a number of distinct cell types in the pars distalis and attempts have been made to relate particular cell types to certain hormones. While this has to some extent been possible, controversy exists regarding gonadotrophs, the cells which produce the hormones LH and FSH. Based on histological staining characteristics, the cells designated δ-(delta) basophils have been shown to be gonadotrophs. Some workers have identified two separate and distinct cell types within this division and have termed them folliculotrophs (FSH producing) and luteotrophs (LH producing) (Schulster, et al., 1976; Holmes and Ball, 1974; Costoff, 1973). Others maintain there is just one type of gonadotroph which can be influenced by extracellular conditions (Franchimont, 1977; Martin, 1976). Evidence for this view is based in part on electron microscopic-immunochemical techniques which show FSH and LH to be present in the same cell (Nakane, 1975). As yet the question of whether the distinct subgroups of gonadotrophs are different cell types, or functional variants of the same cell type, remains unresolved (Farquhar, et al., 1975; Costoff, 1977). A recent study on the ovine pituitary with antibodies to intact LH, and to each of the subunits, α and β, of LH has identified the LH producing cells, which are located near capillaries. The
authors did not localize FSH however, they do suggest their technique for future study to determine if FSH is also located in the LH producing cells of the sheep pituitary (Dacheux and Dubois, 1978).

In the context of this discussion the term pituitary is used to mean the pars distalis part of the adenohypophysis and to indicate that portion of the pituitary gland which is primarily concerned with reproduction.

**B. THE HYPOTHALAMUS IN FEMALE REPRODUCTION**

The hypothalamus is that area of the brain which by definition lies under the thalamus, however its limits are not clearly defined (Daniel and Prichard, 1975). It is generally accepted to encompass the area from the optic chiasma to the mammillary body and includes the tuber cinereum and by some workers, the median eminence and infundibulum. It forms the walls and floor of the lower part of the third ventricle of the brain and its upper border is the hypothalamic sulcus. The lateral boundaries of the hypothalamus are less well defined, and are taken as the optic tracts, cerebral ganglia, and other structures (Martin, et al., 1977; Daniel and Prichard, 1975).

The neurosecretory or hormone producing cells of the hypothalamus, are true neurons in that they develop action potentials, have synaptic vesicles and are dependent on neuroglial elements. There is an extensive nerve fibre system in the hypothalamus for rapid communication within the hypothalamus and between the hypothalamus and other parts of the central nervous system. Neuron cell bodies aggregate in bundles termed nuclei, and various nuclei have been assigned roles in hypothalamic function. This may be an inaccurate designation as the functions of a given region often extends beyond the anatomic margins of the nuclei (Martin, et al., 1977).

The anterior-preoptic hypothalamus appears to regulate the activity of other areas of the hypothalamus. The suprachiasmatic preoptic region or
medial preoptic area seems to be involved with circadian and cyclic reproductive rhythms, including the induction of ovulation. The effects on ovulation are mediated through the basal arcuate area of the hypothalamus. The medial preoptic area receives neural inputs from extrahypothalamic structures of the limbic and midbrain system. Fibres from the anterior-preoptic hypothalamus form the preoptico-tuberal tract which terminates in the area of the arcuate nucleus of the ventromedial (medial basal) hypothalamus. These fibres must be intact in order for the preovulatory surge of LH to occur. The arcuate nucleus which forms the very basal (tuberal) hypothalamus appears to be involved in the tonic secretion of gonadotropins. The medial basal hypothalamus is considered to be the location of releasing and inhibiting-factor producing neurons (Wüttke, 1976). The arcuate nucleus and adjacent area have clusters of dopaminergic fibre projections into the median eminence. These form the tuberoinfundibular (tuberohypophysial) nerve tracts which terminate in the external layer of the median eminence at the capillaries which form the hypophysial portal blood vessels. There is strong evidence for synaptic linkage between these nerve tracts and afferents arising in the preoptic area. Axonal terminals from noradrenergic and possibly serotonergic fibres from mesencephalic structures are also found in the median eminence (Wüttke, 1976). These monoaminergic fibres may modulate activity of releasing hormone neurons but their role is unclear. Fibres from the fornix go to the arcuate nucleus and anterior lateral area as well as to the mamillary body via the hippocampo-hypothalamic tracts. The medial mammillary nucleus of the posterior (caudal, mammillary) hypothalamus is an area of many efferent and afferent pathways and is involved among other things in the mediation of behaviour related to sexual activity, however, it has no known influence on endocrine function (Martin, et al, 1977).
Although the hypothalamus is involved in coordinating many functions in the animal, for the purpose of this discussion only those factors directly involved in reproduction will be discussed.

C. THE PITUITARY-HYPOTHALAMUS RELATIONSHIP

Much evidence has accumulated suggesting a relationship between the hypothalamus and adenohypophysis and ultimately gonadal function (Harris, 1972; Anderson and Haymaker, 1974; Daniel and Prichard, 1975; Dörner, 1976; Fink, 1976; Martin, et al., 1977; Donovan, 1978a). Observations that the pituitary was not autonomous in maintaining cyclic gonadal function, coupled with observations of the effect of external factors on reproduction, led to the implication that the pituitary was under control of the central nervous system. The nature of this control was postulated many years before it was elucidated.

The neurohumoral concept, also called the portal vessel chemotransmitter hypothesis postulated that substances (neurohumoral agents) from the hypothalamus passed by a neural and vascular pathway to control the release of anterior pituitary hormones. This concept is now well established and a factor responsible for the release of LH and FSH from the adenohypophysis has been isolated, characterized as a single decapeptide, and synthesized (Schally, et al., 1971; Matsuo, et al., 1971; Guillemin, 1974). As yet, it appears there is only one factor responsible for the release of both gonadotropins and it is termed variously LH-RH, LH/FSH-RH, or GnRH (gonadotropin releasing hormone) (Reichlin, et al., 1976; Convey, 1973).

The neurovascular link as outlined above forms the critical pathway from the nerve cells of the hypothalamus to the glandular cells of the pars distalis. Daniel and Prichard (1975), investigating the vascular link, have determined that specific regions of the pars distalis receive blood from particular areas in the neurohypophysis (median eminence region) and further that, "The area
in the pars distalis that is fed by any individual portal vessel is strictly circumscribed. These observations are of special interest in relation to the fact that the various types of cells in the pars distalis tend to be grouped in particular regions of the lobe”.

The lack of evidence for a separate releasing hormone for FSH implies that the separation of FSH and LH release by the adenohypophysis must be attributed to differences in the pituitary mechanisms of LH and FSH secretion (Fink, 1976; Vale, et al., 1977). As previously mentioned, there is evidence for only one type of gonadotroph, and therefore the existence of only one releasing hormone would appear reasonable. This aspect will be discussed in a later section.

GnRH also seems to have biological actions within the central nervous system and may affect sexual behaviour (Moss and McCann, 1973; Pfaff, 1973a; Moss and McCann, 1975; Donovan, 1978b). GnRH is found in the pineal, midbrain, and cerebral and cerebellar cortices and brain stem, as well as in the hypothalamus and pituitary. It has an effect on the electrical activity of CNS neurons and is considered a neurotransmitter agent by Wilber, et al., (1976), who state; "(hypothalamic hormones)...may also subserve central nervous system function in the role of synaptic modulators".

D. EXTRAHYPOTHALAMIC STRUCTURES IN REPRODUCTION

Observations of the effect that external factors have on ovulation and other aspects of reproduction suggested the influence of higher neural centres on hypothalamus function. This evidence includes the physical stimulation of the genital area leading to ovulation in animals that are induced ovulators. Psychological stress can lead to amenorrhea in women. The presence of visual olfactory, and auditory stimuli can induce or postpone ovulation (Dörner, 1976). Light plays an important role in initiating reproductive cycles and there is
evidence that the pineal gland may be involved (Lisk, et al., 1972).

I. The Limbic System

The limbic system is anatomically defined to include the medial part of the mesencephalic reticular formation (midbrain limbic system), the hypothalamus, the hippocampus, the septum, the amygdala, and the cingulate and pyriform cortex (Martin, et al., 1977). The main connecting pathway is the medial forebrain bundle, a multi-neuronal, multi-synaptic system. There are no direct sensory inputs into the limbic system with the exception of the retinohypothalamic connection to the suprachiasmatic nucleus. Sensory inputs are probably relayed through the reticular formation of the brain stem and thalamus (Wilber, et al., 1977). The amygdala sends projections from its corticomedial nuclei through the stria terminalis to the septum, medial preoptic hypothalamus, and the external border of the ventromedial nuclei of the hypothalamus. From the basomedial amygdala, the projections of the ventral amygdalofugal pathway pass to the lateral hypothalamus. However the termination and function of this pathway is unresolved (Ellendorf, 1976; Martin, et al., 1977). Stimulation of the amygdala evokes an excitatory response in neurons of the ventromedial nuclei (Wilber, et al., 1976).

The fornix forms the efferent projection of the hippocampus. It has a major distribution to the mammillary body, and also direct inputs into the arcuate and ventromedial nuclei of the hypothalamus. Some of these seem to terminate directly on the tuberoinfundibular neurons (Lammers and Lohamn, 1974). There is evidence that afferent impulses to the medial basal hypothalamus are required to trigger the preovulatory discharge of gonadotropins (Taleisnik and Beltramino, 1975). The amygdala area has been the most studied and there is the indication of both a facilitatory and an inhibitory influence on gonadotropin secretion depending on the area stimulated. The facilitatory
impulses are transmitted via the stria terminalis. The inhibitory pathway is unknown (Taleisnik and Beltramino, 1975). Observations suggest two antagonistic systems; the mesencephalo-hippocampal system which has an inhibitory effect on gonadotropin secretion, and the mesencephalo-amydaloid system which has a facilitatory effect on gonadotropin secretion (Taleisnik and Beltramino, 1975). The limbic-midbrain regions appear to be involved in the onset of puberty. Electrical stimulation of these areas can alter pituitary hormone secretion (Ellendorf, 1976).

The hypothalamic and extrahypothalamic pathways are assumed to mediate the circadian rhythms of hormone secretion, stress induced alterations in hormone secretion, integration of neuroendocrine activity with autonomic nervous system responses, and neuroendocrine effects triggered by olfactory and peripheral sensory responses (Martin, et al., 1977).

II. The Pineal Gland

The pineal gland is a true endocrine gland of neural origin (from neuroepithelium) but is not a neuroendocrine organ, as is the hypothalamus, because synthesis of its secretory products takes place in organ specific cells, the pinealocytes, and not in neurons (Kappers, et al., 1974; Kappers, 1976). The function of the pineal has not been fully elucidated but as Reiter (1976) states: "If any functional position of the pineal gland seems well established, it is probably that of the control of seasonal reproductive events in some mammals". It is evident that light acts through the pineal gland to synchronize reproduction with the seasons. The mechanisms involved are not fully clear. External lighting generates impulses which pass through the accessory optic tracts via sympathetic nerve fibres to the pineal (Kappers, 1976; Wurtman and Moskowitz, 1977). The response to light and dark changes is reflected in a parallel rhythm in enzyme activity concerned with indolamine biosynthesis.
In some manner, the pineal acts as an intermediary between seasonal photo-periodic changes, and the neuroendocrine-reproductive axis (Reiter, 1976). The main function of the pineal appears to be antigonadotropic in that it suppresses reproductive activity. Pineal gland activity is inversely related to the functional status of the gonads in seasonal breeders. The secretory products of the pineal are of two types. The indolamines, especially melatonin, appear to have antigonadotropic activity. However, the pineal also appears to secrete polypeptides with antigonadotropic activity. There two products may interact to produce overall effects (Reiter, 1976). It appears that in the sheep, melatonin is secreted into the systemic blood stream (Rollag, et al, 1978). The pineal effect seems to be on the hypothalamus to influence the secretory activity of the pituitary possibly by affecting synthesis and/or release of GnRH. Reiter (1972) speculates: "it is interesting that hypothalamic inhibitory factors...have not been demonstrated for FSH and LH although the existence of releasing factors (or factor) for these hormones are (is) certain. Perhaps the pineal provides the important neuroendocrine means for inhibiting these reproductively essential hormones".

However, Schally, et al, (1973a), comments that although both releasing and inhibiting factors are known for growth hormone, melanocyte stimulating hormone and prolactin, these hormones do not stimulate their target cells to produce products which could act as auto-regulatory or "feed-back" agents. Adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), LH and FSH, for which inhibiting factors have not been demonstrated, all stimulate their target cells to produce hormones which, carried in the blood stream, can exert a feedback effect on the production and/or release of either the hypothalamic releasing hormones or the pituitary hormones or both.
E. THE HYPOTHALAMIC-HYPOPHYSIAL OVARIAN RELATIONSHIP

The reciprocal relationship between the pituitary and gonads has long been recognized and the mechanism of negative feedback of sex steroids on the release of gonadotropins was formulated in 1932 (Moore and Price, 1932; reviews, Hutchinson and Sharp, 1977; Dörner, 1976). This closed negative feedback loop, or push-pull system is based on observations that removal of the pituitary results in cessation of ovarian function. Removal of ovaries leads to increased gonadotropin synthesis and secretion, and to pituitary hypertrophy. The injection of estrogen to castrates leads to decreased gonadotropin release (Lisk, et al, 1972).

The gonadotropins FSH and LH, separately, and together, bring about maturation of the ovarian follicle, ovulation, and formation of the corpus luteum. In addition, these hormones stimulate steroidogenesis in the ovaries.

It is difficult to assign specific roles to these hormones as they appear together in vivo and, in physiological conditions, they probably act synergistically in many instances. Generally, FSH is assigned the role of transforming ovarian follicles into Graafian (preovulatory) follicles and, with LH, stimulating estrogen synthesis by the theca cells. Both hormones may have a role in ovulation although LH appears to predominate. Postovulation both FSH and LH initiate luteinization and formation of the corpus luteum, the function of which is maintained by LH. LH also stimulates progesterone secretion (Sherwood and McShan, 1977).

