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A total of five individual experiments were conducted with gonadectomized, male sheep to study the influence of estrogen on the plasma level of pituitary activities, with the greatest emphasis given to gonadotropic activity. Estrogen treatments employed were as follows:

1. 12 mg. diethylstilbestrol (DES) implanted subcutaneously.
2. 1 mg. DES injected intramuscularly daily for 4 consecutive days.
3. 1 mg. estradiol benzoate (EB) injected intramuscularly daily for 4 consecutive days.
4. 1 mg. EB injected intramuscularly daily for 10 consecutive days.
5. 0.025 mg. EB injected intramuscularly daily for 10 consecutive days.

To determine sequential changes in plasma activities, samples of cavernous sinus blood were collected at intervals prior to, during and following estrogen treatments and bioassayed as raw plasma or fractions of plasma obtained by pH and acetone precipitations. Hypophysectomized, immature, female rats were utilized as the assay animals.

A significant depression in plasma gonadotropic activity to all estrogen treatments was clearly demonstrated as evaluated by the ovarian and uterine weight responses in the assay animals. Histological examination of the rat ovarian follicles and interstitial tissue revealed that the estrogen inhibition of plasma gonadotropic activity involved both FSH and ICSH components. The gonadotropic depression was apparent for periods of time following estrogen withdrawal, as well as being evident during treatment. Tail length, epiphyseal width, adrenal weight, thymus weight and thyroid weight responses of the assay animals to the injected plasma did not establish marked trends as did the gonadal responses. These findings are discussed in relationship to current concepts of estrogen-pituitary interrelationships.

ESTROGEN REGULATION OF PITUITARY FUNCTION IN THE GONADECTOMIZED SHEEP

by

GORDON JAY COOPER

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Assistant Professor of Animal Science
In Charge of Major

Redacted for Privacy

Head of Department of Animal Science

Redacted for Privacy

Dean of Graduate School

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ESTROGEN REGULATION OF PITUITARY FUNCTION IN THE GONADECTOMIZED SHEEP

I INTRODUCTION

There is much interest among animal scientists as well as animal producers for improving the reproductive performance of farm animals. In order to determine the conditions necessary for improving reproductive performance, one must first understand the underlying mechanisms involved in reproductive processes. For example, there must be a thorough understanding of the hormonal factors involved in reproduction. Only after such underlying mechanisms are understood can maximum progress in improving reproductive performance be accomplished.

One possible route for better understanding hormonal factors in reproduction is the study of pituitary-gonadal interrelationships. It is well known that a close physiological relationship exists between the pituitary gland and the gonads. For instance, the gonads are dependent upon the hormones of the anterior pituitary gland for proper development and function. Likewise, the gonads produce hormones which regulate the secretion of certain anterior pituitary hormones. For these intricate communications to be accomplished, hormones must be transported by the circulatory system to carry out their stimulatory or inhibitory actions. Thus, analysis of pituitary venous blood samples taken from the animal while under the influence of

certain gonadal hormones would allow study of the qualitative and quantitative changes in the hormones being released by the pituitary gland. As this new approach is more direct than those previously used, a more clear evaluation of pituitary function is possible.

This report consists of a review of the literature on estrogen and pituitary interactions, and also the results of a series of experiments conducted to determine the role estrogen has in regulating the release of anterior pituitary hormones by utilizing a subject devoid of its own gonadal hormones.

II REVIEW OF LITERATURE

The precise role of estrogen in regulating pituitary function is not clearly understood although studies of pituitary-gonadal interrelationships have been quite extensive. A most important problem is how estrogen affects the production, storage and especially the release of anterior pituitary hormones. To study the functional activity of the pituitary as affected by estrogen treatment, previous workers have considered pituitary size, pituitary cytology, pituitary hormone content and gonadal responses in estrogen-treated animals as well as pituitary hormone levels in urine and blood.

Pituitary Size

It has been shown by many workers that gonadectomy as well as systemic administration of estrogen to laboratory animals will cause an increase in pituitary weight (Leonard, Meyer and Hisaw, 1931; Ellison and Burch, 1936; Freudenberger and Clausen, 1937; Smith and Smith, 1944; Burrows, 1949). Kitay (1963b) observed an increase in pituitary weight of 70% and 18% after castration of male and female rats, respectively. Estradiol benzoate injected into intact male and female rats increased pituitary weights 165% and 133%, respectively (Kitay, 1963a). Kanematsu and Sawyer (1963c, 1964) observed similar pituitary hypertrophy in ovariectomized

rabbits given intrahypothalamic, estrogen implants.

Pituitary Cytology

Castration of laboratory animals is known to induce cytologic changes in the pituitary gland including hypertrophy of the basophils to form castration cells (Burrows, 1949; Purves, 1961). These enlarged beta cells or castration cells are vacuolated in such a way as to exhibit a characteristic "signet ring" appearance. Systemic estrogen treatment is known to prevent or reverse the formation of castration cells as well as to decrease the number of acidophils and basophils and increase the number of chromophobes (Burrows, 1949). Rahlmann and Cupps (1962b) observed a cytological change in pituitaries of heifers treated with estradiol which was suggested as being similar to the pituitary response of the rat. Bogdanove (1963) suggested that the action of estrogen in preventing castration cell development is a morphological change associated with the inhibition of gonadotropic hormone secretion. He found that estrogen implanted directly in the pituitary caused a localized regression of castration cells. Bogdanove concluded that since appropriate doses of estrogen directly prevented the development of both central and peripheral gonadotrophs, this possibly reflects cessation of both FSH and ICSH synthesis. Kanematsu and Sawyer (1963c) have reported that minute amounts of estradiol benzoate placed in the posterior median eminence

(PME) markedly inhibited castration cell development in ovariectomized rabbits. They distinguished two types of basophils, aldehyde fuchsin (AF) positive and AF-negative,with the latter type shown to be gonadotropic cells.

Pituitary Hormone Content

Effect of Gonadectomy

Early studies on pituitary-gonadal interrelationships involved changes in the gonadotropic hormone content of the anterior pituitary gland after castration. To determine the amount and nature of hormones present in the pituitary, it was either homogenized and injected or simply implanted into recipient animals. Using these techniques, many workers have found a marked increase in gonadotropic content of the pituitary after gonadectomy. Engle (1929) observed increased gonadotropic potency in the pituitaries of castrate male and female rats as evaluated by increased ovarian weights of immature, female rats and mice implanted with the pituitaries. Similarly, Evans and Simpson (1929) found that pituitaries from intact, female rats implanted at the rate of two pituitaries daily for 2 days into immature, female rats produced an average ovarian weight of 19.5 mg. as compared to 113.5 mg. ovaries produced by similar implants from spayed females. Similar increased pituitary gonadotropic potency after gonadectomy has been observed in guinea pigs

(Severinghaus, 1932), rabbits (Smith, Severinghaus and Leonard, 1933) and sheep (Warwick, 1946).

More specific findings are that gonadectomy causes both quantitative and qualitative changes in pituitary hormone content. It is in general agreement that ovariectomy causes an increase in the concentration of FSH and ICSH in the pituitary gland of the rat (Greep and Jones, 1950). Hellbaum and Greep (1943) found that pituitaries of adult, male rats contained mainly FSH. However, after castration, the levels of both FSH and ICSH were markedly increased as evaluated by the ovarian weight response and presence or absence of luteinization in intact and hypophysectomized, immature, female rats. Using an HCG augmentation assay for FSH, Parlow (1964) found that pituitary FSH levels were increased five fold after ovariectomy of adult mice. However, in contrast to the findings of Hellbaum and Greep (1943), ICSH was the same concentration in the pituitaries of normal and ovariectomized mice as determined by the ovarian ascorbic acid depletion (OAAD) assay. It has also been found that gonadectomy causes a major increase of FSH (Gans, 1959a) and ICSH (Ramirez and McCann, 1963) only in the pituitaries of adult female and immature, male rats. Since the pituitary of the intact male contained high levels of FSH and ICSH, gonadectomy caused only a slight increase in these activities. The FSH and ICSH activity in the two latter studies was evaluated by increased uterine and

ovarian weight in hypophysectomized, immature rats and the OAAD assay, respectively.

Hellbaum (1933) found that pituitary extracts of gonadectomized, male horses injected into immature, female rats produced mainly follicular development with slight luteinization occurring in only two of 16 rats. He found this to be in sharp contrast to pituitary powder of sheep as doses sufficient to produce large follicles, invariably stimulated luteinization.

From the above studies, it can be seen that gonadotropic potency of the pituitary is increased after gonadectomy. However, gonadectomy does not increase gonadotropic activities equally under all conditions, and results vary with sex and species.

Effect of Estrogen

Early work by Meyer et al. (1930) showed that pituitaries of estrogen-treated, gonadectomized rats contained less gonadotropic activity than did pituitaries from control rats. Gonadotropic potency of the pituitaries was determined by the ovarian weight response in immature, female rats implanted with the pituitaries. Using the same assay, Meyer et al. (1932) obtained similar results by injecting 4 to 10 r.u. of estrogen for 13 to 37 days into gonadectomized rats. It was concluded that estrogen acted to decrease the gonad stimulating power of the pituitary by inhibiting the production of the gonadotropic

complex. However, the possibility of continued release during estrogen injection was not ruled out. Similar results have been reported with gonadectomized guinea pigs injected with 25 to 100 r.u. of estrogen (Nelson, 1935).

Although lowered pituitary gonadotropic potency after estrogen treatment has been demonstrated by many workers, Lauson, Heller and Severinghaus (1938), Heller and Heller (1939) and Heller, Heller and Severinghaus (1942) were unable to decrease pituitary gonadotropic potency of rats with large doses of estrogen. They determined the gonadotropic hormone content of the pituitary by uterine weight stimulation in immature, female rats given pituitary implants. These three groups of workers suggested that the injected estrogen could have been above the physiological limits required to depress pituitary gonadotropic potency of gonadectomized rats.

Many studies have been conducted to study the qualitative effects of estrogen on pituitary gonadotropic potency of laboratory animals. With an ovarian augmentation test in immature, female rats, Leonard (1937) found a marked increase of FSH and a slight increase of ICSH in pituitaries of rats following ovariectomy. However, treatment with 10 r.u. of estrogen for 10 days gave FSH only slightly above and ICSH slightly below the level found in pituitaries of intact rats. By utilizing the testis weight response in hypophysectomized, immature rats, Paesi et al. (1955) found that 50 mcg. of estradiol benzoate

lowered the FSH content of pituitaries of ovariectomized females, intact males and castrated, male rats to a level only slightly above the low level found in intact females. However, the low pituitary FSH level of the intact female was not affected by estrogen treatment. Hoogstra and Paesi (1957) and Paesi, de Jongh and Engelbregt (1957) found a similar low level of FSH in pituitaries of estrogen-treated rats as evaluated by the testis weight response in hypophysectomized, immature rats.

With the OAAD assay, Ramirez and McCann (1963) found that subcutaneous injections of estradiol benzoate into ovariectomized rats lowered pituitary ICSH levels below that found in untreated controls. It was suggested that estrogen blocked the synthesis of ICSH. Similarly, Gans and Van Rees (1962) reported decreased pituitary ICSH activity in gonadectomized rats treated with 0.5 and 2.0 mcg. of estradiol as evaluated by the ventral prostate response in hypophysectomized, immature rats. However, in contrast to previous findings, they reported an increased ICSH level in pituitaries of ovariectomized rats treated with 0.1 and 0.2 mcg. of estradiol. In the castrate male, ICSH levels were not influenced by the low doses. It was assumed that estrogen if chronically administered depresses the production of ICSH.

The effect of estrogen treatment on pituitary gonadotropic potency of women has been studied by Rowlands and Sharpey-Schafer

(1940). They injected 10 mg. of estradiol daily into four women dying of different diseases. After death, the pituitaries were assayed in hypophysectomized, immature, female rats as were pituitaries of five women not receiving estrogen. Pituitaries of the estrogen-treated women were found to possess much less gonadotropic activity than those of non-treated women as determined by ovarian and uterine weight responses in the assay rats.

