

DEVELOPMENT OF METHODS FOR MULTIPLE STEROID RADIOIMMUNOASSAY
AND APPLICATION TO THE STUDY OF FEMALE RATS
DURING PUBERTY AND DHA
INDUCED OVULATORY FAILURE

by

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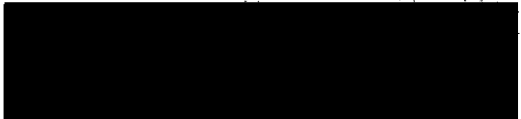
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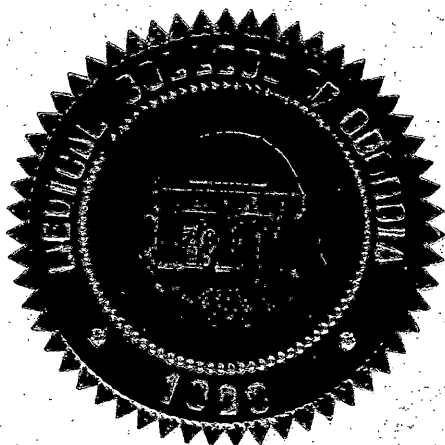
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I. INTRODUCTION

One of the most intriguing problems in the field of reproductive endocrinology has been to identify and develop an understanding of the relationship between steroid hormones and the secretion of anterior pituitary gonadotropins. It has been demonstrated that gonadal steroids may have both a facilitative and inhibitory influence upon the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). The mechanisms involved in the regulation of adenohypophyseal secretions are poorly understood. As a result, points of critical control which apparently involve interaction among the gonads, anterior pituitary, hypothalamus and probably higher brain centers, are the subject of many current investigations.

A great deal of information concerning the interplay between steroids and gonadotropin secretion has been derived from studies dealing with female infertility. Ovulatory failure in the human has been associated most commonly with polycystic ovarian disease - Stein Leventhal Syndrome - and androgen producing tumors of the ovary and adrenal. Characteristics shared by these two disorders are disruption of ovulatory menstrual cycles, aberrant FSH/LH secretion and symptomatic and/or biochemical evidence of excessive androgen production. That the abnormal production of androgens may be related to the ovulatory failure associated with these anomalies has been suggested since ovarian wedge resection in polycystic ovarian disease or tumor excision often result in a decrease in circulating androgens and restoration of fertility. Thus a cause-effect relationship has been proposed between excessive androgens or their metabolites and loss of normal reproductive function in the female.

In order to more fully investigate the influence of androgens on

fertility in the female, several animal models have been developed. Gorski and Barraclough (1961) reported that the administration of testosterone propionate to neonatal female rats induced permanent infertility. Although this experimental model has received much attention, it is perhaps not adequate for comparison to androgen induced infertility in the human female which is reversible. Another, more closely related experimental model has been developed and studied previously in this laboratory. Black and Mahesh (1969) found that short term administration of a weak androgen, dehydroepiandrosterone (DHA) to immature female rats could induce precocious ovulation; chronic treatment however led to subsequent development of polycystic ovaries and ovulatory failure. Withdrawal of androgen treatment permitted a resumption of regular estrous cycles and proven fertility. Pituitary and serum levels of both FSH and LH have been found to be tonic during the period of chronic DHA therapy (Black, 1969; Knudsen, 1974). The precocious ovulations induced by DHA treatment at 27-29 days of age were not synchronized, occurring on one of several days thereafter. The administration of pregnant mares serum gonadotropin (PMSG) on day 30, an agent which itself may cause premature ovulations 72 hours later in the immature rat, could effectively synchronize ovulations to occur only on day 30 after three days of DHA treatment.

From the previous mentioned studies it appears that androgens, or their metabolites, may have both stimulatory and inhibitory actions with regards to gonadotropin secretion. Estradiol-17 β has also been found to exert both positive and negative influence on the secretion of FSH and LH as has progesterone. In general, the actions observed with progesterone have been dependent upon prior exposure to or concomitant administration of estro-

gens. Conclusions concerning the role of estradiol and progesterone are derived from studies in which these steroids have been measured in blood during various reproductive states in addition to conclusions reached as a result of exogenous administration.

It is not entirely clear whether all the effects of DHA in the immature female rat are direct or via metabolic conversion. It has been suggested however, that some conversion is required in the induction of precocious ovulation since the administration of cyanoketone effectively blocks premature puberty while DHT, a non-aromatizable androgen, will not precipitate early ovulation in immature female rats. In the chronically DHA-treated female rats, the primary defect responsible for anovulation is probably not in the ovarian ovulatory mechanism since the administration of exogenous gonadotropins can override the DHA effect and cause ovulation. In addition, there is some evidence to suggest that the pituitary is at least qualitatively responsive to exogenous LRF although the dosage used was high. Thus it would appear that the actions of DHA in the immature female rat are due to some alteration in the normal steroid milieu and the site of action is probably located within the hypothalamic-anterior pituitary complex.

Investigations recently completed in this department have shown that specific estrogen receptors are present in both the anterior pituitary and hypothalamus of male and female rats (Korach and Muldoon, 1974a, 1974b). Although the steroidal state of both sexes and the observed patterns of gonadotropin secretions are different, these receptors may play a key regulatory role in the secretion of FSH and/or LH in both. The studies of Cidlowski and Muldoon (1974) have indicated that the concentrations of cy-

toplasmic (cytosol) receptor in both tissues of male and female rats may be altered by the administration of estrogens. Within one hour after estradiol treatment to castrated rats, cytosol receptor content is significantly reduced while 15-20 hours later, replenishment of receptors to pre-injection concentrations occurs. Similarly, Greeley et al. (1975) found that cytosol estradiol receptor levels in the anterior pituitary and hypothalamus fluctuate during the adult rat estrous cycle: on proestrus, a time of high circulating estradiol, receptor levels were low, while on estrus, a time of decreasing blood estradiol concentrations, cytosol receptor content was replenished. In addition, pituitary responsiveness to exogenous LRF was found to be greatest on proestrus.

Several critical questions concerning the DHA-treated female rat remain to be answered. First, it remains to be determined precisely what changes in blood steroids occur after DHA treatment in relation to the induction of precocious ovulation or with chronic DHA therapy, the development of polycystic ovaries and infertility. A second critical problem is to identify mechanisms existing at the level of the hypothalamic-pituitary unit through which peripheral steroid hormones may alter the synthesis or secretion of FSH and LH. Previous studies have attempted to study this problem, however the exact nature of the interaction between steroids and the hypothalamic-pituitary unit has been difficult to define.

The most logical means of addressing this first question would be to quantify daily changes in several steroids in peripheral blood of individual rats during the various reproductive stages brought about by DHA treatment. The compounds which would be of special interest in such a study would be DHA and potential metabolites such as androstenedione, testosterone, 5 α -

dihydrotestosterone and estradiol-17 β . Other steroids such as progesterone and 17 α hydroxyprogesterone should also be quantified in order to detect changes in endogenous steroid secretion as opposed to alterations which arise through the metabolism of exogenously administered DHA.

At present, there are methods available for the quantification of each of the above mentioned compounds. However, due to the limitations imposed by utilization of small experimental animals, the volume of blood would be insufficient for the analysis of more than one or two steroids from an individual sample.

In light of recent findings, the distinct possibility exists that estrogen receptors of the anterior pituitary and hypothalamus may constitute a locus for the modulatory action of estradiol and possibly other steroid hormones upon adenohypophyseal secretion of LH and FSH. The measurement of cytosol estradiol receptors in these tissues thus would be an important adjunct to the study of the DHA-treated female rat. Alterations in receptor concentrations, brought about by various experimental procedures, coupled with observations on circulating levels of several steroid hormones and gonadotropins should contribute to a more complete understanding of fertility and also ovulatory failure.

The primary aim of this investigation was to more fully evaluate the DHA-treated immature female rat during precocious ovulation, ovulatory failure and androgen withdrawal, at which time a restoration of normal reproductive function would occur. Of particular interest was to identify alterations in blood steroids brought about by DHA treatment and relate these changes to serum gonadotropins and concentrations of cytosol estradiol receptors of the anterior pituitary and hypothalamus. The following

objectives were thus chosen as means to fulfill the basic aim of gaining a more complete understanding of reproductive function in the DHA-treated rat model:

1. the development and validation of methods for multiple steroid radioimmunoassay which would make possible the simultaneous measurement of several steroid hormones from a small serum sample (1 to 2 ml);
2. the measurement of blood steroids and cytosol estradiol receptors of the anterior pituitary and hypothalamus during the natural onset of puberty;
3. the measurement of blood steroids, gonadotropins and cytosol estradiol receptors of the anterior pituitary and hypothalamus during the synchronized onset of puberty in PMSG-primed immature female rats. Experimental findings in this animal preparation plus those during unsynchronized ovulation (2.) would be considered to be representative of normal puberty which is followed by the establishment of regular estrous cycles;
4. the measurement of blood steroids, gonadotropins and cytosol estradiol receptors of the anterior pituitary and hypothalamus during DHA-induced precocious puberty which is synchronized with PMSG;
5. a study of the immediate post-ovulatory period following the induction of ovulation with DHA plus PMSG. Concentrations of blood steroids, gonadotropins and cytosol estradiol receptors would be compared between groups of rats in which DHA administration would be stopped or continued for 10 days after the early onset of puberty;
6. the measurement of blood steroids, gonadotropins and cytosol estradiol receptors of the anterior pituitary and hypothalamus during chronic DHA treatment, which would result in ovulatory failure, and following the

discontinuation of DHA, at which time a restoration of fertility would occur.

It is hoped that the results of this investigation will contribute significantly to a more lucid comprehension of the hormonal interplay during normal female reproductive function and the changes brought about by androgen excess.

II. LITERATURE REVIEW

A. METHODS FOR STEROID HORMONE ANALYSIS

The evaluation of steroid hormone concentrations in biological fluids has been hampered in the past by a lack of sensitive and specific assay techniques. Many methodological advances have contributed to the current capabilities in this field. A brief review will demonstrate the degree of these refinements which has occurred over the past several years.

The earliest methods employed for steroid hormone measurement involved the response of a biological parameter to a hormone as endpoint and were thus called bioassays. Such methods were developed for the quantification of estrogens, (Astwood, 1938) androgens (Eisenberg and Gordon, 1950; Moore, 1939) and progesterone (Astwood, 1939; Hooker and Forbes, 1947). These techniques were utilized primarily for the analysis of crude urinary extracts or whole tissue homogenates. The variability in individual animals, lack of specificity, poor sensitivity and lack of differentiation among compounds of high and low biological activity in a mixture, were contributing factors which necessitated the development of more precise chemical methods.

Newer techniques for steroid hormone assay were perfected which utilized colorimetric endpoints. Most of these techniques were first applied to the analysis of several different classes of urinary steroids rather than specific compounds. Examples are methods for the measurement of 17 ketosteroids (Drechter et al., 1952), 17 ketogenic steroids (Brooks and Norymberski, 1953) and estrogens (Brown, 1952). Although colorimetric steroid assay methods offered some advantages to bioassays, substances were present in urinary extracts which could interfere in the assay. These materials contributed to a low sensitivity and poor specificity and

made colorimetric methods unsuitable for blood steroid assay. While the analysis of the several classes of urinary steroid metabolites was useful, it was at best only a reflection of blood steroid concentrations.

Substantial improvement of these somewhat nonspecific chemical and biological assays was accomplished by the addition of preliminary steroid purification steps. Techniques of paper chromatography (Bush and Mahesh, 1959), thin layer chromatography (Lisboa and Diczfalussy, 1963), celite column chromatography (Preedy and Aitken, 1961; Siiteri and MacDonald, 1963) and Sephadex column chromatography (Beling, 1963) were perfected which allowed the isolation of one or several steroids. These chromatographic techniques contributed specificity to more conventional assays but an inherent lack of sensitivity in colorimetric and biological assays still precluded the possibility of analyzing blood steroid concentrations.

Murphy (1964, 1967) developed the first steroid assay techniques which were sufficiently sensitive and specific for the analyses of blood steroids. Her technique utilized radiolabeled steroids as ligands and corticosteroid binding globulin (CBG) (Muldoon and Westphal, 1967) as a corticosteroid binding agent. This system, termed "competitive protein binding assay", was patterned somewhat after the original protein hormone radioimmunoassay of Berson and Yalow (1959) and was based upon the displacement of ^3H labeled cortisol from CBG by unlabeled standard and unknown corticoids. Soon thereafter, other naturally occurring binding proteins were used for the radioassay of estradiol-17 β (Korenman et al., 1969), progesterone (Baranczuk et al., 1973), estrone (Tulchinsky and Korenman, 1970) and testosterone (Rosenfield et al., 1969). Although Erlanger et al., (1957, 1958) proposed that steroid-protein conjugates might be synthesized in such a manner that

they could serve as specific antigens, steroid radioimmunoassays were not developed until after competitive binding methods were in use. At present, radioimmunoassays are used for the measurement of minute amounts of several blood steroids including estrogens (Emment et al., 1972; Yoshizawa and Fishman, 1971), androgens (Thorneycroft et al., 1973; Buster and Abraham, 1972), and progestins (Abraham et al., 1972 b; Stone et al., 1971). Such radioligand binding assays and radioimmunoassays have made it possible to measure many steroids in peripheral blood which are present normally in minute (picomolar) quantities, thus being far superior to the original biological or chemical analyses of crude urinary extracts.

Although currently used radioassays are highly sensitive, most are not specific enough to assay blood steroids without some preliminary chromatographic purification. Methods used for such purification are primarily Sephadex column chromatography (Carr et al., 1971) or celite column chromatography (Abraham et al., 1971, 1972) which are modifications of originally applied techniques for the separation of urinary steroids. Some investigators have utilized the steroid separation properties of various chromatographic techniques plus steroid antibodies and/or steroid binding proteins to develop multiple steroid assay systems. Such systems allow for the simultaneous analysis of more than one steroid from a single plasma extract. One method, developed by Concolino and Morocchi (1972), utilized chemical modifications of steroids and multiple chromatographic steps while another (Dupon et al., 1973) employed chromatography plus derivative formation prior to assay. Another system utilized paper chromatographic techniques for the isolation of several steroids prior to assay (West et al., 1973). Such multiple assay techniques obviously offer certain advantages over

more conventional single steroid assay methods but often yield poor steroid recovery and thus require substantial volumes of plasma. In addition, the complex nature of the chromatographic technique and derivative formation used are quite time consuming. Another shortcoming in some of these methods are high assay blank values which contribute to a lower assay sensitivity.

B. HORMONAL INTERRELATIONSHIPS IN THE FEMALE REPRODUCTIVE CYCLE

Of the hormones synthesized and secreted by the anterior pituitary gland, FSH and LH are regulators of ovarian function. Fevold et al., (1931) first identified these hormones and proposed that FSH stimulated ovarian follicular growth while LH was responsible for follicular rupture and corpus luteum formation. Greep (1961) proposed that LH was secreted in massive quantities just prior to ovulation. More recently the roles of LH and FSH have come under closer scrutiny. As a result of improved assay methods, it has been demonstrated that both FSH and LH are secreted throughout the female reproductive cycle and that a massive release of both hormones from the pituitary precedes ovulation (Rosemberg and Keller, 1965; Dane and Parlow, 1971). In addition, Goldman and Mahesh (1968, 1969) and Lostroh and Johnson (1966) have raised new questions concerning the actions of FSH and LH on ovulation since they found that FSH alone may cause ovulation in both the rat and hamster.

The anterior pituitary was once considered to be the "master gland", all other endocrine tissues being subservient to its direction. It is now recognized that the pituitary is itself partially dominated by the hypothalamus and perhaps higher brain centers. Specific hormones or factors have been identified or proven to exist within the hypothalamus which regulate the synthesis and/or secretion of gonadotropins and other adeno-

physeal hormones (Porter and Jones, 1956; Talwalker et al., 1963; Wilber and Porter, 1970; Amoss et al., 1971; Matsuo et al., 1971). Observations made during the rat estrous cycle have indicated that an increase in LH-releasing hormone (LH-RH) concentrations in hypothalamic-pituitary portal blood occurs on proestrus which results in a depletion of hypothalamic LH-RH content and is associated with the preovulatory gonadotropin surge (Eskay et al., 1974; Wheaton and Fawcett, 1974).

Temporal relationships between steroid hormones in blood and ovulation have also been determined. Estradiol-17 β levels in peripheral and ovarian vein blood have been shown to increase during ovarian follicular growth reaching peak values and then decreasing just prior to the preovulatory surge of FSH and LH in many species (Abraham et al., 1972b; Shaikh, 1971; Weick et al., 1973). Following ovulation, estradiol is secreted by the corpus luteum in the human (Mikhail, 1970) and monkey (Weick et al., 1973) but is not elaborated in the rat which lacks a luteal phase (Hori et al., 1968).

Progesterone concentrations in blood are low during follicular growth and appear to rise in response to the preovulatory gonadotropin surge (Neill et al., 1967; Barraclough et al., 1971; Butcher et al., 1974). Concentrations of 17 α -hydroxyprogesterone have also been measured and are significantly higher in the luteal phase as compared to the follicular phase of the human menstrual cycle (Strott and Lipsett, 1968). Concentrations of androgens such as androstenedione and testosterone have also been evaluated during the female reproductive cycle. Although no significant cyclic fluctuations are presently recognized, the levels of both hormones parallel somewhat the secretion of estradiol in the human (Horton, 1965; Gandy and

Peterson, 1968) and rat (Dupon and Kim, 1973).

The relationship of blood steroids to the secretion of pituitary gonadotropins is not completely understood however certain observations have been made. Castration of the female leads to a chronic elevation of both FSH and LH in serum (Gay and Midgley, 1969) while replacement with various ovarian steroids may suppress this post-castration rise (Parlow, 1964; Eldridge et al., 1974a,b). These findings have suggested the existence of a negative feedback action of steroids on LH and FSH secretion.

Other experimental findings have suggested that a positive or facilitative action of steroids on gonadotropin secretion also exists. Everett (1948) found that steroids such as progesterone and estradiol could not only inhibit or delay ovulations but could also advance ovulation in the adult rat. These findings have been authenticated more recently (Barraclough and Haller, 1970). Other investigations have shown that steroids may also further increase the release of gonadotropins in the castrate (Caligaris et al., 1971) or intact female Ying and Greep, 1972; Karsh et al., 1973).

It has been postulated that preovulatory secretions of the ovary are necessary for the elaboration of the midcycle FSH and LH surge and thus ovulation. Most evidence suggests that estradiol-17 β concentrations in blood act as a signal indicating that ovarian follicle maturation has proceeded to a state that is ripe for ovulation. Experimental manipulations which tend to abolish or counteract the preovulatory estrogen peak in blood block not only the FSH and LH surge but ovulation as well (Shirley et al., 1968; Ferin et al., 1969a,b; Schwartz, 1969).

The site(s) of steroid feedback control of gonadotropin secretion are

poorly understood at the present. However, it is likely that the anterior pituitary and hypothalamus are involved (McCann et al., 1968; Weick and Davidson, 1970). Following castration, LH-RH concentrations in hypothalamic-pituitary blood are markedly elevated which suggests that some basal, tonic inhibitory influence of ovarian steroids exists at the level of the hypothalamus or in higher centers of the brain (Ben-Jonathan et al., 1973). Imposed upon this basal inhibitory influence may be cyclic increases in anterior pituitary sensitivity to LH-RH caused by increasing titers of certain blood steroids as suggested by the work of several investigators (Greeley et al., 1974; Debeljuk et al., 1972; Weick and Davidson, 1970).

It is suggested, from the previously mentioned studies, that the female reproductive cycle is maintained by an orderly secretion of hormones from the hypothalamus, anterior pituitary and ovary. Each of these glands have regulatory actions on the others and are, therefore, essential for the ovulatory process.

C. ALTERATIONS IN THE ONSET AND MAINTENANCE OF CYCLIC REPRODUCTIVE FUNCTION IN THE FEMALE

Steroid hormones have been shown repeatedly to have profound influences on reproductive processes in both the immature and mature female. Ovulatory failure in the human is most commonly associated with polycystic ovarian disease and steroidogenic tumors of the adrenal and ovary. The pathological characteristics of these anomalies are similar in many respects. Chronically elevated concentrations of androgens were detected in urine and/or blood in cases of ovarian arrhenoblastoma (Mahesh et al., 1970), adrenal tumors (Mahesh et al., 1968) and polycystic ovarian disease (Mahesh and Greenblatt, 1964). The androgens, dehydroepiandrosterone and androstene-

dione, have been identified as the steroids most commonly found in excess in cases of polycystic ovarian disease (Mahesh and Greenblatt, 1964). Tonic, acyclic serum levels of gonadotropins have been reported in many cases of ovulatory failure in association with androgen excess due to polycystic ovarian disease (Gambrell et al., 1973) and androgen secreting tumors (Mahesh et al., 1968). Amelioration of many symptoms associated with androgen excess in the human female, such as hirsutism and ovulatory failure, has been shown to occur when the source of androgen secretion has been suppressed (Greenblatt, 1953) or removed by tumor extirpation or ovarian wedge resection in polycystic ovarian disease (Greenblatt and Coniff, 1968).

The administration of steroids has also been shown to be disruptive to normal reproductive function in experimental animals. The administration of testosterone propionate to neonatal female mice or rats has been demonstrated to result in permanent sterility (Pfeiffer, 1936; Barraclough and Leatham, 1954; Gorski and Barraclough, 1961). As adults, such androgenized mice and rats have acyclic vaginal cytology, cystic ovarian follicles and are anovulatory but retain the capacity to ovulate following the administration of exogenous gonadotropins (Grant et al., 1964).

Although a direct action of androgen in the neonate may be responsible for subsequent ovulatory failure, similar findings have been reported following the administration of estrogen to neonatal female rats (Greene and Burill, 1941; Gorski, 1963). Since many tissues are capable of converting androgens to estrogens (Wotiz et al., 1956; Baggett et al., 1959; MacDonald et al., 1967; Naftolin et al., 1971) the effects of neonatal androgen treatment may be due to subsequent metabolism to estrogen. This hypothesis is strengthened by the studies of Luttge and Whalen (1970), who

found that 5α -dihydrotestosterone did not cause sterilization when administered to a neonatal female rats.

In the prepubertal female rat, the effects of androgen or estrogen treatment are substantially different from those seen in the neonate. Frank et al., (1925) and Ramirez and Sawyer (1965a) have shown that in immature female rats, short-term estrogen therapy could advance the onset of puberty which was followed by the establishment of normal estrus cycles. More recently, Black and Mahesh (1969) reported that short-term treatment of premature female rats with dehydroepiandrosterone, a relatively weak androgen, could also induce precocious ovulation; subsequent reproductive function was not evaluated however. Black (1969) also noted that the administration of other aromatizable androgens such as androstenedione and testosterone could induce precocious puberty.

Knudsen (1974), also studying the immature female rat, found that treatment with dehydroepiandrosterone (60 mg/kg body weight) on day 27-29 of age resulted in ovulations on day 30-31 which could be synchronized to occur on day 30 only following the administration of pregnant mares serum gonadotropin (PMSG) on the AM of day 30. Without the androgen pretreatment, PMSG usually results in ovulations 72 hours later (Cole, 1936) but in the former case, it was presumed to augment the endogenous surge of gonadotropins induced by dehydroepiandrosterone treatment.

Mahesh and Knudsen (1974) attributed the induction of precocious ovulation by dehydroepiandrosterone to prior conversion to estrogen. Cyano-ketone, an inhibitor of 3β -hydroxysteroid dehydrogenase, when administered concomitantly with DHA was capable of blocking uterine ballooning, FSH and LH surge on the afternoon of day 30 and ovulation. 5α -dihydrotestosterone,

a non-aromatizable androgen metabolite of testosterone, was ineffective in inducing precocious ovulation or vaginal patency.

Although short term androgen or estrogen administration to immature female rats resulted in precocious puberty, long term androgen treatment of both immature (Black, 1969; Knudsen, 1974) and mature female rats (Huffman, 1941; Roy et al., 1962) may lead to ovulatory failure which is characterized by constant vaginal cytology and development of cystic ovarian follicles which are similar to those seen in the human polycystic ovarian disease. Differing from neonatal androgen and estrogen treatment, these measures result in ovulatory failure which is not permanent but reversible following discontinuation of androgen treatment (Black, 1969). In this respect, long term androgen treatment of prepubertal or adult female rats, is also similar to conditions of ovulatory failure in the human female related to androgen excess.

The mechanisms through which chronic DHA therapy causes ovulatory failure in the female rat are not presently recognized however it appears that normal functioning of the hypothalamic-pituitary complex is disrupted. Knudsen (1974) has reported that the ovaries of such animals are probably not directly involved in the ovulatory failure since they are qualitatively responsive to exogenous gonadotropin administration. Chronic DHA treatment resulted in tonic, low serum levels of LH and high levels of prolactin. The administration of LH-RH, in large dosages, was capable of inducing increases in serum LH. Similar results have been reported with respect to cases of polycystic ovarian disease in humans (Oettinger et al., 1975).

These findings indicate that chronic androgen administration to prepubertal or adult females, but not neonates, can lead to temporary loss of

fertility. This reversible ovulatory failure is similar in many respects to that seen in several human anomalies. Certain basic differences apparently exist between androgen induced ovulatory failure and that resulting from estrogen treatment (e.g., modern contraceptive drugs) since polycystic ovarian development is associated with the former but not normally with the latter type of steroid induced anovulation in the human female.

The establishment of the chronic androgen treated immature female rat as a model of the human polycystic ovarian disease appears to be valid although some species differences in the response to androgen exist. While neonatal androgen (or estrogen) treatment causes permanent infertility in the female rat no such phenomenon has been described for the human. Countless cases of virilizing congenital adrenal hyperplasia (CAH), which has its origins in embryonic development and in the untreated cases, continues neonatally on to adult life due to a specific genetic defect, have been reported in the human female. Although CAH has been associated with massive androgen production (Rivarola et al., 1967) there are no permanent, irreversible defects produced in the hypothalamic-hypophyseal-ovarian axis. In addition, there have been case reports of severe physical masculinization of the female fetus without induction of sterility following the treatment of pregnant women with progestational agents which possess a high androgenic biological activity as well.

D. ESTROGEN RECEPTORS IN TARGET TISSUES

Information concerning the mechanisms of steroid hormone action has increased significantly during the past several years. It has previously been well documented that the mammalian uterus is a target tissue for estrogens (Astwood, 1938). Although specific biochemical and morphological

changes in this tissue have been attributed to estrogen action, mechanisms involved in these responses have been difficult to evaluate.

The development of high specific activity radiolabeled steroids has provided an invaluable tool in steroid hormone research. Glascock and Hoekstra (1959) injected tritium labeled hexestrol, a synthetic estrogen, into goats and found that the uterus accumulated and retained radioactivity several hours after other non-target tissues had become cleared of the estrogen. Jensen and Jacobson (1962) found a similar uptake and retention of ^3H -estradiol in the rat uterus. Similar studies demonstrated that the uterus in other species also possessed estradiol concentrating mechanisms (Korenman, 1968; Terenius, 1968). In addition to the uterus, uptake and retention of estrogens have been demonstrated in the oviduct of the chick (O'Malley and Means, 1974) and the anterior pituitary and hypothalamus of the rat (Eisenfeld and Axelrod, 1966; Kato and Villee, 1967).

Following the initial descriptions of estrogen retention within the uterus and other tissues, studies were undertaken to evaluate the nature of this binding. Specificity studies revealed that the accumulation and retention of radiolabeled estradiol within target tissues could be depressed in vivo by prior systemic administration of unlabeled estradiol, other estrogens and antiestrogens; non-estrogenic compounds were generally ineffective in this regard (Jensen, 1962; Roy et al., 1964; Terenius, 1968 a,b).

Subsequent to radiolabeled estradiol injections in vivo, radioactivity has been localized in nuclear and cytoplasmic cellular subfractions of the uterus (Gorski, et al., 1968), anterior pituitary and hypothalamus (Attramadal, 1964) of the rat. Stumpf (1968) and Pfaff (1968) have verified these

biochemical findings with autoradiographic analyses.

Studies employing dialysis, Sephadex column chromatography and sucrose density gradient ultracentrifugation have further characterized the association of ^3H -estradiol with its target tissues. Specific estrogen binding macromolecules have been described in the cytosol (105,000 x g supernatant) (Toft and Gorski, 1966) and nucleus (Maurer and Chalkley, 1967) of the rat uterus. Macromolecular binding has also been characterized for estradiol in the cytosol of the anterior pituitary (Korach and Muldoon, 1974a) and hypothalamus (Korach and Muldoon, 1974b) in rats. The evidence to date indicates that the cytoplasmic retention of estradiol involves interaction with high affinity binding sites of an estrogen specific protein.

The accumulation of radiolabeled estradiol in the nucleus, following in vivo administration, requires the prior association of estradiol with the cytoplasmic receptor and transfer of the estrogen-receptor complex to the nucleus. Tissues which are not specific targets for estrogen lack such cytoplasmic binding proteins (Jensen et al., 1968). The transfer of the estrogen-cytoplasmic receptor complex to the nucleus has been found to require molecular transformation (Jensen and DeSombre, 1972) which when blocked, inhibits nuclear accumulation (Muldoon, 1971). In addition, Giannopoulos and Gorski (1971) have shown that as nuclear translocation occurs, a proportional decrease in cytoplasmic estrogen receptor results.

Following nuclear translocation and cytoplasmic depletion of receptors, a replenishment of cytoplasmic binding sites has been demonstrated which involves the synthesis of new receptor proteins (Sarff and Gorski, 1971). It has been suggested that cytoplasmic estrogen receptor replenishment may also involve mechanisms which are independent of RNA and pro-

tein synthesis (Cidlowski, 1975). The concentrations of cytoplasmic estradiol receptors in target tissues such as the uterus, anterior pituitary and hypothalamus are thus a product of utilization (nuclear transfer) and replenishment of receptor. In vivo, conditions of high blood estradiol titers would be expected to result in low cytoplasmic receptor levels whereas with low estradiol levels, cytoplasmic receptor content would be higher.

Much work has been devoted to the development of concepts concerning mechanisms of estrogen (and other hormone) action in target tissues. Biochemical changes following estradiol administration include generalized increases in RNA, DNA and protein synthesis (Hamilton, 1968; O'Malley and Means, 1974). The induction of specific protein synthesis has also been demonstrated in the rat uterus (Barnea and Gorski, 1970) but similar studies have not been undertaken in the anterior pituitary or hypothalamus.

These biochemical manifestations of estrogen action have been presumed to be a result of the interaction of estradiol with cytoplasmic receptors and transfer to the nucleus where some association with chromatin occurs (Hamilton, 1968; O'Malley and Means, 1974). There are, however, other consequences of estrogen treatment which apparently do not require nuclear transfer of an estrogen-receptor complex for manifestation of biochemical response (Muldoon, 1971).

Since sex hormones and estradiol-17 β in particular have been shown to modulate the secretory activity of the hypothalamic-hypophyseal complex, it would seem possible that some steroid actions may involve interaction with specific receptors in these tissues. In initial studies, Korach and Muldoon (1974 a, 1974 b) could detect no difference among cytoplasmic es-

tradiol receptor concentrations of the anterior pituitary and hypothalamus with respect to sex or presence of gonads in rats. These studies however, do not refute the possibility that estrogen receptors may be involved in the regulation of anterior pituitary function. Other investigators have clearly demonstrated that cytoplasmic estradiol receptor concentrations of the uterus fluctuate as a function of blood estradiol titers in female rats (Feherty et al., 1970; Shain and Barnea, 1971; Lee and Jacobson, 1971). Similar fluctuations have also been reported in the anterior pituitary and hypothalamus following exogenous estradiol administration (Cidlowski and Muldoon, 1974) and during the rat estrous cycle (Greeley et al., 1974). These studies have shown that cytoplasmic estradiol receptors are depleted on proestrus, when estradiol is at its peak (Butcher et al., 1974) and are high on estrus, when estradiol concentrations are lowered. On the other hand, total tissue uptake is highest on proestrus (Shain and Barnea, 1971) when maximal uterotrophic responses to estradiol are seen (Lee, 1974); maximal responsiveness of the pituitary to LH-RH is also occurring at this time (Aiyer and Fink, 1974; Greeley et al., 1974).

Other previously mentioned studies utilizing estrogen antagonists (Shirley et al., 1968) and anti-estradiol antiserum (Ferin et al., 1969), both of which effectively diminish the interaction of estradiol with its cytoplasmic receptors have been demonstrated to inhibit the preovulatory surge of FSH and LH necessary for ovulation. The current circumstantial evidence indicates that estradiol receptors of the anterior pituitary and hypothalamus may be involved in the mechanisms of estradiol modulation of gonadotropin secretion. Further work in this area is critically necessary to allow a more complete understanding of hypothalamic-pituitary-ovarian axis.

III. MATERIALS AND METHODS

A. STERIODS

Unlabeled steroids for use in radioimmunoassay were obtained from Schwartz Mann and used without further purification. DHA, for injection in animal studies, was obtained either from Schwartz Mann or Steraloids and was dissolved in propylene glycol (4.5 or 9.0 mg/.2ml). Previous studies utilizing melting point analysis, optical rotation and chromatography have demonstrated the purity of the commercially supplied DHA (Knudsen, 1974). In addition, these findings have been substantiated by radioimmunoassay. The following radiolabeled (^3H) steroids were obtained from New England Nuclear for use in steroid radioimmunoassay and other procedures requiring radioactive tracers: 2,4,6,7- ^3H -estradiol-17 β (100 Ci/mmol), 2,4,6,7- ^3H -estrone (95 Ci/mmol), 7- ^3H -dehydroepiandrosterone (21 Ci/mmol), 1,2- ^3H -5 α -dihydrotestosterone (44 Ci/mmol), 1,2,6,7- ^3H -testosterone (91 Ci/mmol), 1,2,- ^3H - Δ^4 -androstenedione (48 Ci/mmol), 2,4,6,7- ^3H -progesterone (81 Ci/mmol) and 1,2,- ^3H -17 -hydroxy progesterone (49 Ci/mmol). The radioactive steroids were brought to within 99% purity by partition paper chromatography. Solvent systems used in paper chromatography were 1) ligroin equilibrated with an equal volume of 95% aqueous methanol, 2) ligroin and benzene (2:1) equilibrated with an equal volume of aqueous methanol and 3) benzene equilibrated with an equal volume of 50% aqueous methanol. A Packard Model 7201 Radiochromatogram Scanner was used for locating radioactive peaks which were then identified by reference to simultaneously chromatographed unlabeled steroids.

B. CHEMICALS AND REAGENTS FOR STEROID RADIOIMMUNOASSAY AND COLUMN CHROMATOGRAPHY

The following solvents were redistilled prior to use: isooctane, benzene, ethyl acetate, methanol, diethyl ether and petroleum ether (B.P. 75-110°C). Toluene (A.C.S.) ethylene glycol (chromoquality) and propylene glycol (U.S.P.) were used without further purification. Celite, treated for removal of acid soluble materials, was obtained from either Johns Manville Co., or Fisher Scientific Co. and was ignited in a muffle furnace for not less than 24 hours at 1000°F prior to utilization for column chromatography. Scintillation fluid, for liquid scintillation counting of tritiated steroids, was prepared using Packard Permablend II, 5 grams/liter of toluene. Dextran (60,000-90,000 mol. wt.) was obtained from Nutritional Biochemicals Co., while Norit A (Neutral decolorizing charcoal) was obtained from Amend Drug and Chemical Supply Co. Bovine serum albumin (BSA) (Fraction V) was purchased from Miles Laboratories.

C. ANTISERA

Antisera specific for the radioimmunoassay of progesterone, 17-hydroxyprogesterone, testosterone, androstenedione and dehydroepiandrosterone were purchased from Dr. G.E. Abraham and have been characterized previously (Abraham et al., 1971 a,b; Abraham and Chakmakjian, 1974). Antisera for the radioimmunoassay of estradiol-17 β and 5 α -dihydrotestosterone were prepared and characterized in this laboratory. All antiserum were lyophilized and stored at 5°C prior to use.