The steroid hormones, estrogens and progestagens, are secreted into the systemic blood stream and plasma levels of these two hormones show alternate cyclic patterns correlated with the various stages of the estrous cycle.

The progestagen produced by the corpus luteum of the ewe is almost exclusively progesterone although there may be small quantities of 20 \( \alpha \)
dihydroprogesterone and 17α-dihydroxyprogesterone (Baird, 1977).

Estrogen is a general term to encompass numerous compounds which bring about all or some estrogenic effects (*vide infra*). The endogenous estrogens of the ewe, produced by the theca cells in the ovary, are the steroids estradiol 17β and, to a much lesser extent, estrone (Baird, 1977). The ovine ovary also secretes large amounts of androstenedione, peaks being correlated with estradiol secretion (Scaramuzzi, *et al.*, 1974). It appears that androstenedione may play a role in the control of basal LH secretion, possibly through peripheral conversion to estrone (Scaramuzzi and Martensz, 1975).

Estrogens produce diverse effects which can be divided into genital and non-genital categories (Briggs and Brotherton, 1970). The genital effects of estrogen include the stimulation of growth and function of the uterus, ovaries, cervix, fallopian tubes, vagina, external genitalia and mammary gland through stimulation of mitosis and protein synthesis.

The non-genital effects of estrogens include the development and maintenance of secondary sex characteristics, anabolic effects such as increased nitrogen retention, sodium retention, and calcium and phosphorus deposition. Of major importance to this discussion are estrogen feedback effects on cyclic gonadotropin secretion, and effects on the central nervous system in provoking behavioural estrus (Heap and Illingworth, 1977; Briggs and Brotherton, 1970; Herbert, 1977).

Although the reciprocal action of gonadal steroids on gonadotropin release is well established, the site/sites at which the feedback occurs is/are still unclear. The bulk of evidence points to a dual system in which gonadal steroids act directly both on the pituitary and in the brain. Evidence that estrogen exerts a negative feedback effect on, i.e. inhibits, gonadotropin secretion is based in part on castration studies in which removal of ovaries results in
hypersecretion of gonadotropins and hypertrophy of the pituitary. Dörner (1976) postulates that gonadotropin function is kept within physiological limits by negative feedback of estrogen. Implanting estrogen into the median eminence of intact animals leads to ovarian atrophy. It is considered that a tonic secretion of gonadotropins is maintained by estrogen action at the medial basal hypothalamus (Canong, 1977).

The situation regarding the negative feedback of estrogen during parts of the estrous cycle in the intact animal is less clear and is brought into question by Pelletier and Thimonier (1975) and Hauger, et al, (1977) on the basis that estrogen level changes parallel LH changes for portions of the cycle. It is evident that 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 physiological events.

During the early follicular phase, a tonic secretion of GnRH, regulated by the basomedial (tuberoinfundibular) region of the hypothalamus, occurs which stimulates the adenohypophysis to release gonadotropins. FSH predominates and follicular maturation is induced (Dörner, 1976; Ladosky and Wandscheer, 1975). At this time the hypothalamic content of LHRH, and the pituitary LH content, are lowest (Stelmasiak and Cumming, 1977).

In the late follicular, preovulatory phase, the positive feedback effect of estrogen on the preovulatory LH surge and on behavioural estrus, is clearly established.

Scaramuzzi (1976) passively immunized ewes against endogeneous estradiol-17β and noted the following effects. Almost one-third of the treated ewes did not ovulate although they showed normal estrous behaviour. Their ovaries contained large atresic follicles with marked hypertrophy of the theca interna, and a non-existent granulosa layer. Of those ewes that did ovulate, about one-
half also showed the same follicular abnormalities. Treated ewes also had many new corpora lutea indicating an increased number of developing follicles. These observations correlate with evidence that estrogen is involved in a negative feedback loop on pituitary gonadotropin secretion. Interference with this feedback resulted in hypersecretion of FSH and LH, with a resulting hypersecretion of estradiol as indicated by the hypertrophy of thecal cells. Also of interest in this study was the separation of estrogen effects on ovulation from those on estrous behaviour, indicating that the threshold for estrogen to induce estrus is lower than that involved in negative feedback on gonadotropin release, and also lower than that required to induce the preovulatory LH surge, a positive estrogen feedback effect.

When ewes were actively immunized against estradiol$\cdot$17$\beta$ there was complete absence of estrous behaviour (Cox and Wilson, 1976; Rawlings, et al, 1978; Pant, et al, 1978). Ewes actively immunized against estrogen had large Graafian follicles, some of which were haemorrhagic (Rawlings, et al, 1978). Plasma LH levels appeared to parallel the increase in serum antibody titre (Rawlings, et al, 1978) and elevated LH levels were lowered by a high dose of stilboestrol dipropionate (Pant, et al, 1978). FSH levels showed only minor oscillations and differential effects to immunization (Pant, et al, 1978).

The corpus luteum is associated with the absence of ovulation and estrus. Formation of the corpus luteum results in increased progesterone secretion which suppresses the cyclic LH secretion via negative feedback (Hendricks and Mayer, 1977). Prolactin may also be required for corpus luteum secretory activity (Robertson, 1977). Progesterone may also regulate tonic LH secretion by altering the frequency of pulsatile release (Yuthasatrakol, et al, 1977). The negative feedback of progesterone on tonic LH release allows pituitary LH stock to build up (Pelletier and Thiemonier, 1975).
At luteal regression, progesterone levels fall and thereby expose the pituitary to rising estrogen levels rendering the stored LH releasable (Stelmasiak and Cumming, 1977). In the late follicular phase, the final maturation of follicles takes place and the increasing estrogen secretion exerts a positive feedback on the female differentiated, amygdalar-hypothalamus system (Dörner, 1976). The preovulatory surge of gonadotropins evoked by rising estrogen levels requires an intact hypothalamohypophyseal unit. It appears that the positive feedback effect of estrogens on the preovulatory LH peak is facilitated by preceding progesterone levels. This may be due to induction of receptor synthesis which will be discussed in a later section. Stelmasiak and Cumming (1977) postulate that high progesterone levels facilitate a "priming effect mechanism" on the pituitary, which, by preventing LH secretion by estrogen waves during the luteal phase, allow the build up of pituitary LH stores. It appears that progesterone as well as estrogen is responsible for the LH peak and that steroids most likely interact with neurotransmitter-regulating mechanisms at extra- and intra-hypothalamic levels (Wüttke, 1976).

The preovulatory surge of gonadotropins could be a result of estrogen action directly on the brain to cause changes in the discharge of releasing hormones or directly on the pituitary gonadotrophs to alter gonadotropin release either by altering secretory activity or by causing changes in sensitivity to hypothalamic releasing hormones (Finn and Booth, 1977). The hypothalamo-hypophyseal portal system presents technical difficulties for sampling to determine the pattern of GnRH release. Sampling of ewe jugular blood to determine the GnRH-like immunoreactivity in plasma, Jutisz, et al. (1973) found the highest level (6ng/ml) during the preovulatory surge of LH and FSH. These workers did not detect GnRH (i.e. less than 0.5 ng/ml) in plasma from outside the estrous period. Crighton, et al. (1973) sampling
at estrus on a more frequent basis, found that GnRH showed a pulsatile release pattern with peak intervals of 1.5 to 6 hours. Foster, et al, (1974, 1976) sampling ewes via jugular cannulae every two hours for twenty days in the breeding season detected GnRH peaks at various times of the cycle. Peaks increase in frequency near the end of the cycle with peaks detected before, during and after the preovulatory LH peak. After estrus, and at day ten of the cycle, GnRH peaks were unassociated with LH release. The halflife of GnRH is estimated at five minutes (Crighton, et al, 1973) however it may be even shorter (Foster, 1974). Although a single injection of GnRH results in an LH peak, the amplitude and duration of this peak is much less than that which occurs during the cycle. Multiple injections to imitate the episodic release of GnRH, resulted in an LH peak approaching that of a normal cycle, implying that it is the frequency of GnRH stimulation that brings about the preovulatory LH surge (Crighton, et al, 1974; Foster, et al, 1976).

There is now considerable evidence that gonadal steroids alter the response of the pituitary to GnRH (Geschwind, 1972; Aiyer and Fink, 1974; Reeves, et al, 1970; Schally, et al, 1973b). Exogenous estrogen or GnRH treatment shows greater effect on LH release at the onset of behavioural estrus than at any other time in the cycle (Reeves, et al, 1970). However the pituitary response to GnRH may be due to the plasma estradiol/progesterone ratio rather than a response to estrogen alone (Pelletier and Thimonier, 1975). The mechanisms by which estrogen exerts its effects on gonadotropins may involve protein synthesis but they are poorly understood (Mahesh and McPherson, 1977). Estrogen appears to have some stimulating effect on the binding capacity of GnRH receptors (Park, et al, 1976). A negative effect of estrogen on pituitary response to GnRH was not demonstrated by physiologic doses however pharmacological infusion completely inhibited pituitary LH/FSH response to GnRH.
Estrogen effects on extrahypothalamic and intrahypothalamic sites have also been demonstrated. Electrical stimulation of the medial preoptic area leads to gonadotropin release however the magnitude of the response is related to the stage of the estrous cycle, being greatest around estrus (Wüttke, 1976). Taleisnik and Beltramino (1975) state: "...ovarian steroids not only may alter the thresholds of excitability of extrahypothalamic structures but also may determine antagonistic effects on the hippocampus and amygdala".

Estrogens have been shown to influence enzyme activity in the pineal, and progesterone is antagonistic to the effect of estrogen. High doses of estrogen inhibit hydroxyindole-O-methyl transferase (HIOMT), a key enzyme in melatonin synthesis, and low doses of estrogen stimulate HIOMT activity. HIOMT activity is two fold higher at diestrus than at estrus in mature cycling rats (Preslock, 1977). Little is known about the feedback mechanisms involved (Kappers, 1976).

A summary of the various interrelationships discussed is illustrated below, adapted from Dörner, 1976. The positive and negative feedback loops are not shown.
F. ESTROGEN RECEPTORS

It became evident in studies of sex hormones that target cells must possess a mechanism for recognizing and distinguishing the various steroid hormones. The search for the recognition system, termed "receptors" was carried out on many fronts; greatly assisted by the technology of radioactive labelling of steroids to high specific activity enabling identification of hormones in very low physiological amounts. Initial studies involved injecting radiolabelled hormone, in vivo, and then locating the hormone distribution, the target organs being those showing selective retention (Jensen, et al, 1972). Further examination of distribution was carried out either in situ,
by autoradiography, or by subcellular analysis using density gradient centrifugation.

It is now well established that steroid hormones elicit target organ responses by interacting with cytoplasmic receptors, which are macromolecular proteins, to form a receptor-hormone complex. The complex undergoes a transformation process and travels to the nucleus where it interacts with the chromatin and brings about transcriptional changes and the formation of mRNA (reviews: Gorski and Gannon, 1976; Baulieu, et al, 1975).

The model system for estrogen action is the uterus. Many of the biosynthetic changes induced by estrogen acting on this system have been elucidated and in particular the synthesis of a specific protein, "induced protein" which is an early event in estrogen action (Katzenellenbogen and Gorski, 1975).

In order for a cellular component to be designated a receptor, it should exhibit certain characteristics among which are: (Clark, et al, 1977; Baulieu, et al, 1975)

1. finite or saturable binding capacity.
2. high affinity within physiological ranges, as circulating levels of estrogen are usually $10^{-9}-10^{-10}$ M. This implies a high affinity constant ($K_A$) of the receptor for estrogen.
3. specificity for particular steroid hormones or class of hormones.
4. tissue specificity in that target organs are the only ones stimulated.
5. correlation of macromolecular binding with a biological response.

Autoradiographic studies have localized estrogen in the pituitary and brain although there are different intensities in distribution throughout target tissues (Stumpf, 1970; Stumpf, 1971a; Stumpf, 1971b).
I. Pituitary Localization

Stumpf (1971a) found the $^3$H-estradiol concentrated not only in basophils, but also in acidophils and chromophobes of the pars distalis. No localization occurred in the pars intermedia and very little in the neurohypophysis. A combined autoradiographic-immunohistochemical technique by Keefer, et al, (1975) showed that most but not all gonadotrophs, as identified with anti-HCG, showed nuclear retention of estradiol.

II. Central Nervous System Retention

Stumpf (1970, 1971b) summarized the major areas of estrogen accumulation in neurons. They are concentrated in the preoptic area of the hypothalamus, the basal tuberal region of the hypothalamus, and in the central-posterior amygdala. Stumpf and Sar (1976) also include the olfactory lobe and tubercle and smaller portions of the septum and hippocampus. These areas correspond to those reported by Pfaff (1973b).

Stumpf and Sar (1976) suggest that differences in apparent uptake by cells in different tissues reflect differences in tissue response which they formulate as the "principle of differential hormone uptake and response threshold". Under physiological conditions certain target tissues may be stimulated while others are not (also Rosner, et al, 1972). They suggest such a differential uptake and response may be an important factor in regulating endocrine feedback systems and behavioural responses.

Estrogen receptors have been most extensively studied in the uterus, however similar proteins have been identified in the pituitary, hypothalamus and other tissues (Liao, 1975). Kato (1977) has recently reviewed the characteristics of steroid hormone receptors in the brain, hypothalamus and pituitary.

III. Estrogen Receptors of the Pituitary

Several workers have demonstrated that the anterior pituitary shows a

IV. Estrogen Receptors of the Hypothalamus

determined by gel filtration, density gradient centrifugation, displacement studies, etc., are similar to uterus and pituitary receptors (Eisenfeld, 1970; Kahwanago, et al., 1970).