Pituitary potency of pregnant cows has been studied by Nalbandov and Casida (1940). They observed a steady and significant decrease in gonadotropic activity of the cow pituitaries from early to late pregnancy as evaluated by the gonadal weight response in rats and chicks injected with pituitary powder. It was suggested that placental estrogen either inhibited the formation or increased the excretion of gonadotropins, although the possible action of other hormones must not be ruled out. Labhsetwar et al. (1964) bioassayed pituitaries of estrogen-treated, spayed heifers using the OAAD and the ventral prostate response for ICSH, and the testis weight response for FSH. The biological responses obtained in hypophysectomized, immature rats clearly demonstrated a decrease in pituitary FSH, and ICSH was lowered as tested with the OAAD assay, but not by the ventral prostate response. Because of the lack of blood assays, conclusions could not be made with certainty that the reduced pituitary potency was due to a release of gonadotropins into the blood, or whether it was

due to decreased gonadotropin production.

Soliman et al. (1963) have studied the effect of estradiol benzoate administered at the rate of 20 mcg. daily for 2 days on pituitary gonadotropic activity in the ovariectomized ewe. Neither pituitary FSH activity, as measured by the HCG augmentation test, nor pituitary ICSH, as measured by a test based on an increase in the number of corpora hemorrhagica, was changed by estrogen treatment. Using the testis weight response in 2 day old chicks, Kammlade et al. (1952) observed a significant drop in pituitary gonadotropic potency of ewes at the time of first heat with a steady increase in potency after heat to a maximum at days 16 and 17 of the cycle. Similarly, Robinson and Nalbandov (1951) assayed pituitaries of 33 swine killed on almost every day of the estrual cycle using the testis weight response in chicks. Results showed that pituitary gonadotropic content during heat was one-half that of midcycle, with potency remaining low through the first 8 days of the cycle, increasing suddenly and remaining high until day 20. It was suggested that the low gonadotropic potency of the pituitaries during heat was due to inhibition of the pituitary by follicular estrogen. However, they were not sure whether the assay measured the actual secretion rate, storage, or residual amounts of hormones remaining after release. Since there was a high correlation between the day of the cycle and pituitary potency, and between pituitary potency and the number and size of

the ovarian follicles, they concluded that the pituitary assay reflected the hormone secretion rate.

If the true functional activity of the pituitary is to be studied, greater emphasis should be given to the study of pituitary secretion. Studies involving estrogen administration are complicated by dose levels and the period of time of injection, but it is generally concluded that short term, low dose estrogen treatment will stimulate the release of gonadotropins (ICSH), while high dose, long term administration will inhibit pituitary secretion (Frank, 1940; Greep, 1961; Everett, 1961). Hisaw et al. (1934) and Fevold, Hisaw and Greep (1936) postulated that when levels of estrogen are low the pituitary secretes larger amounts of FSH. As the estrogen reaches moderate levels, FSH secretion is decreased and the pituitary is stimulated to secrete larger amounts of ICSH. Following is a discussion of literature either supporting or opposing the theories of pituitary secretion as determined by studies involving either gonadal inspection, parabiosis, urine gonadotropin levels or blood gonadotropin levels.

Gonadal Inspection of Estrogen-Treated Intact Animals

Inhibitory Effect of Estrogen

It was concluded by Moore and Price (1932) that injuries to gonads following gonadal steroid administration to either sex is a

pituitary involvement. Their work showed that the atrophic effects of estrogen on the rat testis could be corrected by implantation of either fresh rat pituitaries or by subcutaneous injections of gonadotropic extracts from pregnant women urine. Similarly, large doses of stilbestrol have been found to cause inactive ovaries in rats (Noble, 1938). However, when the inactive state was observed, administered gonadotropins plus stilbestrol caused the previously inactive ovaries to become functional. It was suggested that the inactive ovaries were associated with an absence of gonadotropic activity in the pituitary due to estrogen inhibiting hormone synthesis.

In a study conducted by Folley and Malpress (1944), they implanted either diethylstilbestrol or hexoestrol subcutaneously into heifers and later inspected the gonads by rectal palpation. Estrogen treatment was found to give inactive ovaries, but removal of the implants gave cyclic periodicity within 2 months. It was suggested that the inactive ovaries were apparently due to decreased gonadotropic function of the pituitary gland. Hammond and Day (1944) similarly observed that implantation of the same estrogenic materials into cows and heifers suppressed ovarian follicular development. After removal of the implants, follicular growth and ovulation were resumed. This was supported by Rahlmann and Cupps (1962a) and Greenstein, Murray and Foley (1958) as they found that 3.5 mg. and 1 to 2 mg. of estradiol, respectively, suppressed follicular development in heifers.

Facilitory Effect of Estrogen

Early work of Hohlweg (1934) supported a release of ICSH from the pituitary gland in response to estrogen treatment as ovaries of immature rats contained numerous corpora lutea after a series of estrogen injections. The work of Fevold, Hisaw and Greep (1936) also supported an ICSH release as they found that estrogen injected into rats augmented the action of a given dose of FSH as shown by an increase in ovarian weight and luteinization. The action was found to be via the pituitary as augmentation of FSH by estrogen did not occur in hypophysectomized rats. Similar gonadal changes supporting an ICSH release after estrogen treatment have been reported by Hisaw et al. (1934), Lane and Hisaw (1934) and Lane (1935). Sawyer, Everett and Markee (1949) injected estrogen into pregnant rats at day 4 of pregnancy and within 48 hours ovulation occurred. If ICSH were injected at day 5 of pregnancy, the same response was observed. With a similar study, Everett, Sawyer and Markee (1949) succeeded in hastening ovulation in normal cycling rats by a properly timed injection of estrogen. However, they suggested that the response was not specific in as much as other substances, including progesterone, could produce the same response. Estrogen treatment has also been found to cause a limited amount of spontaneous ovulation in rabbits (Sawyer and Markee, 1959).

Conflicting results have been obtained as to the ovulatory response in anestrous ewes injected with estrogen. Hammond, Hammond and Parkes (1942) and Hammond (1945) were able to induce estrus and ovulation in anestrous ewes with estrogen injections but the ovulatory response was not consistent. However, Cole, Hart and Miller (1945) failed to observe any ovarian stimulation in four anestrous ewes treated with either 100 r.u. or 400 r.u. of estrogen for 15 days. Similarly, Vander Noot, Reece and Skelley (1949) failed to induce lambing in anestrous ewes with estrogen injections although the ewes came into heat. Administration of estradiol to ewes at a time of the estrous cycle when ovarian follicles would presumably be able to respond to any increased release of ICSH does not hasten the time of ovulation (Dutt, 1953). It was suggested that rising blood levels of estrogen at the time of estrus may not cause a release of ICSH or else the 1 mg. dosage employed was above physiological limits. A similar study in dairy heifers by Hansel, Trimberger and Bearden (1952) was conducted to determine if injecting estrogen causes a release of ICSH. However, administration of 1000 to 3000 I.U. of estradiol to 14 dairy heifers at the onset of heat did not shorten the ovulation interval. The average interval from onset of estrus to the time of ovulation was 32.4 hours for the estrogen treated periods and 29.8 hours for the control period on the same animals. In another study with heifers, Wiltbank, Ingalls and Rowden

(1961) failed to observe consistent ovulation in response to estrogen injections.

Support for an ICSH release in response to estrogen in swine has been reported by Kidder, Casida and Grummer (1955). In an experiment with ten gilts, a 3 mg. injection of diethylstilbestrol at day 11 of the estrous cycle caused a lengthening of the cycle due to luteinization of the ovarian follicles. Injections on day 16 generally shortened the cycle.

Parabiosis Experiments

Effect of Gonadectomy

Since the level of pituitary hormones in blood is low and difficult to detect, indirect studies involving the parabiotic union of rats have added greatly to the study of pituitary secretion. As early as 1931, Martins, Rocha and Silva placed immature, intact, female rats in parabiosis with immature, gonadectomized rats. Within 6 to 7 days, the non-spayed partner showed precocious estrus and ovulation indicating an excessive supply of gonadotropins from the pituitary of the gonadectomized twin. Similarly, Cutuly, McCullagh and Cutuly (1937) joined hypophysectomized, male rats with either castrate or intact, male rats. The degenerated testicles of the hypophysectomized rat were restored to a functional state only if joined to a castrate

partner. Thus, the pituitary gland not controlled by gonads produced or liberated a greater amount of gonad stimulating hormone than pituitaries of intact rats. Hertz and Meyer (1937) and Biddulph and Meyer (1946) similarly found an increased release of gonadotropic hormones from the pituitary of the gonadectomized twin of a pair of intact-gonadectomized, parabiotic rats.

Inhibitory Effect of Estrogen

By use of parabiotic techniques with rats, Hertz and Meyer (1937) found that the excessive supply of gonadotropins from the pituitary of the gonadectomized twin of parabionts could be completely prevented by injections of estrogen into the same twin. This was determined by lack of hypertrophy of the ovaries of the intact, female twin. Kincl, Birch and Dorfman (1964) injected estradiol subcutaneously once daily for 10 days into the ovariectomized twin of a parabiotic pair of intact-ovariectomized rats. A low dose of 0.03 mcg. was the smallest dose needed to produce minimum pituitary inhibition, and 0.2 mcg. was the minimum dose needed to produce maximum inhibition as shown by the ovarian weight of the intact twin. It required 0.2 and 0.9 mcg. of estradiol to produce minimum and maximum uterine weight stimulation, respectively, in the ovariectomized twin. Similar inhibitory actions of estrogen on pituitary function have been reported by Biddulph, Meyer and Gumbreck (1940), Meyer

and Biddulph (1941) and Byrnes and Meyer (1951a, b) with parabiotic rats and Miyake (1961) with parabiotic mice.

To eliminate the endogenous source of gonadotropins, Cutuly and Cutuly (1938) joined hypophysectomized, immature, male rats in parabiosis with castrate males. Daily injections of either estrone or estradiol at the rate of 5 to 100 mcg. into the castrated partner prevented the descent and functional repair of the testis of the hypophysectomized twin. The functional repair of the testis did occur if estrogen were not injected, demonstrating that estrogen inhibits the secretion of gonadotropins from the pituitary.

Facilitory Effect of Estrogen

With parabiotically joined rats, Biddulph, Meyer and Gumbreck (1940) observed corpora lutea on ovaries of the intact twin when estrogen was administered to the gonadectomized partner. It was suggested that quantities of estrogen necessary to prevent the castration hypersecretion of gonadotropins prevents the production and release of FSH while increasing the production and release of ICSH.

Urine Hormone Content

Effect of Gonadectomy

In clinical studies with ovariectomized women, gonadotropic

activity has been detected in the urine. Frank and Salmon (1935) found elevated levels of gonadotropins in the urine of women after ovariectomy as evaluated by ovarian changes in immature, female rats. Using the ovarian and uterine weight responses in hypophysectomized, immature rats, Rowlands and Sharpey-Schafer (1940) similarly found elevated levels of gonadotropins in the urine of women after ovariectomy.

Robertson, MacGillivray and Hutchinson (1963) detected elevated gonadotropic activity in urine of male and female sheep after gonadectomy by the uterine weight response in mice. As compared with the human level of activity, the level in the sheep was considerably lower.

Inhibitory Effect of Estrogen

In the above mentioned work by Frank and Salmon (1935), they injected ovariectomized and post-menopausal women with a total dose of either 4,000 or 22,000 r.u. of estradiol benzoate. They noted a rapid disappearance of gonadotropic activity from the urine with the activity remaining absent for 28 to 70 days. Similarly, Rowlands and Sharpey-Schafer (1940) found that 10 mg. of estradiol given to ovariectomized women caused gonadotropin disappearance from the urine. Further support of these findings was the work of Igarashi, Matsumoto and Hosaka (1965), as they found that 10 mg. of estrogen injected into three spayed women daily for 7 days gave a marked decrease in

urinary gonadotropin as evaluated by the uterine weight response in mice. However, after withdrawal of treatment, the urinary level of gonadotropic activity increased beyond the pre-treatment level. This was considered as being a rebound phenomenon of pituitary function which was found to improve fertility in otherwise infertile females.