Estradiol Antiserum (RP#1)

The 17-0-carboxy methyl oxime of estrone was first synthesized and then conjugated covalently to BSA according to techniques described by Erlanger et al. (1958). The conjugate (2.0 mg/injection) was suspended in a 1:1 mixture of Freund's Adjuvant and glass distilled water and administered

subcutaneously once each week for six weeks to New Zealand White rabbits. After the initial immunization period, animals were injected monthly with blood samples being drawn biweekly.

DHT Antiserum (#A-3)

The 3-O-carboxy methyl oxime of testosterone was prepared by the method of Erlanger et al. (1957) and covalently bound to BSA (by Dr. T.G. Muldoon). Immunization and bleeding schedule for the rabbits was as described for the estradiol antiserum (RP#1).

D. CELITE COLUMN CHROMATOGRAPHY OF STEROIDS

Columns were prepared by tightly packing with a glass rod to a height of 5 cm with a 2:1 mixture (weight:volume) of celite and stationary solvent phase in disposable 5 ml glass pipettes (Kimble). The stationary solvents used were either 1:1 ethylene glycol and propylene glycol (column system A) or ethylene glycol (column system B). After packing, each column was washed with 10 ml isooctane to clear air bubbles from the column. After washing, the columns were then ready for sample application and elution. The mobility of several steroids alone and in combination with others was evaluated on both column systems.

Briefly, ^3H -steroids were added to serum or distilled water in glass test tubes and extracted twice with 5 volumes of ether. The aqueous phase was quick frozen; the organic phase was decanted and dried under a stream of nitrogen or under vacuum. The dried ether extract was then dissolved in 0.5 ml isooctane and transferred to the celite column. This procedure was used for the standardization of the column systems since this same method would be employed when unknown serum samples were processed for assay.

The column, with sample applied, was then eluted under nitrogen pressure (2-3 psi) using isooctane and various mixtures of ethyl acetate and isooctane as mobile solvents. In preliminary studies, each 0.5 ml eluate was collected into counting vials, evaporated and counted for radioactivity following the addition of 10 ml counting solution. Optimal conditions were determined to allow for complete and separate collection of a maximum number of steroids from each column system with a minimum total elution volume. The specific column chromatographic techniques which were eventually employed for multiple steroid radioimmunoassay will be discussed further in Results.

E. METHODS FOR BLOOD STEROID RADIOIMMUNOASSAY

When unknown steroids were analyzed, the following procedures were followed. To 1 or 2 ml of serum (or water) was added 0.1 ml volumes (in phosphate buffer, 0.1 M, pH 7.0) of ^3H -labeled steroids in trace amounts (400-800 cpm/steroid) for computation of methodological losses. After equilibration, the samples were extracted as described earlier and applied to the celite column. Several fractions were then collected from the column into 10 ml vacutainer tubes, evaporated under a nitrogen stream and reconstituted in various volumes of the appropriate buffer. Duplicate aliquots were then taken for assay and a third aliquot was transferred to a counting vial and counted in 10 ml of scintillation fluid for determination of internal tracer recovery.

Standard lines for each steroid were constructed over a range of 0-1000 pg except estradiol, which covered a range of 0-200 pg. The assay procedure used for all steroid assays was the same except for estradiol-17 β and DHT in which standard or unknown volumes were 0.4 ml rather than 0.5 ml and the buffer used for estradiol assay was phosphate buffer (0.1 M, pH 7.0)

rather than a 0.1% gelatin phosphate buffer.

Each standard or unknown was incubated in 10 x 75 mm glass test tubes (Corning) with its specific antiserum (0.1 ml) for 1 hour at 4°C after thorough mixing. Tritium labeled standard (0.1 ml, 6,000-10,000 cpm) in buffer was added and tubes remixed and incubated for an additional hour at 4°C. Bound and free fractions were separated by the addition of 0.2 ml of a dextran coated charcoal suspension (0.625 g charcoal and .0625 g dextran per 100 ml buffer). Tubes were mixed again and incubated for 10 minutes at 4°C and then centrifuged (2000 rpm) for 10 minutes at 4°C. The supernatant (antisera-bound steroid) was decanted into counting vials and mixed well with counting solution (10 ml) before counting.

Standard lines were plotted as log dose standard versus relative percentage bound (the zero dose set at 100%). Standard lines were linearized by a logit transformation and the unknown mass was then determined using a computer generated linear regression program (Hewlett Packard Calculator, Model 9100 B). In all assays, a water blank was processed exactly as unknown plasma samples and blank values obtained from the standard line were subtracted from the unknowns. Final unknown concentration was computed based upon recovery of an internal tracer.

F. CYTOSOL ESTRADIOL RECEPTOR ASSAYS OF THE ANTERIOR PITUITARY AND HYPOTHALAMUS

Anterior pituitaries and hypothalami were removed from rats at sacrifice (5-7 animals per group) and placed directly into ice cold Tris EDTA buffer (T.E.), pH 8.0, 0.01M Tris, 1.5 mM EDTA. Within 30 minutes after necropsy, these tissues were washed twice with T.E. buffer and homogenized in 1.5 - 2.1 ml of cold T.E. buffer using all glass homogenizers (Kontes

Glass Co.) which were similarly kept cold in an ice bath. Homogenates were then centrifuged in a Beckman L2-65B Ultracentrifuge for 1 hour at 4°C (105,000 x g). The supernatant (cytosol) was then decanted and kept at low temperature. Two aliquots (0.1 and 0.2 ml) in duplicate of the cytosol were then mixed with 0.05 ml ³H-estradiol (1 x 10⁻¹² moles/1.41 x 10⁵ dpm or 1 x 10⁻¹² moles/2.09 x 10⁵ dpm per ml of T.E. buffer) plus buffer to equal a total volume of 1.0 ml.

Non-specific binding was quantified by incubating similar aliquots of cytosol with radiolabeled estradiol-17β plus a 100-fold molar excess of unlabeled estradiol. All incubations were performed in 10 x 75 mm plastic culture tubes (Falcon Plastics) for 18-22 hours at 4°C. Cytosol receptor bound ³H-estradiol was then precipitated by the addition of 0.5 ml protamine sulfate solution (4 mg/ml T.E. buffer). Tubes were mixed gently, incubated for 10 minutes at 4°C and then centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was decanted (unbound fraction) and the bottom of the assay tubes (which contained the receptor - ³H-estradiol-17β pellet) were sliced off into counting vials with a heated knife. After the addition of 10 ml of scintillation fluid, vials were shaken for 30 minutes and then counted in a Beckman LS 230 or Packard Liquid Scintillation Spectrometer. The efficiencies of these instruments for unquenched tritium samples are 50% and 40% respectively. Disintegrations per minute (dpm) were calculated by the external standards method.

Specific bound dpm ³H-estradiol-17β was determined by subtracting the dpm bound in the presence of 100-fold excess unlabeled estradiol-17β (non specific binding) from the total bound dpm in the samples incubated with radioactive estradiol-17β only. This method of correction for non

specific binding has been previously validated by Korach and Muldoon (1974 a,b). In addition, cytosol aliquots were evaluated to determine protein concentration by the method of Lowry et al., (1951) and cytosol estradiol-17 β receptor content of the anterior pituitary and hypothalamus were expressed as specific bound moles ^3H -estradiol/mg cytosol protein.

G. STUDIES IN THE IMMATURE FEMALE RAT

Female rats of the Sprague Dawley strain were purchased from the Holtzman Co. and were received no later than 23 days of age, which allowed a minimum period of 4 days acclimation prior to experimental manipulations. Rats were housed 3-4 per cage and received lab chow and drinking water ad libitum. The lighting schedule was 14 hr light and 10 hr darkness/24 hr; 12:00 noon being the mid point of the light period. In all experiments to be described, injections were subcutaneous.

Animals were sacrificed by decapitation or by ether inhalation; there were no differences noted in the various experimental parameters examined which could be attributed to the mode of sacrifice.

At each sacrifice, the following procedures were used: after weighing each animal, blood was collected from the trunk after decapitation or was collected via cardiac puncture under ether anesthesia as a terminal procedure. Blood samples were allowed to stand at room temperature for a minimum of 4 hours to allow full clot retraction and serum was obtained following centrifugation for 15 minutes at 2,000 rpm. Serum was then frozen and stored at -15°C until used for steroid or gonadotropin assays. Hypothalami and/or anterior pituitaries were removed and processed as described earlier for estradiol-17 β receptor analysis. Ovaries and uteri were dissected, cleaned of adventitious tissue and weighed to the nearest

0.5 mg; when present, uterine fluid was drained prior to weighing uteri. Organ weights are expressed as mg/100g body weight. Ovaries were inspected grossly to document the presence of large follicles, cysts and/or corpora lutea. Oviducts were pressed between two glass slides and examined microscopically to determine the presence or absence of ova, which were counted. Vaginal smears of all animals with patent vaginae were taken at sacrifice; in addition, most animals were smeared daily after vaginal opening for the duration of each experiment.

The following experiments were performed in the course of these studies in the immature female rat:

Experiment 1. Female rats were treated with 8 IU PMSG (Equinex, Ayerst)/0.5 ml saline at 10 AM at 30 days of age. Groups of six rats were sacrificed at 11 - 12 noon on days 27-39 of age; additional sacrifice times were at 6 PM on days 30 and 32 of age. Only anterior pituitaries were removed for use in cytosol estradiol receptor assays.

Experiment 2. Female rats were treated with DHA (60 mg/kg body weight/day) in propylene glycol at 8:30 - 9:00 AM on days 27-29 of life; PMSG was injected as in Experiment 1. Additional groups of control animals were treated with vehicle only on days 27-30. Animals were sacrificed daily as in the previous experiment (Exp. #1).

Experiment 3. A large group of immature female rats were treated with DHA and PMSG as in Experiment 2. On day 31, the rats were divided into two groups; one group received additional DHA therapy (60 mg/kg body weight/day) for 10 days, the other received no further treatment. Animals were sacrificed daily as in Experiment #1 with an additional sacrifice scheduled for 6 PM on day 29. Anterior pituitaries were utilized for the evaluation

of cytosol receptor content in these groups.

Experiment 4. Immature female rats were sacrificed daily, with no treatment, as in Experiment #1. Anterior pituitaries and hypothalami were removed for cytosol receptor assays.

Experiment 5. Female rats were treated with PMSG as in Experiment #1. Animals were sacrificed at 11 AM on day 29 and 2 - 3 PM on day 32 and 33. An additional group of saline treated controls were sacrificed on the afternoon of day 32. Anterior pituitaries and hypothalami were utilized in cytosol receptor analyses.

Experiment 6. Female rats were treated chronically (age 27-46) with DHA as in Experiment 2. A group of control rats were also treated with vehicle only. On the AM (11 - 12 noon) of days 27 and 34, groups of DHA and vehicle-treated rats were administered LH-RH (10 ng/100 g body weight) or vehicle (0.05 M acetic acid, 0.9% saline, 1.0% BSA) and were sacrificed 30 minutes later. In addition, DHA-treated rats were administered LH-RH or vehicle at 46 days of age and sacrificed as above. Additional sacrifice times for the DHA-treated rats were at 11 - 12 noon at 32, 48, 50, 54, 58, 61, 66 and 68 days of age after DHA had been discontinued. Anterior pituitaries and hypothalami were removed for use in cytosol receptor assays from all groups.

Experiment 7. Female rats were castrated at 8 - 9 AM on day 30. Intact and castrate rats were then treated with PMSG or saline (11 AM) and sacrificed at 6 PM on day 30; intact females were also sacrificed at 11 AM and 6 PM on days 31-32 and 11 AM on day 33. Anterior pituitaries and hypothalami were utilized for receptor analyses.

H. STUDIES IN THE HUMAN FEMALE

Peripheral blood samples were obtained from 10 normal female volunteers (age 18-28 years) during the various stages of the menstrual cycle. These samples were analyzed by the multiple steroid radioimmunoassay techniques which were developed in this laboratory. Data obtained from such analyses served as an additional validation of the assay method. Other blood samples were obtained from hirsute, anovulatory adult females who displayed symptoms consistent with the diagnosis of Polycystic Ovarian Disease. In addition, blood samples were also obtained from other patients having masculinizing tumors of the ovary. These studies constituted the first time a full spectrum of steroid hormones had been quantified in the normal or hirsute female from single serum samples. Data obtained in these human studies also served as a basis for comparison to findings in the androgen-treated rat.

I. RADIOIMMUNOASSAY OF SERUM FSH AND LH

Serum FSH and LH were quantified by double antibody radioimmunoassay techniques. Materials for these assays were provided through the National Institute of Arthritis and Metabolic Diseases (NIAMD) of the National Institutes of Health. The specific assay techniques utilized have been described previously by Eldridge *et al.*, (1974 b). The sensitivity of these methods is 10 ng/ml when 0.2 ml unknown aliquots are analyzed. By a logit transformation of the LH or FSH standard line, it is possible to quantify gonadotropin concentrations which are less than 10 ng/ml serum. Interassay quality control was maintained by including standardized serum samples from a large serum pool in each assay.

J. STATISTICAL ANALYSES

All group data in this dissertation are expressed as the mean \pm standard error of the mean (S.E.) unless otherwise indicated. Differences in the various parameters quantified between groups were analyzed using an unpaired t test (Steel and Torrie, 1960). The $p < 0.05$ level was chosen as significant in these studies.

IV. RESULTS

A. CELITE COLUMN CHROMATOGRAPHY OF STEROIDS

The chromatographic mobility of the following steroids was established on celite partition columns: progesterone, androstenedione, testosterone, 17-hydroxyprogesterone, dehydroepiandrosterone, 5 α -dihydrotestosterone, estrone and estradiol-17 β . It was found that progesterone, androstenedione, 5 α -dihydrotestosterone and 17-hydroxyprogesterone could be separated and isolated individually following chromatography on column system A (stationary phase - ethylene glycol: propylene glycol, 1:1). This was accomplished by eluting this column with 7 ml of isooctane (column fractions 2-15; each fraction representing 0.5 ml eluted from the column, column fraction 1 was considered to be the void volume), 4.0 ml of 5% ethyl acetate/isooctane (fractions 16-23) and 4.0 ml of 15% ethyl acetate/isooctane (fractions 24-32).

Similarly, the quantitative isolation and separation of dehydroepiandrosterone and estradiol-17 β could be achieved from a fraction which contains both testosterone and estrone by chromatographing on column system B (stationary phase - ethylene glycol). This column was eluted with 3.5 ml of isooctane (fractions 2-8), 8.0 ml of 5% ethyl acetate/isooctane (fractions 9-24) and 4.0 ml of 30% ethyl acetate/isooctane (fractions 25-33). An example of the pattern of elution for the above mentioned steroids on column systems A and B is shown in Figure 1.

This two column technique was adopted as a preliminary purification of blood steroids for subsequent analysis by radioimmunoassay. Due to the low concentrations of some steroids in blood, this method was applicable only when sample volume was not a limiting factor (e.g. greater than 2.0 ml). Modifications of this method were necessary, however, for application to

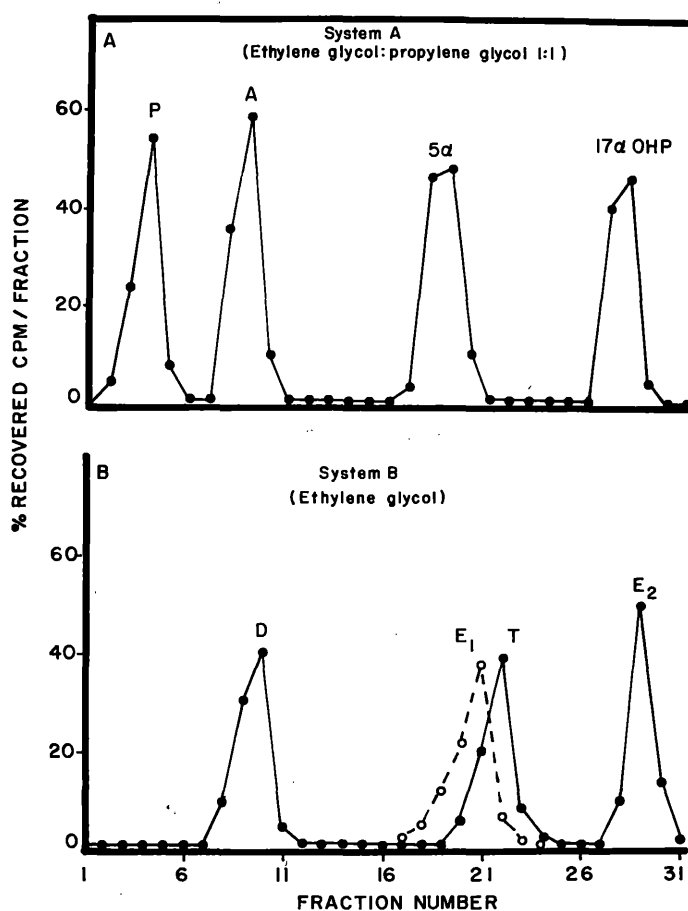
Figure 1. Separation and Recovery of Simultaneously Chromatographed
³H-Steroids Using Column Systems A and B.

Abbreviations:

P	-	progesterone
A	-	androstenedione
5 α	-	5 α -dihydrotestosterone
17 α OHP	-	17-hydroxyprogesterone
D	-	dehydroepiandrosterone
T	-	testosterone
E ₂	-	estradiol-17 β

Each fraction is equal to 0.5 ml eluted from the columns.

SEPARATION AND RECOVERY OF SIMULTANEOUSLY CHROMATOGRAPHED
 H^3 STEROIDS USING COLUMN SYSTEMS A AND B



the quantification of steroid concentrations in small serum volumes such as would be obtained from experimental animals like the immature rat.

Since non-polar steroids such as progesterone and androstenedione could not be easily separated from DHA (or 5α -dihydrotestosterone) on column system B, column system A was chosen for further studies. It was found that estradiol- 17β could be eluted from this column by the addition of a final wash of 4.5 ml of 35% ethyl acetate/isooctane. DHA could be eluted in close proximity to 5α -dihydrotestosterone, yet apart from androstenedione and progesterone, in the 5% ethyl acetate/isooctane wash. In addition, testosterone was found to migrate in a fashion similar to 17-hydroxyprogesterone, being totally eluted in 15% ethyl acetate/isooctane. The chromatographic separation of these seven steroids, using this modified column system A, are shown in Figure 2. Estrone, not depicted, was also eluted in the 15% ethyl acetate in isooctane solvent wash.

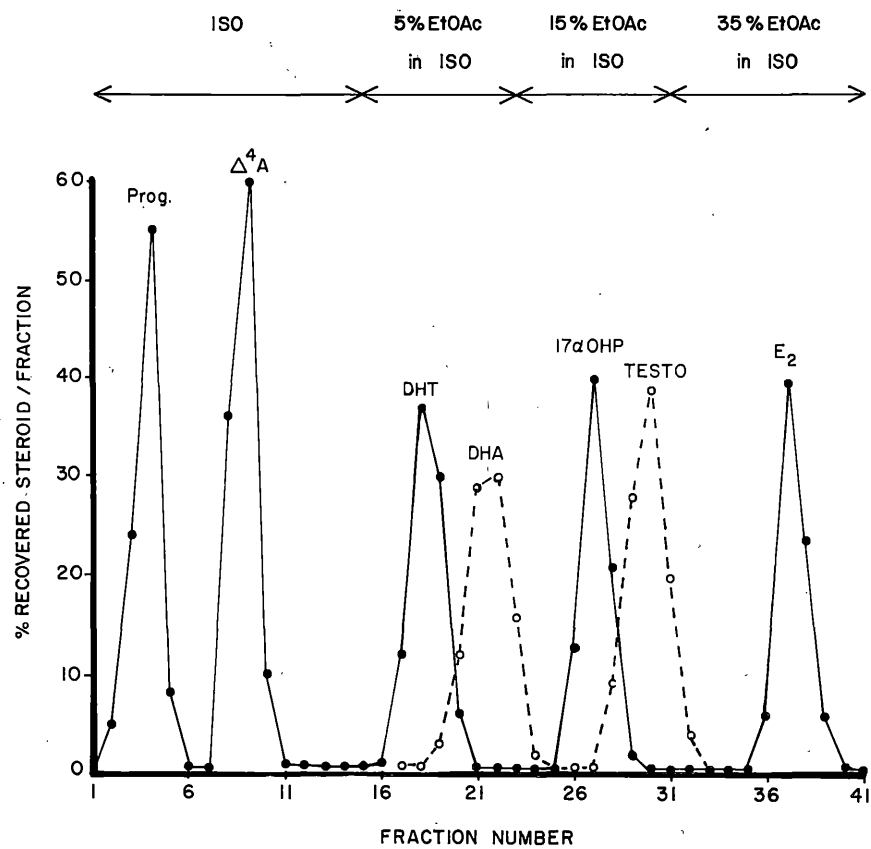
The location of radioactive steroid peaks following celite partition chromatography has been found to be quite consistent over the course of these studies and did not vary significantly among three commercially available brands of celite (Johns Manville Hi Flow (A.W.), Johns Manville L-665-A, and Fisher Scientific). The addition of as much as 100 ng of each of these steroids (unlabelled) plus radiolabeled steroids to a serum sample prior to ether extraction and chromatography did not alter the elution of any steroid on column systems A or B or the modified system A. This would indicate that an overloading of the celite column would be improbable during the analysis of serum samples with high steroid content. These initial studies demonstrated the resolving power of the celite columns and suggested that radioimmunoassay of several steroids from a single blood sample would

Figure 2. Separation and Recovery of ^3H -Steroids Chromatographed on the Modified Column A System

Abbreviations:

Prog. - progesterone
 $\Delta^4\text{A}$ - androstenedione
DHT - 5α dihydrotestosterone
DHA - dehydroepiandrosterone
 $17\alpha\text{OHP}$ - 17 -hydroxyprogesterone
TESTO - testosterone
 E_2 - estradiol- 17β

Each fraction is equivalent to 0.5 ml eluted from the column.



be feasible.

Procedures for the collection of steroids prior to radioimmunoassay using both the two column system (A + B) and the one column modified system A were developed and are described in Tables I and II. Although the buffer volumes mentioned in both Table I and II were applied originally to the analysis of human blood steroids, they have proven equally useful for the quantification of steroid hormones in the female rat as well. The only modifications of these procedures employed during studies in the female rat have been to dilute further those fractions containing androgens (DHA, 5 α -dihydrotestosterone, androstenedione and testosterone) and estradiol-17 β when exogenous steroid (DHA) was administered, as mentioned in the various experimental protocols.

The radioimmunoassay of two compounds from a single column fraction, as proposed in Table II (modified column system A) for DHA and 5 α -dihydrotestosterone or for testosterone and 17-hydroxyprogesterone, was possible under the conditions used because neither compound in either of these two fractions (17-24, 26-31) interfered in the assay of the other. If binding proteins or less specific antisera were used, this procedure would not be advisable nor proper. Under assay conditions, it was found that either radiolabeled 5 α -dihydrotestosterone or DHA could be utilized as an internal recovery tracer for both compounds when added to a serum sample since both steroids were readily soluble in ether (the extraction solvent) and in isooctane (the solvent for sample application to the column), and both compounds were completely eluted from the column within the assay fraction collected (17-24, Table II). The same relationship was found to exist between testosterone and 17-hydroxyprogesterone with respect to solvent solubility

Table I. Elution Procedures with Celite Column Systems A and B for Multiple Radioimmunoassay

MOBILE SOLVENT	VOLUME ADDED	FRACTIONS ¹ AND VOLUME COLLECTED	STEROID ISOLATED	BUFFER VOLUME ² ADDED TO DRIED ELUATE
<u>SYSTEM A</u>				
isooctane	7.0 ml	3-6 (2.0 ml)	progesterone	2.0 ml ³
		8-11 (2.0 ml)	androstenedione	2.0 ml
5% ethyl acetate in isooctane	4.0 ml	16-21 (3.0 ml)	5 α -dihydrotestosterone	2.0 ml
15% ethyl acetate in isooctane	4.0 ml	26-30 (2.5 ml)	17-hydroxyprogesterone	2.0 ml
<u>SYSTEM B</u>				
isooctane	3.5 ml	discard		
5% ethyl acetate in isooctane	8.0 ml	8-12 (2.5 ml)	dehydroepiandrosterone	4.0 ml
		18-23 (3.0 ml)	testosterone	2.0 ml ⁴
30% ethyl acetate in isooctane	4.0 ml	26-31 (2.5 ml)	estradiol-17 β	1.7 ml

¹Column Fractions 1,2,7,12-15,22-25 were discarded with System A while column fractions 1-7, 13-17,24-25 were discarded with System B.

²Buffer volumes added to column eluates apply to plasma or serum samples obtained from men and women.

³Progesterone fraction is dissolved in 5.0 ml buffer when sample is obtained during the luteal phase of the menstrual cycle.

⁴Testosterone fraction is dissolved in 5.0 ml buffer when sample is obtained from a man.

Table II. Elution Procedures Used with Modified Column System A for Multiple Radioimmunoassay

MOBILE SOLVENT	VOLUME ADDED	FRACTIONS ¹ AND VOLUME COLLECTED	STEROID ISOLATED	BUFFER VOLUME ² ADDED TO DRIED ELUATE	
				MALE	FEMALE
isooctane	7.0 ml	3-6 (2.0 ml)	progesterone	2.0 ml	2.0 ml ²
		8-11 (2.0 ml)	androstenedione	2.0 ml	2.0 ml
5% ethyl acetate in isooctane	4.0 ml	17-24 (4.0 ml)	5 α -dihydrotestosterone	2.5 ml ³	2.5 ml ³
			dehydroepiandrosterone		
15% ethyl acetate in isooctane	4.0 ml	26-31 (3.0 ml)	17-hydroxyprogesterone	4.0 ml ⁴	3.0 ml ⁴
			testosterone		
35% ethyl acetate in isooctane	4.5 ml	35-39 (2.5 ml)	estradiol-17 β	1.7 ml	1.7 ml

¹The following column fractions were discarded with the modified column system A: 1,2,7,12-16,25,32-34.

²Progesterone fraction is dissolved in 5.0 ml buffer when plasma or serum sample is obtained during the luteal phase of the menstrual cycle.

³Two 0.4 ml aliquots are removed for 5 α -dihydrotestosterone assay; one 0.5 ml aliquot is counted to determine recovery of internal tracer; 2.0 ml buffer is added to the remainder of the fraction and after mixing, two 0.5 ml aliquots are removed for assay of dehydroepiandrosterone.

⁴0.5 ml aliquots are removed in duplicate for the assay of both compounds and an additional 0.5 ml aliquot is taken and counted to determine recovery of internal tracer.

and column chromatography behavior.

In practice, when the two column technique was to be used for chromatographic separation and isolation of the various steroids for assay, tracer amounts (400-800 cpm) of tritiated DHA, testosterone and estradiol-17 β were added to one aliquot of a single serum (or plasma) sample while tritium labeled progesterone, androstenedione, 5 α -dihydrotestosterone and 17-hydroxyprogesterone tracers were similarly added to a second aliquot of the same sample. Both aliquots were then extracted and chromatographed separately on the two columns. However, when the single modified column system A was to be used, tracer quantities of radiolabeled 5 α -dihydrotestosterone and 17-hydroxyprogesterone were added for calculation of recovery of DHA/5 α -dihydrotestosterone and testosterone/17-hydroxyprogesterone respectively in addition to adding tracer quantities of radiolabeled progesterone, androstenedione, and estradiol-17 β to the same sample prior to ether extraction and column chromatography.

B. COLUMN RECOVERY OF RADIOLABELED STEROIDS

The different fractions chosen for the collection of steroid hormones prior to assay have been mentioned in Tables I and II. Several experiments were carried out in which one radiolabeled steroid at a time was applied to the column which was then eluted with the various mobile solvents (isooctane and mixtures of ethyl acetate in isooctane). Fractions were collected (in total) into counting vials and the recovery of each ^3H -steroid was quantified for each of the assay fractions. Those 0.5 ml fractions normally discarded were also collected separately and counted as well.

Using the column system A, $93 \pm 4.2\%$ (S.D.) of progesterone was recovered in the appropriate fraction; this fraction was contaminated with only 2% or

less of the total androstenedione present. The androstenedione fraction had a $85.5 \pm 9.2\%$ recovery with no progesterone contamination. Recoveries of 5α -dihydrotestosterone and 17-hydroxyprogesterone were $87.2 \pm 5.7\%$ and $88.0 \pm 6.4\%$ respectively in the appropriate fractions. When estradiol was collected, $68.5 \pm 11.1\%$ was recovered. Using column system B, $87.5 \pm 7.8\%$ of DHA was recovered in the proper fractions. The fractional recovery of testosterone was $78.5 \pm 3.5\%$ while that for estradiol was $87.0 \pm 2.8\%$. There was no contamination of any fraction with steroids to be recovered elsewhere, and the recovery of radioactive steroids in those designated to be discarded was quite minimal ($<1\%$).

These studies indicated that in addition to the consistency of the pattern of steroid elution observed, the recovery of the various steroids in the fractions designated to be collected prior to assay was high and was quite reproducible. Following the addition of several tracers (for calculation of methodological losses during extraction and chromatography) to a serum sample, the separate collection of the proposed column fractions would allow for a correct determination of the recovery of each steroid in question, without contamination with radioactivity arising from any other steroid tracer. If this were not the case, there could be no valid correction applied for the loss of steroid from an unknown sample during the procedure of serum extraction and chromatography prior to assay. As a continuous quality control, the chromatographic mobility and fractional recovery of each radiolabeled steroid were regularly checked at 6-8 month intervals, during the completion of these studies. No significant alteration in either of these two parameters were detected during the course of these evaluations.

C. ESTRADIOL ANTISERUM

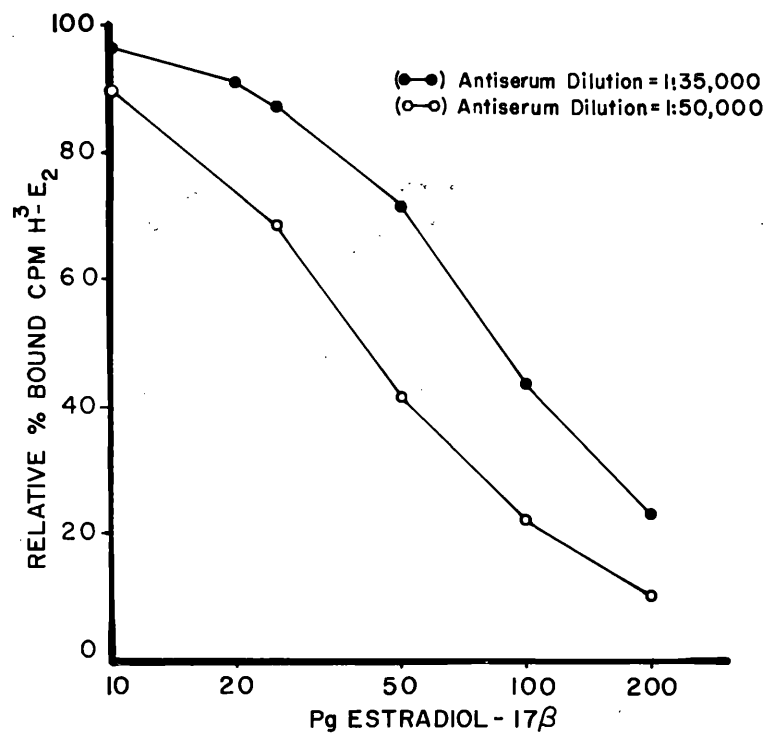
Serum samples obtained from rabbits injected with the estrone-17-BSA conjugate were checked for the presence of antibodies to estrogens. Serum samples were diluted serially with phosphate buffer and aliquots were incubated with 10,000 cpm ^3H -estradiol for 1 hour at 4°C . Separation of antibody bound and free ^3H -steroid was accomplished following the addition of 0.2 ml dextran coated charcoal solution, incubation for 10 minutes at 4°C and centrifugation of assay tubes at 2,000 rpm at 4°C for 10 min. The supernatant was decanted into counting vials and counted. Percent bound was calculated as equaling $(\text{cpm bound} / \text{total added } ^3\text{H-estradiol-17}\beta) \times 100$. Titer was defined as that dilution of serum which bound 50% of the added radiolabeled estradiol (10,000 cpm). Within three months, the titer of antiserum from one rabbit (RP#1) had risen and then plateaued at 1:50,000. The highest titer serum samples obtained from this rabbit were pooled. Further characterization of the antiserum was performed on the serum pool.

Standard lines were constructed for this antiserum (RP#1) as described in Materials and Methods. Examples of standard lines for estradiol-17 β are shown in Figure 3. It should be noted that the standard line has a steeper slope and was therefore more sensitive at the higher antiserum dilution (1:50,000). Specificity of the antiserum was then evaluated by comparing the ability of various steroids to compete with ^3H -estradiol-17 β for binding sites on the antibodies as opposed to unlabeled estradiol-17 β . Percent cross reaction (C.R.) was assumed to be 100% for estradiol-17 β , that of other compounds tested was calculated as follows: $\% \text{ C.R.} = (\text{mass of estradiol-17}\beta \text{ which causes a 50\% or other standard \% reduction in antibody bound } ^3\text{H-estradiol-17}\beta) \div (\text{mass of test steroid which, when incubated with the$

Figure 3. Standard Lines for Estradiol-17 β Using Antiserum RP#1

Standard lines for estradiol-17 β were run with the antiserum (RP#1) by incubating 0.1 ml antiserum solution (in phosphate buffer) over a range of 0-200pg. At a final antiserum dilution of 1:50,000, the sensitivity of the standard line was found to be 2 pg for estradiol-17 β .

STANDARD LINES FOR ESTRADIOL USING ANTISERUM RP-1



antiserum, causes a similar reduction in bound ^3H -estradiol-17 β) $\times 100$. As shown in Table III, the antiserum was specific for estradiol-17 β and estrone (90% C.R.) which are easily separated on the celite partition columns. The antiserum had minimal affinity for synthetic estrogens or non-estrogenic steroids.

D. ANTISERUM FOR 5 α -DIHYDROTESTOSTERONE RADIOIMMUNOASSAY

When the modified column system A is to be used, DHA and DHT are isolated together in one fraction prior to assay (Table II). Only this assay fraction contains two androgens which are present in human blood samples in vastly different quantities, (DHA $> 5 \times$ DHT concentrations); concentrations of these compounds in the serum of female rats are undocumented. Slight cross reaction of a DHT antiserum with DHA would result in erroneous measurement of DHT in human and possibly other serum samples, especially that of the DHA-treated rat.

An antiserum (S-741, prepared in sheep against testosterone-3-oxime-human serum albumin) was purchased which was reported to be useful for the assay of testosterone and DHT (Coyotupa et al., 1972). Similarly, another antiserum had been developed in our laboratory for application to the radioimmunoassay of these two steroids. Specificity of this antiserum (#A-3, prepared against testosterone-3-oxime-BSA) was evaluated in a manner similar to studies on the estrogen antiserum (RP-1). The results of cross reactivity determinations are shown in Table IV. Antiserum #A-3 cross reacted primarily with testosterone and DHT but reacted minimally with other androgens and non-androgenic steroids.

With the availability of two antisera for potential use in the radioimmunoassay of 5 α -dihydrotestosterone, the suitability for such utilization

Table III. Specificity of Estradiol Antiserum (RP#1)

<u>STEROID TESTED</u>	<u>% CROSS REACTIVITY</u>
Estradiol-17 β	100.00
Estrone	90.00
1,3,5(10)-estratriene-3,16 β ,17 β -triol	20.00
16 α -hydroxy-estrone	31.90
11 β -hydroxy-estrone	20.00
Estradiol-17 α	14.3
Estriol	9.0
17 α -ethinyl-estradiol	2.8
mestranol	0.04
5 α -androsterone-3 α ,17 β -diol	1.41
5 α -androsterone-3 β ,17 β -diol	0.72
3 α -hydroxy-5 β -androsterone-17-one	0.50
Androsterone	0.30
11 β -hydroxy-androsterone	0.13
11-oxo-androsterone	0.49
4-androstene-3,11,17-trione	0.004
11 β -hydroxy-androstenedione	0.35
Testosterone	0.04
Dehydroepiandrosterone	0.10
Progesterone	0.001
17-hydroxy-progesterone	0.001
Corticosterone	0.001

Trivial nomenclature used: 17 α -ethinyl-1,3,5(10)-estratriene-3,17 β -diol (17 α -ethinyl estradiol), 3 methoxy-17 α -ethinyl-1,3,5(10)-estratriene-3,17 β -diol (mestranol).