V. Estrogen Receptors of Extrahypothalamic Brain Structures

It has been more difficult to demonstrate macromolecular receptors in other parts of the brain. Although Rosner, et al., (1972) did not isolate a specific receptor from the cerebral cortex, they found that estrogen significantly increased $^3$H-cytidine incorporation into RNA as well as increased protein synthesis. Ginsburg, et al., (1975b) determined that the amygdala contains one hundred times fewer binding sites than the pituitary.

Few studies on estrogen binding in the brain have included the pineal gland. Rosner, et al. (1972) found the uptake of estradiol by the pineal to be significantly higher on a per milligram wet tissue basis than that of the uterus in ovariectomized rats. Marks, et al. (1972) determined that the binding of $^3$H-estradiol by the macromolecular fraction of the pineal cytosol approached that bound by the anterior pituitary, and was about four times that bound by the hypothalamus on a "disintegration-per-minute per milligram protein" basis (DPM/mg. protein).

VI. Binding Sites and the Estrous Cycle

The uptake of $^3$H-estradiol by pituitary and hypothalamus varies with the stage of the estrous cycle (Kato, 1970a). It has been shown that the number of high affinity estrogen binding sites fluctuates with the cycle (Ginsburg, et al., 1975a). A correlation between estrogen receptor content and gonadotropin cyclicity was shown by Parker, et al., (1976) who found that the depletion of cytosol receptors was followed by the gonadotropin surge. The number of estrogen binding sites is related to the pituitary response to GnRH (Greeley, et al., 1975a,b). At proestrus the cytosol receptor content of the rat anterior pituitary
decreases forty percent, and then is replenished during late proestrus and estrus (Greeley, et al, 1975b). The pituitary response to exogenous GnRH during proestrus parallels the changes in the content of estrogen receptor in the cytoplasm and nucleus (Sen and Menon, 1978).

VII. Pituitary and Brain Receptors and the "Steroid Model"

The pituitary and brain estrogen receptors fit the steroid model of the uterus with respect to depletion and replenishment pattern (Cidlowski and Muldoon, 1974; Ginsburg, et al, 1975a). Although the appearance of "induced protein" has not been demonstrated there are some indications that protein synthesis is required for estrogen effects in these tissues (Schneider and McCann, 1970). The restoration of lordosis behaviour in rats requires twenty-four hours thereby implying that neurons are acting as steroid target cells, that is, responding to estrogen signals by biochemical events like protein synthesis (Beyer, 1976).

VIII. Receptor Universality vs. Tissue Specificity

Although the estrogen receptors of the pituitary and brain show many characteristics similar to uterine receptors there may be tissue specific characteristics as yet undetected. Ginsburg, et al, (1975a) found that the dissociation constants ($K_D$) for pituitary tissues were consistently lower than those obtained from hypothalamus and uterus tissue. The rates of reaction to reach equilibrium were also different. It took uterus and hypothalamus less than five minutes (30°C) while pituitary, cortex, and amygdala took more than ten minutes (30°C) (Ginsburg, et al, 1975b). Further evidence that pituitary receptors may be different is presented by Ginsburg, et al, (1976b) who estimated the affinity constants for eleven compounds using cytosols from five different tissues. The ranking orders were the same, however there were differences between the tissues in the absolute values. There was a tendency
toward higher affinity in the pituitary, significantly so for seven of the eleven compounds. It is speculated that in the pituitary there is an additional estrogen binding moiety of even higher affinity of such low quantity as to be undetected by Scatchard analysis. As yet, whether estrogen receptors are identical throughout the reproductive system remains unresolved. Evidence both in favour of, and against, tissue specificity of estrogen receptors is presented by Kato (1977).

IX. Structural Requirements for Binding

There are certain chemical characteristics of estrogenic steroids which are recognized by estrogen receptors in target cells. The binding of a hormone to the receptor to form an activated (transformed) receptor hormone complex, which then translocates to the nucleus, involves various types of chemical interactions between the protein receptor and steroid hormone. Hydrophobic interactions, hydrogen bonding, and steric factors all play a part in high affinity, specific binding (King and Mainwaring, 1974). An estrogenic steroid is mainly non-polar in nature and therefore hydrophobic interactions are important in binding, the specificity being determined by the spacial arrangements of polar substituents (Liao, et al, 1973; King and Mainwaring, 1974).

In order for a compound to exhibit estrogenic activity the presence of at least one aromatic ring is considered critical (Frieden, 1976). The binding affinity of a steroid is strongly dependent on the presence of a phenolic hydroxyl, and on substituents on ring D (Korenman, 1969). The highest affinity occurs for a steroid with a phenolic hydroxyl group on C 3 and an alcoholic hydroxyl at C 17 in a β-configuration (Hähnel, et al, 1973). A 17 β hydroxyl on ring D is common to both androgen and estrogen steroids (Busetta, et al, 1977). The presence of an oxygen function on ring D is important in binding, and its state of oxidation and position influences binding affinity (Hähnel,
et al, 1973). Binding affinity is reduced if the C 17 hydroxyl is changed from β to α configuration or if the 17 β hydroxyl is oxidized to a ketone, as in estrone (King and Mainwaring, 1974). Binding affinity may be enhanced by certain D ring substitutions such as in ethinylestradiol and estrone acetate (Korenman, 1969). The receptor binds the D ring loose enough to permit certain molecular flexibility (Hospital, et al, 1975), and may in fact form two hydrogen bonds with the 17 β hydroxyl (Busetta, et al, 1977). The difference between estrogenic and androgenic steroids occurs in the A ring, which is fully saturated or contains a C 4 – C 5 double bond in androgens, and is aromatic in estrogens (Busetta, et al, 1975). The free phenolic hydroxyl on ring A is essential and its position is of critical importance for estrogen activity (Hähnel, et al, 1973; King and Mainwaring, 1974). Hormone specificity is assigned to the A ring; however Busetta, et al (1977) speculate that it is only the oxygen substituent at C 3 and not the aromatic nature of the ring that is important. Removal of the C 3 hydroxyl blocks binding (King and Mainwaring, 1974) while C 3 methylation results in a reduction in binding affinity (Korenman, 1969; Geynet, et al, 1972; King and Mainwaring, 1974) and acetylation of the C 3 hydroxyl retains activity (Korenman, 1969). Substitutions on C 2 are inhibitory (Korenman, 1969). Addition of alkyl groups or oxygen functions to the steroid leads to less active compounds (Frieden, 1976; Korenman, 1969). A C 11 β hydroxyl or C 16 hydroxyl diminishes binding (King and Mainwaring, 1974; Korenman, 1969). Additional unsaturation on the estrogen steroid nucleus inhibits binding (Korenman, 1969; Hähnel, et al, 1973). C 7 α methoxylation (Geynet, et al, 1972) or the presence or absence of an angular methyl group on C 13 (Hähnel, et al, 1973) have little influence on binding.

In general, additional oxygen functions on ring D, additional substitutions on ring A, and unsaturation of ring B, decrease affinity (Hähnel, et al, 1973).
In order to bind to the estrogen receptor a steroid must possess two potential hydrogen bonding groups about 15 Å apart at either end of the molecule (King and Mainwaring, 1974). Hähnel, et al., (1973) consider that the steroid attaches to the binding site first through a hydrogen bond at the C 3 hydroxyl which may induce a stereospecific conformational change in receptor to permit the C 17 β hydroxyl attachment through a strong hydrogen bond at a less specific site. Busetta, et al (1977) however, consider the first attachment to be via the D ring with estrogenic or androgenic properties related to the orientation of the A ring substituents, a difference of approximately 3 Å. They state that a compound with a D type ring and with the same thickness of a steroid "would have the corresponding activity of an estrogen or androgen depending on the location of the terminal atom of the A ring". Korenman (1969) in studying various compounds found that, with some exceptions, the relative binding affinity of a compound to the uterine receptor parallels the uterotrophic properties of that compound.

X. Non Steroid Estrogens: Antiestrogen Properties

Non steroid compounds possessing some of the characteristics mentioned above also exhibit varying degrees of estrogenicity (Geynet, et al, 1972). A potent non steroid estrogen, diethylstilbestrol (DES), binds to the uterine estrogen receptor with equal or greater affinity than estradiol (Hospital, et al, 1972). A number of classes of compounds which can bind to the same, or part of the same, receptor binding site as does estradiol 17 β can interfere with or compete with estradiol for binding sites when both are present. The various classes of non steroid compounds with these properties have been reviewed by Geynet, et al., (1972), Frieden (1976) and others (Lunan and Klopper, 1975; Baulieu, 1976). Many of these compounds can also exhibit agonist, that is, estrogenic characteristics, often to a lesser extent than estradiol.
Because these compounds can interfere with normal estrogen action *in vivo*, they fall into the broad category termed antiestrogens. Antiestrogenic substances have been defined as compounds which interfere with any actions of an endogenous estrogen like estradiol \(17^\beta\) or estrone (Sankaran and Prasad, 1972). These agents can be subdivided further into two categories: a) those which have no affinity for estrogen receptors, that is, do not compete with estradiol for binding sites, and inhibit estrogen action through means other than the estrogen receptor mechanism; and b) those that interfere with estrogen action directly through the receptor mechanism by binding to the cytoplasmic receptor thereby inhibiting the uptake of estradiol (Rochefort, *et al.*, 1972). Examples of the former group, (a), are androgens, progestagens and enzyme inhibitors. Examples of the latter group, (b), are numerous, and in many cases these compounds mimic the biological actions of estradiol and are competitive inhibitors of estrogen binding in target tissues (Sankaran and Prasad, 1972). It is the latter type of compound that can bind to the estrogen receptors which is of interest to this discussion.

It is becoming increasingly evident that it is not the initial competition for estrogen binding sites in target tissue alone, but the subsequent chain of events which occurs, that determines the anti-estrogenicity of a compound (Clark, *et al.*, 1974; Cidlowski and Muldoon, 1976; Baulieu, 1976). In the target cell there are several potential mechanisms of action or points of interference by antiestrogens. These are outlined in Figure 1, (Jordan, *et al.*, 1978a). Competitive antagonism, 1, could occur for the cytoplasmic receptor. Such competition would be a function of the relative affinities and concentrations of estrogen and competitor. This possibility is considered a simplistic explanation of antiestrogen activity (Clark, *et al.*, 1974; Cidlowski and Muldoon, 1976; Jordan, *et al.*, 1978a).
Antagonists could inhibit the transformation, 2, to the activated receptor hormone complex. This mechanism has yet to be demonstrated.

It is possible that the translocation to the nucleus, 3, of the activated complex could be inhibited, however compounds with anti-estrogenic activity have been shown to induce translocation (Rochefort, et al, 1972).

It appears that there is not necessarily a common mechanism for all classes of antiestrogens and attempts have been made to classify groups of antiestrogenic compounds on the basis of length of nuclear retention (Clark, et al, 1977b) (see Table I).

a. long acting antiestrogens

The most studied group of antiestrogenic compounds is the triphenylethylene derivatives which includes clomiphene, tamoxifen, CI 628, and nafoxidine (see Appendix). These compounds have been termed "long acting" antiestrogens in that they cause abnormally long nuclear retention of the receptor complex (Clark, et al, 1977b). It was postulated that this retention could interfere with cytoplasm estrogen receptor resynthesis, 4, and/or replenishment (Clark, et al, 1974). However, these compounds also have long plasma half lives, e.g. tamoxifen $t_{1/2}$ is several days, and thus even if resynthesis of cytoplasmic receptors does occur, as indicated by Koseki, et al, (1977), little accumulation of receptors in the cytoplasm would result as there would be immediate translocation to the nucleus (Jordan, et al, 1977b). Thus the tissue becomes refractory to subsequent estrogen stimulation. Koseki, et al, (1977), suggest that the inability of the cell to process or remove the receptor/antiestrogen complex from the nucleus may somehow interfere with continuous uterine estrogen stimulation.

b. short acting antiestrogens

Of direct interest to this discussion are the compounds which have been
FIGURE 1: POTENTIAL MECHANISMS OF ACTION OF ANTIESTROGENS IN THE TARGET TISSUE CELL\textsuperscript{a}

<table>
<thead>
<tr>
<th>CYTOPLASM</th>
<th>NUCLEUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. estrogen receptor + estrogen</td>
<td>2. transformed receptor complex</td>
</tr>
<tr>
<td>4. receptor resynthesis (24 hours)</td>
<td></td>
</tr>
</tbody>
</table>

1. competitive antagonism
2. inhibition of transformation
3. inhibition of translocation
4. inhibition of receptor resynthesis

\textsuperscript{a}: as from Jordan, et al, 1978a.
<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</td>
<td>short (1-4 hr)</td>
<td>partial agonist/antagonist</td>
<td>early responses</td>
</tr>
<tr>
<td></td>
<td>DMS</td>
<td></td>
<td>when injected. Agonist when</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 oxo-estradiol</td>
<td></td>
<td>implanted.</td>
<td></td>
</tr>
<tr>
<td>2. long acting</td>
<td>A. estradiol</td>
<td>intermediate</td>
<td>Agonist</td>
<td>early and late responses</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>(6-24 hr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. triphenyl-ethyline</td>
<td>long acting</td>
<td>Agonist - one injection.</td>
<td>early and late responses</td>
</tr>
<tr>
<td></td>
<td>derivatives</td>
<td>greater than</td>
<td>Antagonist - multiple injections.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>eg. Nafoxidine</td>
<td>24-48 hrs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CI 628</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tamoxifen</td>
<td></td>
<td></td>
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</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 II and II activity

Classification based on events that occur after a single injection of the compound.

termed "impeded", based on uterotrophic effects (Terentius and Ljungkvist, 1972) or "short acting", based on subcellular retention (Clark, et al, 1977b).