In contrast to the above findings, Heller, Chandler and Myers (1944) reported that low doses of diethylstilbestrol administered to 23 ovariectomized women failed to prevent the typical rise in urinary gonadotropins following spaying. However, larger doses were found to be completely effective. Their findings were based on the ovarian and uterine weight responses in immature rats.

Facilitory Effect of Estrogen

Funnell, Keaty and Hellbaum (1951) found direct evidence in favor of ICSH release as estrogen given to women with menopausal symptoms increased ICSH activity in urine where only FSH had previously been detected. Gonadotropic activity was evaluated by the ovarian weight response and presence or absence of luteinization in intact and hypophysectomized, immature, female rats.

Blood Hormone Content

Effect of Gonadectomy

Although blood levels of gonadotropins are low and difficult to

detect in intact animals, many workers have been able to detect gonadotropic activity in blood of gonadectomized, laboratory species. Emery (1932) was one of the first workers to detect an increased level of gonadotropic activity in the blood of rats after gonadectomy as evaluated by the ovarian weight response in immature, female rats. Later, Hellbaum and Greep (1943), in contrast to previous reports, found detectable quantities of ICSH and a small amount of FSH in the serum of intact, male rats. After gonadectomy, the blood level of FSH greatly increased while the level of ICSH could not be detected in a 10 ml. sample of plasma. Gonadotropic activity was determined by the ovarian weight response and the presence or absence of luteinization in either intact or hypophysectomized, immature, female rats. The detection of ICSH and FSH in the blood of intact rats was not confirmed by Cozens and Nelson (1958), using ovarian interstitial tissue repair and ovarian follicular growth in hypophysectomized, immature rats. However, Ramirez and McCann (1963), using the OAAD assay, found minimal amounts of ICSH in plasma of intact, male rats.

Cozens and Nelson (1958) did detect FSH activity in plasma of ovariectomized rats as early as 7 days postoperatively by injection of 24 or 32 ml. of plasma into immature, hypophysectomized, female rats for 4 days. The FSH activity continued to increase slowly but progressively as the postoperative interval increased up to a 4 month

period. Similar increased blood levels of FSH and ICSH in gonadectomized rats have been reported by Contopoulos, Simpson and Koneff (1958), Gans (1959a, b), McCann and Taleisnik (1961a, b) and Ramirez and McCann (1963).

Kanematsu and Sawyer (1964) found elevated levels of ICSH in the plasma of rabbits after ovariectomy as evaluated by the OAAD assay. They found that plasma from ovariectomized rabbits produced an ovarian ascorbic acid depletion of 21.6% as compared to plasma from intact rabbits which produced 5.8% depletion.

Levels of FSH have been detected in blood of ovariectomized mice with the HCG augmentation assay, but in contrast to findings in the rat, ICSH could not be detected in the same blood by the OAAD assay (Parlow, 1964). It was suggested that the bioassay method was not sensitive enough, or that ICSH increased immediately after ovariectomy and then returned to normal before the mice were autopsied.

Inhibitory Effect of Estrogen

Almost all available reports on the effect of estrogen on blood levels of gonadotropins deal with laboratory animals. Using an HCG augmentation test and the ventral prostate response for the detection of FSH and ICSH, respectively, Gans (1959a, b) found that a presumably physiological dose of 2 mcg. of estradiol benzoate injected for 4

days into gonadectomized male and female rats lowered the plasma level of FSH and ICSH. However, the low levels of ICSH in plasma of intact rats were not affected by estrogen treatment. It was suggested that ICSH release and production are greatest when no estrogen is present. With the same assay, Gans and Van Rees (1962) found that doses of 0.5 and 2.0 mcg. of estradiol decreased serum ICSH levels of both gonadectomized male and female rats. However, 0.1 and 0.2 mcg. of estradiol resulted in only a slight decrease in serum ICSH in ovariectomized rats, but the same doses significantly decreased serum ICSH in gonadectomized, male rats. It was suggested that estrogen if chronically administered depresses the production and inhibits the release of ICSH. These two effects were suggested as having different threshold levels with the threshold for release being lower in females, and the threshold for inhibition of production being lower in males. Ramirez and McCann (1963) similarly found a lowered level of ICSH in plasma of estrogen-treated, gonadectomized rats with the OAAD assay. They believed that more estrogen was needed to block synthesis than release of the gonadotropins. By use of the OAAD assay, McCann and Taleisnik (1961b) observed decreased ICSH activity in plasma of ovariectomized rats within 1 day after a single subcutaneous injection of 50 mcg. of estradiol benzoate. An injection of 5 mcg. of estradiol benzoate decreased ICSH activity in the plasma within 2 to 3 days and the plasma

activity failed to return to the pre-treatment level for 1 week after withdrawal of estrogen treatment. A dose of 0.1 mcg. was the minimum effective dose of estradiol needed to decrease ICSH activity within 3 days, but ICSH returned to the pre-injection level within 4 days after the initial injection. A slight decrease of serum ICSH activity occurred within 1 hour after an intravenous injection of either 40 or 50 mcg. of estradiol.

Due to limited information, it is difficult to make conclusive statements as to the effect of estrogen on blood levels of gonadotropins in larger mammals. Soliman et al. (1963) directly determined blood levels of ICSH and FSH in ovariectomized ewes treated with 20 mcg. of estradiol benzoate daily for 2 days by assaying the blood in immature mice. Follicle stimulating activity was determined by an HCG augmentation test and ICSH by injecting the test material simultaneously with pregnant mare serum (PMS) and counting the number of corpora hemorrhagica. The estrogen treatment apparently did not cause variations in blood levels of either FSH or ICSH as compared to control ewes.

Facilitory Effect of Estrogen

It was earlier believed by Hohlweg (1934) that estrogen increased the pituitary secretion of gonadotropins as pituitaries of estrogen-treated rats, after being implanted into immature, female

rats, resulted in luteinization of the ovaries of the recipient animals in contrast to only follicular development from untreated pituitary implants. Hellbaum and Greep (1946) and Bradbury (1947) believed that the low gonadotropic hormone content of pituitaries of estrogen-treated rats was due to estrogen causing a release of gonadotropins from the pituitary. Sidki, Badawi and Soliman (1958) observed an increase in plasma ICSH in ovariectomized rats injected with 1 mcg. of estradiol benzoate daily for 30 days. The ICSH activity was evaluated by the number of corpora hemorrhagica on the ovaries of immature, female mice injected with pregnant mare serum plus the test material. McCann and Ramirez (1964) observed an increase in plasma ICSH of intact rats implanted with estradiol in the median eminence area of the hypothalamus, but a depression of plasma ICSH activity was observed in ovariectomized rats similarly implanted. Plasma ICSH activity was evaluated by the OAAD assay. They suggested that estrogen inhibits ICSH release only if the pituitary secretion is elevated and increases ICSH release when the pituitary secretion is low. This suggestion does not explain the increased plasma activity observed by Sidki, Badawi and Soliman (1958) in estrogen-treated, ovariectomized rats. In contrast to the above reports, Gans (1959a, b), Gans and Van Rees (1962), Ramirez and McCann (1963) and McCann and Taleisnik (1961b) found that treatment of rats with estrogen would lower the blood level of gonadotropins.

Effect of Estrogen on Other Pituitary Tropic Activities

Somatotropic Activity

Many workers have found that estrogen will inhibit growth in rats, but reports are conflicting as to the site of estrogen action. Reece and Leonard (1939) and Freudenberger and Clausen (1937) suggested that estrogen inhibited growth by lowering the growth stimulating power of the pituitary. They based the theory on the finding that hypophysectomized, ovariectomized rats implanted with pituitaries of non-treated rats gained significantly more weight than recipient animals implanted with pituitaries of estrogen-treated donors. In contrast, there is evidence that estrogen inhibits growth by acting on the growth center of bones. It was found by Spencer, D'Amour and Gustavson (1932) that injecting the growth factor from the pituitary plus estrogen into immature rats gave growth curves paralleling that of the immature controls. However, average bone lengths of rats treated in such a way were not as great as the controls, but were greater than bone lengths of rats receiving estrogen alone. Noble (1938) similarly found that growth rates were inhibited in rats given stilbestrol, and administration of STH simultaneously with stilbestrol did not improve growth.

In contrast to the action of estrogen in rats, the growth rate of

ruminant animals is stimulated by estrogen (Clegg and Cole, 1954; Hammond, 1958). Hammond (1958) suggests that estrogen acts to increase the production of STH since estrogen implants in ruminants give similar protein anabolism as would be expected to STH injections.

Adrenocorticotropic Activity

Many workers have reported that systemic injections of estrogen into laboratory animals will cause adrenal enlargement (Ellison and Burch, 1936; Allen and Howard, 1942; Smith and Smith, 1944; Greep and Jones, 1950; Kitay, 1963c). Ellison and Burch (1936) and Allen and Howard (1942) believed adrenal hypertrophy was due to an action of estrogen mediated through the pituitary to increase ACTH release. This was supported by the fact that adrenal hypertrophy was dependent on the presence of the pituitary as estrogen did not cause adrenal enlargement of hypophysectomized rats (Ellison and Burch, 1936). Kitay (1963a, b) found that 2 mcg. of estradiol increased the secretion of ACTH in gonadectomized and intact rats as evaluated by changes in pituitary ACTH content and adrenal weight. The estrogen treatment also stimulated adrenal secretion as determined by in vitro study of steroid yields of adrenal slices.

Clegg and Cole (1954) suggest that the acceleration of protein anabolic processes in ruminants implanted with stilbestrol is indirectly due to androgens from the adrenal gland instead of STH

from the pituitary, although both hormones increase nitrogen retention. Since a marked depression of blood eosinophils was observed, Clegg and Cole concluded that estrogen increased the release of ACTH from the pituitary which stimulated the adrenal cortex to produce androgen. Development of masculine characteristics in estrogen-treated animals supported the involvement of androgen.

Thyrotropic Activity

Many workers believe estrogen has a direct effect on the thyrotropic activity of the pituitary (Mazer, Israel and Alpers, 1936; Freudenberger and Clausen, 1937; Victor and Andersen, 1938). It was suggested by Reineke and Soliman (1953) that estrogen exerts its effect on the thyroid via the pituitary, possibly to influence TSH secretion. They injected 6 r.u. of estradiol benzoate into female rats and found that within 48 hours the thyroidal I^{131} uptake was significantly increased. Chronic doses of estrogen at 300 r.u. for 20 days, however, did not increase thyroidal I^{131} uptake. Similarly, Money et al. (1950, 1951) reported that 50 mcg. of estradiol injected into male rats for 10 days increased thyroidal I^{131} uptake, but larger doses had no effect. Reineke and Soliman (1953) found support for the pituitary involvement as hypophysectomized, ovariectomized rats had a greatly reduced thyroidal I^{131} uptake as compared to ovariectomized controls. Injection of either 6 or 300 r.u. of

estradiol gave no significant effect on thyroidal I^{131} uptake in the hypophysectomized, ovariectomized rats, however, the same treatment caused a significant increase in thyroidal I^{131} uptake in the ovariectomized rats.

Site of Estrogen Action

In early reports by Moore and Price (1932) and Meyer, Leonard and Hisaw (1932), it was suggested that gonadal hormones act to regulate pituitary function by a feed back action exerted directly on the pituitary. However, Everett (1948), Sawyer, Everett and Markee (1949), Markee, Everett and Sawyer (1952) and Sawyer and Markee (1959) believed that estrogen acted in some way via the nervous system as they found that estrogen facilitated ovulation in laboratory animals, but this response could be inhibited by nervous system blocking drugs. They (1949, 1952) suggested that estrogen exerted its action by altering the threshold to extrinsic stimulation of a gonadotropic sex center in the hypothalamus. Recent findings support the idea that estrogen acts through a negative feed back action on the hypothalamus to control the secretion of pituitary gonadotropins (Flerko and Szentagothai, 1957; Lisk, 1960; Davidson and Sawyer, 1961; Kanematsu and Sawyer, 1963a, 1964). By examination of the gonads of laboratory animals, these workers found that ovarian or estrogen implants in the hypothalamus caused a decrease

in gonadotropic hormone secretion, but similar implants in the pituitary did not decrease gonadotropin secretion. However, Kanematsu and Sawyer (1964) found that estrogen implants in the pituitary of ovariectomized rabbits stimulated the release of ICSH into the circulation as evaluated by the OAAD assay.