Table IV. Specificity of Antiserum #A-3

<u>STEROID TESTED</u>	<u>% CROSS REACTIVITY</u>
5 α -dihydrotestosterone	100.00
Testosterone	200.00
5 α -androstane-3 α ,17 β -diol	11.08
5 α -androstane-3 β ,17 β -diol	5.47
Androsterone	1.59
Dehydroepiandrosterone	0.20
Androstenedione	1.50
17-hydroxyprogesterone	0.60
Estrone	0.80
11 β -hydroxy-androsterone	0.01
11-oxo-androsterone	0.72
11 β -hydroxy-androstenedione	0.95
4-androstene-3,11,17-trione	0.62
3 α -hydroxy-5 β -androstane-17-one	0.25
16 α -hydroxy-estrone	0.001
11 β -hydroxy-estrone	0.001

was investigated by a series of parallel experiments.

Standard lines, using ^3H -DHT as radioligand were established for DHT, DHA and androstenedione with both antisera, #A-3 and S-741. Assays were performed as follows: 0.4 or 0.5 ml of standards were incubated with 0.1 ml antiserum solution (#A-3 or S-741 respectively) for 1 hr at 4°C . Then 0.1 ml of ^3H -DHT was added and tubes were incubated for an additional hour at 4°C . Dextran coated charcoal suspension (0.2 ml) was then added, tubes mixed and incubated for 10 min at 4°C , after which they were centrifuged at 2,000 rpm for 10 min at 4°C . The supernatants were then decanted and counted after addition of 10 ml of counting solution (0.5% Permablend II in toluene). The results of this comparison are shown in Figure 4. It is apparent that DHA and androstenedione are potentially interfering substances when DHT is assayed with antiserum S-741 whereas there is minimal cross reaction with these steroids when antiserum #A-3 is used. Since androstenedione is well separated from the DHA/DHT fraction in the chromatographic system used, only the interference of DHA was studied in detail for these antisera.

To investigate further the potential for DHA interference in this assay procedure, a 10 ml human peripheral plasma pool was extracted and chromatographed using celite column system A with the following exception: several 0.5 ml column fractions (17-24) which would ordinarily be pooled were collected individually into 10 x 75 mm glass culture tubes (Corning). The fractions were then evaporated and reconstituted in assay buffer. Aliquots of each column fraction were then analyzed to determine the recovery of ^3H -DHT, DHA and DHT. DHT was assayed using both antisera, #A-3 and S-741, while DHA was assayed using an antiserum specific for DHA (S-1502). The

Figure 4. Standard Lines Constructed for DHT, DHA and Androstenedione Utilizing Antisera #A-3 and S-741

Incubation procedures (time, temperature, standard volumes and antiserum volumes) were as described in the text. Each antiserum was reacted with various standard amounts of DHT, DHA or androstenedione (Δ^4 A) then reacted with ^3H -DHT. For ease of graphical representation, standard lines are expressed as log dose vs. relative percent bound CPM ^3H -DHT. Following application of a logit transformation, these standard lines are linear with coefficients of variation (r) greater than 0.990.

CROSS REACTIVITY EVALUATIONS ON ANTISERA #A-3 AND S-74I

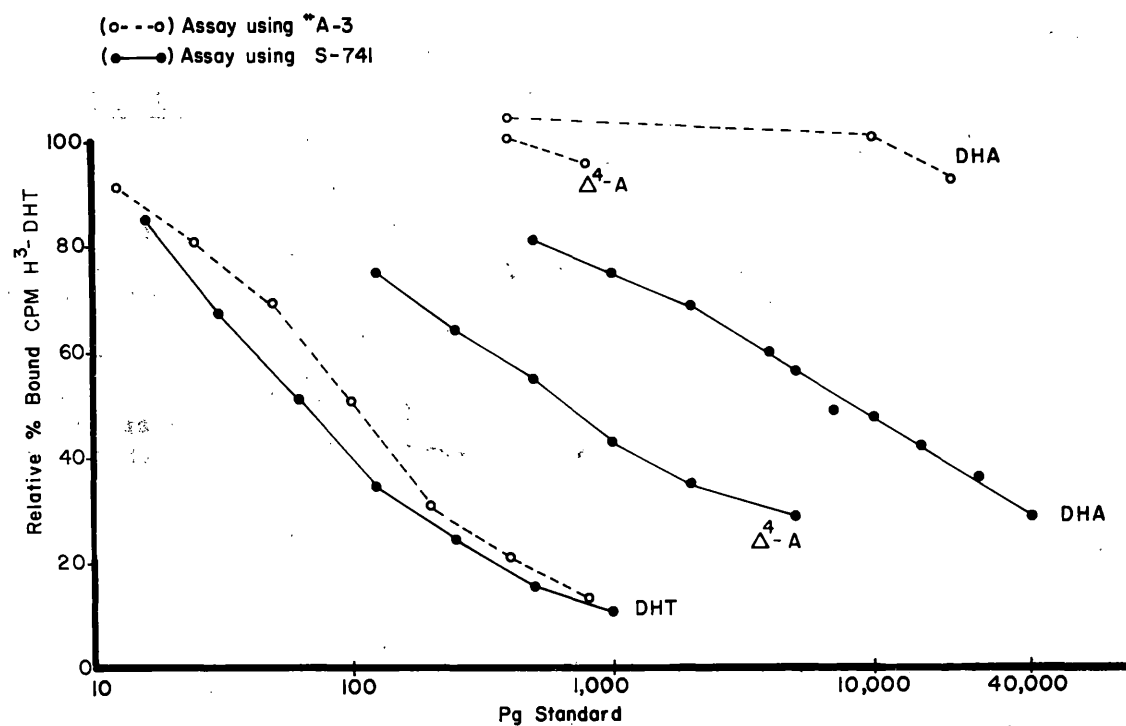
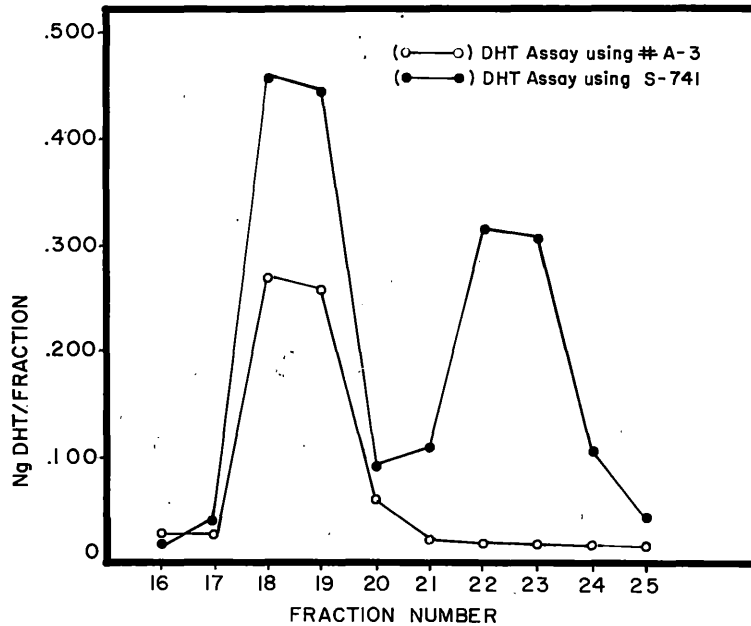
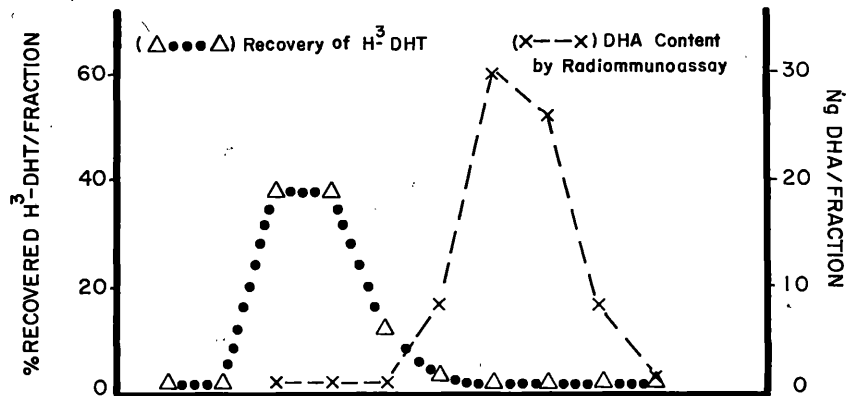


Figure 5. Fractional Recovery of DHT, ^3H -DHT and DHA from a 10 ml Human Plasma Pool

The sample was extracted with two volumes of ether and chromatographed using the modified column A system; fractions 16-25 (0.5 ml each) were eluted and collected separately. Each fraction was evaporated, reconstituted in buffer and evaluated for ^3H -DHT content, DHA content by radioimmunoassay, and DHT content using both antisera, S-741 and #A-3, for radioimmunoassay.



results of this study are shown in Figure 5.

When the peripheral pool was analyzed, the elution of DHT, as measured with antiserum #A-3 paralleled the recovery of the radioactive tracer, ^3H -DHT. However, the elution of DHT as measured by antiserum S-741 paralleled the elution of both ^3H -DHT and DHA (Figure 5). Fraction #21, which contained only 1% of the ^3H -DHT appeared to contain more DHT than fraction #20 (which contained 12% of the recovered ^3H -DHT) when assayed using S-741. The apparent recovery of immunoreactive DHT in fraction 21 was due to the interference with this antiserum by 8 ng of DHA which was found to be present in this fraction. Column #22, which contained no ^3H -DHT but contained 30 ng of DHA, appeared to contain over 0.3 ng of DHA when assayed using antiserum S-741. There was no measurable DHT in this fraction (#22) when antiserum #A-3 was employed. It seems that as little as 8 ng of DHA can interfere significantly with the measurement of DHT when antiserum S-741 is used while there is no detectable interference with antiserum #A-3 by as much as 30 ng of DHA.

As a final evaluation of these antisera, several serum samples with varying DHA concentration were extracted and chromatographed using celite column system A as described earlier. Column fractions 17-24 were pooled as usual whereby DHT and DHA were isolated together for assay. The concentrations of DHT were higher when assayed with S-741 than levels obtained on the same samples assayed with #A-3. The DHT concentrations found using S-741 could be corrected for DHA interference. A comparison of values obtained using both antisera and the corrected values using S-741 are shown in Table V. Included in this table are the DHA concentrations of each sample. Several separate evaluations were made to determine the cross re-

activity of DHA (1-15 ng) with S-741. A mean value for cross reactivity of 1.2% was obtained from these studies and applied to correct the DHT concentrations of plasma samples described above. The formula used was as follows: corrected DHT (S-741) = DHT (S-741) - (0.12 x DHA concentration).

When applied to peripheral samples (#1-5, Table V) with DHA concentrations less than 15 ng/ml, this correction resulted in similar DHT concentrations as measured by #A-3 and S-741. Additionally, when applied to adrenal vein samples (#1,2) this correction also proved useful. That this correction worked with plasma samples with extremely high DHA concentrations may have been coincidental. Nevertheless, it was concluded from these comparisons that antiserum S-741 was unsuitable for the radioimmunoassay of 5 α -dihydrotestosterone while #A-3 would be useful for the quantification of this steroid when isolated in the presence of DHA.

E. STUDIES ON THE RELIABILITY OF MULTIPLE STEROID RADIOIMMUNOASSAY TECHNIQUES

As an index of precision of these multiple steroid radioimmunoassay systems, pooled plasma samples were assayed following extraction and chromatography using the single modified celite column system A or the two column method (A + B). An example of such an evaluation, in which the single column chromatographic step was used, is shown in Table VI. Intra-assay variability, as represented by the coefficients of variation (mean/S.D.) x 100) was found to be low for each steroid assayed. The precision of the modified column system A was similar to that observed when two column chromatographic steps (A + B) were used. This would indicate that there was no loss in precision by using the more rapid column method for

Table V. Comparison of Human Plasma DHT Concentrations Using #A-3 and S-741

PERIPHERAL PLASMA SAMPLE	(ng/ml)			
	DHT S-741	DHT Corrected S-741	DHT #A-3	DHA
1	.209	.085	.099	10.4
2	.222	.166	.170	4.7
3	.108	.042	.046	5.5
4	.299	.222	.234	5.4
Mean \pm (S.E.)	.203 (.031)	.113 (.035)	.121 (.035)	
ADRENAL VEIN SAMPLE				
1	.439	.070	.079	30.8
2	.560	.162	.110	33.2

Table VI. Studies on Precision of Steroid Levels as Determined by Using Modified Column System A

	S T E R O I D A S S A Y E D						
	Estradiol	Proges- terone	5 α -DHT	DHA	Testosterone	17-hydroxy- progesterone	Androstenedione
Mean Concentra- tion (ng/ml)	.036	.253	.647	8.039	6.770	1.949	1.518
Standard Devia- tion	.003	.014	.037	1.030	.376	.165	.065
Standard Error	.001	.007	.015	.421	.153	.067	.027
Coefficient of Variation	8.9%	5.4%	5.8%	12.8%	5.6%	8.5%	4.3%

steroid isolation prior to assay.

Another critical consideration in such a method is the contribution of interfering substances, which may be accumulated during extraction and chromatography, to the calculated value for each steroid assayed. The degree of sensitivity of this multiple steroid radioimmunoassay technique, or any other method, is related inversely to the extent of such interference (method blank). The amount of interference may be quantified after extraction and chromatography by the assay of steroid-free samples such as water or buffer. Summary data describing the method blank in each steroid assay ($n = 10$ or more) are presented in Table VII. No significant difference in blank values (pg) were noted with respect to either the one or two column chromatographic methods.

In addition to the above criteria, the recoveries of the various steroids during actual assay conditions were evaluated. It was previously determined that column recovery of each steroid was both consistent and adequately high. It was found that the overall recovery of blood steroids during the multiple steroid radioimmunoassay was similar to that found when each steroid was individually chromatographed and collected according to the procedures outlined in Table I and II. Using column system A, $85 \pm 10\%$ (S.D.) of progesterone, $76 \pm 18\%$ of androstenedione, $74 \pm 17\%$ of 5α -DHT and $92 \pm 15\%$ of 17-hydroxyprogesterone were recovered ($n = 25$). With column system B, $81 \pm 17\%$ of DHA, $72 \pm 16\%$ of testosterone and $76 \pm 10\%$ of estradiol-17 β were recovered ($n = 33$). When the modified column system A was utilized for the simultaneous separation of the seven steroids for assay, $80 \pm 9\%$ of progesterone, $81 \pm 15\%$ of androstenedione, $78 \pm 12\%$ of DHT/DHA, $88 \pm 10\%$ of 17-hydroxyprogesterone/testosterone and $65 \pm 10\%$ of

Table VII. Studies on Non-Specific Interference (Blanks) In Steroid Radioimmunoassays

S T E R O I D A S S A Y E D							
	Estradiol	Proges- terone	5 α -DHT	DHA	Testosterone	17-hydroxy- progesterone	Androstenedione
Water or Buffer Blank (pg)	4.9	3.5	6.9	14.7	8.2	6.3	11.3
Standard Deviation	4.5	2.8	3.6	2.0	4.5	4.3	4.0

estradiol-17 β were recovered (n = 100).

The accuracy of these methods were studied as a final reliability evaluation of the multiple steroid radioimmunoassay techniques. The most effective means of establishing accuracy was to analyze human blood samples by these assay methods and compare the results to concentrations reported in literature for each steroid measured. Individual serum or plasma samples were obtained from normal adult male and female volunteers and were assayed following chromatographic separation of steroids with both the one and two column methods. The results of this study are represented in Table VIII.

Significant differences in levels of the potent androgens 5 α -dihydrotestosterone and testosterone were noted among men and women; (testosterone - males = 4.7 ± 0.7 ng/ml, females = $0.28 \pm .04$ ng/ml; 5 α -dihydrotestosterone - males = $.62 \pm .04$ ng/ml, females = 0.11 ± 0.2 ng/ml). Peripheral concentrations of the weaker androgens were equivalent in men and women: (DHA - men = 5.7 ± 1.1 ng/ml, women = 4.2 ± 0.4 ng/ml; androstenedione - men = 1.2 ± 0.3 ng/ml, females = 1.3 ± 0.3 ng/ml). Concentrations of 17-hydroxyprogesterone in men (1.6 ± 0.4 ng/ml) were not different from those found in women during the luteal phase of the menstrual cycle (1.3 ± 0.3 ng/ml); both were significantly higher than levels of this steroid in women during the follicular phase of the cycle (0.17 ± 0.05 ng/ml). Progesterone levels in women prior to ovulation were low (0.11 ± 0.02 ng/ml) as were levels in men (0.17 ± 0.04 ng/ml); serum levels of this compound in women during the luteal phase was much higher (5.4 ± 1.6 ng/ml). Plasma levels of estradiol-17 β in men (0.027 ± 0.012 ng/ml) were similar to that found during the early follicular phase of the menstrual cycle in women (0.041 ± 0.015 ng/

Table VIII. Peripheral Blood Steroid Levels in Normal Men and Women

STEROID CONCENTRATIONS (ng/ml)							
	Estradiol	Progesterone	17-hydroxyprogesterone	DHT	Testosterone	DHA	Androstenedione
<u>M A L E S</u>							
	.043	.136	---	.584	4.12	6.9	1.7
	.002	.077	1.2	.705	3.64	3.3	0.3
	.037	.253	1.9	.647	6.77	8.0	1.5
	----	.200	1.6	.535	4.26	4.5	1.2
MEAN \pm (S.E.)	.027 (.013)	.167 (.038)	1.55 (.35)	.618 (.037)	4.70 (.703)	5.68 (1.07)	1.18 (.31)
<u>F E M A L E S</u> (early follicular phase)							
4	.022	.065	.36	.071	1.262	4.0	.45
5	.015	.082	.10	.102	.226	2.9	.40
8	.097	.126	.14	.083	.216	3.9	1.60
10	.033	.081	.14	.023	.076	2.1	.50
11	.040	.190	.13	.073	.252	3.4	2.00
MEAN \pm (S.E.)	.041 (.014)	.109 (.023)	.17 (.05)	.070 (.013)	.206 (.034)	3.26 (.35)	0.99 (.33)
(luteal phase)							
16	.069	4.70	1.42	.211	.491	6.3	1.6
18	.068	6.89	1.73	.185	.360	6.3	1.5
18	.084	10.12	1.96	.094	.320	4.1	2.8
22	.052	4.93	1.11	.134	.331	4.9	1.0
26	.019	0.40	0.32	.118	.216	4.3	0.8
MEAN \pm (S.E.)	.058 (.011)	5.41 (1.58)	1.31 (.29)	.149 (.022)	.344 (.044)	5.18 (0.48)	1.54 (0.34)

ml) but were significantly lower than concentrations in women after ovulation and corpus luteum formation (0.058 ± 0.011 ng/ml). Since the above concentrations are quite similar to the cited levels reported in literature (Table IX), it was felt that the technique developed for multiple steroid radioimmunoassay were accurate in addition to being precise, sensitive and reproducible.

F. HORMONAL STUDIES IN THE HUMAN FEMALE WITH PHYSICAL AND BIOCHEMICAL SYMPTOMS INDICATIVE OF EXCESSIVE ANDROGEN PRODUCTION

The research presented in this dissertation was performed with a prime goal of gaining more insight into previously established androgen induced alterations in the reproductive axis of the female rat as well as those documented in the human female. Although many studies have been reported which dealt with concentrations of various hormones in human females having ovulatory failure associated with excessive androgen production, few investigators have quantified a full spectrum of sex steroids (e.g., androgens, progestins and estrogens) in such patients. To afford a more complete understanding of the reproductive disorders in these patients such studies were carried out. Serum levels of sex steroids in androgenized human females may then serve as an established basis to which studies in the DHA-treated female are compared.

Serum steroids were quantified by multiple steroid radioimmunoassay techniques in 13 untreated patients exhibiting symptoms commensurate with the diagnosis of polycystic ovarian disease. The results of this study are compared to serum steroid concentrations of normally cycling adult women in Figure 6. Concentrations of progesterone in the hirsute, polycystic ovarian disease patients were significantly higher ($p < 0.01$) than levels

Table IX. Peripheral Blood Steroid Concentrations in Normal Adult Men and Women as Reported in Current Scientific Literature

SUBJECT	STEROID CONCENTRATIONS AND (REFERENCES) ng/ml \pm S.D.			
	Testosterone	DHT	DHA	Androstenedione
Males	4.9 \pm 1.6 (1)	0.54 \pm .19 (5)	2.7 \pm .2 (8)	1.3 \pm .4 (2)
	6.7 \pm 2.3 (2)	0.53 \pm .11 (6)	5.1 \pm 2.9 (2)	1.2 \pm .4 (11)
	5.3 \pm 0.5 (3)	0.47 \pm .17 (7)	---	1.1 \pm .3 (6)
Females	0.22 \pm .07 (1)	0.15 \pm .05 (5)	2.5 \pm 0.7 (8)	1.8 \pm .6 (10)
	0.12 \pm .08 (4)	0.17 \pm .03 (6)	7.4 \pm 2.4 (9)	1.1 \pm .5 (4)
	0.17 \pm .38 (3)	0.16 \pm .05 (7)	5.0 \pm 0.9 (10)	1.4 \pm .3 (11)

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Table IX. Continued

SUBJECT	STEROID CONCENTRATIONS AND (REFERENCES) ng/ml \pm S.D.		
	17-hydroxyprogesterone	Estradiol-17 β	Progesterone
Males	0.9 \pm .3 (12)	.024 \pm .007 (15)	.23 \pm .06 (18)
	1.0 (13)	.028 \pm .009 (16)	.12 \pm .04 (19)
	1.2 \pm .6 (14)	---	.28 \pm .15 (20)
Females	.42 \pm .20 (12)	.065 \pm .014 (15)	.54 \pm .10 (18)
	F* .30 (13)	.069 \pm .056 (16)	.32 - .51 (19)
	.40 \pm .2 (14)	.037 \pm .010 (17)	.50 \pm .34 (20)
	1.7 \pm .5 (12)	.137 \pm .036 (15)	8.6 \pm 4.7 (18)
	L** 1.3 (13)	.099 \pm .054 (16)	5.8 - 10.3 (19)
	1.3 \pm .6 (14)	---	13.1 \pm 3.6 (20)

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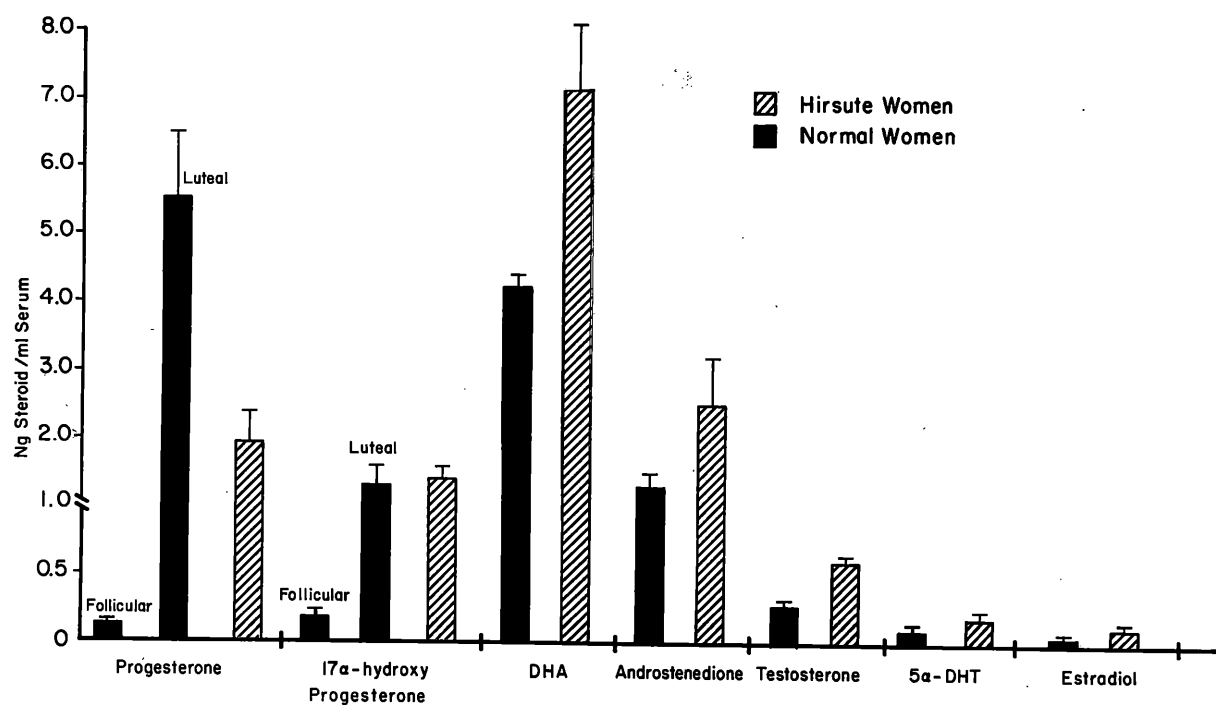
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F* Indicates samples obtained during the early follicular phase of the menstrual cycle (days 1-10).

L** Indicates samples obtained during the luteal phase of the menstrual cycle (days 16-28).

Figure 6. Peripheral Serum Levels of Steroid Hormones in Hirsute Women with Polycystic Ovarian Disease and in Normal, Adult Women

Peripheral blood samples were obtained between 8 and 12 AM from 10 healthy, normally menstruating women and 13 untreated hirsute patients in whom a diagnosis of polycystic ovarian disease had been made. Steroid concentrations were quantified by radioimmunoassay following extraction of 1.0 ml serum samples and isolation of steroid fractions using celite column chromatography techniques described earlier. Steroid concentrations are expressed as the mean \pm S.E. of 10 (normal women) and 13 (hirsute women) determinations.



of this steroid in normal women during the follicular phase of the menstrual cycle. Progesterone levels in such patients were, however not as great as levels found during the luteal phase in normal women. A similar significant difference ($p < 0.01$) was noted with respect to serum levels of 17-hydroxyprogesterone in hirsute women when compared to levels during the follicular phase of normal women. There was no difference between concentrations of 17-hydroxyprogesterone in hirsute or normal luteal phase females. When compared to average serum concentrations of androgens throughout the menstrual cycle in normal women, levels of 5α -dihydrotestosterone, testosterone, androstenedione, and DHA were significantly elevated ($p < 0.01$) in hirsute women. Estradiol- 17β concentrations, though elevated, were not significantly higher in hirsute women than normal cycling females.

In most of these hirsute patients, serum gonadotropins were low, at or below the sensitivity of both the human FSH and LH radioimmunoassays.

Similar studies were conducted in a more limited number of severely masculinized human females with ovarian tumors ($n = 2$) and congenital adrenal hyperplasia (CAH) ($n = 1$). Results obtained from these evaluations are shown in Table X. In the first patient, who was only 13 months old, the most notable finding was serum levels of testosterone which were higher than those found in most normal adult females. Concentrations of steroids in infants are largely undocumented, however androgen levels are generally quite low. Concentrations of all steroids were elevated in the second patient (18 months of age) who was diagnosed as having the 11β -hydroxylase deficiency form of congenital adrenal hyperplasia. Serum concentrations of progesterone, 17-hydroxyprogesterone, androstenedione, testosterone and 5α -dihydrotestosterone were significantly higher ($p < 0.01$) in this child than

Table X. Concentrations of Serum Steroids in Severely Masculinized Patients Having Ovarian Tumors or Congenital Adrenal Hyperplasia

PATIENT	DIAGNOSIS	TREATMENT	STEROID CONCENTRATIONS (ng/ml serum)						
			Estra- diol	Proges- terone	17-hydroxy- progesterone	DHA	A	Testos- terone	DHT
1.	Ovarian Tumor (unclassified)	none	-----	-----	0.7	0.1	0.3	0.30	---
2.	C.A.H.	none	.094	2.37	5.20	3.2	3.6	0.73	.154
3.	Ovarian Adrenal Rest Tumor	none	.034	3.9	3.6	5.7	8.2	3.0	.550
3.	Ovarian Adrenal Rest Tumor	Dexametha- sone	.032	3.1	3.5	2.0	10.0	3.5	.700
3.	Ovarian Adrenal Rest Tumor	1 day post- operative	.022	0.5*	0.9*	9.4	2.2*	0.7*	---
3.	Ovarian Adrenal Rest Tumor	4 days post- operative	.029	0.2*	0.6*	7.9	0.7*	0.5*	.272*

* Indicates a significant decrease in serum steroids in patient #3 following surgical extirpation of the ovarian tumor ($p < 0.01$).

serum levels of these hormones in an adult female. In the third patient, who had secondary amenorrhea of 15 months duration associated with an adrenal rest tumor (or lipoid cell) of the ovary, levels of 17-hydroxyprogesterone, androstenedione, testosterone and dihydrotestosterone were significantly higher ($p < 0.01$) than concentrations of these steroids in normal women. Progesterone concentrations were higher than levels during the follicular phase of normal women, but were not above the normal range of values for women in the luteal phase of the menstrual cycle. Serum levels of DHA were normal in this patient while estradiol concentrations were subnormal. No decrease in the markedly elevated steroids in this patient were detected during adrenal suppression (2 mg of dexamethasone/6 hours x 3 days). Following surgical removal of the ovarian tumor, serum levels of the elevated androgens as well as progesterone and 17-hydroxyprogesterone were decreased. Serum FSH and LH, which were tonically suppressed prior to surgery, increased in the immediate post-operative period and the patient resumed regular, ovulatory menstrual cycles within two months after surgery.

G. STUDIES DURING THE NATURAL ONSET OF PUBERTY IN FEMALE RATS

The onset of puberty in immature female rats is not a synchronized event and therefore does not occur at a specific age for all animals within an experimental group. Because of the normal heterogeneity which exists among experimental animals, discrete biochemical and morphological changes are difficult to document in relation to peripubertal development. Similar difficulties are also encountered when the estrous cycle of the adult female rat is studied although the problems are less profound. Any longitudinal study in untreated immature female rats which attempts to characterize the onset of natural puberty must, therefore, be viewed with normal varia-

tions in mind.

Rather than try to map out specific acute hormonal changes in a group of maturing female rats, this investigation was undertaken in order to determine if a pattern of change occurred. Blood steroid levels, cytosol estradiol receptors, ovarian and uterine weights of untreated female rats from 28-29 days of age were evaluated. Alterations in these experimental parameters were then related to the onset of puberty as evidenced by the occurrence of vaginal opening and ovulation in young, untreated female rats. In addition, the pattern of change, if any, could then be compared to that observed during the synchronized induction of puberty following PMSG or DHA plus PMSG treatment in immature female rats (Experiments 1-3, 5 and 7).

Ovulation, Vaginal Cytology

Ova were first detected in untreated female rats at 36 days of age ($n = 2/6$). Although evidence of ovulation (ova and/or corpora lutea) was found in most animals sacrificed on days 37-39, a few animals showed no evidence of impending or prior ovulation at these times. A similar asynchrony was noted with respect to vaginal opening; 80% of the rats sacrificed at 36 days of age had open vaginae (100% closed at 35 days of age) while some rats still had closed vaginae as late as 39 days of age. On each day of sacrifice, after vaginal patency, female rats had varied vaginal cytology ranging from pure estrus (all cells = cornified epithelial) to those in which a mixture of epithelial, leucocytes and nucleated cells were found.

Ovarian and Uterine Weights

Changes in ovarian and uterine weights in these control female rats (28-39 days of age) are shown in Table XI. No significant daily changes in ovarian weight were detected from 28-33 days of age. Significant in-

Table XI. Ovarian and Uterine Weights During Sexual Maturation in Untreated Female Rats¹

Age At Sacrifice	mg/100 g B.W. mean \pm S.E.	
	Ovarian Weight	Uterine Weight
28 AM	26.1 \pm 1.8	81.4 \pm 5.2
30 AM	28.9 \pm 1.2	93.8 \pm 9.6
32 AM	26.5 \pm 2.4	124.1 \pm 24.3
33 AM	25.5 \pm 1.4	114.4 \pm 23.3
35 AM	32.7 \pm 2.2*	148.7 \pm 16.8*
36 AM	39.1 \pm 2.1*	123.9 \pm 7.7
37 AM	30.5 \pm 2.1	128.0 \pm 14.5
39 AM	30.1 \pm 2.7	123.3 \pm 7.1

¹ Indicates n = 6 animals/day.

* Indicates organ weights are significantly higher ($p < 0.01$) than those prior to 32 days of age.

creases ($p < 0.01$) did occur at 35 and 36 days of age; ovarian weights were however reduced on days 37 and 38. A significant increase ($p < 0.01$) in uterine weight occurred at 35 days of age, one day before the first ovulations were detected. No statistically significant daily changes in uterine weight were noted during the interval of 35-39 days of age. Uteri of these animals were, however significantly heavier ($p < 0.01$) than those sacrificed prior to 32 days of age.

Serum Steroid Concentrations

Peripheral concentrations of steroid hormones in these animals are shown in Table XII. Three serum pools per day (two animals/pool) were assayed by multiple steroid radioimmunoassay techniques described earlier. No significant alterations in any steroid was noted prior to 33 days of age. Estradiol concentrations increased at 33 days of age and then increased further at 35 days of age, levels at both times being significantly higher than that observed at 32 days of age ($p < 0.01$). The increased serum concentrations of estradiol which occurred at 33 and 35 days of age coincided well with the increment in uterine weight observed at 35 days of age (Table XII). No further increase in serum estradiol occurred from 35 to 37 days of age. Progesterone levels ranged from 1.8 to 4.7 ng/ml during the period of 28-33 days of age; no statistically significant differences were noted from day to day at this time. At 35 and 36 days of age, significant increases in progesterone concentrations were noted ($p < 0.01$ as compared to levels at 32 days of age) which coincided with the initial increases in ovarian weight at 35 days of age and presence of ovarian corpora lutea by 36 days of age. Serum levels of 17-hydroxyprogesterone were also fairly tonic from 28 to 33 days of age and then increased at 35 and 36 days of

Table XII. Serum Concentrations of Steroid Hormones Before and During the Onset of Puberty in Untreated Female Rats¹

Age at Sacrifice	Steroid Concentrations (ng/ml) mean \pm S.E.						
	Estradiol	Progesterone	17-hydroxy- progesterone	Testos- terone	Androstene- dione	DHT	DHA
28 AM	.012 \pm .007	4.2 \pm 2.5	.76 \pm .23	.051 \pm .003	.18 \pm .04	.050 \pm .024	1.86 \pm .81
30 AM	.018 \pm .009	2.6 \pm 1.5	.40 \pm .07	.144 \pm .053	.24 \pm .05	.029 \pm .006	1.06 \pm .25
31 AM	.019 \pm .002	3.6 \pm 0.8	.59 \pm .14	.111 \pm .038	.22 \pm .07	.075 \pm .014	
32 AM	.009 \pm .006	1.8 \pm 0.8	.84 \pm .52	.052 \pm .004	.17 \pm .02	.037 \pm .009	1.22 \pm .85
33 AM	.025 \pm .003*	4.7 \pm 1.0	.60 \pm .26	.024 \pm .024	.24 \pm .07	.044 \pm .010	0.86 \pm .19
35 AM	.037 \pm .018*	10.9 \pm 3.3*	1.03 \pm .004	.259 \pm .184*	.37 \pm .09	.058 \pm .003	1.03 \pm .73
36 AM	.038 \pm .001*	22.2 \pm 0.3*	2.20 \pm .56*	.240 \pm .118*	.79 \pm .31*	.073 \pm .026	1.38 \pm 1.01
37 AM	.044 \pm .016*	11.1 \pm 1.2*	1.16 \pm .29	.135 \pm .052	.77 \pm .28*	.103 \pm .018*	1.73 \pm 1.42
39 AM	.027 \pm .009*	14.8 \pm 1.5*	1.66 \pm .29	.082 \pm .052	.88 \pm .09*	.091 \pm .021	0.66 \pm .07

¹ Three 1.0 ml serum pools, composed of sera of two rats each, were assayed in duplicate for each sacrifice.