These compounds bind to the cytoplasmic receptor and translocate to the nucleus, however their nuclear retention is of short duration, and although early events of estrogen stimulation are induced, later events are not, thereby demonstrating the need for sustained estrogen stimulation for full estrogenic effect (Lan and Katzenellenbogen, 1976). If these compounds are administered repeatedly, thereby sustaining plasma concentration for longer periods they can bring about full estrogen stimulation (Baulieu, 1976). Examples of compounds in this category are estriol (Lan and Katzenellenbogen, 1976) and dimethylstilbestrol (DMS) (Capony and Rochefort, 1977). Capony and Rochefort, (1977), using $^3$H-DMS determined directly that the receptor-DMS association rate was slower and dissociation rate faster than for the estradiol-receptor complex. DMS translocated to the nucleus, however the nuclear retention was short. Katzenellenbogen, et al, (1978), compared the actions of diethylstilbestrol (DES) and DMS. This study showed that both DES and DMS rapidly translocated receptor to the nucleus but nuclear receptor levels rapidly returned to control level by 6 hours with DMS while they remained elevated with DES. Methylation of DMS prolonged its effect and the methyl ether become much more like DES.

c. differences in target tissue responses

As indicated, the estrogenic or antiestrogenic properties of compounds have been characterized in the uterine system; depending on physiologic state, compounds can act either as agonists or as antagonists to estradiol. Few studies of antiestrogen action in other target tissues have been reported; however, there are indications that there may be tissue differences in response to these compounds. Cidlowski and Muldoon, (1976), studying DMS, CI 628 and
MER 25, (a triarylalkane estrogen antagonist with no agonist properties) in anterior pituitary, hypothalamus and uterus found the same order of binding inhibition for all tissues but the degree of inhibition varies with tissue, particularly with the long acting compounds. Luine and McEwen (1977) studied the effect of CI 628 on various estrogen-affected enzyme systems in the uterus, brain and pituitary. As an agonist CI 628 equalled estradiol in altering enzyme activity in the brain, (i.e. increased acetyl-CoA-:choline-O-acetyl transferase, E.C.23.3.1.6., CAT; and decreased monoamine:O$_2$ oxidoreductase, E.C.1.4.3.4, MAO), however was less effective (approximately one-half that of estradiol) on increasing G6PDH (D-glucose-6-phosphate:NADPH$^+$ oxidoreductase, E.C.1.1.1.49) activity in the uterus. CI 628 showed even less effect in the pituitary, in that, while estradiol doubled G6PDH activity, there was no significant change with CI 628. As an antagonist CI 628 did decrease nuclear estrogen binding in all three tissues, but the estrogen binding recovery times were different. The pituitary and uterus were slower to recover than the hypothalamus, preoptic and amygdala areas. Antagonist properties were different in each tissue. In the uterus and pituitary CI 628 attenuated the effect of estradiol on G6PDH activity whereas in the brain, CI 628 did not block estrogen changes to CAT or MAO. The authors concluded that CI 628 is a more potent agonist and lesser antagonist in the brain than in the pituitary or uterus. Etgen and Whalen (1978) indicate that DMS induces normal lordosis behaviour in rats at concentrations which are weakly estrogenic on uterine growth. DMS also inhibits compensatory ovarian hypertrophy at the same concentration. The effects of these compounds on receptor replenishment and nuclear binding in the brain and pituitary are not yet clear and it is not known if they act in the same manner as in the uterus.
G. GENISTEIN AND COUMESTROL

Two compounds which appear to act in a similar manner to estriol and DMS are genistein and coumestrol (Newsome and Kitts, 1979). They are representatives of the classes of compounds known as isoflavones and coumestans, respectively. These compounds are of particular interest as they occur in plants but can produce estrogenic effects in animals, (review: Farnsworth, et al, 1975), and have been termed phyto-estrogens.

I. Binding Properties

Genistein and coumestrol have been shown to bind to the uterine estrogen receptors of sheep (Shutt and Cox, 1972) and pregnant rabbits (Shemesh, et al, 1972). These compounds can induce estrogen-like activities in the uterus including water imbibition and incorporation of labelled precursors into protein, lipid and RNA (Notebloom and Gorski, 1963). They also bring about the synthesis of "induced protein" in the rat uterus, an early event characteristic of estrogen stimulation (Sömjen and Kaye, 1976). Recently these compounds have been shown to bind to estrogen receptors in human breast cancer cells and they can enhance tumor cell proliferation (Martin, et al, 1978). The binding ability of coumestrol coupled with its fluorescent characteristics has made this compound a tool in the study of binding properties of calf uterine estrogen receptors (Lee, et al, 1977).

Coumestrol shows greater affinity for the uterine receptor than isoflavones although still less than estradiol. The reported values for coumestrol range from twenty times (Shutt and Cox, 1972) to seventy times (Shemesh, et al, 1972) and genistein at 111 (Shutt and Cox, 1972) to 175 times (Shemesh, et al, 1972) less effective than estradiol in competitive studies. The ranking of various coumestan and isoflavone compounds on a relative binding ability basis confirms the importance of phenolic groups to binding affinity (Shutt and Cox, 1972,

The in vitro binding affinities of coumestrol and genistein are greater and closer together than was expected from their in vivo uterotrophic potencies (Shutt and Cox, 1972). On a uterotrophic basis coumestrol is 160 times and genistein 2500 times less effective than estradiol in rats (Perel and Lindner, 1970). These compounds have been shown to inhibit the uptake and utero-vagino-trophic response to estradiol (Folman and Pope, 1966, 1969; Shutt, 1967).

II. Presence in Plants

Plants containing phyto-estrogens have been implicated in infertility problems in grazing animals, in particular sheep (reviews: Bickoff, 1968a; Braden and McDonald, 1970; Cox and Braden, 1974; Shutt, 1976; Livingston, 1978). The isoflavones are the major phyto-estrogens in clover and the coumestans predominate in the medics (Braden and McDonald, 1970).

a. isoflavones

The isoflavones genistein and formononetin were isolated from subterranean clover (Trifolium subterraneum) following research into the causative agents of severe reproductive disorders in sheep grazing subterranean clover in Australia (Bradbury and White, 1951). The syndrome was characterized by symptoms of hyperestrogenic stimulation therefore estrogen-like compounds in the subterranean clover were suspected. The permanent infertility which resulted from long term grazing of estrogenic pastures became known as "clover disease". Other estrogenic isoflavones subsequently isolated from subterranean clover and red clover (Trifolium pratense) as well as other plants include biochanin A, diadzein and pratensein. Structural formulae are shown in Figure 2a.

For many years confusion existed regarding species different responses to phyto-estrogen compounds depending on route of administration (review: Morley, et al., 1968). After extensive studies on the metabolism of isoflavones in the
**FIGURE 2**

**a: ISOFLAVONES**

- GENISTEIN
- BIOCHANIN A
- DAIDZEIN
- FORMONONETIN

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>$R_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>OH</td>
</tr>
<tr>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>H</td>
</tr>
</tbody>
</table>

(CH$_3$ in 4'-methoxycoumestrol) $\rightarrow$ OH

**b: COUMESTANS**

**Coumestrol**

Others: Psoralidin, Lucernol, Trifoliol, Sativol, Medicagol, etc., (Bickoff, 1968).

*Figure 2: Structural Formulae of Phyto-estrogens*
sheep it was found that biochanin A and genistein are degraded in the rumen to
estrogenically inactive phenols, such as p-ethylphenol (Braden, et al., 1967).
Formononetin, however, is demethylated to diadzein and then metabolized to
equol (7,4'-dihydroxyisoflavan) and, to a much lesser degree, O-desmethyl-
angolensin, compounds which are estrogenically active (Shutt and Braden, 1968).
Equol is considered the agent responsible for "clover disease" in sheep grazing
pasture with high formononetin content (Shutt and Braden, 1968; Shutt, et al.,
1970).

b. coumestans

Reports that ladino clover (Trifolium repens) and alfalfa (Medicago sativa)
also contained estrogenic substances (Engle, et al., 1957; Coop and Clark,
1960) led to the discovery of coumestrol, a representative of a new class of
compounds, the coumestans (Bickoff, et al., 1957). Other coumestans include
trifoliol, 4'-O-methoxycoumestrol, medicagol, lucernol, sativol, 3-methoxy-
coumestrol and 11,12 dimethoxy-7-hydroxycoumestan (Bickoff, et al., 1969). The
major ones are coumestrol and 4'-O-methylcoumestrol. Structural formulae are
shown in Figure 2b.

Little is known about the metabolism of coumestrol; however 4'-O-methyl-
coumestrol is believed to be demethylated to coumestrol in the rumen. (Shutt,
et al., 1969). Absorption of coumestrol from the rumen takes place and little
or no inactivation occurs as reflected in unchanged plasma levels of conjugated
coumestrol (Kelly and Lindsay, 1978).

c. blood levels in animals

Although the phyto-estrogens and their metabolites show relatively low
affinity for estrogen receptors, their concentration in the blood stream can
be several orders of magnitude greater than endogenous estrogen levels (Shutt,
et al., 1967; Shutt, et al., 1970; Lindner, 1967). The greatest proportion of
blood phyto-estrogens are in a conjugated form as glucosiduronates which are considered inactive (Shutt, et al, 1967). A smaller proportion appears as sulpho-conjugates (Wong and Cox, 1971) and less as "free" or unconjugated: these forms being considered biologically active (Shutt, 1976). The plasma levels of "free" and sulpho-conjugated phytoestrogen can reach the nanogram per milliliter range (Wong and Cox, 1971; Shutt, et al, 1967; Braden, et al, 1971; Kelly and Lindsay, 1978) whereas endogenous estrogen levels are in the picogram per milliliter range.

d. infertility problems

Although severe clover disease is now rare due to changes in management practises (Lightfoot, 1974) subclinical fertility problems related to clover disease still exist (Adams, 1977b). Ewes, termed "clover-affected", show a lowered fertility rate believed related to the impairment of fertilization due to decreased sperm motility in the increased fluidity of cervical mucus (Cox and Braden, 1974). Attempts have been made to relate changes in cervical mucus to subfertility and to utilize it as a diagnostic tool, however the variable nature and observation that histologic changes in the cervix are not necessarily associated with the production of abnormal mucus, have so far limited this technique (Adams, 1977a, b).

Another form of reproductive problem is termed "temporary infertility" which occurs in ewes grazing estrogenic pasture prior to and during the mating season (Morley, Axelson and Bennett, 1964). Although having a marginal effect difficult to detect without close study, this type of infertility could nevertheless be important economically (Coop and Clark, 1960; Braden and McDonald, 1970; Scales, et al, 1977).

Of a temporary nature (Engle, et al, 1957) this infertility is characterized by a reduction in the number of ewes showing estrus (Lightfoot and Wroth,
1974; Coop and Clark, 1960; Clark, 1965) and delayed first detectable estrus (Engle, et al, 1957). There is a lowered fertilization rate (Lightfoot and Wroth, 1974; Engle, et al, 1957) and delayed first conception (Engle, et al, 1957; Coop and Clark, 1960; Clark 1965). While impaired sperm transport may be a cause, it is also possible that there is a reduction in ovulation rate (Lightfoot and Wroth, 1974; Wroth and Lightfoot, 1976; Scales, et al, 1977). This is also indicated by a reduced number of ewes twinning (Coop and Clark, 1960; Scales, et al, 1977).

Although most studies into the effects of phyto-estrogens have concentrated on changes in the reproductive tract there are indications that these compounds can interfere with the normal hormone balance between ovaries and hypothalamus/hypophysis.

III. Indirect Evidence for Effects on Hypothalamo-Hypophysis Area

Observations of estrus without ovulation in flocks grazing estrogenic pasture by Firth, et al, (1977) suggest that anovulatory estrus may be a significant contributing factor to the low fertility of clover-affected ewes. Kelly, et al, (1976) noted that some ewes displaying estrus had no recent corpus luteum when laparotomized. Ewes on phyto-estrogens showed follicular abnormalities such as numerous follicles, deficient antrum formation and signs of early atresia (Adams, 1977c; Kelly, et al, 1976). Adams (1976) indicated that there was a failure of ovarian compensatory hypertrophy in clover-affected ewes implying an alteration in hormone balance of the hypothalamus-pituitary-ovarian axis. Kelly, et al, (1976) noted that very high levels of coumestans inhibited the expression of estrus in ewes and suggested this was due to a lack of endogenous estrogen. Newsome and Kitts (1977) reported that ewes consuming forage containing phyto-estrogens had plasma estrogen levels lower and more uniform than controls suggesting an effect on gonadotropin secretion. Kelly, et al,
(1976) reported that histological examination of the pituitary of clover affected ewes showed basophils with relatively enlarged vacuolated nuclei and degranulated cytoplasm. Adams (1977c) also noted degranulation of the δ-(delta) basophils and cells hyperactive in appearance. Hearnshaw, et al, (1972) however, found that there were no consistent changes in the pituitaries of clover-diseased ewes confirming the observations of Gardiner and Nairn (1969). Examination of the hypothalamus revealed groups of shrunken hyperchromatic neurons (Adams, 1977c; Gardiner and Nairn, 1969) possibly associated with the development of permanent infertility in ewes. Hearnshaw, et al, (1971) noted that the plasma LH concentration of ovariectomized ewes rises after ingesting estrogenic subterranean clover similar to the LH response to stilbestrol injection. Hearnshaw, et al, (1972) reported that the estrogen evoked LH release response in ovariectomized ewes is inhibited in ewes with clover disease. Extending these observations, Findlay, et al, (1973) infused clover-diseased ovariectomized ewes with GnRH and did provoke an LH surge thus demonstrating that the pituitary was capable of releasing LH and therefore the failure of estradiol to provoke the LH surge was due to interference with the capability of the hypothalamus to respond to estrogen.