Inhibition of castration cell formation in ovariectomized rabbits given hypothalamic, estrogen implants (Kanematsu and Sawyer, 1963c) offers further support for the feed back action of estrogen on the hypothalamus in controlling gonadotropin secretion. However, Bogdanove (1963) proposed that estrogen inhibits castration cell development by a direct action on pituitary cells. He believed that estrogen from the hypothalamic implants was transported via the portal blood system to the anterior pituitary where estrogen acts directly on the pituitary cells.

Kanematsu and Sawyer (1963b) presented evidence which suggests that estrogen acts via the hypothalamus to increase the release of ACTH. They observed hypertrophy of rabbit adrenals when the rabbits were given intrahypothalamic, estrogen implants. Similar adrenal hypertrophy was not observed with intrapituitary implants.

General Conclusions and Aims of Present Study

From the survey of literature, it is concluded that the precise role of estrogen in regulating pituitary secretion is quite

controversial. Estrogen appears to increase pituitary gonadotropic output under some conditions while decreasing the output under other conditions. The variation appears to be due to factors as length of treatment, dose levels, species, age, sex and reproductive state of the animal. Further complications occur as most of the studies have involved the determination of total gonadotropic activity rather than independent determinations of ICSH and FSH. Another problem has arisen when attempts are made to interpret pituitary hormone content in terms of secretory activity. The direct determination of blood levels of hormones gives a more clear evaluation of pituitary secretion. The development of assay methods which are sensitive enough to measure body fluid levels of pituitary hormones is a possible way of improving methods for the study of pituitary function.

Recently developed techniques for obtaining pituitary venous blood have aided greatly in the study of functional activity of the pituitary gland under various influences. Ganong and Hume (1956) made the first contribution in this area as they developed a technique with the dog whereby blood was collected by puncturing the cavernous sinus. The needle was placed in the sinus with the aid of a stereotaxic instrument after an opening had been made in the dorsal skull. McFarland, Clegg and Ganong (1960) took advantage of this finding and developed a technique whereby pituitary venous blood could be collected very effectively from the cavernous sinus of unanesthetized

sheep by inserting a hypodermic needle in the sinus via the foramen ovale. This technique of cavernous sinus blood collection has now been applied to cattle (Donaldson and Hansel, 1964; Seifart and Hansel, 1965). Foltz, Johnson and Nelson (1966) have obtained presumably cavernous sinus blood by a technique whereby blood is taken from the deep facial vein of sheep.

By use of the blood collection technique of McFarland, Clegg and Ganong (1960), Ellington, Contopoulos and Clegg (1962) detected FSH and ICSH activity in cavernous sinus blood of gonadectomized, male sheep by bioassay of the blood in immature, female rats which were hypophysectomized at 26 to 28 days of age. They used repair of ovarian interstitial tissue and ovarian follicular stimulation for detection of ICSH and FSH, respectively. Recently, McDonald and Clegg (1966) have determined FSH and ICSH activity in cavernous sinus blood of gonadectomized male and female sheep by the HCG augmentation assay and the OAAD assay in immature, female rats. They found that gonadectomy of the sheep did not alter the serum ICSH activity in either sex, but FSH activity increased in both sexes after gonadectomy. As well as observing changes in gonadotropic activity of cavernous sinus blood after castration, changes have also been observed to factors such as progesterone treatment (Ellington, Contopoulos and Clegg, 1964; McDonald and Clegg, 1965) and stage of the estrous cycle (Dierschke and Clegg, 1965; Seifart and Hansel,

(1965). The value of this technique for the study of the effect of various factors on pituitary function is obvious.

The purpose of the present study was to determine the role of estrogen in regulating the secretion of anterior pituitary hormones, particularly the gonadotropins, by utilizing the cavernous sinus technique. The gonadectomized, male sheep was used as the donor animal to eliminate the endogenous source of gonadal steroids as well as for the high level of gonadotropic activity in their blood. Hypophysectomized rats were utilized as the assay animals as they are, of course, uncomplicated by an endogenous source of pituitary hormones.

III EXPERIMENTAL MATERIALS AND METHODS

Experimental Design

A total of five individual experiments were conducted to determine the influence of estrogen administration on pituitary function with the greatest emphasis given to pituitary gonadotropic activity. The experimental design for each of the experiments is summarized in table 1. The animals utilized as donors to furnish plasma for subsequent hormone bioassays were mature, gonadectomized, male sheep of mixed breeding. Orchidectomy had been performed on the donors at approximately 2 to 6 weeks of age. While on experiment, the sheep were provided a daily ration of 1 lb. of grain mix and approximately 2 lb. of alfalfa hay.

The estrogens that were administered to the wethers were estradiol benzoate[1] (EB) and diethylstilbestrol[2] (DES) as shown in table 1. The estrogens for injection were made up in the concentrations of either 1 mg. (experiments II, III and IV) or 0.025 mg. (experiment V) of estrogen per ml. of vegetable oil. All injections were made intramuscularly at the same time of day and were of the same volume. In experiment I, each sheep received a total of 12 mg. of

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[1] Sigma Chemical Company

[2] Chas. Pfizer and Company

DES implanted in the form of four pellets of equal potency. Implantations were made subcutaneously in the ear in such a way that all four pellets would be deposited at the same site. Teat length measurements were taken in experiments IV and V (Braden, Southcott and Moule, 1964).

Cavernous Sinus Plasma Collection

Samples of cavernous sinus blood (pituitary venous blood) were collected from the wethers at intervals before, during and following estrogen treatment (table 1) by the technique described by McFarland, Clegg and Ganong (1960). A total of 100 ml. of blood was collected from each wether at each collecting interval. The collecting equipment consisted of a 100 ml. syringe attached to a 2 inch, 16 gauge, hypodermic needle. The equipment had previously been heparinized with a solution containing 300 I.U. of heparin per ml. of either physiological saline or Tyrode's solution. Each 100 ml. sample was placed into a centrifuge bottle containing 300 I.U. of dry heparin and immediately cooled in an ice bath. Within 30 minutes of collection, the samples were centrifuged under refrigeration for 10 minutes at 9000 r.p.m. The resulting plasma was then decanted from the cellular material, pooled among animals for each collection interval, separated into aliquots and frozen. The plasma was later bioassayed either as raw or fractionated plasma.

TABLE 1. Outline of the experimental design used to study the influence of estrogen on pituitary activity in the gonadectomized, male sheep.

Experiment number	Sheep				Assay Animals	
	Number	Estrogen used	Treatment	Cavernous sinus blood collection days[a]	Plasma preparation[b]	Treatment
I	6	DES[c]	12 mg. subcutaneous implant per sheep	0, 8	Raw	Total dose 20 ml. per rat
II	6	DES	Total dose 4 mg. per sheep; 1 mg. I.M.[d] for 4 consecutive days	0, 4, 7, 9	Raw	Total dose 20 ml. per rat
III	4	EB[e]	Total dose 4 mg. per sheep; 1 mg. I.M. for 4 consecutive days	0, 4, 7, 9	Raw	Total dose 20 ml. per rat
IV	4	EB	Total dose 10 mg. per sheep; 1 mg. I.M. for 10 consecutive days	0, 6, 12, 15, 18	Fractionated	Total dose 30 ml. eq. per rat
V	6	EB	Total dose 0.25 mg. per sheep; 0.025 mg. I.M. for 10 consecutive days	0, 6, 12, 15, 18	Raw	Total dose 20 ml. per rat
					Fractionated	Total dose 40 ml. eq. per rat

[a] Day 0 represents blood collections made before estrogen treatment. Day 1 is considered the first day of estrogen treatment. The plasma samples were pooled among sheep for each interval.

[b] Raw refers to unaltered plasma and fractionated plasma was that prepared by the modified Cole and Goss fractionation method.

[c] Diethylstilbestrol.

[d] Intramuscularly.

[e] Estradiol benzoate.

Fractionation Procedure

When fractionated plasma was bioassayed, the raw plasma was thawed to room temperature and fractionated by means of a modified Cole and Goss (1939) fractionation process. The fractionation procedure employed (figure 1) was as follows: The pH of the plasma was carefully adjusted to 9.0 with 1 N NaOH while stirring vigorously. Cold acetone equal to 90% of the plasma volume was added slowly with vigorous stirring. The solution was then allowed to stand at 5°C. for 18 hours after which it was centrifuged under refrigeration for 10 minutes at 9000 r.p.m. The supernatant liquid was decanted and saved for future use. The precipitate was washed with a volume of cold, 50% acetone equal to the original volume of plasma. The washing was combined with the previously obtained supernatant and carefully adjusted to pH 6.0 with 1 N HCl while stirring vigorously. A volume of cold acetone was slowly added to bring the solution to 70% acetone (sp. gr. 0.897) while stirring vigorously. The resulting mixture was allowed to stand at 5°C. for 2 to 4 days. The precipitate was then collected by centrifugation under refrigeration and dissolved in physiological saline for immediate injection into assay animals.

Prior to the adoption of the above fractionation procedure, preliminary studies were conducted that involved the bioassay of aliquots

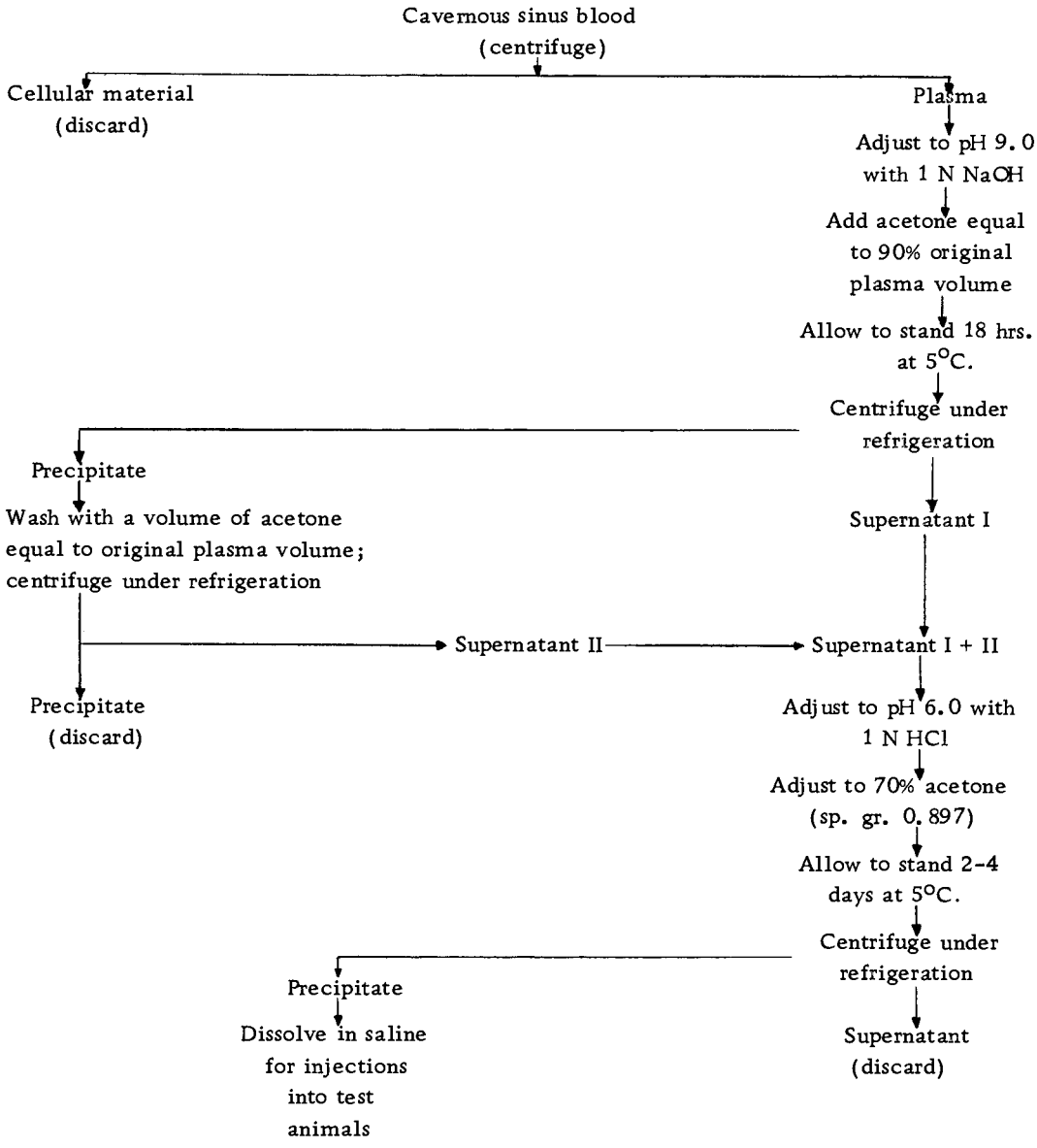

Figure 1. Scheme for concentrating gonadotropic activity in cavernous sinus blood from sheep.