* Indicates that steroid concentrations are significantly higher ($p < 0.01$) than concentrations at 32 days of age.

age. Serum levels of progesterone and 17-hydroxyprogesterone were lower at 37 and 39 days of age than day 36 but were still higher than preovulatory concentrations at 33 days of age.

Peripheral concentrations of testosterone and androstenedione were similarly low at 28-33 days of age but serum levels of both steroids increased at 35 and 36 days of age. Testosterone levels then declined at 37 and 39 days of age but androstenedione levels remained significantly higher ($p < 0.01$) than concentrations at 33 days of age or earlier. Serum dihydrotestosterone (DHT) levels were highest from 36-39 days of age; no significant daily changes in serum concentrations were noted with respect to this compound. DHA levels in female rats from 28-39 days of age did not appear to fluctuate a great deal and due to the rather large daily variation, no statistically significant changes were established. In general, levels of these steroid hormones were higher from 35-39 days of age (when ovulations and ovarian corpora lutea were apparent) than corresponding concentrations in the yet immature female rats sacrificed from 28-33 days of age.

Cytosol Estradiol Receptors

Cytosol estradiol receptor concentrations of the anterior pituitary and hypothalamus are depicted in Table XIII. The binding capacity of both tissues was found to be high and constant at 28, 30 and 32 days of age. A significant decrease ($p < 0.01$) in receptor content of both the anterior pituitary and hypothalamus occurred at 33 days of age, when estradiol levels in serum had increased. Receptor levels of both tissues then remained at a lower level from 35-39 days of age. Since the day of estrus did not occur on a specific day for all animals, a dramatic depletion-replenishment cycle

Table XIII. Cytosol Estradiol Receptor Levels of the Anterior Pituitary and Hypothalamus During Sexual Maturation in Untreated Female Rats¹

Age at Sacrifice	Specific Bound moles ³ H-estradiol/mg cytosol protein mean \pm S.E.	
	Pituitary $\times 10^{-14}$	Hypothalamus $\times 10^{-15}$
28 AM	15.1 \pm 0.2	8.1 \pm 0.3
30 AM	16.1 \pm 2.5	8.8 \pm 0.2
32 AM	12.0 \pm 0.9	8.3 \pm 0.7
33 AM	8.7 \pm 0.8*	5.8 \pm 0.2*
35 AM	7.5 \pm 0.1*	6.3 \pm 0.2*
36 AM	7.0 \pm 0.4*	5.8 \pm 0.1*
37 AM	9.5 \pm 0.8*	6.1 \pm 0.2*
39 AM	7.4 \pm 0.6*	5.4 \pm 0.1*

¹ Two different cytosol aliquots, in duplicate, were assayed for each sacrifice.

* Indicates that receptor levels are significantly lower ($p < 0.01$) than that of animals sacrificed at 32 days of age or earlier.

in receptor levels associated with ovulation was not detected in these studies.

H. EFFECTS OF PMSG TREATMENT IN THE IMMATURE FEMALE RAT

The normal onset of puberty in the female rat does not occur at a precise age but rather may occur over a range of days. Because of this imprecision, significant changes related to ovulation in the pubertal rat are difficult to document. The PMSG-primed immature female was thus studied in detail in order to clearly define changes in blood steroids, gonadotropins and cytosol estradiol receptors which are associated with the synchronized induction of precocious ovulation. Data obtained in this investigation would serve as a basis for comparison to the precocious puberty associated with the administration of DHA.

Ovulation, Vaginal Cytology

The injection of 8 IU PMSG to 30 day old female rats resulted in precocious ovulation on day 33 of life as evidenced by the presence of oviductal ova and ovarian corpora lutea. In the three experiments in which PMSG was administered, no ova were found as late as 6 PM on day 32 of life. Furthermore, none of the control (vehicle-treated) animals showed any evidence of ovarian or uterine stimulation prior to day 35. The number of oviductal ova detected (after PMSG) were fairly consistent among these experiments: Experiment #2 - 8 ova per animal; Experiment #5 - 6.6 ova per animal; Experiment #7 - 11 ova per animal. In addition, the majority of PMSG-treated rats had open vaginae on the morning of day 33 (only one rat had vaginal opening prior to this time); all displayed typical estrus type vaginal smears consisting primarily of cornified epithelial cells.

Following estrus at day 33, the PMSG-treated rats apparently began

cycling regularly. Vaginal smears taken on days 34 and 35 of life were usually composed of leucocytes, indicating vaginal diestrus. Nucleated cells were the principle cell type found on day 36, indicating that the rats were in the proestrus stage of the estrous cycle. Day 37 was characterized mainly by estrus type smears again (4 days after the initial ovulation). Ova were however not found in all animals sacrificed at this time. As on days 34 and 35, the majority of rats again had diestrus type smears on days 38 and 39 of life.

Ovarian Weights

The effects of PMSG administration on ovarian weight are summarized in Table XIV. A consistent finding in these experiments was that ovarian weight increased significantly ($p < 0.01$) by the AM of day 31, 24 hours after PMSG treatment but did not increase further until the PM of day 32. The highest weights were usually recorded on days 33 (estrus) and 34. In the one group which was studied through a second estrus cycle, a second increase in ovarian weight was noted on day 37 (estrus).

Uterine Weights

Changes in uterine weight after PMSG therapy are shown in Table XV. Uterine weights were elevated within 8 hours after PMSG administration however significant increases were not noted until the AM of day 31. Uterine weights rose progressively following PMSG treatment and reached peak values on the morning of day 32. On the day of estrus (33) uterine weights decreased and were lowered further on day 34. A second increase in uterine weight was noted on day 36 (proestrus) followed by a decrease on day 37 (estrus). Uterine and ovarian weights of control animals were not significantly higher on day 33 than in the PMSG-treated rats on the morning of

Table XIV. Changes in Ovarian Weight¹ Following Treatment of Immature Female Rats With 8 IU PMSG or Saline at 10 Am, 30 Days of Age

Age at Sacrifice	Ovarian Weights (mg/100 g B.W) Group Mean \pm S.E.			
	Experiment #2	Experiment #5	Experiment #7	Experiment #7 (Controls) ²
30 AM ²	25.9 \pm 0.7	26.9 \pm 1.9		27.2 \pm 1.3
30 PM	34.5 \pm 1.8*		32.3 \pm 2.6*	25.0 \pm 0.5
31 AM	42.1 \pm 3.3*		40.6 \pm 2.2*	31.4 \pm 1.0
31 PM			40.4 \pm 2.4*	26.8 \pm 0.9
32 AM	41.8 \pm 2.2*		39.3 \pm 3.1*	
32 PM	49.2 \pm 3.6*	32.9 \pm 2.8*	52.2 \pm 3.9*	31.6 \pm 1.0
33 AM	53.6 \pm 2.9*		56.3 \pm 4.7*	26.3 \pm 2.0
33 PM		50.7 \pm 2.2*		
34 AM	56.2 \pm 3.6*			
35 AM	51.5 \pm 2.3*			
36 AM	52.9 \pm 4.8*			
37 AM	54.9 \pm 2.6			
38 AM	43.6 \pm 3.4*			

¹ n = 6-7 animals per experimental group for each sacrifice.

² Ovaries were obtained from saline-treated rats.

* Ovarian weights of PMSG-primed rats are significantly higher than vehicle-treated controls ($p < 0.01$) within the same experiment.

Table XV. Changes in Uterine Weight¹ Following Treatment of Immature Female Rats with 8 IU PMSG or Saline at 10 AM, 30 Days of Age

Age at Sacrifice	Uterine Weights (mg/100 g B.W.) Group Mean \pm S.E.			
	Experiment #2	Experiment #5	Experiment #7	Experiment #7 (Controls) ²
30 AM ²	81.8 \pm 10.9	56.9 \pm 3.9	71.7 \pm 3.9	---
30 PM	103.6 \pm 10.8		91.9 \pm 18.1*	78.5 \pm 5.9
31 AM	110.1 \pm 11.9*		137.8 \pm 7.9*	78.4 \pm 5.4
31 PM			148.2 \pm 15.9*	91.9 \pm 8.3
32 AM	197.2 \pm 8.3*		193.2 \pm 18.4*	
32 PM	212.9 \pm 10.1*	200.6 \pm 7.0*	212.0 \pm 8.3*	78.0 \pm 6.5
33 AM	160.9 \pm 13.7*		169.9 \pm 11.6*	73.9 \pm 10.7
33 PM		189.9 \pm 9.2*		
34 AM	123.9 \pm 8.9*			
35 AM	130.9 \pm 4.7*			
36 AM	174.3 \pm 8.1*			
37 AM	144.9 \pm 13.6*			
38 AM	125.1 \pm 5.4*			

¹ n = 6-7 animals per experimental group for each sacrifice.

² Uteri were obtained from saline-treated control rats.

* Uterine weights of these PMSG-primed rats are significantly ($p < 0.01$) higher than saline-treated controls within the same experiment.

day 30.

Blood Steroids

The administration of 8 IU PMSG to 30 day old female rats caused significant changes in concentrations of all blood steroids measured. Estradiol-17 β levels (Fig. 7) rose steadily following PMSG and reached peak values on the AM of day 32 which were significantly ($p < 0.01$) higher than pre-injection levels or concentrations found in vehicle-treated controls. By 6 PM (day 32) estradiol-17 β levels had begun to fall and were significantly depressed ($p < 0.01$ as compared to concentrations on previous days) on the morning of day 33 (estrus).

After PMSG administration, serum levels of 5 α -dihydrotestosterone (Fig. 7) increased slightly through day 31 but fell on the AM of day 32. 5 α -dihydrotestosterone levels were then increased on the PM of day 32 and declined on the morning of day 33. After PMSG treatment serum concentrations of progesterone and 17-hydroxyprogesterone (Fig. 8) were elevated from the PM of day 30-31 but fell on the AM of day 32. A significant increase ($p < 0.01$) in both steroids was noted at 6 PM on day 32. Levels of these steroids then declined on the morning of day 33 and only progesterone was increased significantly ($p < 0.01$) on day 34 (diestrus). Concentrations of 17-hydroxyprogesterone and progesterone in vehicle-treated rats showed no significant changes from day 30-34.

Changes in concentrations of dehydroepiandrosterone, testosterone and androstenedione following 8 IU PMSG or saline injections are depicted in Figure 9. Each rose, after PMSG, on the next morning (day 31). At 6 PM on day 32, a second significant increase in DHA and androstenedione was noted in the PMSG-primed rats; testosterone also increased but the change

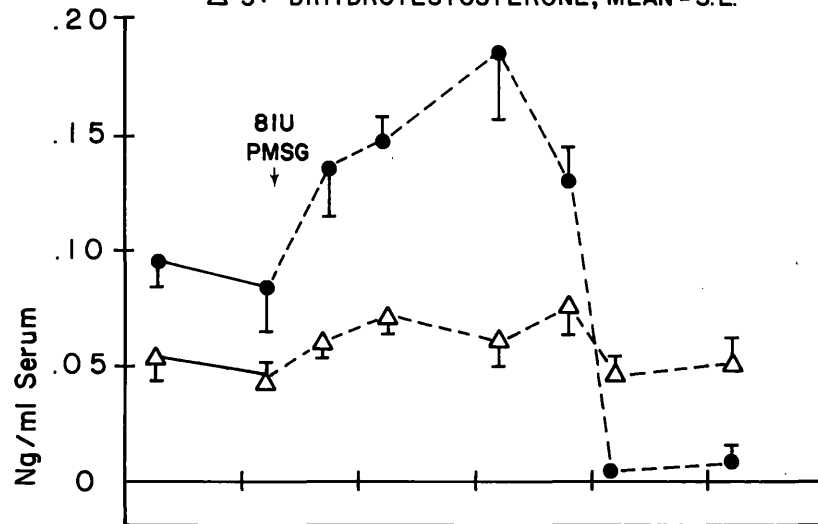
Figure 7. Peripheral Serum Concentrations of Estradiol-17 β and 5 α -dihydro-
testosterone in the PMSG-Primed Immature Female Rat.

Female rats were bled by cardiac puncture under ether anesthesia as a terminal procedure at the indicated times from 29 to 34 days of age. 8 IU PMSG in 0.5 ml saline or vehicle (controls) was administered S.C. at 30 days of age (10 AM) to all animals. Steroids were quantified by radioimmunoassay following extraction and chromatography using the modified celite column system A. All points represent the mean steroid concentrations (\pm S.E.) obtained from 6 to 7 individual serum samples (1.0 ml each) per experimental group for each sacrifice.

PMSG TREATED FEMALE RATS

● ESTRADIOL-17 β , MEAN \pm S.E.

△ 5 α - DIHYDROTESTOSTERONE, MEAN \pm S.E.



VEHICLE TREATED FEMALE RATS

SALINE

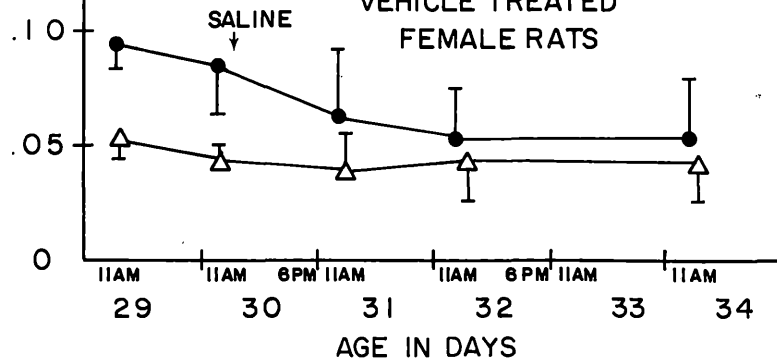


Figure 8. Peripheral Serum Concentrations of Progesterone and 17-hydroxy-
progesterone in the PMSG-Primed Immature Female Rat.

For details, see Figure 7.

PMSG TREATED FEMALE RATS

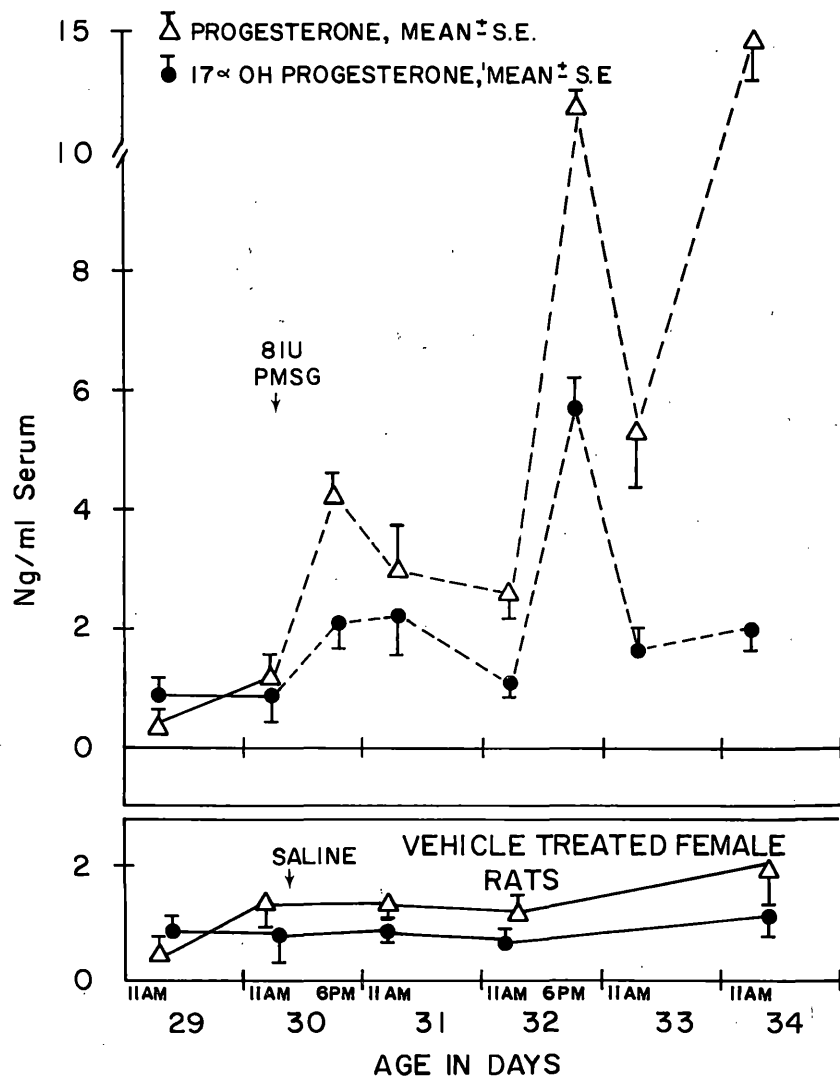
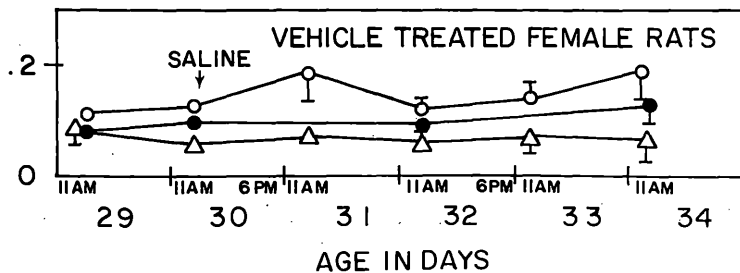
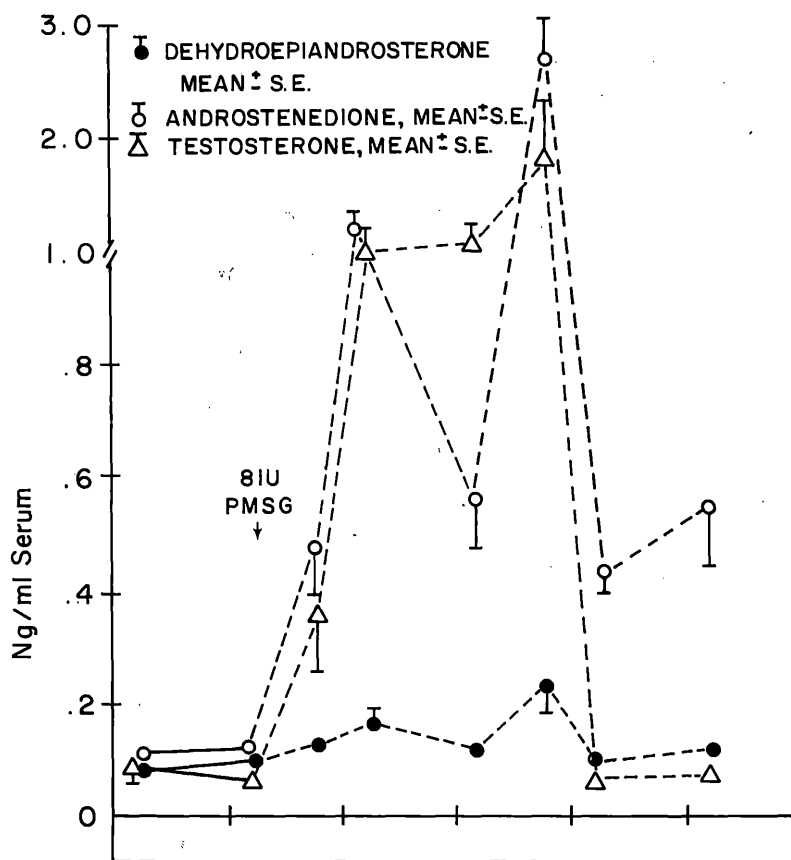


Figure 9. Peripheral Serum Concentrations of Androstenedione, Dehydroepian-
drosterone and Testosterone in the PMSG-Primed Immature Female
Rat

For details, see Figure 7.

PMSG TREATED FEMALE RAT



was not significant.

A significant decrease in all three compounds occurred on the AM of day 33; none increased significantly on day 34 although androstenedione levels were still significantly higher than that prior to PMSG. Concentrations of DHA and testosterone (day 33 and 34) were not different from those levels found in saline-treated controls.

Serum concentrations of these seven steroids were also studied from day 35-39 in the PMSG-treated rats during which a second estrus had been detected. A great deal of variation in vaginal cytology, ovarian and uterine weight was found among the individual animals whose serum was analyzed and significant changes were difficult to establish for any particular day. The failure to obtain samples in the PM, especially on day 36, may be partially responsible for this. A significant ($p < 0.01$) increase in serum estradiol-17 β was noted, however, on the AM of day 36 ($.037 \pm .011$ ng/ml), (proestrus) as compared to the AM of day 35 ($.012 \pm .005$ ng/ml) or day 37 ($.013 \pm .005$ ng/ml).

Serum Gonadotropins

Serum levels of gonadotropins, FSH and LH, were also quantified in female rats after the injection of 8 IU PMSG or saline (controls). The results of these analyses are shown in Table XVI. In the PMSG-primed rats, significant ($p < 0.01$) increases in serum levels of FSH and LH were noted on the afternoon of day 32, 53-57 hours after treatment. No such increases in gonadotropin concentrations were detected in vehicle-treated controls. Serum LH and FSH decreased on estrus (day 33) to levels not significantly different from those of saline-treated controls.

Cytosol Estradiol Receptors

Table XVI. Serum Concentrations of FSH and LH in Female Rats¹ Following Treatment with 8 IU PMSG or Saline at 10 AM, 30 Days of Age

Age at Sacrifice	ng/ml of Serum, Group Mean \pm S.E.			
	LH		FSH	
	PMSG	SALINE	PMSG	SALINE
30, 11 AM	36.9 \pm 9.1		28.5 \pm 2.7	
32, 11 AM		31.2 \pm 13.5		46.5 \pm 7.8
32, 2 PM	349.5 \pm 37.9*		86.5 \pm 16.9*	
32, 6 PM	459.3 \pm 10.0*		132.1 \pm 28.9*	
33, 2 PM	47.0 \pm 2.9		32.1 \pm 6.2	

¹ n = 7 animals per sacrifice.

* Serum FSH and LH in these PMSG-primed rats are significantly higher (p < 0.01) than levels on day 30 or in saline-treated controls.

Changes brought about in anterior pituitary cytoplasmic estradiol-17 β receptor concentrations by PMSG are summarized in Table XVII. Estradiol-17 β receptor content of the pituitary was decreased as early as 8 hours after PMSG treatment in intact female rats. In female rats castrated on the morning of day 30, the injection of PMSG (Exp. #7) did not cause a decrease at 6 PM on day 30, in estradiol receptor levels of the anterior pituitary gland ($9.2 \pm 0.4 \times 10^{-14}$ moles ^3H -estradiol-17 β bound/mg cytosol protein). The depletion of cytosol estradiol-17 β binding capacity continued on day 31 of age and reached a nadir on the PM of day 32. On day 33 (estrus) a significant increase ($p < 0.01$) consistently occurred in levels of cytosol receptors. This replenishment of receptor binding capacity continued on the days of diestrus (34,35) and another depletion was noted on day 36 (proestrus). Receptor levels of the anterior pituitary were again replenished on estrus (day 37).

As shown in Table XVIII, hypothalamic cytosol estradiol receptor concentrations were also decreased following PMSG treatment of intact female rats. Significant differences between PMSG and saline-treated controls were noted by the PM of day 30. PMSG treatment of ovariectomized rats (Exp. #7) had no effect on cytosol receptor concentration ($9.6 \pm 0.2 \times 10^{-15}$ moles ^3H -estradiol-17 β bound/mg cytosol protein). Progressive decreases in receptor binding capacity in PMSG-primed rats were found in day 31, with lowest levels being obtained on day 32. Replenishment of hypothalamic estradiol-17 β receptors occurred on day 33 (estrus). No significant fluctuations in receptor levels of vehicle-treated controls were detected in these studies.

Table XVII. Cytosol Estradiol-17 β Receptor Levels in the Anterior Pituitary Gland¹ Following PMSG (8 IU) or Saline Treatment at 10 AM, 30 Days of Age

Age at Sacrifice	Specific bound moles ³ H-estradiol-17 β /mg cytosol protein Group Mean \pm S.E. $\times 10^{-14}$			
	Experiment #2	Experiment #5	Experiment #7	Experiment #7 (Controls)
30 AM ²	8.2 \pm 0.3	10.5 \pm 1.9		
30 PM	5.8 \pm 0.5*		6.21 \pm 0.3*	8.3 \pm 0.2
31 AM	5.5 \pm 0.4*			8.7 \pm 0.3
31 PM			4.5 \pm 0.7*	10.4 \pm 0.1
32 AM	2.3 \pm 0.2*		3.4 \pm 0.4*	9.0 \pm 0.5
32 PM	1.9 \pm 0.1*	5.9 \pm 0.2*	1.3 \pm 0.1*	12.6 \pm 0.5
33 AM	6.5 \pm 0.1*		8.9 \pm 0.3*	11.4 \pm 1.1
33 PM		10.5 \pm 0.2		
34 AM	7.2 \pm 0.1*			
35 AM	8.2 \pm 0.7			
36 AM	3.3 \pm 0.1*			
37 AM	5.0 \pm 0.1*			
38 AM	6.3 \pm 0.4*			
39 AM	8.0 \pm 0.1			

¹ Two different cytosol aliquots, in duplicate, were assayed per experimental group for each sacrifice.

² Receptor concentrations were quantified in pituitary cytosols obtained from saline-treated control rats.

* Receptor concentrations in these PMSG-primed rats are significantly lower ($p < 0.01$) than saline-treated controls within the same experiment.

Table XVIII. Cytosol Estradiol-17 β Receptor Levels in the Hypothalamus¹
Following PMSG (8 IU) or Saline Treatment at 10 AM, 30
Days of Age

Age at Sacrifice	Specific bound moles ³ H-estradiol-17 β /mg cytosol protein Group Mean \pm S.E. $\times 10^{-15}$		
	Experiment #5	Experiment #7	Experiment #7 (Controls) ²
30 AM ²	9.2 \pm 0.4	10.1 \pm 0.3	
30 PM		7.4 \pm 0.5*	9.0 \pm 0.7
31 AM		5.9 \pm 0.1*	
31 PM		4.5 \pm 0.2*	10.1 \pm 0.5
32 AM		2.6 \pm 0.2*	9.4 \pm 0.2
32 PM	4.6 \pm 0.2*	1.9 \pm 0.1*	9.9 \pm 0.1
33 AM		9.6 \pm 0.8	10.4 \pm 0.5
33 PM	8.7 \pm 0.4		

¹ Two different cytosol aliquots, in duplicate, were assayed per experimental group for each sacrifice.

² Receptor concentrations were quantified in hypothalamic cytosols obtained from saline-treated control rats.

* Receptor concentrations in these PMSG-primed rats are significantly lower (p < 0.01) than saline-treated control values within the same experiment.

I. EFFECTS OF SHORT-TERM DHA TREATMENT ON THE HYPOTHALAMIC-PITUITARY-OVARIAN AXIS OF THE IMMATURE FEMALE RAT.

It has been previously established that DHA treatment can induce the early onset of puberty in the immature female rat (Black and Mahesh, 1969). In an effort to more clearly determine the hormonal changes that are associated with DHA treatment and precocious ovulation, immature female rats were studied during and after treatment with DHA (60 mg/kg BW/day, age 27-29). In two experiments, PMSG was administered to DHA-treated rats on the AM of day 20; PMSG treatment was omitted in experiment #6 (chronic DHA administration). Vehicle-treated female rats were also evaluated during the interval of 27-31 days of age.

Ovulations and Vaginal Cytology

It was found that ovulation could be induced on day 30 in female rats treated with DHA plus PMSG. The number of oviductal ova varied from 3-10 in individual animals; of those rats treated with DHA plus PMSG, 100% were found to have ova and/or corpora lutea by the morning of day 31. Ovulation was found to occur as early as day 30 or as late as day 32 in animals treated with DHA only; the number of ova found at sacrifice were similar to that described earlier. Vaginal patency occurred by day 31 in more than 90% of the DHA-treated female rats (all experiments). No vehicle-treated control animal was ever found to have an open vagina prior to 35 days of age.

Ovarian Weight

Changes in ovarian weight in the DHA and vehicle-treated female rat are presented in Table XIX. Ovarian weight in the groups treated with DHA plus PMSG did not change appreciably from the morning of day 27 until the morning of day 30. Significant increases ($p < 0.01$) were noticed in DHA-

Table XIX. Changes in Ovarian Weight During Short-Term Treatment of Immature Female Rats With DHA

Age at Sacrifice	Ovarian Weight, mg/100 g B.W. mean \pm S.E.				
	DHA plus PMSG ¹	Control ²	DHA ³	Control ³	
27 AM	30.3 \pm 1.3	29.2 \pm 1.9	29.3 \pm 0.8	31.2 \pm 1.7	25.3 \pm 1.4
28 AM		31.8 \pm 2.7			
29 AM	28.5 \pm 1.1	32.1 \pm 1.6	30.8 \pm 0.7	34.1 \pm 1.6	28.5 \pm 1.7
29 PM		32.2 \pm 2.0			
30 AM	30.7 \pm 1.4	39.8 \pm 2.5*	27.4 \pm 0.5	31.3 \pm 2.9	27.0 \pm 1.2
30 PM	38.4 \pm 3.2*	39.4 \pm 2.6*	28.4 \pm 0.9		
31 AM	39.7 \pm 2.7*	59.8 \pm 4.9*	27.3 \pm 1.1	42.5 \pm 3.0*	31.2 \pm 1.2

¹ Rats were administered DHA (60 mg/kg BW/day) in single daily injections (S.C.) at 8:30-9:00 AM at 27-29 days of age. 8 IU PMSG was administered (S.C.) at 10 AM on day 30 of life; n = 6 animals per sacrifice.

² Controls were injected with 0.2 ml propylene glycol (vehicle for DHA) and 0.5 mg saline (vehicle for PMSG) as in ¹ above; n = 6 animals per sacrifice.

³ Rats were treated chronically with DHA (60 mg/kg BW/day) beginning at 27 days of life; controls received daily propylene glycol injections (0.2 ml); n = 6 animals per sacrifice.

* Ovarian weights are significantly higher ($p < 0.01$) than those obtained at 27 days of life (DHA-treated or controls) and corresponding controls of the same age.

treated rats on the morning of day 30 in only one experiment; other groups treated with DHA plus PMSG or DHA alone increased significantly on the PM of day 30 or the AM of day 31, respectively. These dramatic increases in ovarian weight were usually associated with the presence of hemorrhagic follicles or corpora lutea. Ovaries of vehicle-treated immature female rats were consistently small and devoid of such morphological evidence of gonadotropin stimulation. No significant increases in ovarian weight were noted from age 27 to age 31 days in these control groups.

Uterine Weight

Uterine weights of DHA-treated rats (Table XX) were elevated as early as 2½ hours after the first injections on day 27. A progressive increase in uterine weight occurred from day 27 to day 29 in DHA-treated rats. Marked accumulation of luminal fluid was apparent by the morning of day 29 in these groups. Control uterine weights were fairly constant and were significantly lower ($p < 0.01$) than those of DHA-treated rats (ages 27-29). From the morning of day 29 to the morning of day 31, uterine weights in DHA-treated rats remained elevated ($p < 0.01$) and were virtually unchanged during this time. The weights on days 27-31 were quite consistent from experiment to experiment in both vehicle-treated controls and androgen treated groups. It may be noted here that the uterine weights of the DHA-treated animals in these studies on day 29 (Table XX) were almost identical to those achieved in PMSG-treated rats on day 32 (Table XV) before ovulation.

Serum Steroid Concentrations

The alterations in peripheral serum steroid concentrations resulting from short-term DHA treatment are compared to levels in vehicle-treated controls in Tables XXI and XXII. Within two hours after DHA administration,

Table XX. Changes in Uterine Weights During Treatment of Immature Female Rats With DHA Plus PMSG¹ or Vehicle²

Age at Sacrifice	Uterine Weight, mg/100 g BW mean \pm S.E.				
	DHA plus PMSG ¹		Control ²	DHA ³	Control ³
27 AM	71.8 \pm 4.6*	78.1 \pm 6.5*	51.9 \pm 3.8	82.1 \pm 2.9**	54.3 \pm 3.3
28 AM	102.9 \pm 4.4**	149.1 \pm 12.3**	53.1 \pm 2.9		
29 AM	197.6 \pm 10.5**	206.9 \pm 14.3**	81.3 \pm 7.8	209.4 \pm 8.4**	77.0 \pm 4.6
29 PM		203.2 \pm 7.9**			
30 AM	196.2 \pm 10.7**	204.2 \pm 12.5**	78.8 \pm 7.9	226.0 \pm 7.0**	75.0 \pm 4.0
30 PM	255.9 \pm 8.3**	195.1 \pm 9.9**	78.6 \pm 6.4		
31 AM	212.9 \pm 20.2**	215.0 \pm 10.0**	82.5 \pm 7.0	240.0 \pm 17.0**	78.0 \pm 5.1

¹ See Table XIX, 1; n = 6 animals per sacrifice.

² See Table XIX, 2; n = 6 animals per sacrifice.

³ See Table XIX, 3; n = 6 animals per sacrifice.

* Uterine weights are significantly higher ($p < 0.05$) than controls.

** Uterine weights are significantly higher ($p < 0.01$) than controls.

concentrations of testosterone, androstenedione, DHT and DHA were significantly increased ($p < 0.01$) over serum levels in controls (Table XXI). These elevations were maintained at fairly constant concentrations from day 27-29 during DHA therapy. It appeared that the markedly elevated concentrations of androstenedione, testosterone and DHT were the result of peripheral metabolism of the injected steroid, DHA. On the AM of day 30, (24 hours after the last injection of DHA) levels of these steroids had fallen by approximately 75%, but were still significantly higher than concentrations in vehicle-treated controls. The decline in serum testosterone and androstenedione levels was checked following PMSG administration at 10 AM on day 30, whereas DHA and DHT concentrations continued to fall throughout day 30. No significant changes in serum levels of these steroids were detected in vehicle-treated controls from day 27-31.

Changes in serum estradiol, progesterone and 17-hydroxyprogesterone following DHA treatment (or vehicle) are shown in Table XXII. Serum estradiol was significantly elevated ($p < 0.01$) for the three days of DHA treatment and then declined by 50% on the morning of day 30. Concentrations on the AM of estrus were however still significantly higher than levels found in controls. Following PMSG administration, estradiol levels were maintained at concentrations which increased slightly at 6 PM, day 30 and 11 AM, day 31. Progesterone levels were elevated over controls on day 29 but fell on the morning of estrus (day 30) in DHA-treated rats. Following PMSG treatment, progesterone levels were again significantly elevated over vehicle-treated controls on the morning of day 31, coinciding with the appearance of ovarian corpora lutea. 17-hydroxyprogesterone concentrations were also increased on day 29 following DHA treatment. Serum levels of

Table XXI. Effects of Short-Term DHA¹ or Vehicle² Injections on Peripheral Serum Androgen Levels in Immature Female Rats

Age at Sacrifice	Steroid Concentrations (ng/ml serum) mean \pm S.E.							
	Testosterone		Dehydroepiandrosterone		Androstenedione		Dihydrotestosterone	
	DHA	Vehicle	DHA	Vehicle	DHA	Vehicle	DHA	Vehicle
27 AM	4.01 \pm .01*	.056 \pm .004	95.63 \pm 18.61*	.270 \pm .020	4.14 \pm .08*	.59 \pm .18	.921 \pm .148*	.057 \pm .034
28 AM	3.84 \pm .17*	.051 \pm .003	68.79 \pm 2.72*	.428 \pm .199	3.81 \pm .41*	.150 \pm .069	.623 \pm .049*	.039 \pm .019
29 AM	4.91 \pm .24	.065 \pm .018	98.11 \pm 5.74*		3.87 \pm .14*	.268 \pm .067	.589 \pm .056*	.044 \pm .020
30 AM	1.42 \pm .62*	.056 \pm .014	26.17 \pm 5.16*	.155 \pm .061	0.74 \pm .12*	.292 \pm .074	.159 \pm .017*	.041 \pm .016
30, 2 PM	.99 \pm .21*		15.72 \pm 2.73*		1.06 \pm .08*		.097 \pm .019	
30, 6 PM	1.38 \pm .15*	.052 \pm .029	10.17 \pm 1.54*	.156 \pm .052	1.04 \pm .16*	.191 \pm .049	.061 \pm .049	.036 \pm .009
31 AM	.69 \pm .12	.114 \pm .041	6.58 \pm 2.31*	.225 \pm .090	0.75 \pm .13*	.187 \pm .050	.030 \pm .030	.040 \pm .015

¹ See Table XIX, 1; n = 6 animals per sacrifice.