There have been few reports on the effects of isolated phyto-estrogen compounds on the hypothalamus or pituitary. Leavitt and Wright (1965) attempted to determine the role of a plant estrogen on the feedback mechanism of the pituitary of mice and reported that coumestrol simulated the effects of estradiol in the inhibition of gonadotropin release determined by histological examination. Leavitt (1965) determined that the coumestrol increased the number of gonadotrophs in the pituitary of mice before it had uterotrophic effects. Coumestrol also appears to produce persistent anovulatory estrus in adult female rats when injected as neonates (Leavitt and Meismer, 1968).
The foregoing observations imply that phyto-estrogens do act on the pituitary and hypothalamus as well as the reproductive tract. In order for phyto-estrogens to act as estrogens or antiestrogens in the pituitary and hypothalamus they must first interact with estrogen receptors.

**EXPERIMENTAL OBJECTIVES**

This study was undertaken to determine the interaction of the phyto-estrogen compounds, coumestrol and genistein, with the cytoplasmic estrogen receptors of pituitary and hypothalamus tissue from sheep. Information regarding the nature of the interaction was sought. Initially, the estradiol binding characteristics of target tissues were investigated.
A. MATERIALS

I. Buffer composition

Estrogen binding assay buffer: 0.01 M sodium phosphate with 0.25 M sucrose,
pH 7.3, containing 0.02% azide.

Homogenization buffer: the same as above with the additional concentration of 0.1 M 2-mercaptoethanol.

II. Chemicals. Sources are indicated after the compounds.

- Blue Dextran-Pharmacia Fine Chemicals.
- Potassium Ferricyanide - Fisher Chemicals.
- Estradiol 17β (1,3,5,(10) oestratrien-3, 17β-diol) - Sigma Chemical Co..
- Diethylstilbestrol (DES) (α, α'-diethyl-4, 4'-stilbenediol) - Matheson, Coleman and Bell, Manufacturing Chemists.
- Coumestrol (7', 6-dihydroxycoumaro (3', 4'-3, 2) coumarone) - Eastman Chemicals.
- Genistein (4', 5, 7-trihydroxyisoflavone) - ICN-K&K Laboratories, Inc..
- $^3$H-estradiol 17β (2, 4, 6, 7, (n)$^3$H) estradiol - Amersham Corp. Specific activity of batches ranged from 89 Ci/mmol to 104 Ci/mmol.

Stock solutions of estrogen compounds were made by dissolving the compound in freshly distilled ethanol. Stock solutions were stored in a freezer and were diluted with buffer for use. Labelled estradiol (250 uCi) in benzene/
ethanol was dried under nitrogen, dissolved in buffer and stored at 4°C.

III. Tissue Samples

a. Source

All tissues were obtained from mature ewes of undetermined age slaughtered at Richmond Packers, Ltd., Richmond, B.C.. In all, results are given for several groups of ewes sampled over the period of these experiments. A total of fifty-one ewes were sampled.

b. Tissue sampling

The brain was exposed immediately post-slaughter either by a) drilling a hole through the parietal and frontal bones with a 2.5 inch hole saw driven by a commercial duty, 1/2 inch capacity Black and Decker electric drill, or b) by chopping the skull with a large cleaver. The latter method proved to be quicker although damage to brain tissue was more extensive. The average time from slaughter to having the tissue in ice was five minutes.

The cerebral hemispheres were lifted up and back (dorsocaudally) and the olfactory bulbs and optic tracts severed to expose the ventral surface of the brain. (Figure 3).

Hypothalamic tissue was removed from an area approximately 5 mm rostral to the optic chiasma, caudally to the mammillary body and laterally bounded by the hypothalamic fissures, to a depth of approximately 5 mm. Average weight of tissue sample was 0.6 gms. This area encompasses the main centres of the hypothalamus known to contain estradiol receptors as shown by autoradiographic studies (Stumpf, 1970, 1971a,b, 1972; et al., 1975). (Figure 4a,b).

The brain was entirely removed from the skull. The diaphragma pellae was cut and the entire pituitary was listed from the sella turcica. Average weight of the pituitary gland was 0.8 gms. The pituitary was used without
Figure 3: View of the Ventral Surface of the Sheep Brain, (May, 1970).
Figure 4a: Midsagittal View of the Sheep Brain (Ranson and Clark, 1959).
Figure 4b: Midsagittal View of the Sheep Brain, section through hypothalamus and pituitary.

(Daniel and Prichard, 1975).
separation into component parts due to technical difficulties, however no estrogen binding has been detected in the posterior portion (Ginsburg, et al., 1975).

Other tissues obtained were pineal glands, uterus, and amygdala.

All tissues were placed in ice-cold homogenization buffer and kept on ice for transport and subsequent procedures.

c. Cytosol preparation

Pooled samples for each tissue were blotted with cheesecloth and weighed. Homogenization with approximately three volumes of homogenization buffer was done by hand in a glass-Teflon tissue homogenizer immersed in ice. Uterus and pituitary tissue required scissor chopping prior to homogenization. The homogenates were centrifuged at 4°C for 60 minutes at 100,000 x g_ave. in a Beckman Model L5-65 Ultracentrifuge.

The supernatants (cytosols) for each tissue were pooled and distributed in 0.5-0.7 ml portions to glass ampoules, frozen in dry ice (-78°C), flame sealed, then transferred to liquid nitrogen (-195.8°C). The ampoules were stored in liquid nitrogen in a cryostat until use.

For assays, the ampoules were opened and cytosol allowed to thaw at room temperature, then kept in ice for immediate use.

B. ESTROGEN PROTEIN BINDING ASSAY

I. Introduction

The method used in this study is an adaptation of that of Ginsburg, et al., (1974). It involves incubation of cytosol with tritiated estradiol with or without unlabelled estrogen. Separation of free from bound estradiol is accomplished by passing the incubate through a Sephadex LH-20 column at 4°C with a thirty minute dissociation time on the column to permit differentiation of high affinity binding from low affinity, non-specific binding.
The cellular components which bind estradiol with high affinity and mediate the action of estrogen are termed receptors. This interaction is termed specific binding and it is a saturable reaction, that is, the number of specific binding sites is finite. In addition to specific, high affinity binding sites, high capacity binding sites of lower affinity may be present in cytosol preparations. The binding of estrogen to these low affinity sites is termed non-specific binding and the presence of non-specific binding must be taken into account in the interpretation of binding data. (Mester, et al., 1970).

Cochet, et al., (1976), have determined that the non-specific binding components fall in a range of 40,000 to 100,000 molecular weight whereas the specific binding occurs with proteins of approximately 240,000 molecular weight. Both types of sites would elute in the same fractions from Sephadex LH-20. Ginsburg, et al., (1974), has developed a cold dissociation step to overcome this difficulty. Chilling the incubate to around 0°C slows the dissociation of bound hormone from the high affinity sites: however the non-specific sites, that is, those with lower affinity, release bound hormone at greater than an order of magnitude faster than the specific, high affinity sites. (Mester, et al., 1970). The released estrogen is retained by the LH-20 so that at the end of the cold dissociation period, only the high affinity, specific sites, retain labelled estradiol which is then eluted from the column and counted. There are numerous methods available to correct for non-specific binding. Parallel incubations can be carried out with unlabelled estrogen in considerable excess. The unlabelled estrogen displaces labelled estrogen from the high affinity, limited capacity, specific sites while labelled estrogen is not displaced from the non-saturable, high capacity, non-specific sites. Therefore bound label in tubes with excess unlabelled estrogen may be attributed to non-specific binding and specific binding determined by differ-
ence from the total binding. This necessitates using twice as much cytosol. As cytosol in this study was in limited supply, a control experiment was carried out to determine if the cold dissociation period was sufficient to minimize the presence of non-specific binding in the column eluate. Incubation with excess DES resulted in negligible binding of labelled estradiol, as given in Figure 5. This indicates that minimal non-specific binding in the pituitary cytosol remains after the cold dissociation procedure.

Column eluates were counted by liquid scintillation.

II. Columns

Glass tubing of approximately 0.5 mm internal diameter was formed into columns 20-25 cm. long with one end drawn to a small diameter. Teflon tubing was attached to the drawn ends and sealed with metal paper clamps, Double Clips. Glass wool plugs were used to retain the gel.

a. gel

Sephadex LH-20, Pharmacia Fine Chemicals, was swollen in cold assay buffer at least twelve hours before use. Sephadex LH-20 is a hydroxypropylated form of Sephadex G-25 and as well as separating on the basis on molecular size, (exclusion limit 5000 molecular weight), exhibits hydrophobic and hydrophilic properties due to an increased ratio of carbon to hydroxyl groups, (Pharmacia information booklet). The lipophilic character of Sephadex LH-20 permits the preferential retention of steroids, including estradiol, in aqueous solution. This property enabled the use of small columns to minimize dilution of the bound fraction of estradiol while retaining the unbound labelled estradiol. The affinity of the gel for steroids also facilitated the differential dissociation of low affinity binding, (Ginsburg, et al, 1974).

b. characterization of columns

Figure 5: $^3$H-Estradiol Binding of Pituitary Cytosol, Column elution, without and with excess DES, (duplicates) to show presence of specific estradiol binding after thirty minute cold dissociation period on LH-20 column.

- $^3$H-Estradiol at 0.17 nM.
- $^3$H-Estradiol at 0.22 nM and DES at 6.5 nM.
(i.d. 0.45 mm), with Sephadex LH-20 to a bed height of 6 cm. Blue Dextran and potassium ferricyanide, located visually, were used to determine the excluded, (void), volume and included volume, respectively, of the column. Initial studies using Blue Dextran and potassium ferricyanide indicated that there was the possibility of free estradiol eluting from the column in the Blue Dextran region where the large cytosol proteins, including estrogen receptors, were expected. Puca, et al, (1971), used Sephadex G-25 to separate bound estradiol from free at 4°C and found the bound fraction to emerge in the macromolecular peak. The elution of radioactivity, $^3$H-estradiol, from a 6 cm column in the absence of cytosol when 200 ul of a mixture containing 100 ul Blue Dextran, 100 ul potassium ferricyanide and 50 ul of $^3$H-estradiol was layered on top is given in Figure 6.

To avoid this possible overlap of bound and free radioactivity all separations in this study utilized gel heights of 12 cm. Ginsburg, et al, (1974), state that the radioactivity in eluates attributable to the high affinity complexes is independent of column length. Elution profiles of cytosol incubates from 12 cm columns are given in Figure 7. TNBS assay absorbances, vide infra, are plotted as well to show the specificity of the radioactive counts in relation to the protein, peptide, and amine absorbances. Each column was used once, the gel removed, the column decontaminated and repacked with fresh LH-20 at least four hours before reuse.

In a comparison of various assay methods, Jungblut, et al, (1972), determined that Sephadex chromatography and agar electrophoresis both stood out over several other separation methods as the most sensitive.

c. Incubation procedure

Cytosol, 200 ul, was added to tubes containing tritiated estradiol and buffer alone, or with unlabelled estrogenic compound, coumestrol, genistein
Figure 6: Elution of $^3$H-Estradiol from 6 cm Sephadex LH-20 column in absence of cytosol. (cCPM are quench corrected counts per minute).
Figure 7: Elution of $^3$H-Estradiol Bound by Pituitary Cytosol from 12 cm Sephadex LH-20 column, with TNBS absorbances shown. (cCPM are quench corrected counts per minute).
or diethylstilbestrol (DES), in a volume of 50 ul. Therefore total incubation volume was 250 ul. Incubation for fifteen minutes was carried out in a 30°C water bath. Two 100 ul aliquots, duplicates, were each layered on LH-20 columns and run in with 100 ul of cold assay buffer. Columns were maintained at 4°C in ice water baths. After the incubation mix was run into the gel bed, column flow was stopped for thirty minutes to allow dissociation of the low affinity, non-specific binding, (Ginsburg, et al, 1974).

A 25 ul aliquot of the incubation mix was transferred to a scintillation vial for a count of total radioactivity present in the incubate.

Columns were eluted into glass mini-scintillation vials, 7 ml volume, Vialette, Amersham Corp., mounted in test tube racks of a LKB fraction collector fitted with drop counting head. Two columns, duplicates were run at the same time using two fraction collectors.

d. location of protein in column eluates

In order to determine accurately the position of cytosol protein in the column eluates, the collected fractions were subjected to a protein detection procedure. To avoid loss of radioactive counts through transfer to other containers all reagents were added to the mini-scintillation vials containing fractions from the columns. Absorbances were read in a Spectronic 20 spectrophotometer-colorimeter, Bausch and Lomb, using an adapter designed to hold the mini-scintillation vial in the light path while activating the "gate", Appendix Figure A-1.

Initially the method of Lowry, et al, (1951) was used to visually locate the protein peak, Appendix 2. As shown in Figure 8, from an initial experiment, the peak of protein absorbance correlated well with the peak of radioactivity. This method presented difficulties due to the large volume of aqueous reagent involved. The assayed samples did not mix well in the scintil-
Figure 8: Elution of $^3$H-Estradiol Bound by Amygdala Cytosol, with Lowry absorbances shown.
lation fluid, PCS, Amersham Corp., and there was frequent, non-uniform phase separation in the scintillation counter. An additional difficulty was numerous spurious counts resulting from chemiluminescence if samples did not stand for some time before adding scintillation fluid. Chemiluminescence can result from oxidation of unsaturated compounds by molecular oxygen and this can be catalyzed by bases such as are present in the Lowry reagents, (Wang, et al., 1975, pg. 269).

For these reasons an alternate method was sought that would be more sensitive, thereby requiring less reagent to be added to the samples but with an easily detected colour reaction for visual location of protein.