of a plasma sample subjected to variations in the initial pH and acetone concentrations. A subsequent study involved the bioassay of a plasma fraction at graded dose levels of 0, 10, 20 and 40 ml. equivalents (eq.). The results of these efforts will be included in this report. The plasma utilized in these preliminary fractionation studies was pooled, cavernous sinus plasma obtained from six sheep of the previous description and by the previously described technique.

Assay Animals

The assay animals were immature, female rats (Long-Evans strain) which had been hypophysectomized at 26 to 28 days of age. The animals were maintained on a diet of Purina rat pellets which was supplemented with ordinary sugar cubes during the actual injection period. The rats were placed on experiment 2 weeks after hypophysectomy.

Bioassay of Raw and Fractionated Plasma

All injections in assay animals were made intraperitoneally once daily for 4 consecutive days. Experiments I, II, III and Va involved the use of raw plasma at a total dose of 20 ml. per rat with 5 ml. injected per rat per day. Aliquots of sufficient volume for one injection day were thawed to room temperature and used. In

experiments IV and Vb, total doses of 30 and 40 ml. eq. per rat, respectively, of fractionated plasma were injected. The plasma fractions were reconstituted in physiological saline in such a way that each assay animal received 1 ml. daily injections. Following the initial injection, the remaining plasma volumes were stored at 5° C. for subsequent injections. Uninjected and saline injected, hypophysectomized rats were included as controls in each assay series for raw and fractionated plasma, respectively.

The assay rats were autopsied under chloroform anesthesia 24 hours after the last injection and the structures of interest were removed with the aid of a dissecting microscope. Body weights were recorded immediately prior to the first injection and again at autopsy. Tail lengths as measured from the anus to the tip of the tail were taken. Ovaries, oviducts and uteri were removed, trimmed free of adhering tissue, weighed and fixed in Bouin's solution. The thymus, adrenals and thyroids were removed, trimmed and weighed. The right tibia was removed from each rat and trimmed free of all adhering tissue. It was then split with a razor blade in a sagittal plane at the proximal end and fixed in 10% neutral formalin. After all structures of interest had been removed, the sella turcica of the rat was examined for any visible pituitary fragments, and if present the data for that particular rat were discarded.

Determination of Hormonal Potency

The quantitative determination of gonadotropic activity in the plasma samples from estrogen-treated wethers was based upon the increase in ovarian and uterine weights and ovarian histological changes in the assay animals. Histological preparations of the ovaries were made by embedding in paraffin and sectioning with a microtome at a thickness of 8 micra. These sections were mounted on slides and stained with hematoxylin and eosin. The amount of FSH activity was determined by microscopic measurement of the diameter of non-atretic follicles with the aid of a calibrated ocular micrometer (Evans et al., 1939). The largest follicle diameter of one ovary in at least 24 sections was taken as the reading for each assay animal. The ICSH activity was evaluated by examination of the ovarian interstitial tissue for evidence of stimulation (Simpson, Li and Evans, 1942). The hypophysectomized, control rats were considered as having unstimulated or negative ovarian interstitial tissue. Stimulated or positive interstitial tissue showed a slight increase in size of the nucleus which had a more rounded appearance. The chromatin, which appeared as descrete spots in the control, became more evenly distributed throughout the nucleus to give a lighter hematoxylin stain. The cytoplasm, which was hard to distinguish in the unstimulated, hypophysectomized control, increased

in amount, became more eosinophilic and distinct.

The quantitative determination of growth stimulating activity was based on the tibial epiphyseal plate response of the assay animal. The method used was that of Greenspan et al. (1949) in which the tibia was stained with silver nitrate. Ten width readings along the entire length of the epiphyseal plate were taken under a microscope at low power magnification with the aid of a calibrated ocular micrometer. The average of these readings, expressed in micra, was taken as the reading for each assay animal.

Due to the nature of the assay procedure utilized in this study, biological responses of a non-gonadal nature could be observed. The advantages of the assay in this respect are obvious.

Analysis of Data

The data were analyzed by the analysis of variance for detection of gross differences among treatment means in a particular study. The least significant difference (LSD) method was used within studies for comparing a given value with the appropriate pre-treatment value.

IV RESULTS

Experiment I

Experiment I was conducted to determine the influence of diethylstilbestrol, a non-steroid estrogen, administered as a 12 mg. implant on the anterior pituitary activities in cavernous sinus plasma. Gonadotropic responses of the bioassay animals to pre-treatment plasma (day 0) and plasma collected following (day 8) the DES implantation are shown in table 2.

There were apparent differences in the gonadotropic activity of the two plasma samples. As indicated by the ovarian weight response, plasma collected after the DES had been implanted (day 8) contained significantly less gonadotropic activity than did the pre-treatment plasma ($P < .05$). The uterine weight responses to the two plasma samples did not differ significantly. Histological examination of the ovaries revealed a decrease in plasma FSH activity ($P < .01$) in the day 8 plasma but a suppression of ICSH activity was not apparent.

There was no apparent effect of estrogen on plasma activities as associated with the tail length, epiphyseal width, adrenal weight and thymus weight responses of the recipient animals (table 3).

TABLE 2. Gonadotropic responses in recipient rats to pooled samples of wether, cavernous sinus plasma collected before and following treatment with 12 mg. of diethylstilbestrol implants. Experiment I.

Sampling interval (days)[a]	Number of rats	Ovarian weight (mg.)	Uterine weight (mg.)	Ovarian follicle diameter (μ)	Ovarian interstitial tissue[b] (+)	(-)
0	5	10.5 ± 1.0[c]	29.5 ± 2.3	528 ± 54	3	2
8	5	7.1 ± 0.8*	37.1 ± 2.4	322 ± 19**	3	2

[a]Day 0 represents blood collections made before estrogen treatment. Day 1 is considered the first day of estrogen treatment.

[b]Number of rats showing a positive (+) or negative (-) interstitial cell response.

[c]Mean ± standard error of the mean.

*Significantly different from the control collection (day 0) at $P < .05$.

**Significantly different from the control collection (day 0) at $P < .01$.

TABLE 3. Tail length, tibial epiphyseal width, adrenal weight and thymus weight responses obtained in recipient rats to pooled samples of wether, cavernous sinus plasma collected before and following treatment with 12 mg. of diethylstilbestrol implants. Experiment I.

Sampling interval (days)[a]	Number of rats	Tail length (cm.)	Epiphyseal width (μ)	Adrenal weight (mg.)	Thymus weight (mg.)
0	5	11.4 ± 0.2[b]	206 ± 5	8.9 ± 0.5	207 ± 21
8	5	11.3 ± 0.2	195 ± 16	9.4 ± 0.3	207 ± 25

[a]See footnote a to table 2.

[b]Mean ± standard error of the mean.

Experiment II

The second experiment was designed to determine the effect of diethylstilbestrol administered at a smaller total dose (1 mg. daily for 4 days) on the pituitary hormonal activities in plasma. To determine if DES treatment caused any sequential changes in plasma activities, samples of plasma were collected at intervals before, during and following treatment. The bioassay results are given in tables 4 and 5.

Gonadotropic activity of the plasma samples is shown in table 4. The estrogen treatment appeared to have an inhibitory effect on plasma gonadotropic activity during and following treatment (days 4, 7 and 9). Plasma collected during and following the estrogen treatment produced ovarian weights in the assay animals which were significantly less than those to plasma collected prior to estrogen treatment ($P < .01$). Although the uterine weight responses indicated the same trend as did the ovarian weight responses, significant differences among the means were not demonstrated. Histological examination of the ovaries further demonstrated a suppression of gonadotropic activity during and following the estrogen treatment. Average follicle diameters of the assay rats injected with plasma collected during and following treatment were significantly less than that for the pre-treatment plasma group ($P < .01$). The ovarian

TABLE 4. Gonadotropic responses in recipient rats to pooled samples of wether, cavernous sinus plasma collected at intervals before, during and following treatment with 1 mg. of diethylstilbestrol daily for 4 consecutive days. Experiment II.

Sampling interval (days)[a]	Number of rats	Ovarian weight (mg.)	Uterine weight (mg.)	Ovarian follicle diameter (μ)	Ovarian interstitial tissue[b] (+)	(-)
0	8	12.4 ± 1.5[c]	39.7 ± 8.7	469 ± 39	4	4
4	9	7.8 ± 0.7**	35.1 ± 2.8	312 ± 18**	0	9
7	10	8.4 ± 0.6**	28.7 ± 2.0	321 ± 12**	0	10
9	9[d]	8.1 ± 0.5**	31.3 ± 2.4	308 ± 13**	1	7

[a, b]See footnotes a and b to table 2.

[c]Mean ± standard error of the mean.

[d]Ovarian histological information was available on eight of the nine rats.

**Significantly different from the control collection (day 0) at $P < .01$.

TABLE 5. Tail length, tibial epiphyseal width, adrenal weight and thymus weight responses in recipient rats to pooled samples of wether, cavernous sinus plasma collected at intervals before, during and following treatment with 1 mg. of diethylstilbestrol daily for 4 consecutive days. Experiment II.

Sampling interval (days)[a]	Number of rats	Tail Length (cm.)	Epiphyseal width (μ)	Adrenal weight (mg.)	Thymus weight (mg.)
0	8	12.0 ± 0.2[b]	184 ± 6	10.6 ± 0.5	194 ± 34
4	9	11.8 ± 0.2	166 ± 8	9.7 ± 0.6	204 ± 13
7	10	11.9 ± 0.2	168 ± 8	10.3 ± 0.6	193 ± 12
9	9	11.8 ± 0.2	158 ± 7*	10.4 ± 0.7	177 ± 15

[a] See footnote a to table 2.

[b] Mean ± standard error of the mean.

*Significantly different from the control collection (day 0) at $P < .05$.

interstitial tissue responses showed the same trend. Thus, the observed suppression of gonadotropic activity included both FSH and ICSH components.

Plasma activities as associated with the tail length, adrenal weight and thymus weight responses of the assay animals were not significantly changed by the estrogen treatment (table 5). The epiphyseal width response to the sample of plasma collected 5 days (day 9) after withdrawal of treatment was lower than that to the pre-treatment sample ($P < .05$).

Experiment III

Experiment III was of the same experimental design as the preceding experiment with the exception that estradiol benzoate, a steroidal estrogen, was used, but administered at the same rate of 1 mg. daily for 4 days. The bioassay results for the different blood sampling intervals are shown in tables 6 and 7.