² See Table XIX, 2; n = 6 animals per sacrifice.

* Indicates significant differences between DHA and vehicle-treated controls at $p < 0.01$.

Table XXII. Effects of Short-Term DHA¹ or Vehicle² Treatment on Peripheral Serum Levels of Estradiol, Progesterone and 17-hydroxyprogesterone in Immature Female Rats

Age at Sacrifice	Steroid Concentrations (ng/ml serum) mean \pm S.E.					
	Estradiol		Progesterone		17-hydroxyprogesterone	
	DHA	Vehicle	DHA	Vehicle	DHA	Vehicle
27 AM	.252 \pm .063*	.035 \pm .022	2.45 \pm 1.20	2.09 \pm 1.31	.796 \pm .045	.805 \pm .220
28 AM	.208 \pm .051*		3.05 \pm 0.95	1.00 \pm 0.66	.745 \pm .105	.843 \pm .275
29 AM	.222 \pm .022*	.034 \pm .013	7.72 \pm 0.77*	4.19 \pm 1.63	1.529 \pm .159*	.936 \pm .154
30 AM	.109 \pm .018*	.020 \pm .009	1.44 \pm 0.78	5.18 \pm 1.23	1.159 \pm .412	1.432 \pm .295
30, 2 PM	.100 \pm .023*		9.72 \pm 2.71*		2.340 \pm .538*	
30, 6 PM	.120 \pm .032*	.004 \pm .003	5.37 \pm 0.97	3.57 \pm 1.03	2.082 \pm .486*	.817 \pm .195
31 AM	.146 \pm .032*	.064 \pm .029	7.38 \pm 1.38*	1.35 \pm 0.39	1.609 \pm .397*	.870 \pm .136

¹ See Table XIX, 1; n = 6 animals per sacrifice.

² See Table XIX, 2; n = 6 animals per sacrifice.

* Indicates significant differences between DHA and vehicle-treated controls at $p < 0.01$.

this steroid were similar in DHA and vehicle-treated groups on the morning of day 30 but following PMSG administration, 17-hydroxyprogesterone concentrations were significantly higher than controls on the PM of day 30 as well as the AM of day 31.

Serum Gonadotropin Levels

The treatment of immature female rats with DHA was found to result in a preovulatory surge of FSH and LH in serum on the PM of day 29 (Table XXIII). An additional significant increase ($p < 0.01$) in serum concentrations of gonadotropins was found on the PM of day 30 in these animals. No elevations in serum FSH was detected on the morning of days 27-30. LH levels were similarly constant on the morning of day 27-29 but were significantly elevated on the morning of day 30 as compared to days 27-29. These nocturnal elevations plus the increase in circulating gonadotropic hormone resulting from PMSG administration were undoubtedly responsible for the ovulations which were detected throughout day 30 in these groups.

Cytosol Estradiol Receptors

Concentrations of cytosol estradiol receptors in the anterior pituitary were significantly depressed ($p < 0.01$) in DHA-treated rats as compared to vehicle-treated controls (Table XXIV). A progressive lowering of receptor content of the anterior pituitary (DHA-treated group) was noted from day 27-29. There was no replenishment of receptors on the day of ovulation (30) or day 31 in these androgen-treated groups as compared to that established on estrus in the PMSG-primed immature female rat (Table XVII and XVIII). A similar decrease was found in concentrations of hypothalamic cytosol estradiol receptors during DHA treatment ($p < 0.01$) as compared to vehicle-treated controls, Table XXIV. As with the anterior pituitary re-

Table XXIII. Effects of Short-Term DHA¹ Treatment on Serum Gonadotropins in Immature Female Rats

Age at Sacrifice	n	Serum Gonadotropin Concentrations (ng/ml) mean \pm S.E.	
		FSH	LH
27 AM	4	49.9 \pm 6.7	15.0 \pm 0.7
28 AM	4	21.8 \pm 4.1	13.7 \pm 1.2
29 AM	4	39.9 \pm 5.8	16.4 \pm 2.5
29 PM	4	155.7 \pm 64.9*	235.5 \pm 105.8*
30 AM	4	32.0 \pm 3.0	45.9 \pm 9.1*
30 PM	4	170.6 \pm 39.5*	339.0 \pm 47.4*

¹ See Table XIX, 1.

*Gonadotropin concentrations are significantly higher ($p < 0.01$) than concentrations in DHA-treated rats ages 27-29 AM.

Table XXIV. Changes in Anterior Pituitary and Hypothalamic Cytosol Estradiol Receptor Concentrations Following Short-Term DHA¹ or Vehicle² Treatment of Immature Female Rats

Age at Sacrifice	Specific bound moles ³ H-estradiol/mg cytosol protein mean \pm S.E.			
	Anterior Pituitary $\times 10^{-14}$		Hypothalamus $\times 10^{-15}$	
	DHA	Vehicle	DHA	Vehicle
27 AM	2.80 \pm .20*	7.70 \pm .85		
28 AM	2.11 \pm .02*			
29 AM	1.83 \pm .09*	7.08 \pm .14	4.96 \pm .15*	8.07 \pm .29
29 PM	2.17 \pm .06*			
30 AM	2.38 \pm .16*	8.23 \pm .03	5.43 \pm .44*	10.1 \pm .03
30 PM	2.36 \pm .16*			
31 AM	1.79 \pm .13*		5.78 \pm .04*	8.65 \pm .04

¹ See Table XIX, 1; Two different cytosol aliquots, in duplicate, were assayed for each sacrifice.

² See Table XIX, 2; Two different cytosol aliquots, in duplicate, were assayed for each sacrifice.

*Receptor concentrations in these groups are significantly lower ($p < 0.01$) than in vehicle-treated controls.

ceptors, there was no replenishment of hypothalamic estradiol receptor concentrations on the day of estrus, day 30, or the next day.

J. STUDIES IN THE IMMATURE FEMALE RAT FOLLOWING INDUCTION OF OVULATION
WITH DHA PLUS PMSG

Although both natural and PMSG induced puberty are followed by the establishment of regular estrous cycles in the rat, no thorough examination has been made of the post-ovulatory period in female rats after short-term DHA plus PMSG treatment. The interval of 31-40 days of age in such animals was therefore a critically important period in these studies. Vaginal cytology, ovarian and uterine weights, blood steroid and gonadotropin levels, and cytosol estradiol receptor content of the anterior pituitary were established for female rats in which puberty had been advanced by short-term DHA (60 mg/kg BW, 27-29 days of age) plus 8 IU PMSG (11 AM, 30 days of age). Experimental findings in these animals (from 31-40 days of age) were compared to those of female rats in which DHA plus PMSG treatment was followed by additional daily DHA administration (60 mg/kg BW) from 31-40 days of age.

Ovulation, Vaginal Cytology

Unlike the PMSG-treated or untreated female rat, the first ovulation in those female rats administered DHA and PMSG was not followed by the immediate establishment of regular estrous cycles. No differences in vaginal cytology were detected between those groups of rats in which DHA was continued or discontinued after day 30 of life. From day 32-40, 85-100% of animals checked each day had diestrus vaginal smears; the number of rats exhibiting pure estrus type smears was 10% or less during this interval. The high percentage of diestrus vaginal smears in these groups could not be

attributed to a mechanically induced pseudopregnancy since all animals were not smeared daily; vaginal smears of one-half of all animals were not taken until the time of sacrifice. The percentage of diestrus and estrus smears was similar regardless of when vaginal cytology was evaluated. In addition to the constant pattern of vaginal cytology, no ova were ever found at sacrifice in the oviducts of rats in these studies (ages 32-40).

Ovarian Weight

Daily changes in ovarian weight are presented in Table XXV for those animals in which DHA treatment was continued and those in which no further treatment was given. Ovarian weights were at a peak on the mornings of day 31 and 32 in both groups and tended to decrease thereafter. Those animals judged to be in diestrus had large ovaries with numerous corpora lutea; those in estrus had smaller ovaries with few, if any, corpora lutea. The ovaries of rats in which DHA was continued were generally heavier than those in which androgen administration had been discontinued throughout the interval of day 31-40. The exceptions, at ages 34 and 37 were due primarily to an excessive number of constant estrus type animals sacrificed on these days in the DHA-treated group.

Uterine Weight

Uterine weights of these rats are compared in Table XXVI. Uteri were quite heavy and their weights were fairly consistent through day 34 in those animals in which DHA was continued and in those in which DHA was discontinued. From day 35 onward, uterine weights of the animals which received no further androgen treatment fell progressively. Those of the DHA-treated rats remained the same or increased slightly and were significantly heavier ($p < 0.01$) from day 33-40 than those in which DHA treatment was

Table XXV. Ovarian Weights¹ in Female Rats (Age 31-40) in Which DHA was Continued or Discontinued Following Precocious Ovulation Induced by DHA and PMSG Treatment

Age at Sacrifice	Ovarian Weight (mg/100 g B.W.) mean \pm S.E.	
	DHA Continued	DHA Discontinued
31	59.8 \pm 4.9	49.3 \pm 3.1
32 AM	61.0 \pm 4.9	53.8 \pm 3.8
32 PM	56.3 \pm 2.8*	44.2 \pm 3.4
33	50.1 \pm 1.0	48.0 \pm 3.0
34	45.0 \pm 3.6	47.9 \pm 2.9
35	43.5 \pm 1.4	41.2 \pm 2.4
36	38.5 \pm 2.2	34.9 \pm 1.8
37	32.9 \pm 1.6	37.4 \pm 3.4
38	44.8 \pm 4.4*	34.5 \pm 2.5
39	42.5 \pm 2.6*	33.8 \pm 1.3
40	39.8 \pm 2.4*	32.4 \pm 1.0

¹ \bar{n} = 6 animals per treatment group for each sacrifice.

* Indicates a significant difference ($p < 0.01$) between ovarian weights of rats in which DHA was continued or discontinued following the induction of ovulation with DHA plus PMSG.

Table XXVI. Uterine Weight Changes in Rats¹ in Which DHA Treatment Was Continued or Discontinued Following the Induction of Precocious Ovulation with DHA and PMSG

Age at Sacrifice	Uterine Weight (mg/100 g B.W.) mean \pm S.E.	
	DHA Continued	DHA Discontinued
31	227 \pm 35	218 \pm 8
32 AM	211 \pm 9	195 \pm 9
32 PM	217 \pm 4	199 \pm 19
33	216 \pm 6	194 \pm 14
34	223 \pm 9*	192 \pm 11
35	217 \pm 17**	165 \pm 9
36	231 \pm 10**	140 \pm 7
37	228 \pm 7**	135 \pm 8
38	270 \pm 19**	119 \pm 4
39	239 \pm 17**	128 \pm 6
40	249 \pm 10**	110 \pm 6

¹ n = 6 animals per treatment group for each sacrifice.

* Indicates significant difference at $p < 0.05$ among groups sacrificed the same day.

** Indicates significant difference at $p < 0.01$ among groups sacrificed the same day.

discontinued at age 29 days. The increase which occurred at day 38 was in all probability due to an over adjustment of the DHA dosage which was calculated on the basis of group body weights every second day.

Serum Steroid Concentrations

Blood steroid concentrations of these two experimental groups following precocious ovulation in the DHA and PMSG-treated rat are shown in Tables XXVII and XXVIII.

By 31 days of age, serum DHA concentrations in rats receiving no further DHA administration were significantly lower ($p < 0.01$) than corresponding rats in which DHA treatment was continued (Table XXVII). In the following days, serum concentrations of DHA were maintained at very high levels in rats in which DHA treatment was continued. DHA levels fell continuously from age 31 onward in those rats in which androgen treatment was discontinued and only reached normal levels after a 10-day withdrawal period. By age 31 days, serum concentrations of testosterone and DHT (Table XXVII) were significantly higher ($p < 0.01$) in rats receiving continued DHA treatment as compared to those in which androgen treatment was stopped. By 35 days of age, peripheral serum levels of testosterone and DHT had declined to normal limits in rats treated with DHA and PMSG only; serum concentrations of these compounds were maintained at fairly constant levels from age 31-39 in DHA-treated rats.

Androstenedione levels at 31 days of age were also significantly decreased in rats receiving DHA and PMSG treatment only as compared to those receiving DHA and PMSG plus additional DHA treatment (age 31-39) (Table XXVII). Concentrations attained by age 33 in rats in which DHA was discontinued were similar to levels found after ovulation on day 34 in PMSG-treated rats.

Table XXVII. Peripheral Serum Androgen Concentrations in Rats¹ Pretreated with DHA Plus PMSG During Androgen Withdrawal or Continued DHA Therapy

Age at		Steroid Concentrations* (ng/ml) mean \pm S.E.			
Sacrifice	Treatment	Testosterone	Androstenedione	DHA	DHT
31	DHA	3.109 \pm .373	4.484 \pm .375	86.27 \pm 10.33	.439 \pm .144
31	---	0.840 \pm .207	1.111 \pm .182	14.49 \pm 2.34	.118 \pm .057
33	DHA	5.396 \pm .678	2.202 \pm .558	56.20 \pm 6.96	.308 \pm .071
33	---	0.451 \pm .106	0.674 \pm .067	2.44 \pm 1.45	.043 \pm .024
35	DHA	3.836 \pm .651	4.462 \pm .372	95.75 \pm 7.74	.767 \pm .233
35	---	0.057 \pm .030	0.737 \pm .165	1.27 \pm .42	.076 \pm .022
37	DHA	4.436 \pm .169	4.187 \pm .271	92.34 \pm 8.44	.535 \pm .041
37	---	0.017 \pm .009	0.521 \pm .115	.61 \pm .15	.084 \pm .028
39	DHA	5.334 \pm .527	2.927 \pm .454	55.43 \pm 4.64	.278 \pm .064
39	---	0.038 \pm .013	0.626 \pm .104	.36 \pm .17	.104 \pm .016

¹ n = 6 animals per treatment group for each sacrifice day.

* Concentrations of each steroid were significantly higher (p < 0.01) in rats treated continuously with DHA, age 31-39 days.

(Fig. 9). No further decline was found in serum levels of androstenedione from age 33-39 in these rats. Elevated concentrations of androstenedione were maintained in those rats receiving DHA treatment from ages 31-39 and daily fluctuations of androstenedione paralleled the rise and fall of DHA levels in these rats.

Serum levels of estradiol-17 β , progesterone and 17-hydroxyprogesterone in these experiments are shown in Table XXVIII. Concentrations of estradiol-17 β were not different in either experimental group at 31 days of age but by 33 days of age, levels of this steroid were significantly lower ($p < 0.05$) in rats during androgen withdrawal as compared to those receiving continued DHA treatment. By 37 days of age, serum levels of estradiol-17 β in rats receiving no additional treatment were equivalent to post-ovulatory levels in the PMSG-treated rats (Fig. 7).

Serum concentrations of progesterone and 17-hydroxyprogesterone were similarly elevated in both experimental groups at 31 and 33 days of age. From age 35-39, levels of progesterone and 17-hydroxyprogesterone remained elevated in those rats receiving continued DHA treatment; levels of these steroids in rats receiving no additional treatment began to decline and were significantly lower ($p < 0.01$) at 35, 37 and 39 days of age. When considered as an individual group, rats with constant estrus type vaginal smears had low serum levels of progestins. Concentrations of progesterone were significantly lower ($p < 0.01$) in the constant estrus animals (mixed ages) than in animals ages 31-39 of either experimental group. In constant estrus animals, 17-hydroxyprogesterone levels were lower (N.S.) than levels in rats 31 and 33 days of age (DHA continued or discontinued). The significant differences in serum progesterone levels between constant estrus

Table XXVIII. Peripheral Concentrations of Estradiol, Progesterone and 17-Hydroxyprogesterone During Androgen Withdrawal or Continued DHA Treatment in Rats Pretreated with DHA and PMSG

Age at Sacrifice	Treatment	n	Steroid Concentrations (ng/ml) mean \pm S.E.		
			Estradiol	Progesterone	17-Hydroxyprogesterone
31	DHA	6	.053 \pm .030	11.32 \pm 1.51	1.408 \pm .209
31	---	6	.076 \pm .026	10.49 \pm 1.18	1.892 \pm .374
33	DHA	6	.122 \pm .037	13.07 \pm 0.65	1.832 \pm .339
33	---	6	.031 \pm .009 *	13.56 \pm 0.60	1.213 \pm .174
35	DHA	6	.107 \pm .019	15.00 \pm 1.59	1.767 \pm .239
35	---	6	.039 \pm .019 *	12.98 \pm 1.10	0.883 \pm .159 **
37	DHA	6	.078 \pm .020	13.40 \pm 0.70	1.794 \pm .219
37	---	6	.008 \pm .007 **	10.99 \pm 1.10 *	0.683 \pm .238 **
39	DHA	6	.190 \pm .035	14.29 \pm 1.07	1.812 \pm .355
39	---	6	.005 \pm .004 **	7.08 \pm 1.03 **	0.652 \pm .078 **
Constant Estrus	---	6	---	4.66 \pm 1.23***	1.046 \pm .243

*Indicates significant difference at $p < 0.05$ in steroid levels of rats on the same day.

** Indicates significant difference at $p < 0.01$ in steroid levels of rats on the same day.

*** Progesterone levels in constant estrus rats are significantly lower ($p < 0.01$) than in any other group.

animals and animals primarily in diestrus are a reflection of the high steroidogenic activity of ovaries with corpora lutea (diestrus rats) as opposed to those in estrus (prior to complete luteinization of hemorrhagic follicles).

Gonadotropin

Serum levels of FSH and LH in post-ovulatory rats during androgen withdrawal or chronic DHA administration (age 31-39) are depicted in Table XXIX.

At 37 and 39 days of age, serum levels of FSH in rats treated with DHA were significantly higher ($p < 0.05$) than in those receiving no further treatment. By 40 days of age, there was no difference in serum levels of FSH between the two groups. Serum levels of LH were suppressed in both groups at 37 days of age. By 39 and 40 days of age, serum LH in animals receiving no further DHA treatment had increased to concentrations significantly higher than that found in the DHA-treated rats, in which LH remained suppressed from age 37-40.

Cytosol Estradiol Receptors

As shown in Table XXX levels of pituitary cytosol estradiol receptors were significantly depressed following the precocious ovulation induced by DHA and PMSG. A gradual increase from 32-40 days of age in levels of these receptors was noted in those rats in which DHA treatment was discontinued. There was no increase in estradiol receptor levels during this period in rats treated continuously with DHA. In fact, a significant decrease was detected at ages 39 and 40 which paralleled the increased estradiol levels detected in serum of these rats at 39 days of age (Table XXX). A significant difference ($p < 0.01$) in receptor binding between those rats chroni-

Table XXIX. Serum Gonadotropin Levels in Rats Pretreated with DHA and PMSG During Androgen Withdrawal or Continuous DHA Treatment

			Serum Gonadotropin Concentrations (ng/ml) mean \pm S.E.	
Age at Sacrifice	Treatment	n	FSH	LH
37	DHA	5	61.8 \pm 2.7	8.4 \pm 0.5
	---	5	27.1 \pm 3.9*	9.8 \pm 1.2
39	DHA	5	46.2 \pm 4.8	6.8 \pm 0.4
	---	4	33.1 \pm 8.9**	19.1 \pm 8.9**
40	DHA	6	46.2 \pm 3.3	7.2 \pm 0.3
	---	6	43.0 \pm 3.0	11.7 \pm 1.1*

* Indicates significant differences at $p < 0.01$ in gonadotropin levels of rats sacrificed the same day.

** Indicates significant differences at $p < 0.05$ in gonadotropin levels of rats sacrificed the same day.

Table XXX. Levels of Cytosol Estradiol Receptors¹ of the Anterior Pituitary in Female Rats Pretreated with DHA and PMSG During Androgen Withdrawal or Chronic DHA Treatment

Age at Sacrifice	Anterior Pituitary Cytosol Estradiol Receptor Concentrations (Specific bound moles $\times 10^{-14}$ ³ H-Estradiol/mg cytosol protein) mean \pm S.E.	
	DHA Chronic	DHA Discontinued
32	1.24 \pm .10	1.51 \pm .18
33	1.39 \pm .18	1.62 \pm .09
34	1.44 \pm .03	1.82 \pm .06*
35	1.71 \pm .09	2.11 \pm .00*
37	1.55 \pm .05	2.49 \pm .11*
39	0.79 \pm .01	2.08 \pm .11*
40	0.71 \pm .01	3.00 \pm .09*

¹ Two different cytosol aliquots, in duplicate, were assayed for each treatment group per sacrifice.

* Cytosol estradiol receptor concentrations are significantly higher ($p < 0.01$) than groups of similar aged rats receiving continued DHA treatment.

cally treated with DHA and those undergoing androgen withdrawal was apparent by day 34; this was a consistent finding through 40 days of age.

K. EFFECTS OF CHRONIC DHA TREATMENT ON THE REPRODUCTIVE AXIS OF IMMATURE FEMALE RATS.

This study was carried out in order to evaluate changes in several experimental parameters associated with chronic DHA treatment and the subsequent ovulatory failure (blood steroids and gonadotropins, cytosol estradiol receptors, vaginal cytology and organ weights). In addition, the androgen withdrawal period was closely studied, during which resumption of regular estrous cycles was anticipated. Starting at 27 days of age, and continuing through 46 days of age, DHA (60 mg/kg BW) was administered daily to female rats. The response to LH-RH (10 ng/100 g BW) was also established before (27 days of age) and after DHA induced precocious ovulation (34 days of age) and at the end of chronic DHA administration (46 days of age).

Ovulations and Vaginal Cytology

By 32 days of age, vaginal patency occurred in 100% of the DHA-treated female rats. Greater than 90% of all animals (n = 58) examined at age 32 were found to have estrus type vaginal smears. From day 33 until 46 days of age, 75% of the DHA-treated rats exhibited constant diestrus vaginal patterns; the remaining 25% had constant estrus vaginal smears. In no instance did the vaginal cytology of any animal change from constant diestrus (or vice versa) during the period of 33-46 days of age. Following the last DHA injection (age 46), the vaginal cytology of each animal remained constant for a short but variable length of time subsequent to the resumption of regular estrous cycles. The interval between androgen with-

drawal and initial resumption of regular cycles was found to be 9.3 ± 2.7 (S.D.) days (calculated as the number of days required for diestrus animals to change to estrus, or the number of days for constant estrus animals to change to diestrus). Equivalent capacities to resume regular estrous cycles, as judged by vaginal cytology were found in both constant estrus and constant diestrus animals. The induction of constant vaginal cytology and the return to regular estrous cycles is graphically demonstrated for a representative group of rats in Figure 10. It should be noted that on a group basis there was a great deal of variation in the vaginal smears of the rats on any given day during androgen withdrawal.

Ovarian Weight

Ovarian weight during and following chronic DHA treatment is shown in Table XXXI). Ovarian weights were highest at 32 and 34 days of age, at which time numerous corpora lutea were found in those animals exhibiting diestrus vaginal smears. By 46 days of age (after 20 days of DHA administration) ovarian weights in constant diestrus rats were significantly heavier than those recorded in constant estrus animals whose ovaries weighed less on a mg/100 g BW than those of untreated controls at 32 days of age. Ovarian cysts were apparent in both constant estrus and constant diestrus animals at 46 days of age but not in 34 day old DHA-treated rats. Following the discontinuation of DHA treatment, ovarian weights of previously constant diestrus animals declined accompanying a regression of corpora lutea. By 61 days of age, ovarian weights were significantly lower ($p < 0.01$) than ovarian weights of 48 day old rats (2 days post DHA). Considerable variation was noted among morphology (corpora lutea, hemorrhagic follicles, cysts, etc.) and weight of ovaries from individual rats within

Figure 10. Vaginal Cytology of a Group of Representative Female Rats During and After Chronic DHA Administration.

Note the chronic vaginal smears of each rat during and shortly after DHA treatment and the non-synchronized resumption of estrous cycles during androgen withdrawal.

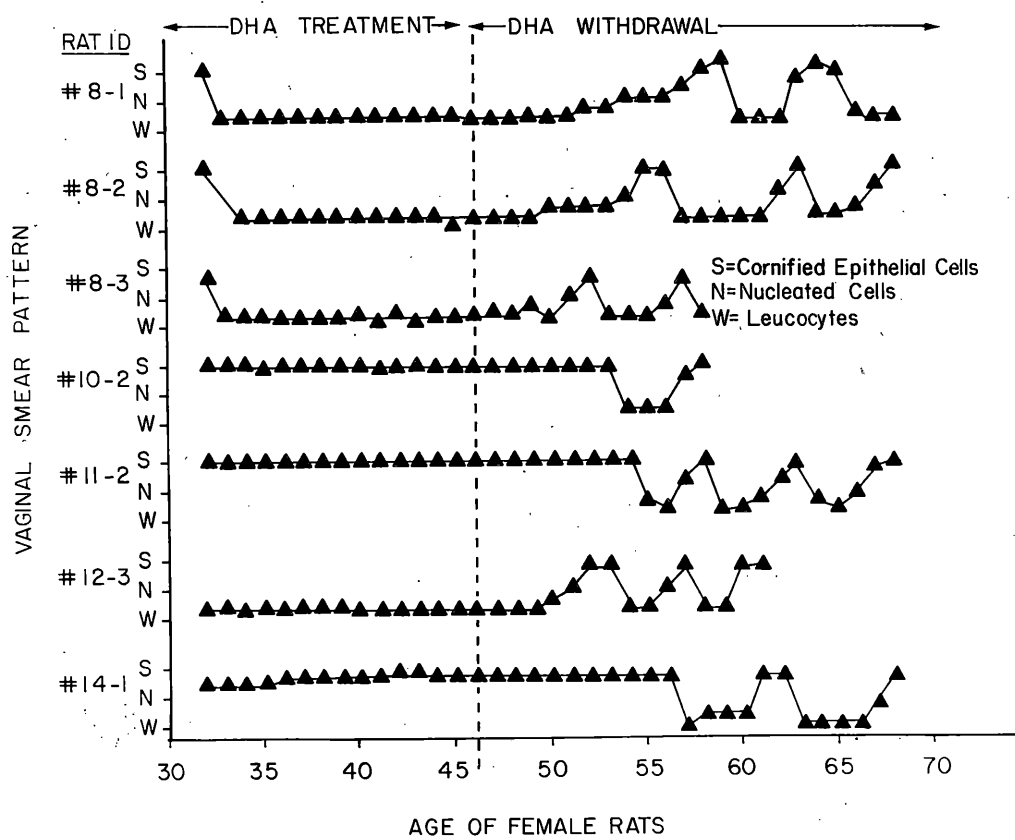


Table XXXI. Ovarian and Uterine Weights in Female Rats During Chronic DHA Administration and During Androgen Withdrawal¹

Age at Sacrifice	Group	Organ Weights (mg/100 g B.W.) mean \pm S.E.	
		Ovary	Uterus
32 PM	DHA	37.8 \pm 2.6	176.6 \pm 12.5
	Control	28.1 \pm 1.5 *	67.7 \pm 5.8 *
34 AM	DHA	43.6 \pm 4.9	215.0 \pm 7.8
	Control	29.3 \pm 1.6 *	127.0 \pm 16.8 *
46 AM	DHA, Constant Estrus	17.7 \pm 2.8	196.7 \pm 12.0
	DHA, Constant Diestrus	38.5 \pm 4.1 *	240.3 \pm 13.3
48 AM	2 days post-DHA	34.7 \pm 4.5	208.4 \pm 17.3
50 AM	4 days post-DHA	29.9 \pm 4.4	201.1 \pm 7.6
54 AM	8 days post-DHA	31.5 \pm 3.7	193.3 \pm 9.6
58 AM	12 days post-DHA	30.4 \pm 3.5	158.0 \pm 9.0 **
61 AM	15 days post-DHA	23.5 \pm 1.3 **	156.0 \pm 9.7 **
66 AM	20 days post-DHA	24.9 \pm 3.1	122.6 \pm 17.4 **
68 AM	22 days post-DHA	32.5 \pm 1.5	132.2 \pm 11.9 **

¹ Female rats were administered DHA (60 mg/kg BW) daily from age 27-46 days; control rats were treated with propylene glycol (vehicle) only; n = 6 animals per treatment group for each sacrifice.

* Indicates a significant difference between ovarian and/or uterine weights of groups sacrificed on the same day (p < 0.01) ages 32-46.

** Indicates that ovarian or uterine weights are significantly lower (p<0.01) than those obtained from rats age 48 days, previously treated with DHA for 20 days.

each sacrifice group during the androgen withdrawal period. As a result, no statistically significant changes could be demonstrated from day 50 through day 60, at which time all animals had undergone at least two consecutive estrous cycles. On a gross morphological basis, a progressive regression of ovarian cysts was apparent, however these findings were not verified by histological examination.

Uterine Weight

Uterine weights of DHA-treated rats, quite high at 32 and 34 days of age, were maintained through age 46 days (Table XXXI). No significant differences in uterine weight could be established between constant estrus and constant diestrus animals at 46 days of age. Following DHA withdrawal, uterine weights remained elevated from 46-54 days of age. A significant decrease occurred at 58 days of age ($p < 0.01$) and no further statistically significant alterations in uterine weight were noted from 58-68 days of age.

Serum Steroid Concentrations

Pooled serum samples (serum from two animals used to form each pool) were analyzed by multiple steroid radioimmunoassay techniques ($n = 3$ pools per sacrifice day). This was done in order to minimize the expected variations in steroid levels of individual rats during this time of androgen withdrawal and non-synchronized resumption of regular estrous cycles. The results of these evaluations are shown in Tables XXXII and XXXIII.

Serum levels of androgens (DHA, androstenedione, testosterone and DHT) were significantly higher ($p < 0.01$) in DHA-treated rats as compared to vehicle-treated controls at 32 days of age (Table XXXII). The levels of these androgens were similarly high at 46 days of age (following 20 days

Table XXXII. Serum Concentrations of Androgens in Female Rats¹ During and After Chronic DHA Administration²

Age at Sacrifice	Steroid Concentrations (ng/ml) mean \pm S.E.			
	DHA	Androstenedione	Testosterone	DHT
32 PM (Control)	9.78 \pm 2.2	0.07 \pm 0.04	0.13 \pm 0.04	0.04 \pm .01
32 PM	148 \pm 20.0*	4.01 \pm 0.72*	4.88 \pm 0.38	0.44 \pm .10*
46 AM, Constant Estrus	158 \pm 8.0	5.13 \pm 1.50	6.63 \pm 0.84	0.46 \pm .11
46 AM, Constant Diestrus	157 \pm 4.0	9.99 \pm 0.97**	8.93 \pm 0.83	0.47 \pm .10
48 AM	99 \pm 27.0†	4.20 \pm 0.35	4.97 \pm 0.82	0.29 \pm .14
54 AM	23 \pm 7.7†	1.66 \pm 0.43†	0.65 \pm 0.13†	0.14 \pm .01†
58 AM	21 \pm 5.5†	1.66 \pm 0.41†	0.44 \pm 0.09†	0.10 \pm .01†
61 AM	7.6 \pm 3.5†	3.14 \pm 1.94	0.31 \pm 0.18†	0.09 \pm .02†
66 AM	4.1 \pm 1.6†	0.69 \pm 0.32†	0.23 \pm 0.07†	0.07 \pm .01†

¹ Three 1.0 ml serum pools, sera from two rats per pool, were assayed in duplicate for each treatment group per sacrifice.

² See Table XXXI, ¹.

*Indicates that steroid levels are significantly higher ($p < 0.01$) than levels in 32 day old vehicle-treated controls.

**Indicates that serum levels are significantly higher ($p < 0.05$) than concentrations in 46 day old constant estrus rats.

†Indicates that serum levels following DHA withdrawal are significantly lower ($p < 0.01$) than constant estrus and constant diestrus rats at 46 days of age.

Table XXXIII. Concentrations of Serum Estradiol, Progesterone and 17-Hydroxyprogesterone in Female Rats During and After Chronic DHA Administration^{1,2}

	Steroid Concentration (ng/ml) mean \pm S.E.		
	Estradiol	Progesterone	17-Hydroxyprogesterone
32 PM (Controls)	0.019 \pm .009*	3.29 \pm 2.37*	0.85 \pm 0.22*
32 PM	0.090 \pm .030	19.90 \pm 2.90	1.69 \pm 0.15
46 AM, Constant Estrus	0.070 \pm .020	13.11 \pm 6.60*	2.43 \pm 0.51
46 AM, Constant Diestrus	0.090 \pm .020	31.48 \pm 4.00	2.82 \pm 0.37
48 AM	0.220 \pm .080	34.29 \pm 3.30	2.89 \pm 0.73
54 AM	0.130 \pm .010	24.48 \pm 0.92	1.26 \pm 0.21**
58 AM	0.050 \pm 0.10**	6.93 \pm 2.18***	1.64 \pm 0.34
61 AM	0.010 \pm .003***	9.90 \pm 2.91**	1.71 \pm 0.23
66 AM	0.023 \pm .005***	1.43 \pm 0.53***	0.61 \pm 0.14***

¹ Rats were treated chronically with DHA (60 mg/kg B.W.) starting at 27 days of age.

² Three 1.0 ml serum pools, sera from two rats per pool, were assayed in duplicate for each treatment group per sacrifice.

* Indicates that steroids are significantly lower ($p < 0.01$) than levels in rats sacrificed the same day.

** Indicates that steroids are significantly lower ($p < 0.05$) than levels at age 48 days.

*** Indicates that steroids are significantly lower ($p < 0.01$) than levels at age 48 days.

of DHA administration). No significant differences among serum levels of DHA, testosterone or DHT were apparent between constant estrus and constant diestrus animals. Androstenedione levels were, however, higher ($p < 0.05$) in constant diestrus rats sacrificed at 46 days of age.

Two days after DHA treatment was discontinued, age 48, an approximate 50% reduction in serum levels of the above mentioned four steroids was detected. Further reductions in peripheral serum concentrations of DHA, androstenedione, testosterone and DHT occurred from 48 to 66 days of age, during which a resumption of regular estrous cycles occurred. No differentiation in the contribution to peripheral steroid levels was possible between endogenous secretion and metabolic conversion of the injection steroid, DHA. However, the clearance of DHA and androstenedione was noticeably slower than that of testosterone and DHT, which suggests that some secretion of the former steroids may have occurred during the androgen withdrawal period. In addition, the return to basal DHA and androstenedione levels in the long term DHA-treated rats was retarded as compared to that observed following short-term DHA plus PMSG treatment (Table XXVII).

Serum levels of estradiol, progesterone and 17-hydroxyprogesterone (Table XXXIII), were also significantly higher ($p < 0.01$) in 32 day old DHA-treated rats than vehicle-treated controls. The marked elevation in progesterone and the lesser increase in serum levels of 17-hydroxyprogesterone were undoubtedly the result of enhanced ovarian secretion due to the presence of large, apparently functional corpora lutea in most DHA-treated rats sacrificed at this time (age 32 days). The increased estradiol concentrations in these rats was, however probably due to the metabolism of DHA.