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) method of Snyder and Sobocinski, (1975), for the determination of amines, gives a yellow coloured product with small amounts of reagent and is considered by the authors to be twice as sensitive as the Lowry method, Appendix 3. This method is particularly suitable for locating protein peaks as it requires the addition of only one reagent to the sample vial. As a qualitative, not quantitative, assessment of eluates was required, reagents were mixed and added in minimum amounts, usually 75 ul per vial. As shown in Figure 7, the first peak absorbance correlated with peak radioactivity. For routine use the position of protein peaks was visually determined. Assayed samples mixed readily in 6 ml scintillation fluid, Biofluor, New England Nuclear, and there was no chemiluminescence observed.

e. measurement of radioactivity

The mini-scintillation vials containing column eluates and scintillation fluid were placed in glass Vialette adapters, Amersham Corp., for counting in a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. Counting efficiency for tritium was approximately forty percent with a background of
20 cpm. Quenching was monitored by the instrument external standard ratio. Background corrected counts per minute were standardized using a tritium quench series and expressed in terms of corrected counts per minute, cCPM. Bound cCPM were obtained by summing the counts in those vials containing the macromolecular protein peak eluates and unbound, "free", cCPM were obtained as the difference between the total cCPM in the incubate and the bound portion.

f. multi-channel elution system

The latter portion of this study involved a modified assay system. Incubations were carried out as above except that five incubates were run simultaneously, in duplicate, using a ten column elution system. Ten glass columns, Econo-columns, Biorad Laboratories, with Luer tips, packed with Sephadex LH-20, were connected to 18 gauge, 1/2 inch needles, Yale, B-D, set into a plexiglass tank containing ice and water. Columns were eluted using a Desaga Multi-channel Peristaltic pump, Brinkman, and a modified Gilson Micro Fractionator, Model FC-100K, with a plexiglass bar containing ten needles as drop tubes which permitted the simultaneous elution of the ten columns into ten fractions each, (adapted from Webb, 1978). Subsequent procedures were as outlined above.

C. TREATMENT OF DATA

I. Introduction

Apparent dissociation constants, \( K_D \), were determined by double reciprocal analysis, (Lineweaver and Burk, 1934). Apparent inhibition constants, \( K_I \), were determined by Dixon plots, (Dixon, 1953). The slopes and intercepts of lines were determined by simple linear regression using the method of least squares. Intercepts, \( K_I \), for Dixon plots were determined graphically and algebraically from the equations of the two intersecting lines.
II. Methods

The application of enzyme kinetics plotting methods to steroid hormone-receptor interactions has been outlined by Rodbard, (1973), in a review of the analysis of bimolecular reactions. Some of these plots were used in this study to gain insight into the interaction of estradiol 17β and phytoestrogen, genistein or coumestrol, with estrogen receptors in cytosol prepared from sheep pituitary and hypothalamus and to determine the competitive nature of these interactions.

The actual hormone-receptor interaction is possibly more complex than the assumed simple bimolecular reaction on which these plots are based. Sanborn, et al, (1971), have noted the presence of at least two interdependent binding sites with similar affinities and the presence of positive cooperativity at low estradiol concentrations. However, Rodbard, (1973), states: "until both the hormone(s) and the receptor(s) are available in homogeneous form it will be nearly impossible to delineate the intricacies of the reaction mechanisms, and obtain realistic mathematical and physical-chemical models".

In order to evaluate competitive interactions, an estimate of the affinity of specific receptors for estradiol 17β is required as competition between two molecules for the same binding site on a receptor is a function of both the concentrations of the molecules and the affinity of the binding site for each of the competing species.

a. the determination of apparent dissociation constants, \( K_D \).

The determination of an apparent \( K_D \), dissociation constant, value for the estrogen receptors was carried out for each cytosol preparation using some of the plotting methods described by Rodbard, (1973). The nomenclature equivalents to enzyme kinetics parameters are given below:
### Enzyme kinetics
- substrate
- enzyme
- velocity
- \( V_{\text{max}} \)
- \( K_m \)

### Binding reactions
- "free", unbound hormone
- receptor
- bound hormone
- total concentration of binding sites
- \( K_D \), apparent dissociation constant

#### i. saturation analysis
The equivalent of the Michaelis-Menten plot of enzyme kinetics for binding analysis is the saturation binding curve.

The dissociation constant is defined as that concentration of ligand at which the binding sites are one half saturated. This is analogous to \( K_m \) of enzyme kinetics where a substrate concentration equal to \( K_m \) yields a reaction velocity equal to one half the maximal or saturation velocity. Both the dissociation constant, \( K_D \), and its reciprocal, the association constant \( K_A \), are measures of the affinity of a binding site for a particular ligand molecule. The rate at which the binding reaction approaches saturation with increasing ligand (hormone) concentration is related to the affinity of the receptor for the ligand. The level of binding at saturation is a measure of the number of binding sites present.

#### ii. double reciprocal analysis
In a simple binding system obeying Michaelis-Menten kinetics the double reciprocal plot, (Lineweaver-Burk, 1934) may be used to determine apparent binding affinities, (Edsall and Wyman, 1958 , pp. 620-622). In the double reciprocal plot the apparent affinity constant \( K_A \), is obtained from the absolute value of intersection on the x axis of the extrapolated line joining the data points. The apparent dissociation constant \( K_D \), is the reciprocal of this value.
The determination of $K_D$ values permits the selection of appropriate experimental conditions for competitive studies. Incubations with genistein or coumestrol were carried out with labelled estradiol concentrations approximately equal to $K_D$ or to six times $K_D$ in order to determine the intersection point on Dixon plots, vide infra, from which the apparent dissociation constants, or inhibition constants, $K_I$ values, for the phytoestrogens were obtained, (Dixon, 1953).

The $K_I$, inhibition constant, is the dissociation constant of the inhibitor-receptor complex, and thus is a measure of the affinity of the receptor for the inhibiting compound. $K_I$ or inhibition constants for genistein and coumestrol were obtained by the use of Dixon plots, a graphical procedure in which the reciprocal of the concentration of bound ligand (hormone) in the presence of inhibitor is plotted versus the inhibitor (phyto-estrogen) concentration. Dixon plots have been used to determine $K_I$ values for a number of estrogens and anti-estrogens at the uterine receptor by Geynet, et al, (1972). This plot also distinguishes between competitive and non-competitive inhibition. Binding data is obtained for a range of inhibitor concentrations at two ligand concentrations and graphed in the manner described. In the case of non-competitive inhibition the intersection point of the two lines thus obtained occurs on the x axis at a value equal to minus $K_I$. For the competitive case the intersection occurs above the x axis but still at an x value equal to minus $K_I$, (Webb, 1963).
RESULTS

A number of experiments to determine estrogen and/or phyto-estrogen binding properties were carried out on ewe hypothalamus, pituitary, amygdala, pineal and uterus cytosol preparations. The binding assay involves incubation of cytosol prepared from the test tissue with a range of concentrations of tritiated estradiol to determine the estrogen binding parameters. Phyto-estrogen binding assays were carried out with a range of competitor concentrations in the presence of a constant amount of tritiated estradiol. Results of estradiol and phyto-estrogen binding experiments for ewe hypothalamus, pituitary, amygdala, pineal and uterus are presented below.

A. HYPOTHALAMUS

Estrogen binding experiments were carried out on four different cytosol preparations of ewe hypothalamus tissue. Experiments using DES, coumestrol and genistein were included to determine the inhibitory activity of these compounds with respect to estradiol in binding to the estrogen receptor proteins in hypothalamus cytosol. The results for separate studies of each hypothalamus cytosol preparation are presented below.

I. Preparation 1, Hypothalamus, September, 1976.

The amount of $^3$H-estradiol binding in this hypothalamus preparation was low, the maximum binding observed being 0.2 percent of the total estradiol present, without a thirty minute dissociation time to minimize non-specific binding, Figure 9. DES added at a concentration greater than one hundred times that of the $^3$H-estradiol present decreased by approximately twenty percent the binding of $^3$H-estradiol in this preparation. Genistein at 4.12 uM decreased $^3$H-estradiol binding by the same amount, as shown in Figure 10. These observations indicate that of the total $^3$H-estradiol bound approximately
Figure 9: Preparation 1, Hypothalamus.

E₂: $^{3}$H-estradiol binding (nM) at concentration $=7.5$ nM (6 experiments); DES: $^{3}$H-estradiol binding (nM) at 7.5 nM in presence of DES=84.8 nM (2 experiments); Gen: $^{3}$H-estradiol binding (nM) at 7.5 nM in presence of Genistein=4.12 nM (2 experiments).

Figure 10: Preparation 1, Hypothalamus.

Percent $^{3}$H-estradiol binding in presence of DES or Genistein, at concentrations in Figure 9. 100% binding $^{3}$H-estradiol=$0.0168$ nM.
20 percent was due to specific binding and this specifically bound $^3$H-estradiol represented 0.04% of the total $^3$H-estradiol present in the incubation. This amount of specific estradiol binding of approximately 0.0034 nM was near the minimal detectable binding level and thus estradiol binding parameters could not be determined for this hypothalamus cytosol preparation. Large excesses of DES and genistein inhibited this specific binding of $^3$H-estradiol.

II. Preparation 2, Hypothalamus, December, 1976

Preliminary binding experiments, omitting the 30 minute dissociation time to minimize non-specific binding, yielded low levels of total $^3$H-binding in this hypothalamus preparation. The maximum $^3$H-estradiol bound represented less than 0.6 percent of the $^3$H-estradiol present at incubation. The $^3$H-estradiol binding data without and with a thirty minute dissociation time are shown in Figure 11.

Competition assays with genistein and coumestrol reduced the $^3$H-estradiol binding approximately 20 percent as shown in Figure 12. An attempt to determine the specific estradiol binding characteristics of this cytosol preparation by using parallel incubations without and with greater than one hundred times molar excess of DES is shown in Figure 13. Curve C represents the difference between the total binding, curve A, and the binding in the presence of excess DES, curve B, and is considered to be the amount bound to the estrogen receptor (Ginsburg, et al, 1974). Maximal specific $^3$H-estradiol binding was approximately 0.005 nM, again too low to accurately assess estradiol binding parameters (Clark and Peck, 1977). The data curve C, Figure 13, is shown in double reciprocal form in Figure 14. This cytosol preparation showed low levels of specific $^3$H-estradiol binding similar to preparation 1 and insufficient data was obtained to present complete estradiol binding information. The specific $^3$H-estradiol binding was inhibited by high levels
Figure 11: Preparation 2, Hypothalamus, $^3$H-Estradiol Binding, nM.

- $^3$H-estradiol binding levels obtained without cold dissociation time.
- $^3$H-estradiol binding levels obtained with thirty minute dissociation time to minimize nonspecific binding.
Figures 12 & 13

**FREE **

H-ESTRADIOL (nM)

**BOUND **

H-ESTRADIOL (nM)

- Specific (A-B)
- Non-Specific
- Excess DES
- Total (no DES)
- 3H-Estradiol Bound
- 3H-Estradiol plus 270nm genistein
- 3H-Estradiol plus 350nm commercial

100% binding of 3H-Estradiol at concentrations shown.

In presence of estradiol, percent 3H-Estradiol bound at 6.5 nM.

In absence, percent 3H-Estradiol bound at 0.099 nM.

Figure 13: Preparation 2, Hypo-

Figure 12: Preparation 2, Hypo-

(above)
of DES, genistein and coumestrol.

III. Preparation 3, Hypothalamus, May 1877, Fresh

A binding assay was conducted using hypothalamus cytosol the day of preparation without liquid nitrogen freezing. Maximal binding was 0.7 percent of the total estradiol present in the incubation. Coumestrol at 69 nM reduced $^3$H-estradiol binding to 63 percent of the control.

IV. Preparation 3F, Hypothalamus, May 1977, Frozen in liquid nitrogen

Additional experiments were conducted with the above preparation after storage in liquid nitrogen. No binding was detected at a total $^3$H-estradiol concentration less than 0.5 nM estradiol. Maximal binding was less than 0.5 percent of the total estradiol present. Attempts to determine the amount of specific binding with excess DES gave erratic results. Competition with coumestrol was inconclusive. Results are presented in Figure 15.

V. Preparation 4, Hypothalamus, May 1978

Estradiol binding in this preparation was less than 0.5 percent of the total present. No further experiments were conducted.

The amount of $^3$H-estradiol bound by these four hypothalamus cytosol preparations ranged from 0.2 to 0.7 percent of the total $^3$H-estradiol present in the incubations. It was concluded that this low level of total detectable $^3$H-estradiol binding coupled with the even lower specific binding component did not permit the reliable determination of estradiol binding parameters. Likewise, reliable determination of inhibition parameters of coumestrol and genistein was not possible at these low levels of $^3$H-estradiol binding due to the difficulty in detecting degrees of inhibition. Coumestrol at a concentration approximately equal to the apparent $K_I$ determined for pituitary cytosol (vide infra) inhibited extradiol binding in hypothalamus cytosol preparations to approximately 60 percent while genistein at a concentration greater than a hundred times apparent $K_I$ as determined in pituitary cytosol (vide infra)
completely suppressed specific estradiol binding in hypothalamus cytosol, preparations 1 and 2. These results suggest that the competitive efficiency of both coumestrol and genistein in hypothalamus cytosol is probably comparable to that determined for the pituitary (vide infra).

B. PITUITARY

The close hormonal relationship between the pituitary and the hypothalamus coupled with its known response to estrogen suggested this tissue as a suitable experimental material to study the binding of estrogen and phyto-estrogens. Experiments on three pituitary cytosol preparations were carried out both to determine estradiol binding characteristics and to determine the competitive action of coumestrol and genistein. Results are presented first for estrogen binding parameters followed by phyto-estrogen binding characteristics.