Estradiol benzoate inhibited plasma gonadotropic activity (table 6), as did the treatments with DES in the previous experiments. The ovarian weight response of the test animals to plasma samples collected during and following estrogen treatment was significantly less than that observed with the pre-treatment plasma ($P < .01$). The same trend was not observed as to the uterine weight response, although the response obtained at day 4 was significantly less than at

TABLE 6. Gonadotropic responses in recipient rats to pooled samples of wether, cavernous sinus plasma collected at intervals before, during and following treatment with 1 mg. of estradiol benzoate daily for 4 consecutive days. Experiment III.

Sampling interval (days)[a]	Number of rats	Ovarian weight (mg.)	Uterine weight (mg.)	Ovarian follicle diameter (μ)	Ovarian interstitial tissue[b] (+)	(-)
0	9	12.2 ± 0.7[c]	31.8 ± 1.5	506 ± 25	5	4
4	10	8.7 ± 0.4**	27.8 ± 1.0*	343 ± 13**	2	8
7	7	9.4 ± 0.9**	32.1 ± 2.0	331 ± 15**	3	4
9	10	8.0 ± 0.6**	30.8 ± 1.3	360 ± 15**	2	8

[a, b]See footnotes a and b to table 2.

[c]Mean ± standard error of the mean.

*Significantly different from the control collection (day 0) at $P < .05$.

**Significantly different from the control collection (day 0) at $P < .01$.

TABLE 7. Tail length, tibial epiphyseal width, adrenal weight and thymus weight responses obtained in recipient rats to pooled samples of wether, cavernous sinus plasma collected at intervals before, during and following treatment with 1 mg. of estradiol benzoate daily for 4 consecutive days. Experiment III.

Sampling interval (days)[a]	Number of rats	Tail length (cm.)	Epiphyseal width (μ)	Adrenal weight (mg.)	Thymus weight (mg.)
0	9	12.1 ± 0.1[b]	173 ± 9	10.9 ± 0.7	236 ± 16
4	10	11.5 ± 0.2*	198 ± 4*	10.2 ± 0.6	220 ± 14
7	7	11.7 ± 0.2	199 ± 7*	9.2 ± 0.5	241 ± 28
9	10	11.8 ± 0.2	193 ± 10	11.8 ± 2.3	222 ± 11

[a]See footnote a to table 2.

[b]Mean ± standard error of the mean.

*Significantly different from the control collection (day0) at $P < .05$.

day 0 ($P<.05$). Histological examination of the ovaries showed that gonadotropic activity was suppressed below the pre-treatment plasma in all samples collected during and following estrogen treatment. In this regard, the ovarian follicle response was less at days 4, 7 and 9 than at day 0 ($P<.01$). As well as a definite suppression of plasma FSH activity by estrogen, there is a definite tendency for a suppression of ICSH activity in plasma samples collected during and following treatment as demonstrated by the ovarian interstitial tissue response in the test animals.

The adrenal weight and thymus weight responses of the test animals to all plasma samples appears unaltered by estradiol benzoate treatment while the epiphyseal width responses to the plasma samples appears increased (table 7). Significant epiphyseal width responses above the pre-treatment plasma collection were observed at collection days 4 and 7 ($P<.05$). The tail length response of the test animals for the plasma sample collected at day 4 was significantly less than that observed with the pre-treatment plasma ($P<.05$).

pH-Acetone Precipitation Studies

Before describing the results of the final two estrogen studies which both involve the bioassay of plasma fractions, it is pertinent to describe the results of preliminary work involved in developing the fractionation procedure. The fractionation method was so

developed to remove the bulk of plasma proteins from the hormones of interest. Such a development is of value because a smaller quantity of total material need be administered to the assay animals. It would facilitate the study of gonadotropin levels in plasma when the pituitary secretion is low in that larger volumes of plasma on the equivalent bases could be tested.

The gonadotropic responses of the assay animals to fractionated, cavernous sinus plasma as well as to raw, cavernous sinus plasma are given in table 8. The first part of the table presents bioassay data for an initial fractionation study conducted by introducing variations in the initial pH and initial concentration of acetone of four aliquots of a sample of cavernous sinus plasma (see figure 1 for basic procedure). Initial pH values of 9.0 and 10.5 and initial acetone concentrations of 60% and 90% (6 and 9 volumes of acetone per 10 volumes of plasma) were used in the four possible combinations. As compared to responses in saline injected, control rats, all methods resulted in a preparation with gonadotropic activity with the exception of method 2.

Method 1 resulted in a preparation with gonadotropic activity as high or higher than the other methods tried and also resulted in a greater quantity of precipitate that could be discarded (table 8). Because of these reasons this method was selected for additional study. The second part of table 8 gives the gonadotropic responses

TABLE 8. Summary of gonadotropic responses in recipient rats to raw and fractionated, wether, cavernous sinus plasma.

Study	Plasma fractionation method[a]	Precipitate[b] discarded (gm./100ml. plasma)	Total dose plasma (ml.eq.)	Number of rats	Ovarian weight (mg.)	Uterine weight (mg.)	Ovarian follicle diameter(μ)	Ovarian interstitial tissue[c] (+)	(-)
Fractionation method study	(1) pH 9; 90% with acetone	8.36	20	4	11.9 ± 2.4[d]	27.5 ± 7.6	473 ± 48	4	0
	(2) pH 10.5; 90% with acetone	7.73	20	3	7.7 ± 0.9	25.0 ± 1.2	399 ± 7	0	3
	(3) pH 9; 60% with acetone	5.79	20	4	13.1 ± 2.8	26.2 ± 3.4	461 ± 17	2	2
	(4) pH 10.5; 60% with acetone	6.36	20	4	9.8 ± 0.6	21.4 ± 0.6	453 ± 33	0	4
	Control rats (saline injections)		0	4	7.7 ± 0.8	19.2 ± 1.7	338 ± 18	0	4
Fractionated graded dose study	Control rats (saline injections)		0	3	8.0 ± 0.5	21.0 ± 5.4	354 ± 10	0	3
	Method 1 above		10	3	11.3 ± 1.0	20.3 ± 1.6	416 ± 16	1	2
	Method 1 above		20	3	12.7 ± 3.4	30.1 ± 6.6	506 ± 28	2	1
	Method 1 above		40	3	22.8 ± 2.4	96.7 ± 3.7	673 ± 27	3	0
Raw plasma study	Total dose: 20 ml. plasma per rat			8	12.4 ± 1.5	39.7 ± 8.7	469 ± 39	4	4
				5	10.5 ± 1.0	29.5 ± 2.3	528 ± 54	3	2

[a] All plasma used represents pooled, cavernous sinus blood collected from the same six crossbred wethers. The plasma used for the initial fractionation study, for the graded dose study and for the two raw plasma studies represents four different collection times from the same animals. See figure 1 for basic fractionation scheme.

[b] Precipitates were oven dried at 110°C. until constant weights were obtained. Represents precipitates formed to the following pH and acetone treatments.

[c] Number of rats showing a positive (+) or negative (-) interstitial cell response.

[d] Mean ± standard error of the mean.

of the assay rats to graded doses of the plasma fraction prepared by method 1. As shown by the ovarian and uterine weight responses, graded gonadotropic activity to doses ranging from 0 to 40 ml. eq. of cavernous sinus plasma was present. Graded FSH and ICSH responses were apparent as indicated by the ovarian follicular and interstitial tissue responses, respectively.

The final part of table 8 shows the bioassay responses to two individual samples of cavernous sinus plasma not subjected to the fractionation procedure. It should be noted that the plasma used for the initial fractionation study, for the graded dose study and for the two raw plasma studies represent pooled plasma from the same six wethers, but collected at four separate times. It is of interest to note that the gonadotropic responses to the 20 ml. of raw plasma are similar to those for 20 ml. eq. of plasma for method 1 as seen in both the first and second part of table 8. However, exceptions are the uterine weight response which appears to be larger for the one sample of unaltered plasma.

As compared to saline injected, control rats at the 20 ml. eq. dose tested there appeared to be no activity in any of the four preparations assoicated with the tail length, tibial epiphyseal width, adrenal weight, thymus weight and thyroid weight responses as shown in the first part of table 9. Similarly, as shown in the second part of table 9, graded activity as measured by the same responses in the

TABLE 9. Summary of tail length, tibial epiphyseal width, adrenal weight, thymus weight and thyroid weight responses obtained in recipient rats to raw and fractionated, wether, cavernous sinus plasma.

Study	Plasma fractionation method [a]	Precipitate[b] discarded (gm./100 ml. plasma)	Total dose plasma (ml. eq.)	Number of rats	Tail length (cm.)	Epiphyseal width (μ)	Adrenal weight (mg.)	Thymus weight (mg.)	Thyroid weight (mg.)
Fraction-ation method study	(1) pH 9: 90% with acetone	8.36	20	4	11.7 ± 0.3[c]	160 ± 8	8.5 ±0.7	191 ±15	5.7 ± 0.5
	(2) pH 10.5; 90% with acetone	7.73	20	3	11.5 ± 0.3	153 ± 19	7.2 ± 0.5	174 ± 2	5.8 ± 0.4
	(3) pH 9; 60% with acetone	5.79	20	4	12.0 ± 0.2	166 ± 10	10.0 ± 1.1	209 ±19	6.5 ± 0.4
	(4) pH 10.5; 60% with acetone	6.36	20	4	12.0 ± 0.3	154 ± 10	8.8 ± 0.7	165 ±28	5.5 ± 0.3
	Control rats (saline injections)		0	4	12.0 ± 0.1	121 ± 11	9.1± 0.5	204 ± 29	6.3 ± 0.1
Fraction-ated graded dose study	Control rats (saline injections)		0	3	11.1 ± 0.1	164 ± 8	8.5 ± 0.6	170 ± 5	3.9 ± 0.8
	Method 1 above		10	3	10.9 ± 0.1	155 ± 5	8.6 ± 0.7	154 ±18	4.1 ± 0.6
	Method 1 above		20	3	11.1 ± 0.4	161 ± 7	7.3 ± 0.3	199 ± 22	4.0 ± 0.8
	Method 1 above		40	3	11.2 ± 0.6	164 ± 10	10.8 ± 0.4	231 ± 14	5.1 ± 1.1
Raw plasma study	Total dose: 20 ml. plasma per rat			8	12.0 ± 0.2	184 ± 6	10.6 ± 0.5	194 ± 34	---
				5	11.4 ± 0.2	206 ± 5	8.9 ± 0.5	207 ± 21	---

[a, b] See footnotes a and b to table 8.

[c] Mean ± standard error of the mean.

assay animals were not demonstrated. The epiphyseal width responses to all preparations of fractionated plasma appear to be lower as compared to the responses obtained with the two samples of raw plasma.

Experiment IV

The experimental design of experiment IV was similar to that of experiment III as estradiol benzoate was administered at the daily rate of 1 mg., but for 10 consecutive days instead of 4. As compared to experiment III, more blood samples were collected during and following estradiol benzoate treatment in experiment IV. Plasma samples for all intervals were subjected to the fractionation procedure (figure 1) previous to bioassay, and administered at a rate of 30 ml. eq. of plasma per rat. Bioassay results for all plasma samples collected before, during and following estradiol benzoate treatment are given in tables 10 and 11.

The suppressive effect of estrogen on plasma gonadotropic activity (table 10) is in agreement with the three previous experiments. Ovarian weights of the test animals significantly below the response obtained with the pre-treatment plasma were observed for samples obtained at 2 days ($P<.01$), 5 days ($P<.05$) and 8 days ($P<.01$) after withdrawal of treatment. The uterine weight responses were also less for samples obtained at the same three times ($P<.01$)

TABLE 10. Gonadotropic responses in recipient rats to pooled samples of wether, cavernous sinus plasma collected at intervals before, during and following treatment with 1 mg. of estradiol benzoate daily for 10 consecutive days. Experiment IV.