Similarly, increased serum concentrations of these steroids were found at 46 days of age in the chronic DHA-treated rats. There was no significant difference in levels of estradiol and 17-hydroxyprogesterone in the constant estrus and diestrus animals. Progesterone concentrations were, however, significantly higher ($p < 0.01$) in the constant diestrus rats, which also had much larger ovaries (Table XXXIII) studded with many corpora lutea. No immediate decrease in serum levels of any of these steroids was found following withdrawal of DHA treatment at 46 days of age. By 58 days of age, 12 days after the last DHA injection, a significant decrease in serum levels of estradiol and progesterone occurred. A further lowering of estradiol levels was noted at 61 days of age whereas progesterone concentrations increased at this time. An initial decrease in 17-hydroxyprogesterone occurred at 54 days of age, however no further decreases occurred through 61 days of age. By 66 days of age, serum levels of estradiol, progesterone and 17-hydroxyprogesterone were equivalent to levels in 32 day old control female rats.

Pituitary Responsiveness to LH-RH

The response to LH-RH (10 ng/100 g B.W., administered S.C.) or vehicle was evaluated before and soon after the induction of precocious ovulation in DHA-treated rats; similar evaluations were conducted in control animals of the same age. In addition, 46 day old DHA-treated rats (constant estrus plus constant diestrus) were administered LH-RH or vehicle alone. The results of these studies are shown in Table XXXIV. At 27 days of age, LH-RH caused a significant increase within 30 minutes in serum LH ($p < 0.01$) and FSH ($p < 0.05$) in control animals. There was no detectable increase in LH or FSH over control or vehicle injected levels in the 27 days old

Table XXXIV. Serum Gonadotropin Concentrations Following LH-RH or Vehicle Administration¹ in Female Rats Treated With DHA²

				Gonadotropin Levels (ng/ml) mean \pm S.E.	
Age at Sacrifice	n	Group	Treatment	LH	FSH
27 AM	6	Control	---	8.2 \pm 0.4	69.0 \pm 14.2
	6	Control	Vehicle	7.9 \pm 1.0	81.2 \pm 11.4
	5	Control	LH-RH	35.4 \pm 9.2*	142.4 \pm 19.9**
	4	DHA	---	9.6 \pm 0.6	43.1 \pm 12.3
	6	DHA	Vehicle	7.7 \pm 0.4	64.9 \pm 7.4
	6	DHA	LH-RH	9.7 \pm 1.2	78.7 \pm 16.2
	6	Control	---	18.3 \pm 6.4	63.5 \pm 15.0
	5	Control	LH-RH	169.5 \pm 63.7*	104.6 \pm 34.0
34 AM	6	DHA	---	7.6 \pm 0.4	44.3 \pm 4.9
	6	DHA	LH-RH	7.2 \pm 0.3	50.5 \pm 14.8
	6	DHA	---	6.7 \pm 0.4	67.7 \pm 4.1
46 AM	6	DHA	Vehicle	7.1 \pm 0.2	62.4 \pm 3.3
	6	DHA	LH-RH	8.2 \pm 0.5	67.6 \pm 5.5

¹ Rats were sacrificed by decapitation before and 30 minutes after treatment with LH-RH (10 ng/100 g B.W.) or vehicle.

² Rats were treated continuously with DHA (60 mg/kg B.W./day, age 27-46 days) or propylene glycol.

* Indicates significant difference in serum levels of FSH or LH in LH-RH-treated rats as compared to controls (time 0 and/or vehicle-treated, time 30 minutes), ($p < 0.01$).

** As above at $p < 0.05$.

DHA-treated rat. At 34 days of age, near the time of natural onset of puberty, basal LH levels in control rats were increased as compared to control levels in 27 day old rats; the response to LH-RH was greater in the 34 day old group as compared to 27 day old rats. The apparent increase in FSH levels in 34 day old controls after LH-RH was not statistically significant. There was no perceptible increase in LH or FSH in the 34 day old DHA-treated rats. Basal LH and FSH levels in the DHA-treated group at this age were noticeably lower than those obtained for 34 day old controls; they were however not different from levels in 27 day old DHA-treated rats. On the last day of chronic DHA administration, (age 46) there was no increase in serum LH or FSH 30 minutes after LH-RH. The data for 46 day old rats are comprised of values obtained in both constant estrus and constant diestrus rats; no differences were noted in LH or FSH levels of those rats obtained at time zero minutes (control), 30 minutes after LH-RH, or 30 minutes after vehicle. It appears that the pituitary response to low dosages of LH-RH is suppressed by DHA treatment. This impairment was evident four hours after the first DHA injection on day 27, in 34 day old rats at which time corpus luteum development was apparent and at 46 days of age after 20 days of DHA administration.

Serum Gonadotropins

The changes in serum levels of gonadotropins, FSH and LH, following DHA withdrawal are shown in Table XXXV. A gradual increase in serum LH was found to occur throughout the withdrawal period. The first statistically significant increase was obtained in 61 day old rats. This increase was further sustained at 66 and 68 days of age when serum LH levels were significantly higher ($p < 0.01$) than levels at 46 days of age. Serum levels of FSH declined slightly from 46-61 days of age but were increased significantly ($p < 0.01$) at 66

Table XXXV. Serum Gonadotropin Concentrations During Androgen Withdrawal in Long-Term DHA-Treated Female Rats¹

Age at Sacrifice	Number of days after Last DHA Injection	n	Serum Gonadotropins (ng/ml) mean \pm S.E.	
			LH	FSH
46 AM	0	6	6.7 \pm 0.4	67.7 \pm 4.1
48 AM	2	6	7.6 \pm 0.6	61.6 \pm 5.6
50 AM	4	6	8.6 \pm 0.4	60.0 \pm 4.3
54 AM	8	6	9.0 \pm 0.9	53.2 \pm 7.8
58 AM	12	6	9.3 \pm 1.1	55.2 \pm 6.4
61 AM	15	6	10.1 \pm 1.3*	51.6 \pm 6.8
66 AM	20	6	14.9 \pm 3.7*	108.2 \pm 28.8*
68 AM	22	6	12.4 \pm 0.8**	72.3 \pm 15.6

¹ Female rats were treated chronically with DHA (60 mg/kg B.W./day, age 27-46 days).

*Indicates a significant increase in serum levels of LH or FSH as compared to levels at 46 days of age, (p < 0.05).

**Indicates a significant increase in serum levels of LH as compared to levels at 46 days of age, (p < 0.01).

days of age. Concentrations of FSH at 68 days of age were not significantly different than those found at 46 days of age. The increasing titers of serum LH during androgen withdrawal were associated temporally with the decreasing levels of most blood steroids quantified during the interval of 46-61 days of age, when a resumption of regular estrous cycles was documented.

Cytosol Estradiol Receptors

Cytosol estradiol receptor content of the anterior pituitary and hypothalamus following DHA withdrawal are shown in Table XXXVI. Both anterior pituitary and hypothalamic estradiol receptor levels were significantly depleted at 46 days of age (as compared to cytosol receptor content in untreated female rats in other experiments). A gradual replenishment of binding capacity was noted for both tissues from age 46-54 days of age. The first significant increase did not occur until 58 days of age in the anterior pituitary and hypothalamic cytosol estradiol receptor concentrations. Hypothalamic estradiol receptor levels were fairly consistent from 58-68 days of age whereas anterior pituitary receptor levels were further replenished at these times. By 68 days of age, cytosol estradiol receptor concentrations of both the anterior pituitary and hypothalamus were equivalent to concentrations found on estrus and diestrus in PMSG-primed rats (Table XVIII). The replenishment of the cytosol estradiol receptors (age 46-68) paralleled the increases in basal levels (AM) of serum LH during the DHA withdrawal period (Table XXXV). In addition, the first significant increment in cytosol estradiol receptor levels of the anterior pituitary and hypothalamus (age 58 days) occurred at the same time that a significant decrease in serum estradiol was detected (Table XXXIII). These bio-

Table XXXVI. Changes in Cytosol Estradiol Receptor Concentrations of the Anterior Pituitary and Hypothalamus During Androgen Withdrawal in Long-Term DHA¹-Treated Female Rats²

Age at Sacrifice	Number of Days After Last DHA Injection	Cytosol Estradiol Receptor Concentrations (specific bound moles ³ H-estradiol/mg cytosol protein) mean \pm S.E.	
		Pituitary $\times 10^{-14}$	Hypothalamus $\times 10^{-15}$
46	0	3.34 \pm .06	3.05 \pm .03
48	2	3.76 \pm .26	3.39 \pm .09
50	4	4.19 \pm .22	3.37 \pm .38
54	8	3.96 \pm .83	4.58 \pm .58
58	12	4.90 \pm .40*	8.35 \pm .15**
61	15	6.04 \pm .01**	7.55 \pm .64**
66	20	6.20 \pm .72**	7.85 \pm .25**
68	22	7.95 \pm .86**	8.09 \pm .16**

¹ Female rats were treated chronically, beginning at 27 days of age, with DHA (60 mg/kg B.W.) for 20 days.

² Two different cytosol aliquots, in duplicate, were assayed on each sacrifice.

* Significant increases in estradiol receptors ($p < 0.05$) as compared to age 46.

**Significant increases in estradiol receptors ($p < 0.01$) as compared to age 46.

chemical changes also coincide well with the average number of days required for resumption of estrous cycles in the chronic DHA-treated rat (9.3 ± 2.7 days).

V. DISCUSSION

MULTIPLE STEROID RADIOIMMUNOASSAY TECHNIQUES

The most significant technical advance in endocrine research during the past 20 years has been the development of new methods for hormone quantification. The most successful of the current techniques are radioimmunoassays which offer sensitivity, precision and reproducibility. Since the introduction of such methods for the measurement of individual steroid hormones, investigators have recognized the potential of radioimmunoassay for quantification of several steroid hormones from single blood samples.

A multiple steroid radioimmunoassay method was developed during these studies and was applied to the measurement of estradiol-17 β , progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, dihydrotestosterone and dehydroepiandrosterone in serum of human volunteers and female rats. The development of two antisera for use in the measurement of estradiol-17 β and dihydrotestosterone was a necessary prerequisite for this method due to the unavailability of specific antisera to these compounds. The antisera developed for estradiol, RP#1 (Table III, Figure 3) and for dihydrotestosterone, #A-3 (Table IV), plus those antisera obtained from commercial sources were found to be suitable for use in the context of a multiple steroid radioimmunoassay method. The requirement in such methods or in single steroid assay methods for specificity of the antisera was demonstrated at length for the dihydrotestosterone antiserum, #A-3, when compared to another antiserum, S-741, currently employed for the radioimmunoassay of this compound (Figures 4, 5 and Table V).

Chromatographic separation of the seven steroids, isolated initially by ether extraction of 1-2 ml of serum (or plasma), was achieved using

celite partition columns. When the serum volume was sufficient (greater than two ml) and thus the quantity of each steroid hormone to be measured was adequate, a two column technique (column system A + B) was adopted for the isolation of the seven compounds (Figure 1, Table I). However, when the sample volume was limited, as expected with small animals, a single celite column method (modified column system A) (Figure 2) was developed and utilized for the separation and collection of the seven steroids from a single serum extract prior to assay as detailed in Table II.

The overall recovery of all steroid hormones following ether extraction of serum and column chromatography was quite high (68-93%) and was reasonably consistent for each compound, regardless of which column method was employed. The precision of these methods (Table VI) was comparable to that reported for single steroid assay methods (Korenman *et al.*, 1969; Buster and Abraham, 1972; Horton, 1965; Abraham *et al.*, 1971; Strott and Lipsett, 1968; Mayes and Nugent, 1968) and the accuracy is acceptable as judged by comparing values obtained in normal male and female serum by these methods (Table VIII) to those reported in literature by a variety of steroid assay methods (Table IX). The method blanks for this multiple steroid RIA are very low (Table VII) which contributes greatly to the high degree of sensitivity possible with this technique. The chromatographic method is simple enough to allow for the simultaneous processing of as many as eight columns and requires about two hours for the complete separation and individual collection of all seven steroids for subsequent radioimmunoassay.

Other multiple steroid radioimmunoassay methods have also been developed recently by other investigators. One such technique was described by

Concolino and Marocchi (1972) and allows the simultaneous quantification of estradiol, estrone, testosterone and androstenedione from a single plasma extract. This method, which is quite complex, required the extraction of 4-6 ml of plasma plus three column chromatographic separations for the isolation of estrone, estradiol and testosterone (plus DHT). Androstenedione was also isolated but had to be converted (sodium borohydride reduction) to testosterone and rechromatographed on thin layer plates prior to assay. All four compounds were then quantified by competitive protein binding assay. The recovery of compounds ranged from 45-60% for androstenedione and 65-90% for estrone, estradiol and testosterone.

Another multiple steroid assay technique was recently reported by Dupon et al. (1973). Estrone, estradiol, testosterone, androstenedione and progesterone were measured from a single 4 ml plasma sample. The method required several chromatographic steps (one Sephadex column plus two thin layer separations) in addition to enzymatic conversion of androstenedione to testosterone prior to assay. Steroids were then quantified by radioimmunoassay (estrogens) or competitive binding assay (androgens plus progesterone). The complexity of the chromatographic steps, high blank value associated with thin layer chromatography, and requirement for large sample volume make this technique somewhat less than optimally desirable for routine use. In addition, this method had a poor recovery of the androgens and progesterone which ranged from 32% to 60%.

The multiple steroid radioimmunoassay systems described in this dissertation are superior to these two methods with respect to the critical areas of sensitivity, resolving power (separation of steroids) and time required for sample preparation prior to actual assay. In addition, the

multiple steroid radioimmunoassay methods (Systems A, B and modified System A) allow for the measurement of a broader spectrum of steroids than the above mentioned methods or any other steroid assay method currently in use.

PERIPHERAL STEROID LEVELS IN THE NORMAL AND ANDROGENIZED HUMAN FEMALE

Studies of steroid levels in normal human females and those with endocrinopathies (polycystic ovarian disease, ovarian tumors, congenital adrenal hyperplasia) yielded a great deal of informative data. Concentrations of weak androgens - dehydroepiandrosterone and androstenedione - were significantly higher in patients with polycystic ovarian disease than in normal women (Figure 6). In addition, levels of more potent androgens - testosterone and dihydrotestosterone - were elevated, to a lesser degree, in the hirsute patients (Figure 6). Elevations in serum levels of androgens described in these patients confirm the studies of Mahesh and Greenblatt (1964) who found that ovaries and ovarian vein blood of such patients contained high concentrations of DHA and/or androstenedione. Other investigators have reported similar elevations in peripheral blood levels of various androgens in hirsute women (Kirschner and Bardin, 1972; Osborn and Yannoni, 1971).

An unexpected finding in evaluations of steroids in patients with polycystic ovarian disease was the significantly elevated concentrations of 17-hydroxyprogesterone and progesterone. As shown in Figure 6, levels of these compounds were considerably higher than concentrations found in normal women during the follicular phase of the menstrual cycle. Abraham and Chakmakjian (1974) have recently evaluated a series of hirsute patients and also report increased serum levels of progesterone and 17-hydroxypro-

gesterone, in addition to the expected elevations of androgens in such patients.

These findings indicate that a generalized increase in serum levels of most androgen precursors occurs in polycystic ovarian disease. However, estradiol levels were not specifically increased and in some instances were reduced in individual patients having polycystic ovarian disease, substantiating the hypothesis that some forms of this disorder may be related to a reduction in the ovarian aromatizing enzymes necessary for estrogen formation from androgen precursors (Mahesh and Greenblatt, 1964).

In addition to the patients with polycystic ovarian disease, it was found that increased androgen secretion occurred in the limited study of patients with ovarian tumors and congenital adrenal hyperplasia (Table X). Previous studies of androgen levels in immature females indicate that concentration of testosterone is lower than that in adult women, as are the concentrations of DHA and androstenedione (Gandy and Peterson, 1968). The levels of these compounds in the child with congenital adrenal hyperplasia and that of testosterone in the infant with an ovarian tumor were equivalent to or higher than levels of testosterone, androstenedione and DHA in the adult female (Table X). The extremely elevated serum levels of progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, and DHT with apparently normal serum concentrations of DHA found in the anovulatory patient with adrenal rest tumor (Table X) confirm previous in vitro analysis of the biosynthetic capacity of this neoplasm (Sandberg et al., 1962). A dramatic reduction of serum levels of the previously elevated steroids was noted following surgical extirpation of the tumor. The subsequent resumption of ovulatory menstrual cycles in this patient offers additional proof

of the inhibitory effects of excessive serum steroid levels on ovulation in the human female as has been found previously in many patients with polycystic ovarian disease (Greenblatt, 1953; Greenblatt and Coniff, 1968).

These analyses of human steroid concentrations represent the first time that a full spectrum of compounds, including androgens, progestins and estradiol, have been measured from a single serum extract. The results obtained in these studies indicate that the multiple steroid radioimmunoassay methods which were developed during the completion of this research endeavor are a potentially useful tool in both routine clinical as well as experimental endocrine research.

STUDIES IN CONTROL AND PMSG-TREATED IMMATURE FEMALE RATS

Pertinent data previously described in the Results section in tabular form, will be presented graphically for the purpose of this discussion in an Appendix (Figures 11-19). Parameters discussed, such as organ weights and serum concentrations of a particular steroid are presented concomitantly for all experimental treatment groups - untreated controls, PMSG, short-term DHA plus PMSG, and long-term DHA in individual figures. When viewed in this fashion, correlative changes in individual parameters are more easily compared from one experiment to another. In addition, similarities as well as disparities which were documented during the onset of puberty and ovulatory failure in female rats are more easily identified.

Studies in Untreated Immature Female Rats

The hormonal events associated with the natural onset of puberty in the female rat are at present only partially understood. Because of the paucity of information concerning this period in the female rat, untreated female rats were studied before and during the expected time of the first ovulation

(which defines the event of puberty). Uterine and ovarian weights were determined, oviducts were examined to detect ova, vaginal patency was documented and steroid levels were quantified in peripheral blood of female rats from 28-39 days of age. In addition, concentrations of anterior pituitary and hypothalamic cytosol estradiol receptors were quantified during the peripubertal period.

The first indications that puberty was imminent were increases in uterine weight (Figure 11) which followed a subtle increase in circulating levels of estradiol beginning at 33 days of age (Figure 13). Although no detectable increase in ovarian weight was apparent at 33 days of age (Figure 12), previous studies have demonstrated that a maturational or developmental change in the ovary begins at 32 days of age, prior to any increment in ovarian weight (Knudsen et al., 1974). These same researchers reported that an increase in uterine weight occurred at 32 days of life and continued through 36 days of age thereby following an increase in the number of large (type 6-7) ovarian follicles at 32 days of age.

In these studies, the first increase in ovarian weight occurred at 35 days of age followed by another increment the next day (Figure 12). The increased ovarian weight occurred at a time when gonadotropin levels would be expected to be elevated (Knudsen et al., 1974). Serum levels of estradiol, progesterone, 17-hydroxyprogesterone, testosterone, androstenedione levels were significantly increased at 35 days of age when compared to levels at 32 days of life (Figures 13-17). A further increase in peripheral serum levels of progesterone and 17-hydroxyprogesterone was detected at 36 days of life, at which time ova and corpora lutea were first noted in these studies. Serum levels of progesterone, 17-hydroxyprogesterone, estradiol

and androstenedione remained elevated from 36-39 days of life during which time the majority of animals were found to ovulate from the first time.

The role of blood steroids during puberty of the female rat are not clear at this time. Previous studies by Presl et al. (1969) have shown that an increase in peripheral estradiol concentrations occurs at the time of puberty in female rats, as have these studies. It has been further demonstrated that progesterone levels are low (1-4 ng/ml) in female rats at 28 and 33 days of life, prior to the onset of puberty (Ramaley and Bartosik, 1974), substantiating the findings of relatively low levels of progesterone in these studies (Figure 14) prior to 35 days of life. There are no known studies to which the serum levels of other steroids (17-hydroxyprogesterone, testosterone, androstenedione, DHT (Figure 18) or DHA (Figure 19) may be compared in the immature female rat. It would appear from this investigation that serum levels of all of these compounds are relatively stable from 28-33 days of life, increasing only after the first increment in serum estradiol at 33 days of life. The marked elevations of most of the steroids quantified from 36-39 days of life was probably due to the preovulatory surges of gonadotropins in individual rats sacrificed during this time.

Of all the steroids studied in relation to sexual maturation of the female rat, estradiol has received the most investigation. A great deal of evidence has been put forth which would implicate estradiol as being important for prepubertal development of the female rat. Administration of estradiol antibodies for four days starting at 10 through 25 days of age was found to effectively impair the normal increase in ovarian and uterine weight found in untreated female rats (Reiter et al., 1972a). The site of

action of estradiol in immature female rats was found to be in part a local effect on the ovary itself (Bradbury, 1961). The mechanism of this local estrogen action was shown to be an enhancement of the ovarian growth response to exogenously administered gonadotropins in hypophysectomized female rats (Meyer and Bradbury, 1969; Reiter et al., 1972b) or endogenous gonadotropins as well in the intact female rat (Reiter et al., 1972a). The increasing levels of estradiol secreted by the maturing ovary may also interact with the hypothalamic-pituitary complex to induce puberty. Estradiol administration has been shown to advance vaginal opening and ovulation in the immature female rat (Frank et al., 1925), as have other steroids which may be converted to estrogen (Black and Mahesh, 1969).

An increase in serum levels of estradiol was detected at 33 days of age in this study of the untreated female rat (Table XII) which coincided with a significant decrease in cytosol estradiol receptor concentrations of both the anterior pituitary and hypothalamus (Table XIII). The increase in serum estradiol levels and depletion of estradiol receptors preceded any increase in other steroid hormones measured and also preceded the expected preovulatory surge of gonadotropins reported to occur from 34-38 days of life in Holtzman rats (Knudsen et al., 1974). A similar depletion of hypothalamic estradiol receptors (cytosol) has been reported by Kato et al. (1974) in peripubertal female rats at 35 days of age as compared to levels at 21 and 28 days of age. They found that the hypothalamic cytosol estradiol receptors remained decreased in rats at 38 days of age which had not ovulated (closed vagina), but was replenished in those following vaginal opening. No distinction was made in the current study between those rats with closed or open vaginae with respect to cytosol receptor levels; however, the majority of rats studied had ovulated during the period of 36-39 days

of age. No replenishment of estradiol receptors was found subsequent to the initial minor depletion at 33 days of age. There was no significant decrease in serum levels of estradiol during this time of unsynchronized ovulations either, which would make complete replenishment of cytosol receptors unlikely.

Studies in the PMSG-Primed Immature Female Rat

Although a pattern of hormonal changes was established by the evaluations conducted in female rats during natural puberty, it was of interest to more clearly identify specific changes associated with the synchronized onset of puberty. Pregnant mares serum gonadotropin (PMSG) has been used extensively by others to induce precocious, synchronized ovulation in the immature female rat since its first description by Cole (1936). The administration of 8 IU PMSG at 10 AM to 30 day old female rats was found, in these studies, to cause ovulation at 33 days of age (mean number of ova = 6-11) in all treated animals. Subsequent reproductive function was apparently not impaired by PMSG treatment since regular estrous cycles ensued after the initial ovulation at 33 days of age.

Ovarian weight increased initially at 6 PM on day 30 in PMSG-primed female rats (Figure 12), saline treatment being ineffective. Ovarian weight then increased further at 31 days of age but did not increase again until the PM of day 32 (technically proestrus). Uterine weight was increased following PMSG administration from day 30 - the AM of day 32 (Figure 11) at which time uterine ballooning was evident. Uterine weight then declined on the day of estrus (33) and decreased further the next day (34, diestrus I). Ovarian weight remained elevated on estrus and numerous corpora lutea were found at this time on diestrus, day 34.

Current concepts concerning the PMSG induced precocious ovulation state that ovarian steroid synthesis and secretion following PMSG administration has a facilitative effect on the preovulatory release of FSH and LH on the evening before ovulation (Schwartz, 1969; Strauss and Meyer, 1962). In these studies, PMSG was found to have an immediate steroidogenic effect, causing a significant ($p < 0.01$) increase in peripheral serum levels of estradiol (Figure 13), progesterone (Figure 14) and 17-hydroxyprogesterone (Figure 15) within 8 hours. Serum levels of testosterone (Figure 16) and androstenedione (Figure 17) were also increased on the PM of day 30 following PMSG administration, but the increases were not statistically significant. Progesterone and 17-hydroxyprogesterone levels declined on the morning of day 31 whereas estradiol, androstenedione and testosterone levels increased further ($p < 0.01$). Maximal uterine weight and accumulation of luminal fluid correlated with the maximal serum levels of estradiol on the AM of day 32. Peripheral levels of androstenedione, progesterone and 17-hydroxyprogesterone were all depressed on the morning of day 32 while estradiol and testosterone increased further. On the PM of day 32, serum concentrations of progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, DHA and DHT were significantly higher than levels in saline-treated controls or in PMSG primed rats sacrificed prior to this time; estradiol levels declined in relation to those levels found on the morning of day 32. The increase in serum levels of the former steroids at 6 PM of day 32 (proestrus) and further ovarian growth (Figure 12) occurred after a significant increase in serum FSH and LH (Table XVI). The decline in estradiol at this time (during and after the FSH/LH surge) supports the in vitro studies of Chatterton et al. (1969) who found that LH inhibited estro-

diol synthesis in ovaries removed at proestrus and estrus from adult rats. Serum levels of each steroid (Figures 13-19) and gonadotropins (Table XVI) declined on day 33, estrus and only progesterone levels were increased on day 34, diestrus I.

The timing of the preovulatory discharge of FSH and LH on the afternoon of day 32 in these studies (Table XVI) is in agreement with the findings of others who studied the PMSG-primed immature female rat (Costoff et al., 1974; Strauss and Meyer, 1962). The progressive increases in blood steroids following PMSG administration are also similar to findings in previous studies. Wilson et al. (1974), using PMSG-primed (PMSG at day 30) Wistar and Sprague Dawley rats, found that estradiol concentrations in peripheral blood increased progressively, reaching peak concentrations on the morning of day 32 prior to ovulation at 33 days of age. Estradiol levels then declined during the evening of day 32 and were not significantly higher on estrus (day 33) than levels found in saline-treated controls. Progesterone levels (Wilson et al., 1973) increased initially after PMSG administration, fell on the morning of day 32, rose sharply on the PM of day 32 and then declined on estrus. Similar patterns of steroid levels after PMSG treatment have been documented by others in the immature female rat (Horikoshi and Weist, 1971; Meyer et al., 1971; Zarrow et al., 1971) and in adult hamsters (Baranczuk and Greenwald, 1973). No previous studies have evaluated levels of 17-hydroxyprogesterone or androgens (testosterone, androstenedione, DHA or DHT in untreated or PMSG-primed immature female rats or hamsters. In another species, McCracken and Baird (1969) have demonstrated an increase in ovarian secretions of androstenedione following PMSG treatment of female sheep.

The dramatic elevations in serum steroids observed on the PM of day 32 (56 hours after PMSG administration) are undoubtedly the results of the preovulatory surge of FSH/LH. Meyer et al. (1971) have previously demonstrated the requirement of this preovulatory LH/FSH surge for the elaboration of a progesterone "surge" on the PM of proestrus in PMSG-primed immature female rats. The massive increase in progesterone and other steroids at the time of the LH/FSH surge may be characteristic only of the PMSG primed immature female rat. However, several studies have shown the acute effects of gonadotropins on ovarian steroid secretion in adult rats (Ichikawa et al., 1972), rabbits (Oxender et al., 1971; Mills et al., 1972) and dogs (Aakvaag and Eik-Nes, 1969).

The decline in peripheral steroid levels on estrus (day 33, Figures 13-19) and the apparent major secretion of only progesterone on diestrus (day 34) is in agreement with the proposed biosynthetic capacity of the post-ovulatory rat ovary (Huang and Pearlman, 1960; Horikoshi and Weist, 1971).

In view of the rather poor documentation of changes in steroid hormone levels during natural and induced puberty in the female rat, a comparison of these results to findings during the adult rat estrous cycle would seem appropriate. Many similarities appear to exist between alterations in steroid concentrations in blood at the time of puberty and those changes noted previously in normal adult cycling rats.

Estradiol concentrations have been found to rise in both ovarian vein blood (Hori et al., 1968; Shaikh, 1971) and peripheral blood (Butcher et al., 1974) beginning on diestrus II (of a four day estrous cycle) and reach peak values in the morning of proestrus. The increasing titer of blood estradiol

correlates well with the increase in uterine weight and uterine fluid accumulation (ballooning) which precede ovulation in both PMSG-primed and adult female rats (Schwartz, 1969). Similar increasing levels of estradiol and elevated uterine weight with fluid accumulation (Figures 13 and 11) were found to occur in both peripubertal control and PMSG-primed immature female rats in this study.

17-Hydroxyprogesterone (Shaikh and Shaikh, 1975) and progesterone concentrations have been shown to be low on the morning of proestrus in the adult female rat, increasing only after the initiation of the preovulatory gonadotropin surge on the afternoon of proestrus (Uchida *et al.*, 1969; Barraclough *et al.*, 1971; Butcher *et al.*, 1974). Progesterone and 17-hydroxyprogesterone levels were also found to be elevated in both PMSG-primed (day 32) and in pubertal control female rats in this study (Figures 13 and 14). Serum estradiol levels declined throughout the afternoon and evening of proestrus in the adult female rat (Butcher *et al.*, 1974; Hori *et al.*, 1969; Shaikh, 1971) and 17-hydroxyprogesterone (Shaikh and Shaikh, 1975), estradiol and progesterone concentrations were lower on estrus than levels found on the PM of proestrus (Butcher *et al.*, 1974). A similar decline in serum estradiol on the PM of proestrus (day 32) and decrease in 17-hydroxyprogesterone, estradiol and progesterone on estrus (day 33) was documented in the PMSG-primed immature female rat (Fig. 13 and 15). Serum estradiol, 17-hydroxyprogesterone and progesterone concentrations did not fall dramatically during the time of ovulations in the group of untreated control rats at puberty (Fig. 13 and 14); however, this was probably due to the lack of synchrony among individual animals within the experimental group. A decline in levels of estradiol, 17-hydroxyprogesterone and progesterone was noted at age

39, however (Figures 13-15), a time at which most rats had already ovulated.

Blood levels of testosterone (Falvo et al., 1972; Dupon and Kim, 1973) and androstenedione (Dupon and Kim, 1973) have been shown to be highest on proestrus in the adult cycling female rat. These findings are in agreement with the pattern of secretions observed in the PMSG-primed female rat (Figures 16 and 17). Serum levels of both compounds were also elevated during non-synchronized ovulations in untreated control female rats at the time of natural puberty (Figures 16 and 17).

In the adult female rat (and other species), the ovary, through its steroid secretions, appears to induce the preovulatory surge of FSH and LH. Most experimental results tend to implicate estradiol as the steroid responsible for the preovulatory gonadotropin release (Schwartz, 1969). Ferin et al. (1969a) demonstrated that the administration of estradiol antibodies on diestrus II and proestrus could block ovulation in the adult female rat. Neill et al. (1974) found that the inhibitory effects of estradiol antiserum could be reversed by the concomitant administration of estrogen. Similar inhibition of ovulation (Ferin et al., 1969b) has also been observed in the PMSG-primed immature female rat: anti-estradiol antiserum, but not antiserum to testosterone or normal rabbit serum, prevented ovulation, which was reversible following the administration of stilbestrol, a synthetic estrogen. Others have also demonstrated the requirement for estradiol secretion prior to ovulation since administration of estrogen antagonists also blocked ovulation and the preovulatory surge of gonadotropins in the adult female rat (Shirley et al., 1968; Labhsetwar, 1970). The facilitative effects of estradiol on gonadotropin secretion have also been demonstrated in the monkey (Karsch et al., 1973) and in man (Yen et al., 1973).

Progesterone, another steroid studied extensively in relationship to gonadotropin secretion, appears to have a facilitative action on FSH/LH secretion only when administered to, or secreted in, an estrogen-primed animal (Everett, 1948; McPherson et al., in press). It is doubtful that progesterone plays a physiological role in the elaboration of the preovulatory surge of gonadotropins in the female rat since peripheral serum concentrations do not rise appreciably on proestrus prior to massive gonadotropin release (Butcher et al., 1974). In addition, administration of progesterone antiserum, unlike estradiol antiserum, has no effect on the timing of ovulation in adult female rats (Ferin et al., 1969a).

The site and mechanism of steroid (estradiol in particular) action on the secretion of gonadotropins is not clear. Both the anterior pituitary and hypothalamus have been implicated as sites of steroid feedback action on gonadotropin secretion (Weick et al., 1971; McCann et al., 1964). The facilitative or stimulatory action of estradiol appears to reside primarily in the anterior pituitary. Weick and Davidson (1970) observed that the implantation of estradiol pellets in the anterior pituitary of rats two days in advance of ovulation (on diestrus II) caused a premature surge of gonadotropins. Further work indicated that estradiol probably acts by sensitizing the anterior pituitary to hypothalamic stimulation (Weick et al., 1971; Aiyer and Fink, 1974). This theory of sensitization has received further verification by the studies of Greeley et al., (1974). They observed that estradiol treatment of castrated female rats in which the anterior pituitary had been removed from hypothalamic influence, potentiated the response of FSH and LH to exogenous LH-RH. Although the previously mentioned studies indicate that estradiol may act at the level of the an-

terior pituitary, the role of the hypothalamus in the induction of the preovulatory gonadotropin surge cannot be overlooked.

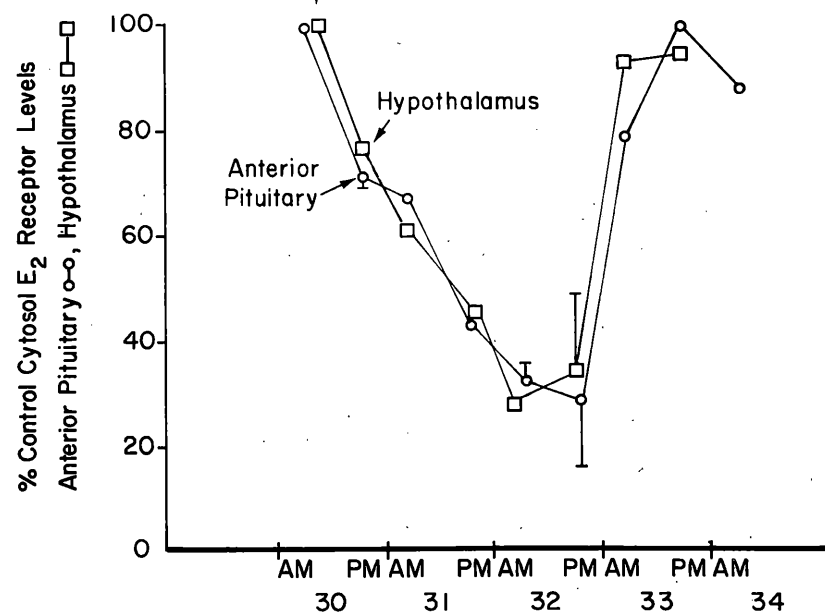
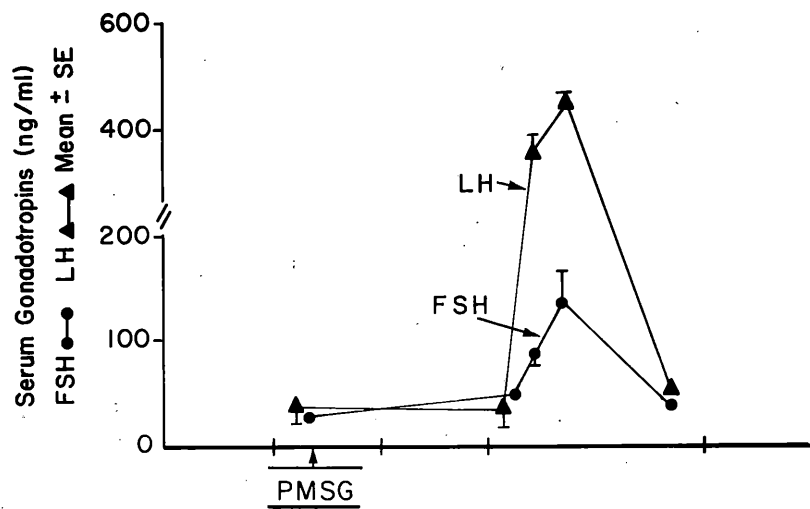
Several investigators have reported on the presence of luteinizing hormone releasing factor (LH-RH) in the hypothalamus. These studies have shown that a depletion of hypothalamic content of LH-RH precedes the preovulatory surge of FSH/LH on proestrus in the adult female rat (Wheaton and Fawcett, 1974; Eskay et al., 1974; Ramirez and Sawyer, 1965). Administration of pentobarbital on the afternoon of proestrus blocks the surge of gonadotropins and thus ovulation by preventing the depletion of hypothalamic LH-RH (Meyer et al., 1974).