I. Determination of Apparent $K_D$ Values for Pituitary Estrogen Receptor

The dissociation constant, $K_D$, is a measure of the binding affinity between a hormone and its receptor and is a characteristic of a particular hormone receptor pair. Apparent $K_D$ determinations for the receptor-estradiol interaction were obtained from double reciprocal analysis of the $^3$H-estradiol binding data for each pituitary cytosol preparation and are presented below.

a. Preparation 1, Pituitary, May 1977

The saturation binding curve for this preparation is shown in Figure 16. The double reciprocal analysis of this data is shown in Figure 17. The apparent $K_D$ for estradiol which is the reciprocal of the X intercept was determined to be 0.39 nM and reciprocal maximal binding, ($B_{\text{max}}^{-1}$), as determined by the Y intercept was 3.1 nM$^{-1}$, equivalent to 0.32 nM.

b. Preparation 2, Pituitary, September 1977

Saturation analysis for this preparation is shown in Figure 18. Double reciprocal analysis is shown in Figure 19. The apparent $K_D$ for estradiol of this preparation as above was determined to be 0.26 nM and reciprocal maximal
Figure 16: Preparation 1, Pituitary, Saturation Curve, (points are means of duplicates). Data presented in Double Reciprocal form in Figure 17.
Figure 17: Preparation 1, Pituitary, Double Reciprocal plot, (r=0.95, n=14), Apparent $K_D=0.39$ nM.
Figure 18: Preparation 2, Pituitary, Saturation Curve, (points are means of duplicates). Data presented in Double Reciprocal form in Figure 19.

Figure 19: Preparation 2, Pituitary, Double Reciprocal plot. (r=0.94, n=19), Apparent $K_d=0.26$ nM.
binding, \( \left( \frac{B_{\text{max}}}{E_{\text{max}}} \right)^{-1} \), was 1.19 nM\(^{-1}\), equivalent to 0.84 nM, therefore the amount of \(^3\)H-estradiol binding in this cytosol preparation was approximately 2.5 times greater than preparation 1. The reciprocal \( E_{\text{max}} \) values were used for the Dixon plots of phyto-estrogen compounds to determine \( K_I \) values (vide infra) in the cases of inhibition data being available for only one \(^3\)H-estradiol concentration.

c. Preparation 3, Pituitary, May 1978

Saturation analysis for this preparation is shown in Figure 20. The amount of maximal \(^3\)H-estradiol in this preparation was similar to preparation 1. Double reciprocal analysis in Figure 21 determined the apparent \( K_D \) for estradiol to be 0.14 nM.

The amount of \(^3\)H-estradiol binding present in pituitary cytosol preparations 1 and 3 were similar, however preparation 2 showed double the amount of binding capacity. In all three cases the amount of specific estradiol binding was sufficient to assess estradiol binding parameters and thus binding inhibition characteristics of phyto-estrogens. The apparent \( K_D \) values for estradiol determined for these three pituitary cytosol preparations when considered together indicate an apparent \( K_D \) of 0.26 ± 0.12 nM, a high affinity of pituitary estrogen receptor for estradiol.

Following the above determinations, studies to assess the competitive efficiency of phyto-estrogen compounds were carried out.

II. Determination of Apparent \( K_I \) Values for Phyto-estrogens

The \( K_I \), inhibitor constant, determined for a compound from competitive studies with hormone present, equals the \( K_D \) of the hormone receptor for the inhibiting compound in the case of competitive inhibition and thus can be used to evaluate the affinity of a receptor for various compounds which may show different degrees of binding ability.

Apparent \( K_I \) values for two phyto-estrogen compounds were determined from Dixon plot analysis for coumestrol and genistein with different pituitary
Figure 20: Preparation 3, Pituitary, Saturation Curve, (points are means of duplicates). Data presented in Double Reciprocal form in Figure 21.

Figure 21: Preparation 3, Pituitary, Double Reciprocal plot. (r=0.74, n=16), Apparent $K_D=0.14$ nM.
cytosol preparations and results are presented separately for each compound.

a. Coumestrol

Apparent $K_I$ determinations were made on two pituitary cytosol preparations.

i. **Preparation 1, Pituitary, May 1977**

Dixon analysis for coumestrol in this preparation as determined by $^3$H-estradiol binding with various coumestrol concentrations is shown in Figure 22. As analysis at only one $^3$H-estradiol concentration (0.5 nM) was completed, the apparent $K_I$ was determined at the point of intersection of the experimental line with a line drawn at reciprocal maximal binding, $(B_{\text{max}})^{-1}$, as determined from double reciprocal analysis for this cytosol preparation (**vide supra**). The apparent $K_I$ for coumestrol was determined to be 61 nM.

ii. **Preparation 2, Pituitary, September 1977**

Dixon analysis for coumestrol on this cytosol preparation is shown in Figure 23. Analysis at only one concentration of $^3$H-estradiol was available (2.8 nM). The apparent $K_I$ was determined at the point of intersection with the reciprocal maximal binding, $(B_{\text{max}})^{-1}$, line as determined from double reciprocal analysis of this cytosol preparation (**vide supra**). The apparent $K_I$ determined for coumestrol was 58.6 nM, that is 59 nM.

b. Genistein

Genistein apparent $K_I$ values were determined on two cytosol preparations. Inhibition curves to show the parallel binding inhibition of genistein at different $^3$H-estradiol concentrations are also presented.

i. **Preparation 2, Pituitary, September 1977**

The inhibition curves for genistein with $^3$H-estradiol at 0.46 nM and 2.9 nM are presented in Figure 24. Dixon analysis, shown in Figure 25, determined the apparent $K_I$ for genistein in this preparation to be 130 nM.
Figure 22: Preparation 1, Pituitary, Dixon Plot, Coumestrol, 
$^3$H-Estradiol at 0.5 nM ($r=0.8, n=10$), Apparent $K_i$ for 
coumestrol determined at $(B_{max})^{-1}$ from Figure 17.

$\text{Apparent } K_i = -61 \text{nM}$

$\frac{1}{B_{max}} = 3.1$
Figure 23: Preparation 2, Pituitary, Dixon Plot, Coumestrol, 
$\text{^3H-estradiol at 2.8 nM (r=0.86, n=8), Apparent } K_I $ for 
coumestrol determined at $(B_{\text{max}})^{-1}$ from Figure 19.
Figure 24: Preparation 2, Pituitary, Inhibition Curves, Genistein with $^3$H-estradiol at concentrations of

2.9 nM •
and 0.46 nM ▲
Figure 25: Preparation 2, Pituitary, Dixon Plot, Genistein, Apparent $K_i$ for genistein determined to be 130 nM. $^3$H-estradiol binding inhibition determined with concentration of

$^3$H-estradiol at 0.46 nM $\triangle$ (r=0.97, n=6)

$^3$H-estradiol at 2.9 nM $\bullet$ (r=0.9, n=6)
Inhibition curves for genistein with estradiol at 0.4 and 2 nM are shown in Figure 26. Dixon plot analysis, shown in Figure 27, determined the apparent $K_I$ for genistein in this preparation to be 210 nM.

Results recorded in Figures 16-21 show the apparent dissociation constant, $K_D$ for estradiol in ewe pituitary cytosol as determined for three cytosol preparations to be $0.26 \pm 0.12$ nM. Apparent $K_I$ determinations on two pituitary cytosol preparations each for coumestrol and genistein revealed coumestrol (apparent $K_I$=59-61 nM) to be about three times more efficient a competitor than genistein (apparent $K_I$=130-210 nM) in ewe pituitary cytosol, that is, the affinity of the estrogen receptor for coumestrol is about three times greater than its affinity for genistein. The receptor affinity for coumestrol is approximately 230 times less, and for genistein approximately 650 times less, than for estradiol.

In addition to the hypothalamus and pituitary, for comparative purposes other possible estrogen target tissues were chosen to examine estradiol and/or phyto-estrogen interaction with cytosol estrogen receptors. In this supplementary study two extrahypothalamic brain structures, the amygdala and the pineal, were examined. In addition, the ewe uterus, in particular the caruncles of the endometrium, was studied for estrogen binding parameters.

C. AMYGDALA

The amygdala was chosen as a representative of extrahypothalamic brain structures believed involved in reproduction, particularly onset of puberty and ovulation. An experiment to determine $^3$H-estradiol binding and competition with 2.71 uM and 5.42 uM genistein in amygdala cytosol was conducted. Results are shown in Figure 28. Although estradiol binding was reduced, the concentrations of both estradiol and genistein in this experiment were high. The
Figure 26: Preparation 3, Pituitary, Inhibition Curves, Genistein, with \(^3\)H-estradiol concentrations of

- \(\bullet\) 2.0 nM
- \(\triangle\) 0.4 nM
Figure 27: Preparation 3, Pituitary, Dixon Plot, Genistein. Apparent $K_I$ for genistein determined to be 210 nM. $^3$H-estradiol binding inhibition determined with concentration of

- $^3$H-estradiol at 0.4 nM  ▲  (r=0.91, n=6)
- $^3$H-estradiol at 2 nM  ▪  (r=0.8, n=7)
Figure 28: Amygdala, $^3$H-Estradiol Binding alone and in presence of Genistein.
closeness of the reduction, even though the genistein concentration was
doubled, indicates that the amount of specific binding is low, that is approx­
imately 10 percent of total binding detected and that genistein completely
inhibited this specific binding. The remaining bound estradiol detected is in
the non-specific fraction. In this experiment the genistein dissolved in
ethanol was added to incubation tubes, then dried under nitrogen. It is
possible that all did not redissolve in the cytosol. For all other competi­
tions with other tissues the genistein or coumestrol was added in ethanol
directly to the cytosol to ensure accurate competitor concentration. As this
preliminary experiment determined that the amount of estradiol binding was
low in amygdala cytosol, this particular aspect was not pursued. An elution
profile for amygdala binding is shown in Figure 8.

D. PINEAL

The pineal gland has been implicated in the control of reproductive
rhythms and is possibly influenced by steroid hormones. An experiment to
determine the possibility of specific estradiol binding in the pineal was
conducted using parallel incubations with 100 times molar excess diethylstil­
bestrol. In addition, competitions with 300 nM and 500 nM genistein were
carried out. As shown in Figure 29, saturation binding was not reached.
Excess DES did not detectably decrease \(^{3}H\)-estradiol binding indicating that
either specific binding was not present or was not detected by this assay
system. This observation is confirmed in the Dixon plot of the genistein
competitions as zero slope indicates the absence of detectable competition,
Figure 30.

E. UTERUS

Experiments were carried out to study the estrogen binding character­
istics of sheep uterine cytosol prepared from caruncles of endometrium. In
Figure 29: Pineal, $^3$H-Estradiol Binding in Presence of DES and Genistein.

Figure 30: Pineal, Dixon Plot, Genistein
order to determine the amount of non-specific binding present in this uterine cytosol preparation a binding experiment with $^3$H-estradiol and parallel incubations containing 100 times molar excess DES was conducted. Results as shown by column elution profile in Figure 31 indicate that the amount of non-specific binding present was less than ten percent of total binding. Saturation analysis is shown in Figure 32 with a maximal $^3$H-estradiol binding greater than 4 nM. Determination of the apparent $K_D$ for estradiol by double reciprocal analysis is presented in Figure 33. The apparent $K_D$ for estradiol in uterine cytosol was determined to be 0.6 nM.
Figure 31: Uterus, Elution of Bound $^3$H-Estradiol from LH-20 column, (cCBM are quench corrected counts per minute),
with

- $^3$H-Estradiol at 0.46 nM
- $\triangle$ as above with excess DES.
Figure 32: Uterus, Saturation Curve, $^3$H-estradiol binding, nM. (Points are means of duplicates). Data shown in Double Reciprocal for $m$ in Figure 33.
Figure 33: Uterus, Double Reciprocal Plot (r=0.93, n=16), Apparent $K_D=0.6$ nM.
Results of $^3$H-estradiol binding studies for the various tissues are discussed in terms of estrogen binding parameters. The results of the phyto-estrogen experiments are then discussed with possible physiological implications mentioned.

I. Estradiol Binding Parameters

It was determined that the amount of specific $^3$H-estradiol binding in these hypothalamus cytosol preparations was low, maximum detected being 0.003 nM in preparation 1 and about 0.008 nM in preparation 2. The non-specific binding component of the total $^3$H-estradiol binding was high (Figure 13). In such a system in which the quantity of non-specific binding is great with respect to total binding the probability of accurate determination of specific binding is very low (Clark and Peck, 1977). For this reason the determination of specific estradiol binding parameters in ewe hypothalamus cytosol was not pursued. It therefore follows that inhibition studies under these binding conditions "can lead to highly erroneous results", (Clark and Peck, 1977).

Because the main emphasis of this study was to determine estrogen receptor affinities for phyto-estrogens no attempt was made to quantitate the amount of estrogen binding for the different tissues. However, it was obvious that cytosol preparations of pituitary and uterus contained greater estrogen binding capacity than cytosol of hypothalamus amygdala and pineal and therefore estradiol binding parameters could be more closely examined in these two tissues.

Apparent estradiol $K_D$ values in this study were determined on cytosol pools of pituitary tissue from intact ewes. The reports of other workers are based on results from individual ovariectomized ewes. The apparent $K_D$ values for estradiol determined in this study, 0.14-0.39 mean 0.26±0.12 nM, are in
the range reported by other workers. Wise and Payne (1975) reported on five individual ewes a mean $K_D$ of 0.103 nM for estradiol and 0.14 nM for estrone, using density gradient centrifugation. These workers also reported a difference in $K_A$ values equivalent to apparent $K_D$ between anoestrus, breeding and estradiol-treated anoestrus ewes ranging from 0.02 nM to 0.08 nM (Wise, et al., 1975). It is possible that the ewes which formed the cytosol pools for this study were in varying reproductive states and thus if pituitary estradiol $K_D$ changes with phase of reproduction then slight differences in individual ewes may influence the apparent $K_D$ value determined on a cytosol pool. Tang and Adams (1978) reported $K_A$ values which are equivalent to $K_D$ values of 0.02 nM for pituitaries from both control and clover affected ewes using the dextran-charcoal assay. Depending on the method of determination, the estradiol $K_D$ values reported for female rat pituitaries range from 0.07 to 0.32 nM (Ginsburg, et al., 1974) and Kato (1977) reported the $K_D$ of rat pituitaries to be 1.4 nM. As is evident from the figures quoted for the female rat, the species most thoroughly studied with regard to hypothalamus and pituitary estrogen binding characteristics, the determination of apparent $K_D$ values is subject to some variation due to method, reproductive state of experimental animals and possibly other factors and thus although absolute values appear different they fall within the same order of magnitude, indicating the high affinity of the receptors for estradiol.