Sampling interval (days)[a]	Number of rats	Ovarian weight (mg.)	Uterine weight (mg.)	Ovarian follicle diameter (μ)	Ovarian interstitial tissue[b] (+)	(-)
0	7	13.5 ± 1.7[c]	40.3 ± 7.8	506 ± 18	5	2
6	6	10.4 ± 1.5	21.0 ± 2.2**	333 ± 9**	1	5
12	7	8.8 ± 0.5**	22.1 ± 2.2**	328 ± 13**	0	7
15	7	9.6 ± 0.8*	21.2 ± 1.3**	371 ± 17**	1	6
18	8	8.2 ± 0.7**	19.5 ± 1.0**	322 ± 13**	0	8

[a, b]See footnotes a and b to table 2.

[c]Mean ± standard error of the mean.

*Significantly different from the control collection (day 0) at $P < .05$.

**Significantly different from the control collection (day 0) at $P < .01$.

TABLE 11. Tail length, tibial epiphyseal width, adrenal weight, thymus weight and thyroid weight responses obtained in recipient rats to pooled samples of wether, cavernous sinus plasma collected at intervals before, during and following treatment with 1 mg. of estradiol benzoate daily for 10 consecutive days. Experiment IV.

Sampling interval (days)[a]	Number of rats	Tail length (cm.)	Epiphyseal width (μ)	Adrenal weight (mg.)	Thymus weight (mg.)	Thyroid weight (mg.)
0	7	11.9 ± 0.2[b]	149 ± 8	10.1 ± 0.5	195 ± 7	6.4 ± 0.4
6	6	11.8 ± 0.4	168 ± 10	9.4 ± 0.6	226 ± 36	6.2 ± 0.8
12	7	11.6 ± 0.2	166 ± 7	11.2 ± 0.5	215 ± 17	5.3 ± 0.5
15	7	12.2 ± 0.3	165 ± 1	9.1 ± 0.5	221 ± 16	6.1 ± 0.8
18	8	11.8 ± 0.2	153 ± 7	8.7 ± 0.7	200 ± 12	5.5 ± 0.6

[a] See footnote a to table 2.

[b] Mean ± standard error of the mean.

and for the day 6 sample as well ($P<.01$). The ovarian follicular responses support the suppression of gonadotropic activity as all plasma samples collected during and following estrogen treatment show significantly less follicle stimulating activity than the pre-treatment plasma ($P<.01$). The interstitial tissue response of the assay rats showed the same trend with a definite suppression of ICSH activity in all plasma collected during and following treatment.

No significant differences among intervals were seen with respect to the tail length, epiphyseal width, adrenal weight, thymus weight and thyroid weight responses in the recipient rats (table 11).

During the period of time when the wethers were on experiment, teat length measurements were taken as a rough quantitative indication of estrogenic activity (Braden, Southcott and Moule, 1964). The teat length measurements of the four wethers in experiment IV are given in the first part of table 16. To detect differences among treatment means for either the right or left teat, the F-value obtained in the analysis of variance was used. In this study, there were apparent differences among measuring intervals for either the right or left teat. The teat length for either the right or left teat appeared to be greatest after 9 estrogen injections with an apparent decline in teat length 5 days after withdrawal of estrogen treatment.

Experiment V

Experiment V was of the same experimental design as experiment IV with the exception that a daily dose of 0.025 mg. of estradiol benzoate was administered and the total dose of plasma equivalents injected into the assay animals was 10 ml. eq. higher. Plasma samples for each interval were assayed as both raw and fractionated plasma at a total dose of 20 ml. (experiment Va) and 40 ml. eq. (experiment Vb) per assay animal, respectively.

Experiment Va

The gonadotropic responses of the test animals to the samples of raw plasma are given in table 12. Even at this low dose level, estradiol inhibited plasma gonadotropic activity during treatment ($P < .01$) and up to 5 days following withdrawal of treatment ($P < .01$) as indicated by the ovarian and uterine weight responses in the test animals. At 8 days after withdrawal of estrogen treatment (day 18), the ovarian ($P < .05$) and uterine ($P < .01$) weight responses were less than at day 0, but a tendency for gonadotropic activity to return to the pre-treatment level was observed. The histological data were in agreement with the transitory inhibition of plasma gonadotropic activity and indicated that it involved both FSH and ICSH components.

No differences among intervals were apparent with respect to

TABLE 12. Gonadotropic responses in recipient rats to pooled samples of raw, wether, cavernous sinus plasma collected at intervals before, during and following treatment with 0.025 mg. of estradiol benzoate daily for 10 consecutive days. Experiment Va.

Sampling interval (days)[a]	Number of rats	Ovarian weight (mg.)	Uterine weight (mg.)	Ovarian follicle diameter (μ)	Ovarian interstitial tissue[b] (+)	(-)
0	5	16.8 ± 2.0[c]	60.8 ± 4.8	672 ± 33	5	0
6	3	9.5 ± 0.6**	23.4 ± 5.7**	454 ± 37**	0	3
12	5	7.6 ± 0.2**	23.8 ± 1.2**	329 ± 11**	0	5
15	5	9.2 ± 0.6**	26.0 ± 1.4**	379 ± 23**	0	5
18	4	13.3 ± 0.8*	35.0 ± 5.6**	557 ± 20**	2	2

[a, b]See footnotes a and b to table 2.

[c]Mean ± standard error of the mean.

*Significantly different from the control collection (day 0) at $P < .05$.

**Significantly different from the control collection (day 0) at $P < .01$.

the tail length, epiphseal width, thymus weight and thyroid weight responses of the assay animals (table 13). Adrenal weight responses from plasma collected at 5 and 8 days after withdrawal of estrogen treatment were significantly less than the response produced by the pre-treatment plasma ($P < .05$).

Experiment Vb

The responses obtained in the assay animals to fractionated aliquots of the raw plasma used in experiment Va from each interval are given in tables 14 and 15.

As before, the gonadotropic activity was significantly depressed by estradiol benzoate during treatment and up to 5 days following withdrawal of treatment (table 14). As indicated by the ovarian and uterine weight responses of the assay animals, the day 6 ($P < .05$) and days 12 and 15 ($P < .01$) plasma samples produced responses significantly less than the responses obtained with pre-treatment plasma. However, 8 days after withdrawal of treatment, the ovarian and uterine weight responses indicated that the gonadotropic activity had returned to a level comparable to that of pre-treatment. Ovarian follicular and interstitial tissue responses of the test animals were in agreement with the above findings as the greatest amount of FSH and ICSH activity was present in the day 0 and day 18 plasma samples.

TABLE 13. Tail length, tibial epiphyseal width, adrenal weight, thymus weight and thyroid weight responses obtained in recipient rats to pooled samples of raw, wether, cavernous sinus plasma collected at intervals before, during and following treatment with 0.025 mg. of estradiol benzoate daily for 10 consecutive days. Experiment Va.

Sampling interval (days)[a]	Number of rats	Tail length (cm.)	Epiphyseal width (μ)	Adrenal weight (mg.)	Thymus weight (mg.)	Thyroid weight (mg.)
0	5	11.2 ± 0.2[b]	197 ± 12	11.6 ± 0.7	193 ± 12	5.2 ± 0.5
6	3	11.4 ± 0.5	168 ± 4	10.3 ± 0.6	183 ± 58	5.1 ± 0.5
12	5	11.7 ± 0.3	208 ± 12	10.2 ± 0.5	177 ± 24	5.4 ± 1.1
15	5	11.5 ± 0.3	186 ± 5	9.6 ± 0.7*	180 ± 13	5.2 ± 0.4
18	4	12.0 ± 0.2	195 ± 11	9.5 ± 0.7*	197 ± 12	5.6 ± 0.2

[a]See footnote a to table 2.

[b]Mean ± standard error of the mean.

*Significantly different from the control collection (day 0) at $P < .05$.

TABLE 14. Gonadotropic responses in recipient rats to pooled samples of fractionated, wether, cavernous sinus plasma collected at intervals before, during and following treatment with 0.025 mg. of estradiol benzoate daily for 10 consecutive days. Experiment Vb.

Sampling interval (days)[a]	Number of rats	Ovarian weight (mg.)	Uterine weight (mg.)	Ovarian follicle diameter (μ)	Ovarian interstitial tissue[b] (+)	(-)
0	5	15.8 ± 1.7[c]	65.6 ± 10.1	703 ± 29	4	1
6	6	12.2 ± 0.8*	45.0 ± 8.8*	575 ± 35**	1	5
12	5	9.9 ± 1.1**	22.4 ± 2.6**	450 ± 19**	1	4
15	6	9.9 ± 1.0**	25.2 ± 2.9**	482 ± 31**	1	5
18	6	16.3 ± 1.0	73.3 ± 6.4	633 ± 23	3	3

[a, b] See footnotes a and b to table 2.

[c] Mean ± standard error of the mean.

* Significantly different from the control collection (day 0) at $P < .05$.

** Significantly different from the control collection (day 0) at $P < .01$.

TABLE 15. Tail length, tibial epiphyseal width, adrenal weight, thymus weight and thyroid weight responses obtained in recipient rats to pooled samples of fractionated, wether, cavernous sinus plasma collected at intervals before, during and following treatment with 0.025 mg. of estradiol benzoate daily for 10 consecutive days. Experiment Vb.

Sampling interval (days)[a]	Number of rats	Tail length (cm.)	Epiphyseal width (μ)	Adrenal weight (mg.)	Thymus weight (mg.)	Thyroid weight (mg.)
0	5	11.6 ± 0.3[b]	150 ± 8	11.5 ± 0.7	150 ± 13	5.2 ± 0.7
6	6	11.8 ± 0.2	157 ± 9	10.0 ± 0.8	206 ± 38	5.7 ± 0.5
12	5	11.6 ± 0.2	166 ± 10	10.1 ± 0.6	185 ± 34	5.7 ± 0.2
15	6	11.8 ± 0.2	169 ± 3	10.2 ± 0.8	201 ± 17	5.4 ± 0.3
18	6	10.8 ± 0.2*	176 ± 7*	9.2 ± 0.6*	160 ± 22	4.7 ± 0.5

[a] See footnote a to table 2.

[b] Mean ± standard error of the mean.

*Significantly different from the control collection (day 0) at $P < .05$.

There was no apparent effect of estrogen treatment on activities as associated with thymus weight and thyroid weight responses of the recipient rats (table 15). However, the tail length response, epiphyseal plate response and adrenal weight response at day 18 were significantly different from the responses at day 0 ($P < .05$). In agreement with previous experiments involving the use of fractionated plasma, the epiphyseal plate responses of the assay rats were generally low.

Teat length measurements of the six wethers receiving the daily dose of 0.025 mg. of estradiol benzoate for 10 consecutive days are shown in the second part of table 16. A significant F-value was obtained when comparing average teat lengths among estrogen treatment intervals for either the left or right teat. The results indicate an increase in teat length during estrogen treatment (day 10) with a partial decline following treatment.

TABLE 16. Teat length measurements of wethers taken at intervals before, during and following treatment with 1 mg. and 0.025 mg. of estradiol benzoate daily for 10 consecutive days.

Estradiol dose level	Measuring interval (days)[a]	Number of wethers	Teat length	
			Right (cm.)	Left (cm.)
1 mg. (experiment IV)	0	4	2.1 ± 0.1[b]	2.1 ± 0.2
	10	4	2.7 ± 0.3	3.1 ± 0.1
	15	4	2.4 ± 0.2	2.5 ± 0.1
	Variance ratio, F		5.81 (P<.05)	21.40 (P<.01)
0.025 mg. (experiment V)	0	6	2.2 ± 0.1	2.2 ±0.1
	10	6	2.9 ± 0.1	3.0 ±0.1
	15	6	2.7 ± 0.1	2.8 ± 0.1
	18	6	2.7 ± 0.2	2.8 ± 0.1
	25	6	2.7 ± 0.1	2.6 ± 0.1
	Variance ratio, F		7.63 (P<.01)	16.43 (P<.01)

[a]Day 0 represents teat length measurements taken before estrogen treatment. Day 1 is considered the first day of estrogen treatment.

[b]Mean ± standard error of the mean.