The finding of cyclic fluctuations in anterior pituitary and hypothalamic estradiol receptors in the PMSG-primed female rat suggested a possible relationship of the receptor to both serum estradiol and gonadotropins. There seemed to exist a temporal relationship between fluctuations of serum estradiol (Figure 13), serum levels of FSH and LH (Table XVI), and the depletion/replenishment of cytosol estradiol receptors (Tables XVII, XVIII). As estradiol titers rose in blood after PMSG administration (Figure 13), cytoplasmic estradiol receptors of the anterior pituitary and hypothalamus were progressively depleted (Tables XVII, XVIII). When depletion of receptors was maximal (day 32) the preovulatory surge of FSH/LH occurred (Table XVI). On estrus, day 33, serum estradiol levels declined significantly (Figure 13), cytosol estradiol receptors of the anterior pituitary and hypothalamus were replenished (Tables XVII, XVIII), and the preovulatory gonadotropin surge was completed. The temporal relationship between depletion and replenishment of cytosol estradiol receptors and serum gonadotropin levels in the PMSG-primed immature female rat is demonstrated more clearly

in Figure 20. The initial depletion of cytosol estradiol evident as early as 8 hours after PMSG treatment, was dependent upon the presence (and presumably the secretory response) of the ovary; in rats ovariectomized prior to PMSG administration there was no decrease in cytosol estradiol receptor concentration of either tissue. The studies of Cidlowski and Muldoon (1974) have demonstrated that in ovariectomized female rats, the administration of estradiol causes a rapid depletion in estradiol receptors of anterior pituitary, hypothalamus and uterus. The time required for restoration of cytosol estradiol binding capacity in these tissues was approximately 15-20 hours after estradiol treatment. This interval between depletion and replenishment correlates well with the replenishment in anterior pituitary and hypothalamus on estrus (day 33) in PMSG-primed female rats following maximal depletion at 32 days of age.

Following the initial precocious ovulation induced by PMSG at 33 days of age, the rats began regular estrous cycles. Three days later, at 36 days of age, most rats were in proestrus again (as on day 32) at which time a significant increase ($p < 0.01$) in serum estradiol levels (Figure 13), uterine ballooning and significant depletion ($p < 0.01$) of the pituitary cytosol estradiol receptor concentrations (Table XVII) were documented. Many female rats then ovulated the next day, age 37 days, as evidenced by oviductal ova, fresh corpora lutea and vaginal estrus. These studies demonstrated a temporal relationship between preovulatory increases in serum estradiol, depletion of estradiol receptors of the hypothalamic-pituitary complex and the ensuing preovulatory gonadotropin surge required for ovulation. They further demonstrated that in those rats in which regular estrous cycles follow precocious ovulation (induced by PMSG), a cyclic depletion/

Figure 20. Correlative Changes in Serum Gonadotropins and Cytosol Estradiol Receptor Concentrations of the Anterior Pituitary and Hypothalamus Following PMSG Administration to 30 Day old Immature Female Rats



replenishment of cytosol estradiol receptors occurred.

Cyclic changes have been previously demonstrated for levels of uterine estradiol receptors during the estrous cycle of the rat. The fluctuations observed appear to be related to changing levels of estradiol in blood. DeHertogh et al. (1971) found an increase in total uterine uptake of radio-labeled estradiol after in vivo administration, on proestrus. In vitro studies show that cytosol estradiol receptors of the uterus are depleted on proestrus (Lee and Jacobson, 1971). In addition, Greeley et al. (1974b) found that cytosol receptors of the anterior pituitary and hypothalamus were depleted on proestrus, a time of enhanced response to exogenous LH-RH, and were replenished on estrus in adult female rats. The apparent discrepancy between results obtained in vitro and in vivo arises from technical differences which may be reconciled. Jensen et al. (1968) and also Giannopoulos and Gorski (1971) have shown that, after estradiol interacts with its cytoplasmic receptor in vivo, the receptor-estradiol complex is transported to the nucleus, thus rapidly decreasing the available binding sites in the cytoplasm. It has also been shown that estradiol stimulates the replenishment of its own receptor (Sarff and Gorski, 1971; Cidlowski and Muldoon, 1974). Thus, if receptor utilization and translocation to the nucleus (in vivo) occurs at a rate in excess of receptor replenishment (as may be the case when estradiol levels are rising in blood) there will be a net decrease in available estradiol receptors in the cytosol (in vitro) although an increased total tissue uptake (in vivo) may occur.

The depletion of cytosol estradiol receptors in target tissues of the adult female rat correlates well with the findings in the PMSG-primed immature female rat on proestrus, (day 32 and 36, as shown in Figure 20 and

Table XVII). These findings, in addition to the previously established temporal relationship between peripheral serum estradiol and the preovulatory gonadotropin surge in immature and adult female rats, strengthen the hypothesis that facilitative estradiol action on gonadotropin secretion may be modulated by an initial interaction with cytosol receptors of the anterior pituitary and/or hypothalamus. The exact biochemical nature of estradiol action in the anterior pituitary and hypothalamus are however, still undocumented at the present.

STUDIES IN THE DHA-TREATED IMMATURE FEMALE RAT

Consistent with the findings of others (Black and Mahesh, 1969; Knudsen, 1974), DHA was found to induce precocious puberty in immature female rats. Treatment with DHA (age 27-29) plus PMSG (age 30) yielded a high percentage of ovulation which occurred primarily at 30 days of age. Treatment of such rats with DHA alone was also capable of inducing precocious puberty in immature female rats, but ovulation was less synchronized, occurring from 30-32 days of age. During the three days of DHA treatment (age 27-29), uterine weight increased progressively, reaching peak proportions at 29 days of age (Figure 11) when uterine ballooning was evident. Similar preovulatory increases in uterine weight were previously demonstrated in control female rats undergoing natural puberty as well as in PMSG-primed female rats (Figure 11). Unlike the control and PMSG-primed female rats, uterine weight in rats treated with DHA plus PMSG did not decline on the day of estrus (day 30) as shown in Figure 10. The first increment in ovarian weight occurred at 30 days of age (Figure 12). Ovarian weight then rose progressively to reach peak values at 32 days of age, at which time numerous corpora lutea were present in the majority of animals. A similar increase in ovarian weight, coinciding with corpus luteum formation was

found in the rats treated with PMSG only at 33 and 34 days of age (Figure 12).

Changes in blood steroids, as the result of DHA plus PMSG treatment of immature female rats, were also evaluated. Within three hours after the first DHA injections (at 27 days of age) serum levels of estradiol (Figure 13), testosterone (Figure 16), androstenedione (Figure 17), DHT (Figure 18) and DHA (Figure 19) were significantly elevated ($p < 0.01$) when compared to vehicle-treated controls sacrificed at 27 days of age. Serum levels of progesterone (Figure 13) and 17-hydroxyprogesterone (Figure 15) were, however, not affected by DHA treatment and remained within normal limits at 27 days of age. Serum concentrations of DHA, DHT, testosterone, androstenedione and estradiol remained significantly elevated from 27-29 days of age during DHA administration; serum levels of progesterone and 17-hydroxyprogesterone increased only at 29 days of age. The preovulatory elevation of these steroids in the DHA-treated rat is similar (although different in absolute quantities) to that documented in the PMSG-primed immature female rat at 30-32 days of life, prior to ovulations at 33 days of age. While the marked elevations of steroids in the latter experimental model arose due to the trophic action of PMSG on the ovary, elevated serum levels of estradiol, testosterone, androstenedione, DHT and DHA were undoubtedly the result of metabolic conversion of DHA rather than induced ovarian activity at 27 and 28 days of life. Prior studies have demonstrated that DHA may be converted readily to other androgens as well as to estrogens (Knapstein, *et al.*, 1968; Weisz and Gibbs, 1974; Naftolin *et al.*, 1971; Kalvert and Block, 1968). In addition, the uterotrophic action of DHA has been found to be dependent upon metabolic conversion of DHA itself (Knudsen,

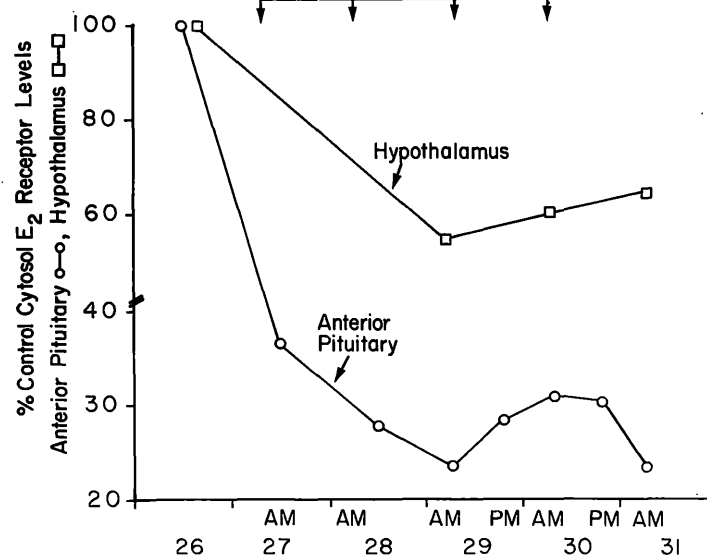
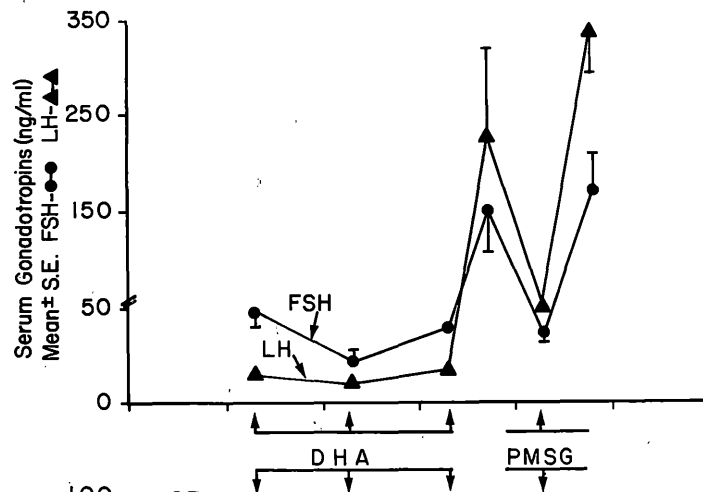
1974). In support of this hypothesis that elevations of estradiol, androstenedione, testosterone and DHT arose from metabolism of DHA instead of ovarian secretion is the finding that serum levels of progesterone (Figure 14) and 17-hydroxyprogesterone (Figure 15), both of which are not by-products of DHA metabolism but which were increased after PMSG treatment, were not initially elevated during DHA administration. Following the last injection of DHA at 29 days of life, serum levels of DHA as well as concentrations of testosterone, androstenedione, DHT and estradiol (Figures 13, 16-19) declined dramatically, further implicating DHA as the principal source of the latter compounds.

Following PMSG administration to rats previously treated with DHA, serum levels of progesterone (Figure 14) and 17-hydroxyprogesterone (Figure 15) were elevated on the PM of day 30, as was the case in rats receiving PMSG treatment only. The rapid decline in serum concentrations of DHA and its metabolites was slowed by the injection of PMSG, with serum estradiol and androstenedione actually increasing on the PM of day 30.

Preovulatory elevations in serum steroids (day 27-29) in the DHA-treated rat were consistent with findings in the other experimental models discussed so far. It would seem that the increased levels of steroids in the DHA-treated female rat were probably responsible for the precocious ovulation observed at 30 days of age via some facilitative feedback action on gonadotropin secretion. Although PMSG was found to have some steroidogenic actions in rats primed with DHA (e.g., estradiol, progesterone, 17-hydroxyprogesterone), the ovulations which occurred at 30 days of age were probably only synchronized by PMSG administration in which PMSG acted in concert with endogenously released gonadotropins (Table XXIII).

As in the previously presented experimental models (normal puberty and PMSG induced precocious ovulation), a temporal relationship between elevated serum steroids and the preovulatory gonadotropin surge was found in the DHA-treated female rat. In addition, a depletion of hypothalamic and anterior pituitary cytosol estradiol receptors, which coincided with increased serum estradiol titers, preceded ovulation. In Figure 21, the relationship between cytosol receptor depletion and serum FSH/LH levels in the DHA plus PMSG-treated rat is documented. A progressive decline in cytosol estradiol receptors in the anterior pituitary occurred on day 27 and 28 and significant depletion of the anterior pituitary and hypothalamus was detected at 29 days of age. On the evening of day 29 and day 30, serum levels of FSH and LH were significantly increased. This depletion of cytosol estradiol receptors and the subsequent preovulatory surge of gonadotropins seen in the androgen-treated female rat (Figure 21) is almost identical to that previously shown to occur in the PMSG-primed immature female rat (Figure 20). A major difference is apparent thereafter between the two experimental models however. Cytosol estradiol receptor content of the anterior pituitary and hypothalamus were replenished on estrus (day 33) in PMSG-treated female rats (Figure 20) while there was no restoration of cytosol estradiol receptor concentrations on day 30 or 31 in rats treated with DHA plus PMSG (Figure 21). The failure of the latter group to replenish cytosol estradiol receptors was probably due to the fact that serum levels of estradiol (Figure 13) remained elevated at 30 and 31 days of age in contrast to the dramatic decline in peripheral estradiol concentrations on estrus (day 33) in the PMSG-primed rats (Figure 13).

Figure 21. Correlative Changes in Serum Gonadotropins and Cytosol Estradiol Receptor Concentrations of the Anterior Pituitary and Hypothalamus Following DHA (Day 27-29) Plus PMSG (30 Days of Age) Treatment of Immature Female Rats



Studies in the Immature Female Rat Following Induction of Ovulation with Short-Term DHA Plus PMSG

Because of the immediate post-ovulatory period (31-40 days of age) following short-term DHA (age 27-29) plus PMSG (age 30) had not been evaluated in the past, this interval was studied and compared to that in PMSG-primed female rats. As previously pointed out, female rats primed with PMSG began normal estrous cycles following the initial precocious ovulation at 33 days of age. In contrast to these findings, rats treated with DHA plus PMSG became acyclic after the early onset of puberty. In an effort to develop an understanding for this ovulatory failure, rats were studied extensively during the period of androgen withdrawal. As an additional comparison, another group of rats were treated with DHA plus PMSG and then treated daily (age 31-40) with additional DHA.

It was found that neither group of DHA-treated rats ovulated or displayed cyclic vaginal smear changes associated with normal estrous cycles as found in rats treated with PMSG only. From 32-40 days of age, the majority of female rats (85%) were in constant diestrus; the remainder were found to have constant estrus vaginal patterns. No differences were noted with respect to vaginal smear patterns which could be related to the differences in treatment during this time (discontinued DHA or further androgen administration). Since special precautions had been taken to avoid mechanical induction of pseudopregnancy, it was felt that the constant vaginal cytology, primarily diestrus, was due to some other factor.

Ovaries of rats treated continuously with DHA (31-40 days of age) were found to be heavier than those of rats during androgen withdrawal (Table XXV). Ovarian weights of both groups were heaviest at 31-32 days of age, declining slowly thereafter (Figure 12). No regression of corpora lutea

was evident in rats receiving continued DHA treatment and only minimal regression was noted in rats in which DHA has been discontinued.

Uterine weights were elevated (approximately 200 mg/100 g B.W.) in both groups at 32 days of age but began to decline slowly in rats receiving no further DHA administration. The decline in uterine weight was noticeably retarded as compared to that found beginning at 32 days of age in rats treated with PMSG only (Figure 11).

It was felt that patterns of peripheral steroids in female rats during androgen withdrawal might reveal clues as to why these animals failed to cycle after the initial ovulation at 30 days of age. By 31 days of age, a significant ($p < 0.01$) decrease in serum levels of androgens (DHA, DHT, testosterone, androstenedione) had occurred in those rats as compared to the group treated with DHA (31-40 days of age) as shown in Table XXVII. By 35 days of age, serum levels of testosterone and DHT had declined to concentrations equivalent to those found after ovulation in PMSG-treated rats. At this time, DHA was still significantly higher in blood (1.3 ng/ml) than levels found before and after ovulation in rats primed with PMSG only. In fact, serum DHA concentrations did not return to basal levels (.3 ng/ml) until 39 days of age, 10 days after the last DHA injection. Serum levels of androstenedione at 35 days of age were less than levels at 27-29 during DHA treatment but were still significantly higher than concentrations prior to ovulation in vehicle-treated controls. Insufficient evidence exists from these evaluations to determine at what stage androstenedione concentrations did reach basal levels.

Estradiol levels were significantly depressed by 33 days of age as compared to rats in which DHA treatment was continued; however basal estro-

diol concentrations (levels found on days other than proestrus) were not attained until 37 days of age (Table XXVIII). It should be noted that although serum levels of estradiol were low, female rats did not begin to cycle at this time during the androgen withdrawal period. Serum levels of progesterone were high (in excess of 10 ng/ml) after ovulation from 31-37 days of age in female rats during androgen withdrawal as well as in female rats treated continuously with DHA (31-40 days of age). The sustained elevations in serum progesterone are compatible with the predominant finding of constant diestrus vaginal smears and morphological appearance of the ovary in most rats (enlarged with numerous corpora lutea). Progesterone concentrations did begin to decline at 35 days of age in rats in which DHA had been discontinued. However, concentrations at 39 days of age were still in excess of levels found on estrus in PMSG-primed female rats (Figure 13). When compared to the majority of rats in constant diestrus at all ages (31-39) progesterone levels in the small number of constant estrus rats (mixed ages) were significantly lower ($p < 0.01$) as shown in Table XXVIII. 17-hydroxyprogesterone concentrations were maintained by continued DHA treatment whereas a significant decline was noted in rats during androgen withdrawal by 35 days of age (Table XXVIII). The fluctuations of these steroids during androgen withdrawal are depicted in Figures 13-19 and may be compared to the other experimental groups more readily by examination of these graphs.

The chronically elevated serum levels of progesterone and 17-hydroxyprogesterone in rats treated chronically with DHA are consistent with findings in pseudopregnant female rats (constant diestrus) reported by Bartosik and Szarowski (1973). Knudsen (1974) has reported that prolactin levels are elevated during chronic DHA administration. Previous studies have demonstrated

that prolactin is luteotrophic in the rat (MacDonald et al., 1970) and that prolactin is increased during pseudopregnancy in adult female rats (Freeman et al., 1974). Since serum DHA levels were still elevated as late as eight days after the last injection of DHA at 29 days of life, it is possible that the prolonged elevations in peripheral blood of DHA may act to maintain corpus luteum function in rats after DHA withdrawal (as evidenced by sustained elevations in serum progesterone). A previous report (Singer et al., 1971) has demonstrated that DHA administration to female rats, following induction of ovulation with PMSG plus HCG (human chorionic gonadotropin), resulted in ovarian enlargement and apparent increased luteal biosynthetic activity. They proposed that the observed ovarian stimulation was due to prolactin secretion under the influence of DHA. This finding is in agreement with the report of Knudsen (1974) and also the observations on steroid secretions during the post-ovulatory period in this dissertation.

Black (1969) and Knudsen (1974) have both concluded that prolonged DHA treatment (after the induction of precocious ovulation) results in a suppressed secretion of LH. In the present investigation, it was found that serum levels of LH were suppressed during the post-ovulatory period in rats treated with DHA plus PMSG (plus or minus additional DHA administration). At 37 days of age, serum LH was found to be less than 10 ng/ml in both groups; LH was however slightly higher in rats receiving no additional DHA administration. It should be recalled that by 37 days of age, estradiol concentrations were within normal limits during androgen withdrawal. At 39 days of age, when serum levels of DHA and estradiol were low, in addition to the declining progesterone levels, LH levels were significantly higher than that found in rats during continued DHA treatment (Table XXIX). A

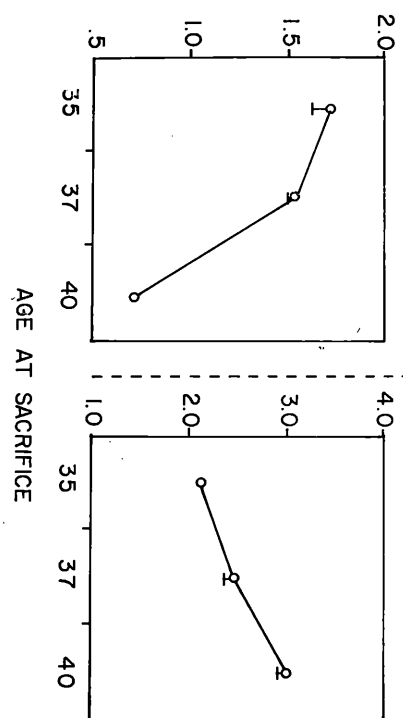
similar relationship was found to exist at 40 days of age with respect to serum LH levels. FSH concentrations were significantly higher in rats during continued DHA administration at 37 days of age, but not at 39 and 40 days of age due to rising serum levels of FSH in those rats receiving no DHA treatment (Table XXIX).

Cytosol estradiol receptor levels of the anterior pituitary were depressed at 32 days of age, similar to preovulatory concentrations at 29 days of age (Table XXX). As previously mentioned, there was no immediate replenishment of receptor concentrations of the anterior pituitary or hypothalamus at the time of ovulation in DHA plus PMSG-treated rats; replenishment was found to occur in rats receiving PMSG only at the time of ovulation. No statistically significant differences were detected in cytosol estradiol receptor content of the anterior pituitary of rats treated with DHA plus PMSG or those receiving additional DHA treatment (31-40 days of age) until 34 days of age. From this day onward, a gradual replenishment of receptors was noted in rats receiving no further DHA treatment; further depletion of anterior pituitary estradiol receptors occurred in chronic DHA-treated rats. As noted earlier, LH levels were depressed after the precocious ovulation and did not begin to increase until serum levels of previously elevated steroids were normalized.

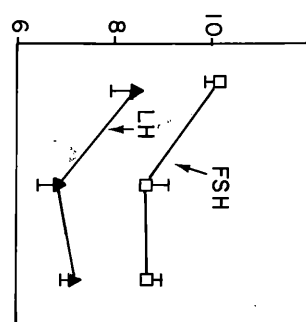
A temporal relationship between estradiol receptor replenishment and basal (AM) LH levels was apparent in this study. This association is graphically presented in Figure 22. As cytosol receptor levels of the anterior pituitary were replenished (during androgen withdrawal) serum LH concentrations rose; as cytosol estradiol receptor levels remained low, depleting even further, a decline in serum LH was noted in rats treated with DHA

Figure 22. Correlative Changes in Serum Gonadotropin Concentrations and Cytosol Estradiol Receptor Levels in the Anterior Pituitary of Female Rats Induced to Ovulate with DHA Plus PMSG Treatment As Determined During DHA Withdrawal or Continued DHA Administration (Age 31-39).

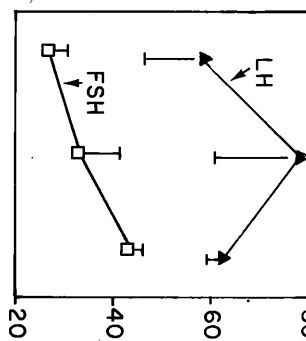
Cytosol E_2 Receptor Content of
Anterior Pituitary, \circ $\times 10^{-14}$ Mean \pm SE
(Specific Bound moles $^3H-E_2$ /mg
cytosol protein)



Serum Gonadotropin Levels
Ng/ml LH, \blacktriangle Mean \pm SE



Continued DHA Treatment



DHA Treatment Stopped
(29 Days of Age)

Serum Gonadotropin Levels
Ng/ml FSH, \square Mean \pm SE

(31-40 days of age). Although hypothalamic receptor levels were not documented in this study, it is anticipated that fluctuations in this tissue paralleled that of the anterior pituitary.

Studies in the Immature Female Rat During and After Chronic DHA Administration

Chronic DHA treatment has previously been shown to cause ovulatory failure and development of polycystic ovaries in immature (Knudsen, 1974; Black, 1969) and mature female rats (Roy *et al.*, 1962). The findings of ovulatory failure and formation of polycystic ovaries in this investigation are consistent with the report of Knudsen (1974) and Black (1969). Chronic DHA treatment (as with short-term DHA plus PMSG) resulted in precocious ovulation (day 30-32) in immature female rats. Following this induction of puberty, female rats experienced ovulatory failure as evidenced by constant vaginal cytology (75% constant diestrus, 25% constant estrus). At 46 days of age, ovarian cysts were found in rats exhibiting both types of vaginal cytology.

Ovarian and uterine weights were significantly higher at 32 and 34 days of age in DHA-treated rats than in controls (vehicle-treated) of the same age (Table XXXI). By 46 days of age, ovarian weights of constant diestrus rats were significantly heavier than those of constant estrus rats (Table XXXI). There was no statistically significant difference in uterine weights of either group. As demonstrated in rats pretreated with DHA plus PMSG (Table XXVIII), progesterone and 17-hydroxyprogesterone concentrations were significantly higher after ovulation (day 32) than serum concentrations of vehicle-treated controls (prepubertal), as shown in Table XXXIII. Progesterone and 17-hydroxyprogesterone concentrations remained elevated through-

out the DHA treatment period (27-46 days of age). At 46 days of age, serum progesterone levels were significantly higher in constant diestrus rats (who had heavier ovaries with many corpora lutea) than in constant estrus rats (smaller ovaries which appeared to be devoid of corpora lutea). Serum levels of all steroids were elevated during the DHA treatment period (Tables XXXII and XXXIII) and were roughly equivalent at 46 days of age to levels noted previously from age 31-39 in rats treated with DHA following DHA plus PMSG priming (Tables XXVII and XXVIII) in another experiment.

As mentioned in the introduction, Knudsen (1974) concluded that the sensitivity of the anterior pituitary to tropic influence was not impaired by chronic DHA treatment. However, since the dosage of LH-RH used to corroborate this hypothesis was high (100 ng), it was felt that impairment or lack thereof in pituitary responsiveness to LH-RH would best be identified with a lower dosage. Two to three hours after DHA administration (8:30 - 9:00 AM) it was found that serum levels of LH were equivalent to controls at 27 days of age, but were substantially lower at 34 days of age in the DHA-treated group (Table XXXIV). There was no detectable increase in LH or FSH 30 minutes after a low dose of LH-RH (10 ng/100 g BW) in DHA-treated rats at 27 or 34 days of age. A significant increase in serum LH occurred at 27 and 34 days of age in control rats; FSH levels were also increased but the increment noted at 34 days of age was not statistically significant. At 46 days of age, basal LH levels were low in the DHA-treated rats and neither LH nor FSH responded to LH-RH administration. From this evaluation, it is apparent that at a low dose of LH-RH, significant differences do exist between the pituitary responsiveness of DHA-treated and untreated immature female rats. A complete comparison of these findings to that of Knudsen

(1974) is difficult because he did not concomitantly assess the responsiveness of control female rats to 100 ng LH-RH.

With discontinuation of chronic DHA treatment, female rats resumed normal ovulatory estrous cycles as has been graphically demonstrated in Figure 10. Although the return to normalcy was not a synchronized event within the experimental group, the interval between DHA withdrawal and resumption of estrous cycles was calculated to be 9.3 ± 2.7 days for the experimental group. Because of the lack of synchrony, animals sacrificed on selected days during the post-DHA treatment period were in various stages of the estrous cycle. For this reason, no attempt was made to relate steroid hormone levels to a particular stage of the estrous cycle during the period of androgen withdrawal. In addition, it was felt that a more meaningful analysis of the androgen withdrawal period could best be performed by comparing, on a day to day basis, clearance of steroids arising from DHA administration to the other parameters quantified (i.e., serum gonadotropins, cytosol estradiol receptors, ovarian and uterine weights).

A slow decline in peripheral serum concentrations of DHA was detected from 48-66 days of age after the last DHA injection at 46 days of age (Figure 19). Serum concentrations of testosterone (Figure 16) and DHT (Figure 18) appeared to decline at a faster rate whereas androstenedione levels declined initially at 48 and 54 days of age but no further significant fluctuations occurred from 54-66 days of age. Serum levels of estradiol (Figure 13) were also elevated for a protracted period of time as compared to testosterone and DHT concentrations. It is possible that endogenous secretion of DHA, androstenedione and estradiol by the cystic ovaries could have contributed to the retarded decline in serum of these

steroids. Evidence for substantial endogenous steroid synthesis and secretion was demonstrated by the significant elevations in serum levels of progesterone and 17-hydroxyprogesterone before and for several days after DHA withdrawal at 46 days of age (Figures 14, 15). Previous in vivo studies in the human female with polycystic ovarian disease (Figure 6) have indicated that cystic ovaries are extremely active in steroid secretions. Studies in the female rat treated neonatally with testosterone (Weisz and Lloyd, 1965; Rosner et al., 1969; Falvo et al., 1972) also demonstrate that cystic ovaries are biosynthetically active. The sustained elevation in uterine weights from 46-54 days of age is further indication of prolonged exposure of this tissue to high serum titers of estradiol and other steroids.

At 58 days of age (12 days after the last DHA injection) serum levels of estradiol (Figure 13) and progesterone (Figure 14) were significantly lower than concentrations at 48 days of age; uterine weight was also significantly reduced at this time (Figure 11). The number of days required for "normalization" of peripheral steroid concentrations correlated well with the length of time required for resumption of estrous cycles in these rats. This suggests that one or a number of the elevated serum steroids had been exerting an inhibitory influence on the hypothalamic-pituitary complex, preventing cyclic secretion of adequate amounts of FSH and LH required for ovulation.

Serum concentrations of LH were found to be suppressed by chronic DHA treatment as predicted from the studies of Black (1969) and demonstrated by Knudsen (1974). Similar suppression of serum LH had been found during continued DHA administration (age 31-39) of rats induced to ovulate by DHA (27-29 days of age) plus PMSG at 30 days of age (Table XXIX). Twenty days

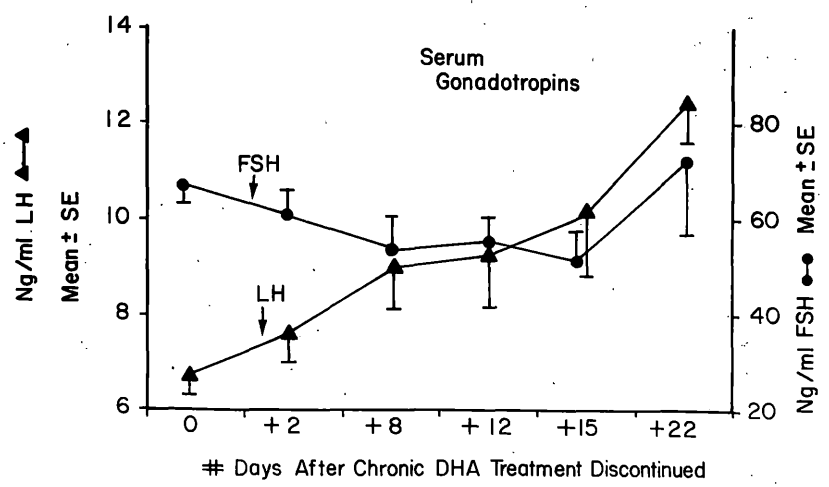
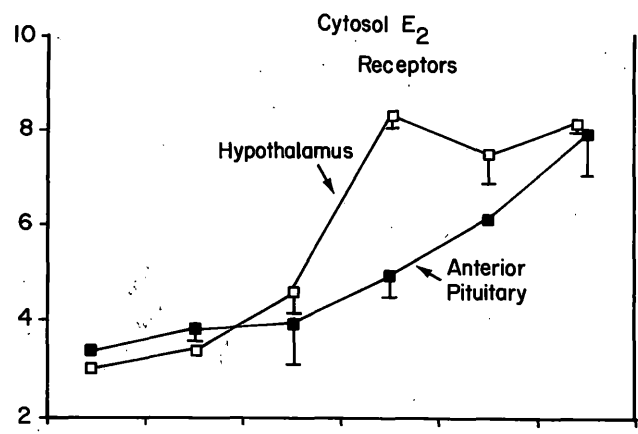
after chronic DHA administration, serum LH was low (46 days of age) while FSH levels were normal or slightly elevated at this time. A noticeable gradual increase in serum levels of LH occurred from 0-8 days after the last DHA injection; FSH levels declined slightly during this time (Table XXXV). By the morning of day 61, a significant increase in serum LH had occurred and at 66 days of age serum levels of FSH and LH were significantly higher than those found at 46 days of age.

Associated with the decreasing titers of peripheral steroids and perhaps related to the observed pattern of gonadotropin secretion was a gradual replenishment of cytosol estradiol receptor levels of the anterior pituitary and hypothalamus (Table XXXVI). The temporal relationship of cytosol estradiol receptor replenishment and serum levels of FSH and LH is demonstrated graphically in Figure 23. In this regard, it should be noted that the first significant increase in estradiol binding capacity of the anterior pituitary and hypothalamus was detected at 58 days of age. From 61 days of age onward, receptor concentrations of the anterior pituitary and hypothalamus were equivalent to those reported by Cidlowski and Muldoon (1974) in intact adult female rats, irrespective of estrous cycle day. These studies suggest that a significant temporal correlation exists between the decline in previously elevated serum steroids, replenishment of cytosol estradiol receptor content, normalization of serum LH and FSH, and resumption of regular estrous cycles in previously anovulatory female rats.

In rats treated continuously with DHA (10 or 20 days), there appeared to exist differential effects of DHA administration on serum levels of FSH as opposed to LH during the period of ovulatory failure. LH was chronically suppressed whereas FSH was normal or slightly elevated as previously reported

Figure 23. Correlative Changes in Serum Gonadotropin Levels and Cytosol Estradiol Receptor Concentrations of the Anterior Pituitary and Hypothalamus of Female Rats During Androgen Withdrawal After Chronic (20 Days) Administration of DHA.

Specific Bound Moles $^3\text{H-E}_2$ /mg Cytosol Protein
 Anterior Pituitary, $\times 10^{-14}$ \blacksquare Hypothalamus, $\times 10^{15}$



by Knudsen (1974). No unimpeachable explanation for these findings is possible at the present; however, the findings of other investigators lends support to these data. Histological and biochemical evidence has been provided by the work of Costoff (1973) and Steinberger et al. (1973) which demonstrates that two different cell types exist in the rat anterior pituitary which synthesize and secrete gonadotropins, one for FSH and another for LH. In addition, Costoff et al. (1974) found that in rats treated with PMSG at 30 days of age, a degranulation of LH cells of the anterior pituitary occurred beginning early on the afternoon of day 32. Concomitant with this degranulation, serum levels of LH increased dramatically. A degranulation of FSH cells occurred later on the afternoon of day 32 which also coincided with an increase in serum levels of FSH at that time. Additional evidence for the possibility that FSH and LH may be regulated by separate control mechanisms has come from the work of McCann et al. (1968) who found two hypophysiotropic substances in the hypothalamus, one eliciting primarily LH secretion and the other causing secretion of FSH.