The apparent $K_D$ for estradiol determined in this study for ewe uterus, in particular the caruncles of the endometrium, was 0.6 nM and this tends to confirm the value reported by Shutt and Cox (1972) of 0.11 to 0.16 nM for the estrogen-primed, ovariectomized sheep uterus. Values reported for other species are also in the same range. The $K_D$ values reported for calf and rat uterus are 0.2-0.5 nM (Geynet, et al., 1972) and Senior (1975) reports the $K_D$ of cow
uterus to be 0.05-0.25 nM. The values for rat uterus have been reported as 0.18-0.46 nM (Ginsburg, et al., 1974) 0.14-0.34 nM (Peherty, et al., 1970), and 1.4 nM (Kato, 1977). Again the high affinity of estrogen receptors in the uterus for estradiol was evident.

Specific estradiol binding was detected in the ewe amygdala, confirming the reports of such binding in this part of the brain in the rat. As with the hypothalamus, the amount of detectable specific estradiol binding in the amygdala cytosol preparation was too low for reliable determination of estradiol binding parameters and was not pursued.

The presence of specific estradiol binding receptors in the pineal gland, as has been previously reported for the rat (Cardinali, et al., 1975), was not detected in the ewe pineal under the conditions of these experiments. As the volume of cytosol prepared was limited due to the small size of the pineal gland, it was not possible to continue experiments on this aspect of estrogen binding.

The determination of estradiol binding parameters in the pituitary permitted the selection of experimental conditions to assess the affinity of estrogen receptors of this tissue for the phyto-estrogens, genistein and coumestrol. Additional experiments on other tissues are reported.

II. The Determination of Phyto-estrogen Binding Characteristics

The nature of the interaction between estradiol and phyto-estrogens with the estrogen receptors of the pituitary was determined to be competitive, that is, these compounds interact with the same binding site as estradiol. This conclusion was drawn from Dixon plot analysis (Figures 25,27). The intersection of the experimental lines for coumestrol and genistein at a point above the abscissa to the left of the ordinate is consistent with competitive inhibition taking place. This implies that at concentrations high
enough, relative to the appropriate affinities involved, coumestrol or genistein can effectively compete with estradiol for estrogen receptors. Furthermore in the absence of estradiol these compounds can bind to the estrogen receptors of the pituitary and possibly act in the manner of estradiol. This competition with estradiol by the phyto-estrogens coumestrol and genistein has been demonstrated in this study to occur with estrogen receptors in cytosol from ewe hypothalamus, pituitary and amygdala. Previously this competition has been reported at uterine receptors of various species and at estrogen receptors from human breast cancer cells (Shemesh, et al, 1972; Shutt and Cox, 1972; Geynet, et al, 1972; Martin, et al, 1978).

In order to quantitate the binding of phyto-estrogen compound to pituitary estrogen receptors the determination of inhibitor constants, $K_I$ values, by Dixon plots was carried out for genistein and coumestrol. In the case of competitive inhibition, as has been herein determined for genistein and coumestrol, the $K_I$ is equal to the $K_D$ of the inhibitor and thus is a quantitative measure of the affinity of the receptor for inhibitor. The apparent $K_I$ values determined for coumestrol and genistein at the ewe pituitary estrogens receptors in this study are similar to those reported by Geynet, et al, (1972) for the same compounds in rat and calf uterine cytosol. The apparent $K_I$ for genistein determined in this study is 130-210 nM, comparable with the figure quoted by Geynet, et al, (1972) of 360 nM. These results suggest a slightly greater affinity for genistein in the ewe pituitary cytosol than that of calf or rat uterine cytosol however not enough information is available to consider if this difference is significant. The apparent $K_I$ for coumestrol determined for pituitary cytosol as 59-61 nM closely approaches the figures of Geynet, et al, (1972) of 42-50 nM for uterine cytosol of calf and rat suggesting similar affinity of pituitary and uterine estrogen receptors for coumestrol.
The qualitative studies of phyto-estrogen binding in hypothalamus and amygdala did not determine competitive characteristics or estradiol binding inhibiting efficiency and therefore the determination of affinity of the estrogen receptors of these tissues for the phyto-estrogens was not possible. The indication for hypothalamus cytosol was that a similar affinity for coumestrol was present as was determined in the pituitary.

A comparison of the various binding characteristics determined for the tissues of this study is shown in Table II.

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>CYTOSOL POOL</th>
<th>$K_D$ ESTRADIOL</th>
<th>$K_i$ GENISTEIN</th>
<th>$K_i$ COUMESTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus</td>
<td>n.d.*</td>
<td>&lt;2710 nM</td>
<td>similar to pituitary</td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>0.26±0.12 nM</td>
<td>130-210 nM</td>
<td>59-61 nM</td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td>n.d.</td>
<td>&lt;2710</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Pineal</td>
<td>Specific estradiol binding not detected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>0.6 nM</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

* n.d. means not determined in this study

The $K_D$ is defined in physical terms as the product of the concentrations of the interacting species, i.e. receptor and estrogen, divided by the concentration of the bound form, i.e. estrogen-receptor complex at equilibrium. Therefore at an estrogen concentration such that the concentration of receptor in free and bound form is equal, then the $K_D$ is equal to the concentration of unbound ligand.*
\[ E + R \not\rightarrow ER, \quad K_D = \frac{(E)(R)}{(ER)}, \text{ so that when } (R) = (ER), \text{ then } K_D = (E), \text{ i.e., } K_D \text{ is equal to the concentration of estrogen at which the receptors } (R) \text{ are one-half saturated.} \]

As the \( K_I \) of a competitive inhibitor is equal to the \( K_D \) of receptor for that inhibitor, then the same conditions apply.

Therefore the lower the \( K_D \) for a compound/receptor pair, the greater the affinity of the receptor for that compound. Thus a comparison of \( K_D \) or \( K_I \) values can give a quantitative assessment of the affinity of the estrogen receptor for various compounds. As determined in this study the pituitary shows approximately three times greater affinity for coumestrol than for genistein, and 230 times less affinity for coumestrol and 650 times less affinity for genistein than for estradiol. The significance of these observations in relation to physiological conditions will be discussed presently.

Other reports of genistein and coumestrol competitions with estradiol at the uterine estrogen receptors have determined relative binding affinities or the concentration of phyto-estrogen necessary to reduce estradiol binding 50 percent (Shemesh, et al., 1972; Shutt and Cox, 1972). Such determinations are a function of estradiol concentration present and thus are a qualitative measure of inhibiting efficiency. The apparent \( K_D \) values for phyto-estrogens determined in this study show a similar trend in the relative magnitude of inhibition reported for genistein and coumestrol by Shemesh, et al., (1972) and Shutt and Cox (1972).

The apparent \( K_I \) values herein reported are equivalent to 16 ng/ml for coumestrol and 35-57 ng/ml for genistein. Assuming that the intracellular levels of these compounds are similar to blood levels, that is, there is no
specific intracellular concentration of compound, then the reported levels of unconjugated, that is, "free" phyto-estrogens in sheep blood are somewhat below the levels necessary for half maximal saturation of receptors as calculated from the data in this study. However, in relation to circulating estradiol levels in the picogram per milliliter range, unconjugated phyto-estrogen levels can reach up to a thousand fold greater and thus can be assumed to exert considerable effect. Particularly as competition between estrogen and phyto-estrogen for receptor is a function not only of relative affinity but also concentration.**

** For estrogen and inhibitor (phyto-estrogen), the fractional binding (f) of each to receptor is represented by

\[
f_E = \frac{(E)}{(E) + K_D} \quad \text{and} \quad f_I = \frac{(I)}{(I) + K_I}\]

(Weatphal, 1971)

A physiological example is presented in the Appendix.

In addition, while the major amount of phyto-estrogen is in the form of inactive glucosiduronates, a portion of the phyto-estrogen conjugates are in the form of sulphoconjugates and the levels in this form exceed plasma "free" levels (Wong and Cox, 1971). Sulphate conjugates of estrogen play an important role in steroid activity (Brooks, et al, 1978) and thus sulphonconjugates of phyto-estrogens may also be of biological significance, (Wong and Cox, 1971).

As determined in this study, phyto-estrogen can interact with estrogen receptors in ewe pituitary, hypothalamus and amygdala. In the case of severe clover disease it has been demonstrated that permanent effects occur in the hypothalamus, which no longer is able to respond to estrogen stimulation.
although the pituitary still responds to exogenous LH-RH (Hearnshaw, et al., 1972; Findlay, et al., 1973). It has been speculated that changes in the estrogen receptors of the hypothalamus and pituitary are responsible for this effect however no mechanism has been proposed (Tang and Adams, 1978). Slightly reduced expression of behavioural signs of estrus has also been observed in clover diseased ewes (Adams, 1978). It is possible that the permanent changes brought about by phyto-estrogens in the hypothalamus are a result of these compounds interacting with estrogen receptors in these tissues.

Direct evidence that phyto-estrogens interfere with gonadotropin release is difficult to demonstrate, however numerous reports imply that this is the case and thus may be an important factor in temporary infertility. Certainly the fact that these compounds have been shown in this study to bind to estrogen receptors in ewe pituitary and hypothalamus lends strong evidence to the view that phyto-estrogens can interfere with the delicate feedback mechanisms involved in gonadotropin release.
BIBLIOGRAPHY


Appendix Figure 1: Miniscintillation vial adapter for Spectronic 20.
APPENDIX 2

Lowry Protein Assay

Reagents

A 2.0% Na$_2$CO$_3$ in 0.1 N NaOH
B 0.5% CuSO$_4$·5H$_2$O in 1% Na$^+$ or K$^+$ tartrate made from mixing on part of 1% CuSO$_4$·5H$_2$O with one part of 2% tartrate.
C mix 50 ml. A plus 1 ml B, fresh each day
D mix 1 part phenol, Folin-Ciocalteau, reagent plus 2 parts water.

Method for Qualitative Determination

Six hundred ul of reagent C was added to scintillation vials containing column eluates, mixed and allowed to stand at room temperature for ten minutes. Then 60 ul of reagent D was added and mixed immediately. Vials were allowed to stand twenty minutes and absorbances read, $A_{600}$, in a Spectronic 20 spectrophotometer-colorimeter with adapter system as in Appendix Figure A1. Alternatively, peaks were located visually.

APPENDIX 3

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 used, as modified for Qualitative Determination

Twenty five mg. of TNBS was dissolved in 100 mls. of 0.1 M sodium tetraborate buffer, pH 9.3. Seventy-five microlitres of this reagent was added to each scintillation vial of column eluate. Vials stood at room temperature for thirty minutes to permit colour development. Location of protein peaks was visually determined.

Adapted from Snyder and Sobocinski, (1975).
APPENDIX FIGURE 2: STEROID ESTROGENS

 Estradiol 17β
 Estrone
 Estriol

<table>
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<th>R₂</th>
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<td>H</td>
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<tr>
<td>=O</td>
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<td>H</td>
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<tr>
<td>OH</td>
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APPENDIX FIGURE 3: DIETHYLSILBESTROL
APPENDIX II  Anti Estrogen Compounds

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<tr>
<th>Common Number</th>
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<tbody>
<tr>
<td>MRL 41</td>
<td>Clomiphene</td>
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<tr>
<td>ICI 46, 474</td>
<td>Tamoxiphen</td>
</tr>
<tr>
<td>CI 628</td>
<td>Nitromophene citrate</td>
</tr>
<tr>
<td>U 11, 100A</td>
<td>Nafoxidine</td>
</tr>
<tr>
<td>MER 25</td>
<td>Ethamoxypitehol</td>
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</table>

* These compounds are reported in the literature either by name or number. The most common usage has been used in the text.

APPENDIX III  Physiological Example of Estrogen and Phyto-Estrogen Interactions with Ewe Pituitary Cytosol Estrogen Receptors

Assuming maximal peripheral plasma estradiol level to be 15 pg/ml as reported in Hauger, et al (1977) and Rawlings, et al (1978) and the \( K_D \) for estradiol to be 0.26 nM, then the fractional occupancy of binding sites, \( f_E \), would be as follows:

\[
f_E = \frac{E}{(E) + K_D} = \frac{0.055}{0.055 + 0.26} = 0.17 \text{ or 17% of the receptors sites would be occupied by estradiol at this concentration.}
\]

Considering the highest preovulatory estradiol levels reported by Scaramuzzi and Land (1978) or 3.9 pg/ml (equivalent to 0.014 nM) then,

\[
f_E = \frac{E}{(E) + K_D} = \frac{0.014}{0.014 + 0.26} = 0.051 \text{ or 5% of the receptor sites would be occupied with estradiol at this concentration.}
\]
For the phyto-estrogen, coumestrol, assuming a blood level of 5 ng/ml (Lindner, 1967) with a $K_I = 60 \text{ nM}$, then the fractional occupancy of binding sites, $f_I$, would be: (5 ng/ml being equivalent to 19 nM)

\[
f_I = \frac{(I)}{(I) + K_I} = \frac{19 \text{ nM}}{19 \text{ nM} + 60 \text{ nM}} = .24 \text{ or 24\% of the estrogen receptors}
\]

of the ewe pituitary would be occupied with coumestrol at this concentration and it can be assumed that this would exert considerable effect on the estrogen dependent mechanisms present.