V DISCUSSION

The reliability of the cavernous sinus technique for the study of pituitary function is supported by previous work (Ellington, Contopoulos and Clegg, 1964; Dierschke and Clegg, 1965; Seifart and Hansel, 1965; McDonald and Clegg, 1965; McDonald and Clegg, 1966). By utilizing hypophysectomized, immature, female rats as the assay animals, blood levels of total gonadotropic activity can be quantitated by the ovarian and uterine weight responses. Furthermore, histological study of the ovaries of the assay animal allows for qualitative as well as quantitative determination of FSH and ICSH (Evans et al., 1939; Simpson, Li and Evans, 1942).

By use of the above methods for the present study, it was found that both estradiol benzoate and diethylstilbestrol significantly depressed plasma levels of gonadotropins in the gonadectomized sheep. Histological examination of the ovaries of the recipient rat revealed that the depression of plasma gonadotropic activity always involved FSH, and ICSH in all studies except experiment I. In all studies, an inhibition of plasma gonadotropic activity was apparent for periods of time following withdrawal of the estrogen treatment. In experiment V, which involved the low daily dose of 0.025 mg. of estradiol benzoate, the plasma gonadotropic activity had returned to a level comparable to the pre-treatment plasma level at 8 days after

treatment withdrawal. The return of plasma activity to approximately the control level was found to involve both FSH and ICSH. The inhibitory patterns in plasma gonadotropic activity were demonstrated with both raw plasma and plasma subjected to a modified Cole and Goss (1939) fractionation process. From the results obtained with the dose levels of estrogen employed, it can be concluded that estrogen acts in some way to inhibit the pituitary release of gonadotropins into circulation.

From previous work with laboratory animals, it is concluded that estrogen acts to control the secretion of gonadotropins through a negative feed back action on the hypothalamus (Flerko and Szentagothai, 1957; Lisk, 1960; Davidson and Sawyer, 1961; Kanematsu and Sawyer, 1963a, 1964). That estrogen acts to regulate pituitary function is supported by alterations in gonadotropic content of the pituitary (Greep and Jones, 1950) and in the blood (Gans, 1959a, b; McCann and Taleisnik, 1961a, b; Ramirez and McCann, 1963) after removal of the source of gonadal estrogen by ovariectomy. Furthermore, administration of estrogen to gonadectomized, laboratory species will alter the pituitary gonadotropic potency as well as the gonadotropic level in the blood (Burrows, 1949; Greep and Jones, 1950; Greep, 1961).

From the studies with predominately laboratory animals, it is usually concluded that short term, low dose estrogen treatment will

stimulate the release of ICSH while high dose, long term treatment will inhibit pituitary secretion (Frank, 1940; Greep, 1961; Everett, 1961). The assumption that estrogen acts to inhibit as well as to stimulate pituitary secretion has been referred to extensively in explaining sexual periodicity in the female. It has been suggested that when levels of estrogen are low the pituitary will secrete larger amounts of FSH, and as estrogen reaches moderate levels the FSH secretion is decreased and the pituitary is stimulated to secrete increased amounts of ICSH (Hisaw et al., 1934; Fevold, Hisaw and Greep, 1936).

A great many of the previous studies from which the above ideas were developed involved indirect determination of pituitary secretion rather than directly measuring systemic activity. Estrogen inhibition of castration cell development in the rat has been interpreted as a morphological change associated with the inhibition of gonadotropic secretion (Bogdanove, 1963). Changes in the pituitary content of gonadotropins after estrogen treatment have been interpreted to indicate an alteration in the secretory activity of the pituitary. However, because of the lack of blood assays, conclusions cannot be made with certainty that pituitary hormone content always reflects secretory activity (Ellington, Contopoulos and Clegg, 1964). Since the circulatory system is the communicating link for hormones of the pituitary and gonads, it seems logical that to determine the

precise role of estrogen in regulating pituitary function, blood levels of pituitary hormones must be determined. In the present study where gonadotropic activity in the plasma was determined directly, it was demonstrated that estrogen had an inhibitory and not a stimulatory effect on plasma gonadotropic activity. Admittedly, four of the five experiments employed involved estrogen dose levels at which an inhibition of gonadotropic secretion would seem likely. However, particularly helpful in resolving the problem at hand is the experiment in which a low dose of estrogen, presumably at or near the physiological level (Robinson, Moore and Binet, 1956), was administered. The daily dose of estradiol benzoate employed was 0.025 mg., which also produced an inhibitory effect on plasma gonadotropic activity. Of interest would be the effect of even lower dosages of estrogen administered either as daily injections or as a single injection on the gonadotropic hormone levels of blood collected at sequential intervals.

It is evident that there are problems involved in interpreting the results of indirect studies with laboratory animals, but the problem is not completely solved by directly measuring blood levels of gonadotropins in estrogen-treated, laboratory species. It has been reported that presumably physiological doses of estrogen will lower the plasma level of FSH and ICSH in gonadectomized rats (Gans, 1959a, b; Gans and Van Rees, 1962; Ramirez and McCann, 1963;

McCann and Taleisnik, 1961b) indicating that pituitary secretion of gonadotropins is greatest when no estrogen is present. However, Sidki, Badawi and Soliman (1958) reported an increase in plasma ICSH of estrogen-treated, ovariectomized rats. The results were not confirmed by McCann and Ramirez (1964) as they observed a depression of plasma ICSH in ovariectomized rats given hypothalamic, estrogen implants, but similar implants in intact animals increased plasma ICSH activity. McCann and Ramirez (1964) offered the suggestion that estrogen acts to suppress pituitary release of ICSH activity when there is an elevated level of plasma activity, as in the castrate animal, while stimulation of ICSH release occurs when plasma gonadotropic levels are low, as in the intact animal. This, of course, does not explain the increase in plasma ICSH activity reported by Sidki, Badawi and Soliman (1958) with the ovariectomized rat.

Support for transitory inhibition of plasma gonadotropic activity by low dose levels of estrogen, as found in the present study with sheep, have been reported by McCann and Taleisnik (1961b). They found that as the dose level of estrogen administered to ovariectomized rats was decreased, the period of time for the complete suppression of plasma gonadotropic activity was increased. At lower dose levels, plasma activity returned to the pre-treatment level in a shorter period of time after withdrawal of estrogen treatment. In

the present study, maximum inhibition of gonadotropin release into the blood was apparent shortly after treating with high doses of estrogen, while the low dose of estrogen used in experiment V did not produce maximum inhibition of plasma activity until ten injections of estradiol benzoate had been administered. In contrast to the results obtained with the high doses of estrogen, withdrawal of the low dose estrogen resulted in a return of plasma activity to approximately the pre-treatment level within 8 days.

As in the case in the present study with sheep, other reports dealing with the same species have not reported a stimulatory effect of estrogen on pituitary gonadotropic secretion. Estrogen administration to anestrous ewes does not stimulate ovulation consistently or with any high degree of frequency (Hammond, Hammond and Parkes, 1942; Hammond, 1945; Cole, Hart and Miller, 1945; Vander Noot, Reece and Skelley, 1949). Similarly, estrogen failed to hasten ovulation in ewes when administered at the onset of natural heat when follicles are at a stage that they should respond to increased levels of ICSH (Dutt, 1953). It was suggested that either estrogen does not cause a release of ICSH in sheep or the doses employed were above physiological limits. Soliman et al. (1963) directly determined blood levels of ICSH and FSH in ovariectomized ewes treated with 20 mcg. of estradiol benzoate for 2 days by assaying the blood in immature mice. Follicle stimulating activity was

determined by an HCG augmentation assay and ICSH activity was determined by injecting the test material plus pregnant mare serum (PMS) and counting the number of corpora hemorrhagica. Their estrogen treatment had no apparent stimulatory or inhibitory effect on either FSH or ICSH. The lack of plasma gonadotropic inhibition observed by Soliman et al. (1963) could possibly be due to the length of estrogen treatment, for in the present study a comparable dose of estradiol benzoate depressed plasma gonadotropic activity after 5 estradiol injections. However, it must be emphasized that the two studies involve different types of gonadectomized sheep.

It may be possible that both estrogen and progesterone function in the stimulatory release of gonadotropins from the pituitary (Everett, 1948). According to this hypothesis, progesterone hastens the time of ovulation when estrogen levels are high and inhibits the same response when estrogen levels are low. However, in a study using the same type of sheep and assay animals as used in the present study, progesterone stimulated the release of both FSH and ICSH into the blood stream on the fifth and sixth day of daily treatment (Ellington, Contopoulos and Clegg, 1964). From this study, it would appear that progesterone stimulates the release of gonadotropins from the pituitary when other gonadal steroids are absent, while in the present study estrogen had an inhibitory action under similar conditions. It would be of interest to determine the effect of progesterone and estrogen administered either in combination or alternately on the

plasma level of gonadotropic activity.

By use of a modified Cole and Goss (1939) fractionation process, gonadotropic activity was successfully concentrated in sheep, cavernous sinus blood. Graded gonadotropic responses to increasing doses of plasma fractions so prepared were observed as evaluated by ovarian and uterine weight responses in the assay animals. The gonadotropic activity consisted of both FSH and ICSH components. However, all plasma fractions produced tail length, epiphyseal width, adrenal weight, thymus weight and thyroid weight responses which were essentially the same as the responses observed in saline injected, control rats. In comparison with the 20 ml. dose of two individual samples of raw plasma, the 20 ml. eq. of the plasma fraction produced similar gonadotropic responses. However, at such dose levels, the uterine weight response appears greater for the raw than fractionated plasma which may be an effect related to the volume of liquid material administered to the test animals.

Although not the primary purpose of this study, observations were also taken on tail length, epiphyseal width, adrenal weight, thymus weight and thyroid weight responses of the assay animals to determine if estrogen treatment had any effect on plasma levels of non-gonadotropic activities. Since it could be established that the non-gonadotropic activities were absent in fractionated plasma, only studies involving the bioassay of raw plasma are relevant to this

discussion. From the results obtained with the raw plasma preparations, it could not be established that estrogen had a growth stimulating effect through the release of either STH (Hammond, 1958) or ACTH (Clegg and Cole, 1954). However, in experiment III, a significant increase in epiphyseal width of the assay animals to plasma from the estrogen-treated wethers did occur, but the same effect was not observed in the other experiments involving the bioassay of raw plasma. No apparent stimulation of TSH release was observed with the estrogen treatments employed as determined by the thyroid weight response in the assay rats. Due to a sensitivity factor, significant alterations in plasma activities as associated with tail length, adrenal weight, thymus weight and thyroid weight responses in the assay rats could have occurred in response to estrogen treatment without being detected.

VI SUMMARY

A total of five individual studies were conducted with gonadectomized, male sheep to determine the effect of estrogen on the secretion of anterior pituitary activities into circulation, with the main emphasis given to gonadotropic activity. The estrogens studied included diethylstilbestrol and estradiol benzoate. The technique of obtaining cavernous sinus blood (pituitary venous blood) via the foramen ovale was utilized. Samples of cavernous sinus blood were collected from the sheep at intervals prior to, during and following estrogen treatment to determine any sequential changes in plasma activity. The plasma was bioassayed in either the raw form or as fractions of plasma in immature, hypophysectomized, female rats.

All estrogen treatments resulted in a significant depression in plasma gonadotropic activity as determined by the ovarian and uterine weight responses in the assay animals. Ovarian follicular and interstitial tissue examination revealed that the depression always involved FSH and usually ICSH. This inhibition of plasma gonadotropic activity was apparent during treatment as well as for periods of time following withdrawal of estrogen treatment. In contrast to the gonadal responses of the assay animals, no marked trends were established with respect to tail length, epiphyseal width, adrenal weight, thymus weight and thyroid weight responses.

The method utilized for fractionating plasma involved pH and acetone precipitations. By use of such a method, plasma gonadotropic activity was successfully concentrated. The plasma fractions which were bioassayed at a total dose of 20 ml. eq. produced gonadotropic responses in the assay rats which were comparable to responses obtained with a 20 ml. dose of raw plasma.

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