In a different type of study, various investigators have noted that following castration of immature and mature female (and male) rats, a significant elevation of serum FSH and LH occurs. Replacement therapy with androgens, estrogens, and estrogens plus progesterone in castrated female rats has revealed that the post castrational rise in serum LH is more readily suppressed than that of FSH. At high doses of replacement steroids, an increased secretion of FSH occurs at a dosage that completely suppressed serum LH (Eldridge et al., 1974 a; Eldridge et al., 1974 b; McPherson et al., 1974; McPherson et al., in press, 1975).

These reports, in addition to that presented herein, suggest that the

secretion of FSH and LH in the female rat may be under separate control mechanisms and that the suppression of LH at a time of normal or enhanced FSH secretion may arise due to differential sensitivities of these control mechanisms to steroid hormone feedback at the anterior pituitary, hypothalamus and/or higher brain centers. The particular steroid (or steroids) responsible for the aberrant, tonic secretion of FSH and LH in long-term DHA-treated rats cannot be specifically identified from these experiments. A significant elevation of several compounds was documented during DHA administration, much of which probably arose from metabolism of DHA itself. A chronic depletion of cytosol estradiol receptors of the anterior pituitary and hypothalamus was noted in these studies in association with tonically elevated serum steroids and acyclic, abnormal gonadotropin secretion. Although high serum concentrations of estradiol alone can cause acute depletion of cytosol estradiol receptors (Cidlowski and Muldoon, 1974; Giannopoulos and Gorski, 1971), the chronic depression of cytosol estradiol receptors may arise through two separate circumstances. Continual utilization of available cytosol receptor and translocation to the nucleus as well as failure to replenish cytosol estradiol receptors in the cytoplasm can both cause chronic depletion of receptors as measured in vitro.

In addition to elevated serum levels of estradiol, there was significant elevation of androgens and progesterone (in most animals) during long-term DHA administration. Studies in other laboratories as well as in our department indicate that steroids other than estradiol may modify the interaction of estradiol with its cytoplasmic receptor. Ruh et al. (1975), have shown that DHT, a steroid found in high quantities following DHA treatment could cause depletion of uterine cytosol estradiol receptors. This was ac-

complished through a translocation of estradiol-free estradiol receptor to the nucleus. Korach and Muldoon (in press, 1975) have studied the interaction of DHT with the estradiol receptor in vitro and found that it can bind to low affinity sites on the estradiol receptor and impede the formation of the estradiol-receptor complex in the cytosol. Another study has shown that progesterone could block certain stimulatory effects of estradiol on oviductal growth in adult monkeys (Brenner et al., 1974). This inhibition was found to be associated with a depression of estradiol binding capacity of estradiol receptors in the cytosol of the oviduct. There was no demonstration of direct competition of progesterone for estradiol binding sites on the receptor and therefore the depletion may have been due to an inhibition of receptor synthesis (replenishment).

These studies point out the potential importance of steroid hormones, in addition to estradiol, which are present in peripheral blood of normal and experimentally altered female rats. It also appears that compounds other than estradiol alone should be quantified when one evaluates the relationship between steroids and gonadotropin secretion. As further studies are completed which evaluate the relationship between steroids (and perhaps other hormones) and the estradiol receptor, a more precise understanding of mechanisms through which steroids modulate the secretion of gonadotropins may be forthcoming.

VI. SUMMARY

Multiple steroid radioimmunoassay methods, which combined the use of a very reliable celite column chromatographic separation and reasonably specific antisera, two of which were developed in this laboratory, were perfected. With these techniques, it was possible to simultaneously quantify as many as seven steroids following an ether extraction of a single serum (or plasma) sample. These methods, which were shown to yield results comparable to that achieved by single steroid assays, are superior to other techniques currently employed for the analysis of several steroid hormones in a single blood sample. In addition, the simplicity, sensitivity and reproducibility of the multiple steroid radioimmunoassay method make this technique equally applicable to routine as well as experimental analyses.

Studies conducted in the untreated immature female rat revealed that puberty, which may occur from 36-40 days of age in Holtzman rats, is preceded by a significant increase in serum estradiol levels as well as other steroid hormones including testosterone, androstenedione, progesterone and 17-hydroxyprogesterone. In conjunction with increasing titers of these steroids, a significant decrease in cytosol estradiol receptor levels of the anterior pituitary and hypothalamus occurred prior to the expected preovulatory release of anterior pituitary gonadotropins, FSH and LH.

In a more synchronized animal model, the PMSG-primed immature female rat, a similar temporal relationship was found to occur before ovulation at 33 days of age, 3 to 7 days before natural puberty. PMSG treatment resulted in an acute increase in serum levels of estradiol, progesterone, 17-hydroxyprogesterone, androstenedione and testosterone within 8 hours. Secretion of estradiol and its immediate precursor, testosterone, was sustained through

the morning of day 32 (proestrus). A progressive depletion of cytosol estradiol receptors of the anterior pituitary and hypothalamus paralleled the increasing titers of serum estradiol. When maximal depletion was reached, a preovulatory discharge of FSH and LH occurred which evoked a dramatic but transient increase in blood levels of each steroid assayed except estradiol on the evening of day 32. Day 33, estrus, was characterized by a replenishment of cytosol receptors and a decline in serum levels of all steroids. The cycle of increased serum estradiol and cytosol estradiol receptor depletion was repeated at 36 days of age (proestrus) prior to a second day of estrus at 37 days of age, at which time serum estradiol declined and receptors were again replenished.

Changes in steroid hormones, cytosol estradiol receptors and gonadotropin levels in the DHA-treated immature female rat prior to precocious ovulation were very similar to those demonstrated during normal puberty and PMSG induced ovulation. A significant increase in serum levels of estradiol, testosterone, androstenedione, DHA and DHA was found within two hours after DHA administration to 27 day old female rats. The increased serum levels of these steroids were maintained through 29 days of age at which time progesterone and 17-hydroxyprogesterone concentrations were also increased as compared to vehicle-treated controls. Cytosol estradiol receptors of the anterior pituitary and hypothalamus were depleted during DHA treatment and preovulatory gonadotropin surges were found to occur on the afternoon of day 29 and 30.

In contrast to the actions of PMSG, steroid levels remained elevated and cytosol estradiol receptors remained depleted for a prolonged time following precocious ovulation induced by short-term DHA plus PMSG. No ovu-

lations or cyclic changes in vaginal cytology were found to occur for at least 10 days after such precocious puberty. During the 10 day interval, a slow decline in peripheral steroid levels occurred which coincided with a gradual restoration of cytosol estradiol receptor binding capacity of the anterior pituitary and increasing serum levels of LH.

In rats treated continuously with DHA (10 or 20 days), all parameters studied remained constant: serum levels of steroids were abnormally high; cytosol estradiol receptors of the anterior pituitary and hypothalamus were depleted; serum levels of LH were low whereas FSH levels were normal or slightly high; no ova, fresh corpora lutea or cyclic changes in vaginal cytology were detected. During chronic DHA administration, a relative insensitivity of the anterior pituitary to low doses of LH-RH, as compared to untreated control rats, was demonstrated.

Following discontinuation of DHA administration (short-term or chronic) serum levels of testosterone and DHT fell to within normal limits within a few days whereas the decline in DHA and androstenedione was more prolonged. In addition, serum levels of progesterone were found to be chronically elevated in most animals and declined slowly after DHA withdrawal as a result of prolonged corpus luteum function. Estradiol concentrations in serum fell more rapidly following short-term DHA administration than after 20 days of DHA treatment, which also resulted in the formation of ovarian cysts.

Although an association was found to exist between serum levels of estradiol and concentrations of anterior pituitary and hypothalamic estradiol receptors, the possible involvement of other steroids, which were also affected by DHA treatment cannot be ruled out at the present. As cytosol estradiol receptor concentrations were being replenished during DHA withdrawal,

serum levels of LH, which had been suppressed were also found to gradually increase.

It is suggested in these studies that estradiol receptors may play a role in modulating the response of the hypothalamic-anterior pituitary unit to changing levels of serum estradiol and possibly other compounds in the female rat. Direct proof for such an involvement has not been established; however it is hoped that the evidence provided will be considered provocative enough to induce further study by others.

VII. LITERATURE CITED

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VIII. APPENDIX

Figure 11. Uterine Weights in Female Rats: Fluctuations Associated With the Onset of Puberty, Ovulatory Failure and Androgen Withdrawal.

UTERINE WEIGHT

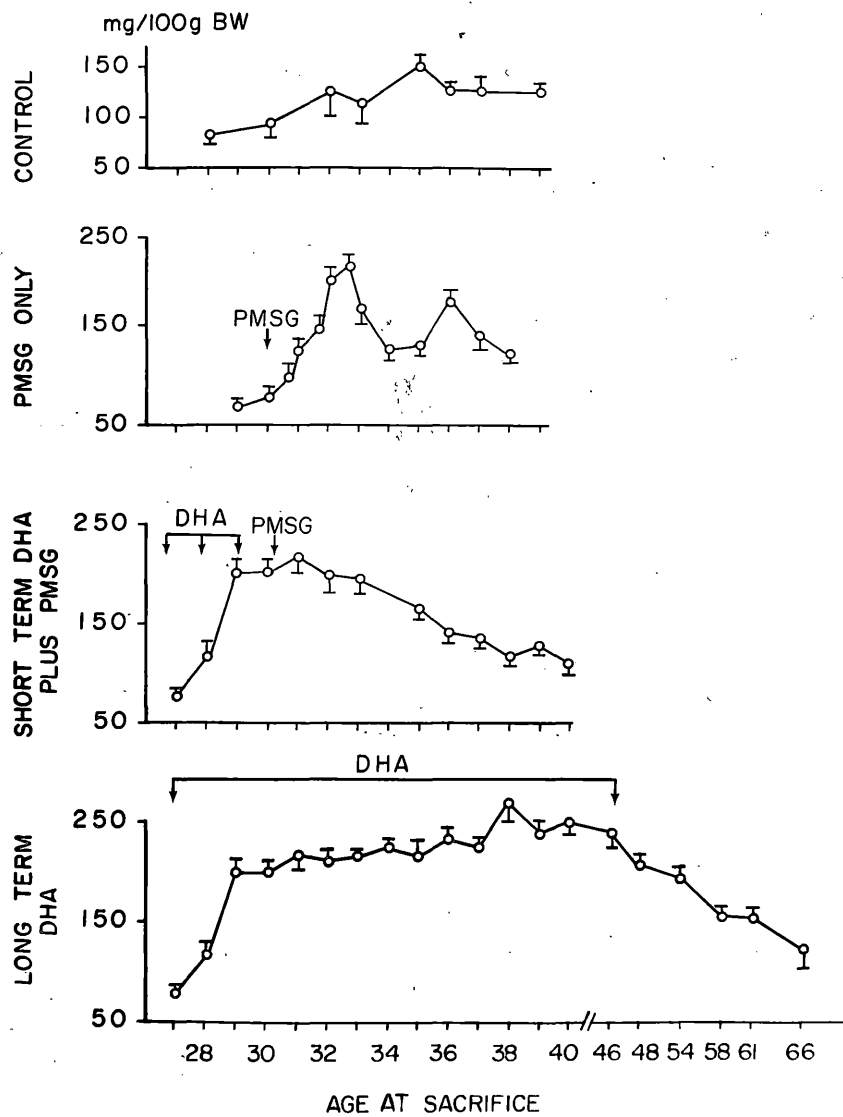


Figure 12. Ovarian Weights in Female Rats: Fluctuations Associated With the Onset of Puberty, Ovulatory Failure and Androgen Withdrawal

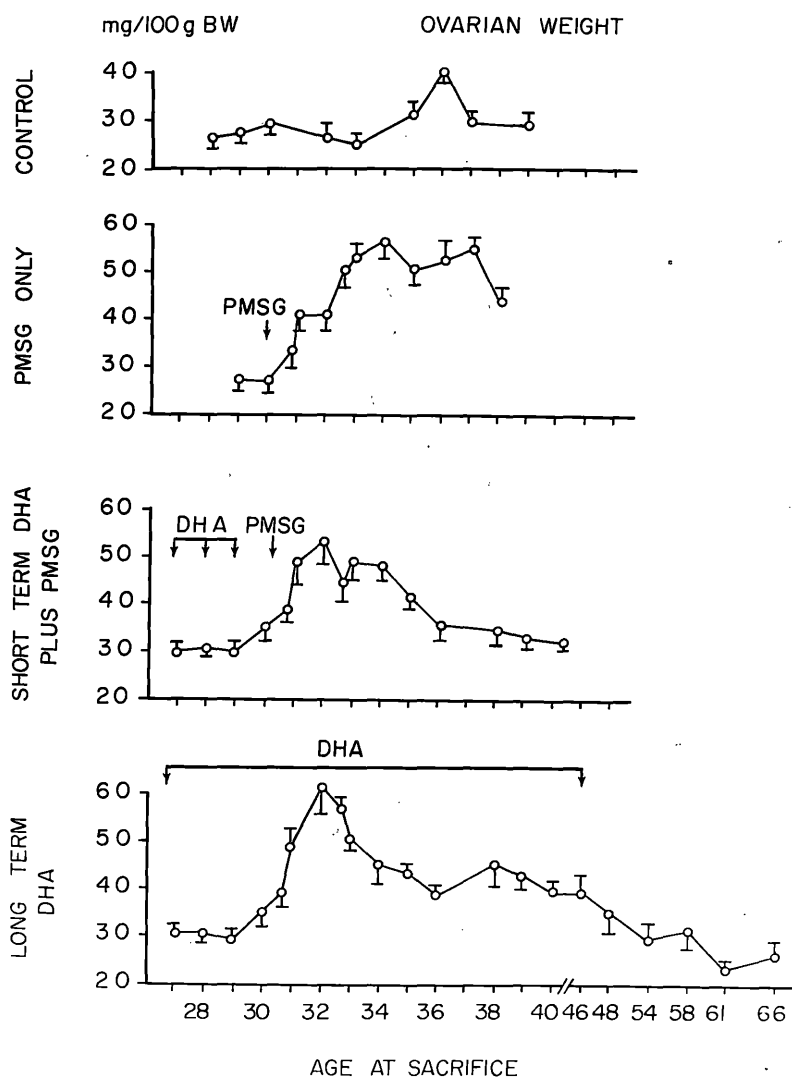


Figure 13. Serum Concentrations of Estradiol-17 β in Female Rats:
Fluctuations Associated with the Onset of Puberty, Ovulatory
Failure and Androgen Withdrawal.

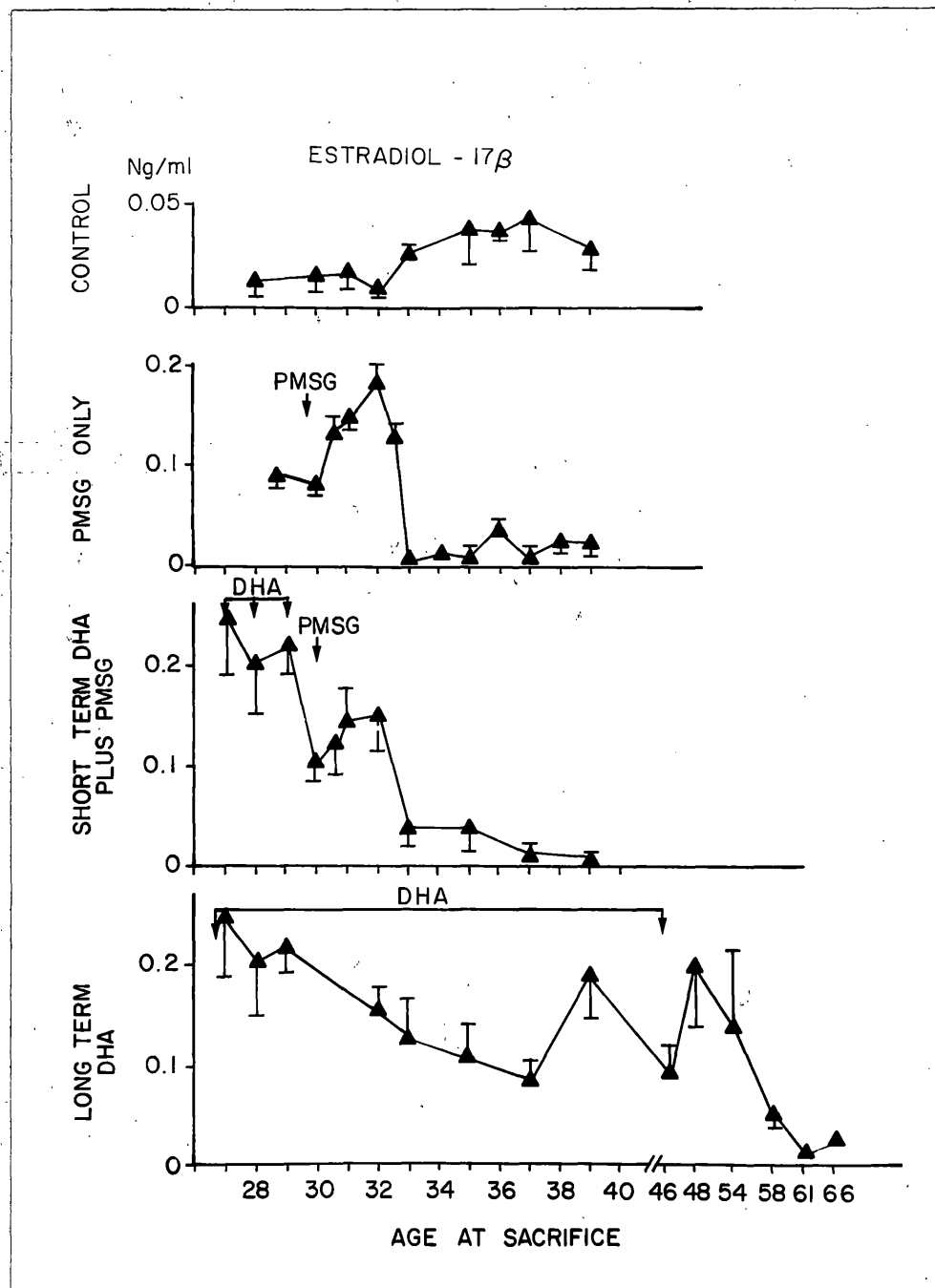


Figure 14. Serum Concentrations of Progesterone in Female Rats:
Fluctuations Associated with the Onset of Puberty, Ovulatory
Failure and Androgen Withdrawal

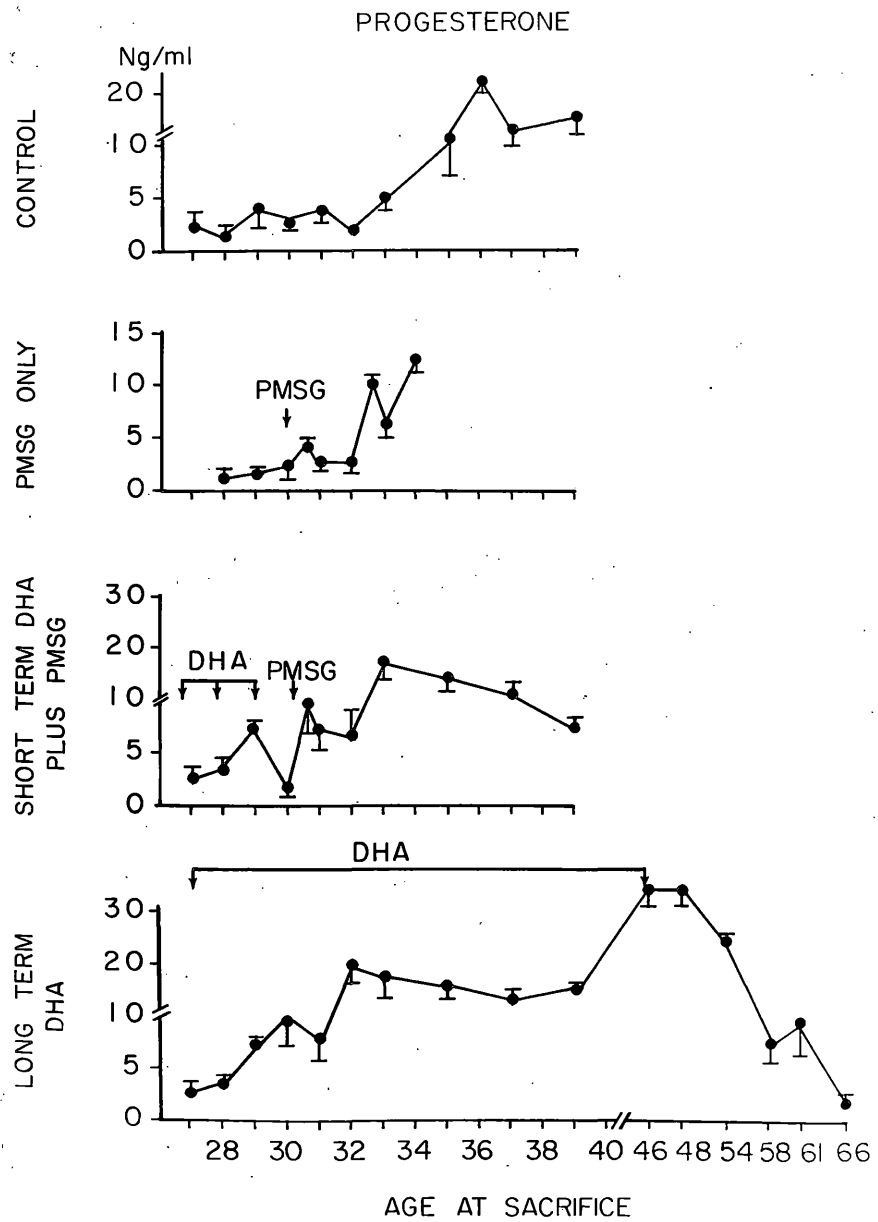


Figure 15. Serum Concentrations of 17-Hydroxyprogesterone in Female Rats:
Fluctuations Associated with the Onset of Puberty, Ovulatory
Failure and Androgen Withdrawal

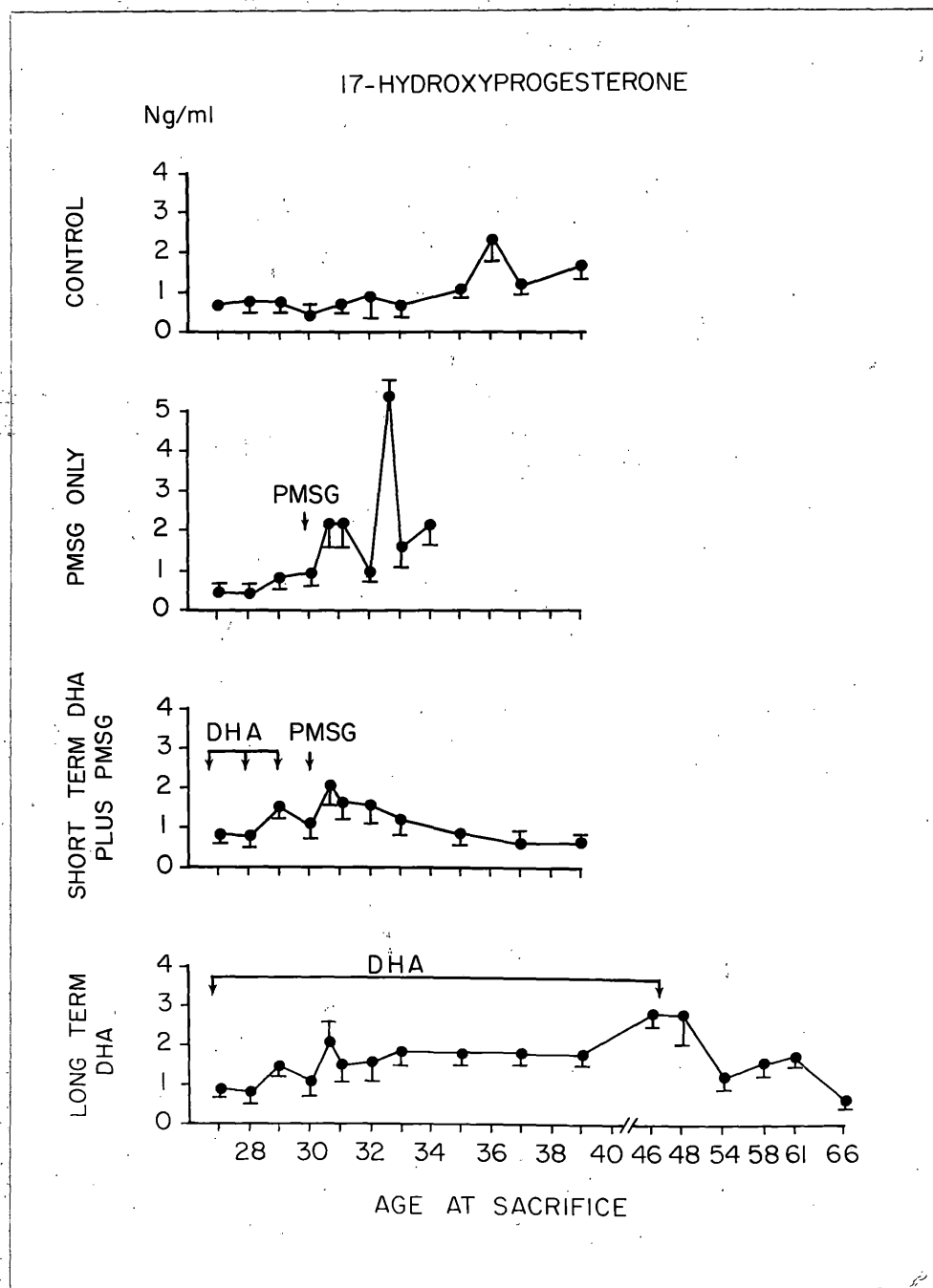


Figure 16. Serum Concentrations of Testosterone in Female Rats:
Fluctuations Associated with the Onset of Puberty, Ovulatory
Failure and Androgen Withdrawal

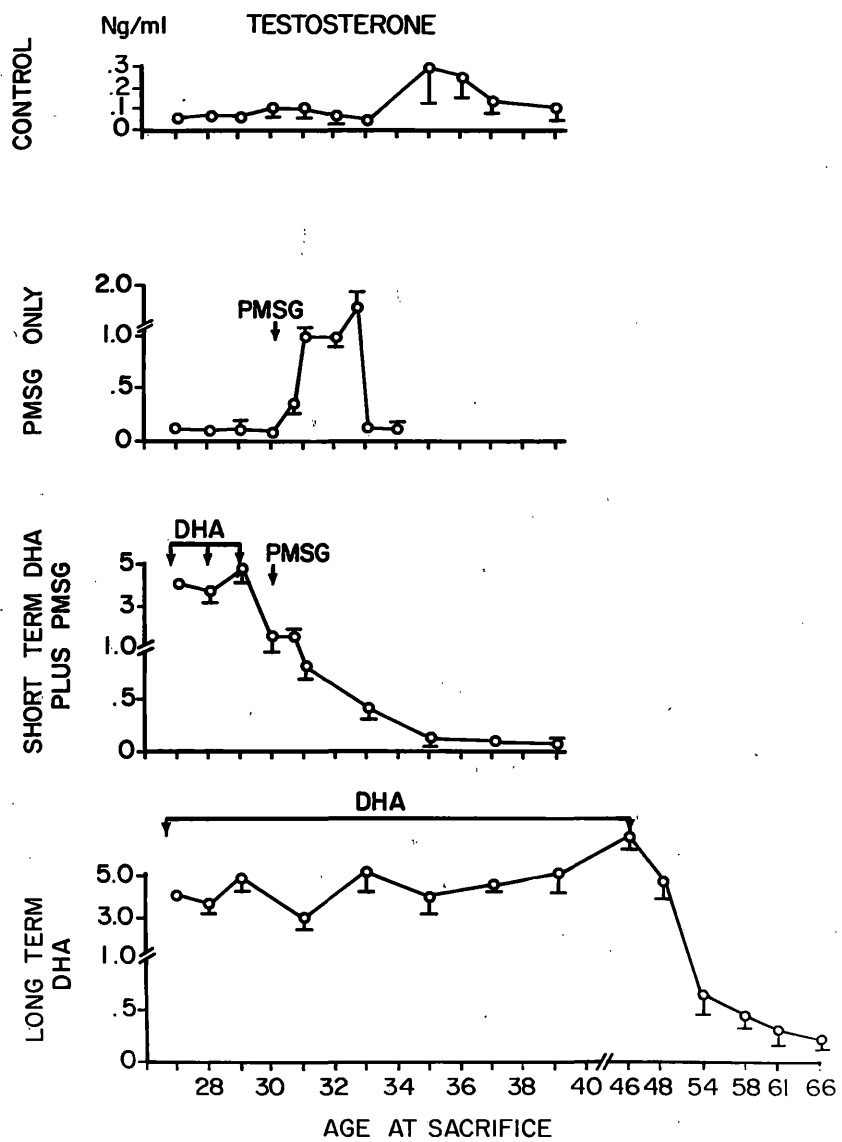


Figure 17. Serum Concentrations of Androstenedione in Female Rats:
Fluctuations Associated with the Onset of Puberty, Ovulatory
Failure and Androgen Withdrawal

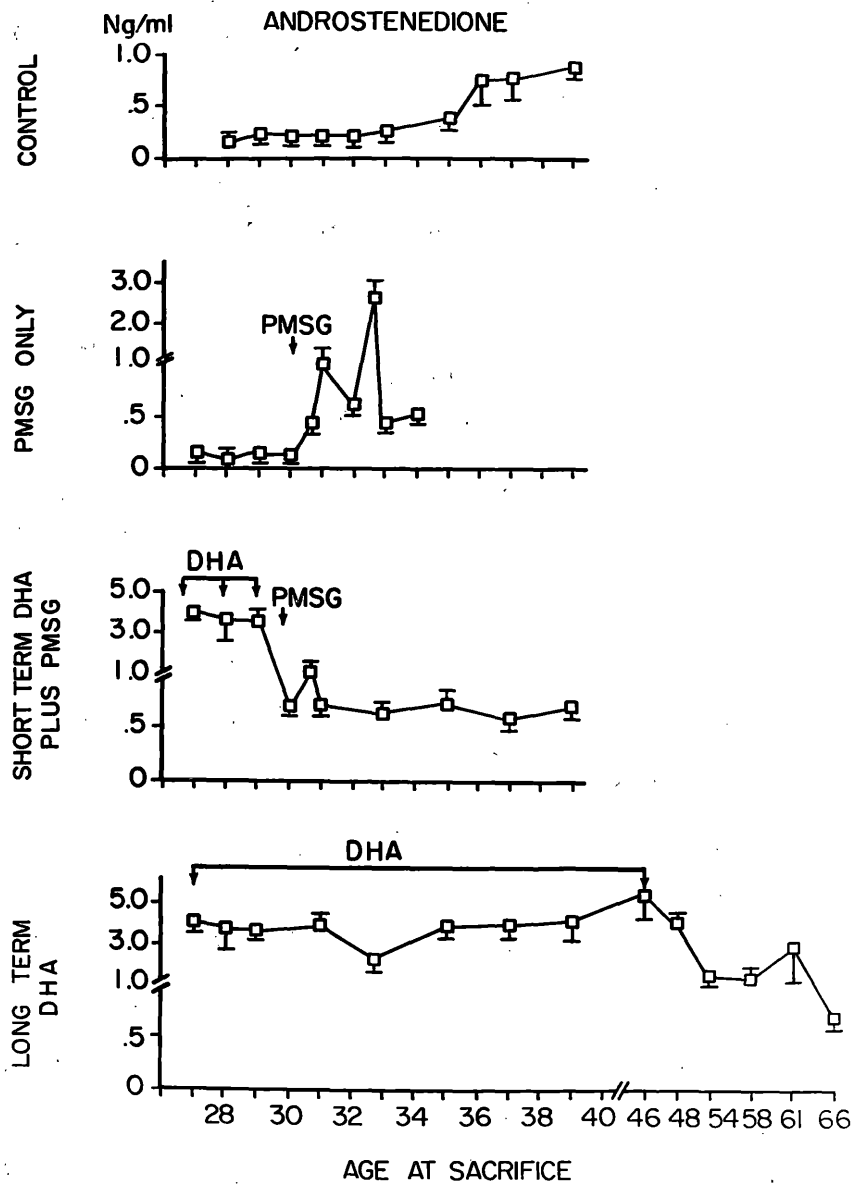


Figure 18. Serum Concentrations of Dihydrotestosterone in Female Rats:
Fluctuations Associated with the Onset of Puberty, Ovulatory
Failure and Androgen Withdrawal

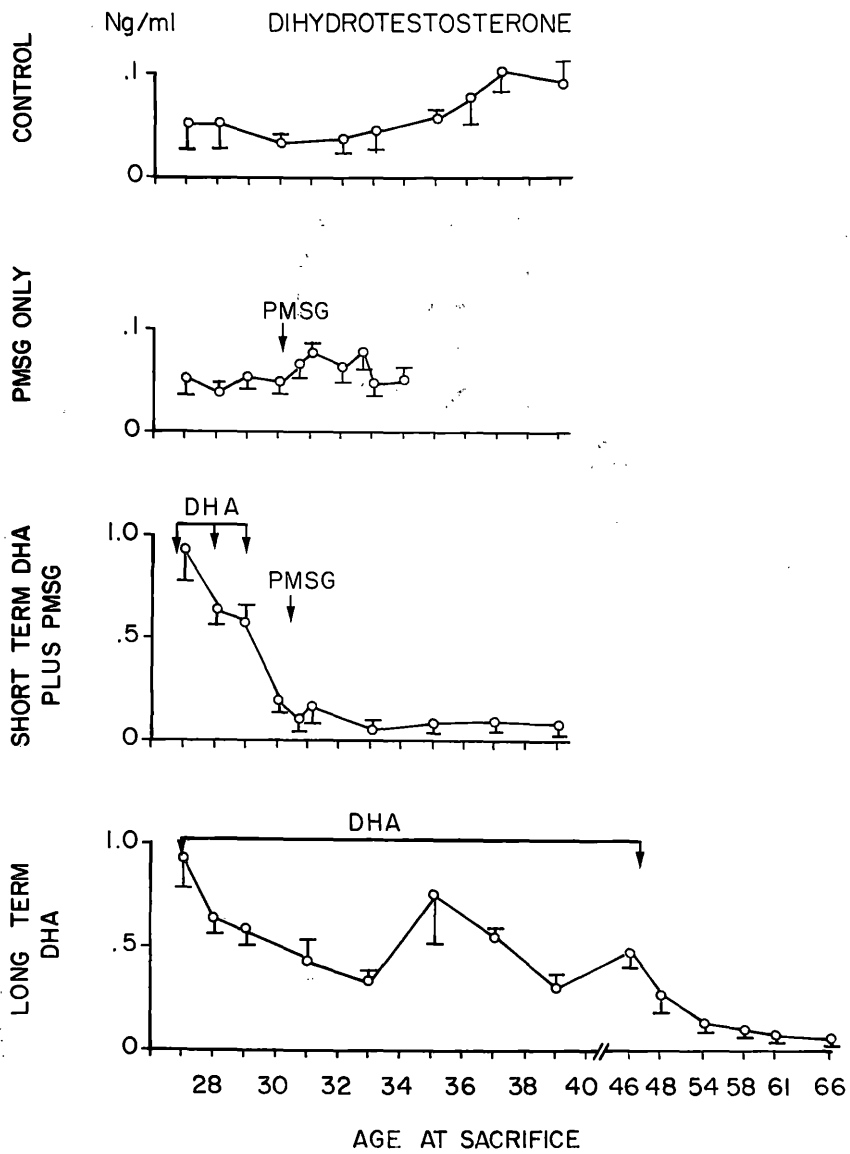


Figure 19. Serum Concentrations of Dehydroepiandrosterone in Female Rats:
Fluctuations Associated with the Onset of Puberty, Ovulatory
Failure and Androgen Withdrawal

DEHYDROEPIANDROSTERONE

