# THE EFFECTS OF PROGESTERONE ON THE PATTERNS OF SEX STEROIDS IN THE SYSTEMIC PLASMA OF THE RHESUS MONKEY DURING THE INTERMENSTRUAL PERIOD

by

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A THESIS

Presented to the Department of Physiology

and the Graduate Division of the University of Oregon Medical

School in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

August, 1972

APPROVED:

[redacted]

(Professor in charge of thesis)

[redacted]

(Chairman, Graduate Council)

## ACKNOWLEDGEMENTS

I wish to thank the various members of the staff at the Oregon Regional Primate Research Center for their editorial, surgical, illustrative, and animal handling assistance. I am especially indebted to Jan Ploem, Henry Stadelman, Mary Sullivan, and Marie Partlow for their tender care of a neophyte steroid chemist and for their excellent technical assistance.

I am also grateful for the encouragement and advice from the members of the Physiology Department at the University of Oregon Medical School. In particular, Dr. John Brookhart was always available for discussion and constructive criticism. Singular thanks are tendered to Dr. John Resko, who as my major advisor spent uncounted hours both in and out of the laboratory and who provided a superb example of scientific excellence.

Finally, this thesis is dedicated to my wife Joi for her enduring understanding, devotion, and love, although words alone can never express my appreciation for her patience during this period.

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# CHAPTER I

## GENERAL INTRODUCTION AND STATEMENT OF THE PROBLEM

Very little is known about the physiology of ovarian secretion in the primate. However, the secretory capacity of the ovary is suggested by the cyclical patterns of estradiol[1] and progesterone in the systemic circulation during the intermenstrual period (1-4). The basal level of estradiol in the peripheral circulation during the early follicular phase of the cycle is associated with the tonic release of gonadotropic hormones. The circulating level of estradiol increases as the follicles develop and during the late preovulatory period reaches a peak concentration that is eight times greater than that of the early follicular period. After the peak, the systemic concentration is drastically reduced within 12 to 24 hours. Initially the late preovulatory period is associated with a slight decrease in peripheral gonadotropins that is consonant with a negative feedback role for the increasing amounts of estradiol. However, during and after the estradiol peak, the preovulatory surge of luteinizing hormone (LH)

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[1] Systematic chemical names of the steroids discussed in the text are found in Appendix A.

is thought to be the result of a positive feedback effect of estradiol. Concurrently progesterone appears in the peripheral plasma and remains throughout the luteal phase after ovulation.

The current explanation of the relationship between the ovary and pituitary contains at least one inconsistency. If the estradiol in the general circulation is from the ovary, why does it decrease rapidly just before ovulation when the ovary is under intense trophic stimulation from the preovulatory surge of LH? The administration of LH in vivo or in vitro generally results in an increased production of all ovarian steroids so that LH is not likely to play an inhibitory role. Moreover, the fact that quantities of estrogen in the peripheral circulation decrease at least 24 hours before the follicle ruptures argues against ovulation being an important factor in the decline of estrogen measured systemically. The observation that progesterone is secreted from the preovulatory ovary in response to gonadotropin stimulation and the fact that it regulates the biosynthesis of androgen in other endocrine glands suggested that it or a progestin is involved in the regulation of the biosynthesis of estrogen by the primate ovary.

Therefore, studies reported here were designed to investigate the role of progesterone in steroid secretion by the primate ovary during the periovulatory period. (1) A method was developed for reproducibily manipulating the concentration of progesterone in the systemic circulation. Ordinarily, progesterone is present in

quantities greater than 2 ng/ml only during the luteal phase of the ovulatory cycle. Consequently, it was necessary to find a way of administering exogenous progesterone that furnished immediate physiological concentrations in the general circulation for the desired treatment period. (2) The peripheral quantities of testosterone were measured throughout the normal menstrual cycle. This descriptive experiment interrelated the systemic pattern of this important androgen with the patterns of other ovarian steroids. Furthermore, assaying it in blood provided an additional end point for analyzing the effects of progesterone, since the androgens are precursors in the ovarian synthesis of estrogens. (3) The effects of exogenous progesterone during the periovular period were tested on the pattern of androgens and estrogens found in the systemic and ovarian venous plasma. If progesterone is acting on the ovary, elevations in the concentrations of this hormone in the systemic plasma by exogenous administration during the midfollicular and periovulatory period should inhibit the secretion of estrogens and androgens. The reduction in the concentration of these compounds in the peripheral blood and the temporal relationships observed will give some insight into the regulatory action of progesterone on the control of hormone secretion by the ovary near the time of ovulation.

# CHAPTER II

## REVIEW OF THE PHYSIOLOGICAL PARAMETERS IN THE PRIMATE OVARIAN CYCLE

The most characteristic external feature of the reproductive cycle in the female primate is menstruation. Initially, the divisions of the cycle were described and defined by sequential changes in the lining of the human endometrium (5). When it was later discovered that ovulation occurred near the midpoint between menses after a period of follicular growth and before the development of a corpus luteum, the cycle was described as having a follicular phase and a luteal phase (5). The first studies of cyclic menstrual periods in the rhesus monkey provided an experimental model with many of the physiologic attributes of man (6, 7). The cycle is 28 days (mode) long, with ovulation occurring between the 11th and 14th days. The dominant hormones in the follicular and luteal phases are estradiol and progesterone respectively (8); the secretion of these hormones in a cyclical fashion by the ovary presumably triggers the morphological changes in the primate endometrium (9).

It was generally assumed that the presence of menstrual flow was associated with the preceding ovulation (5). However,

identification of anovulatory cycles in the monkey followed by menses suggested that ovulation and menstruation are not necessarily related (10). Moreover, the fact that endometrial degeneration, usually found before the onset of menses, occurs in either the follicular or the luteal phase and that menstruation can be induced in the ovariectomized female by various steroid treatments (5) indicated that a more precise physiological correlate of ovarian development than menstruation was desirable. The appearance of vaginal bleeding, however, still serves as a convenient external marker.

The most significant feature in the reproductive cycle is the periodic release of a mature ovum. The ovarian cycle, unlike the menstrual cycle, is divided into follicular and luteal periods by the physical process of ovulation. The definition of the two phases need not be restricted to this event, and any other reference point can be selected that divides the cycle near the time of ovulation, e.g. the preovulatory peak of estradiol or the preovulatory surge of LH.

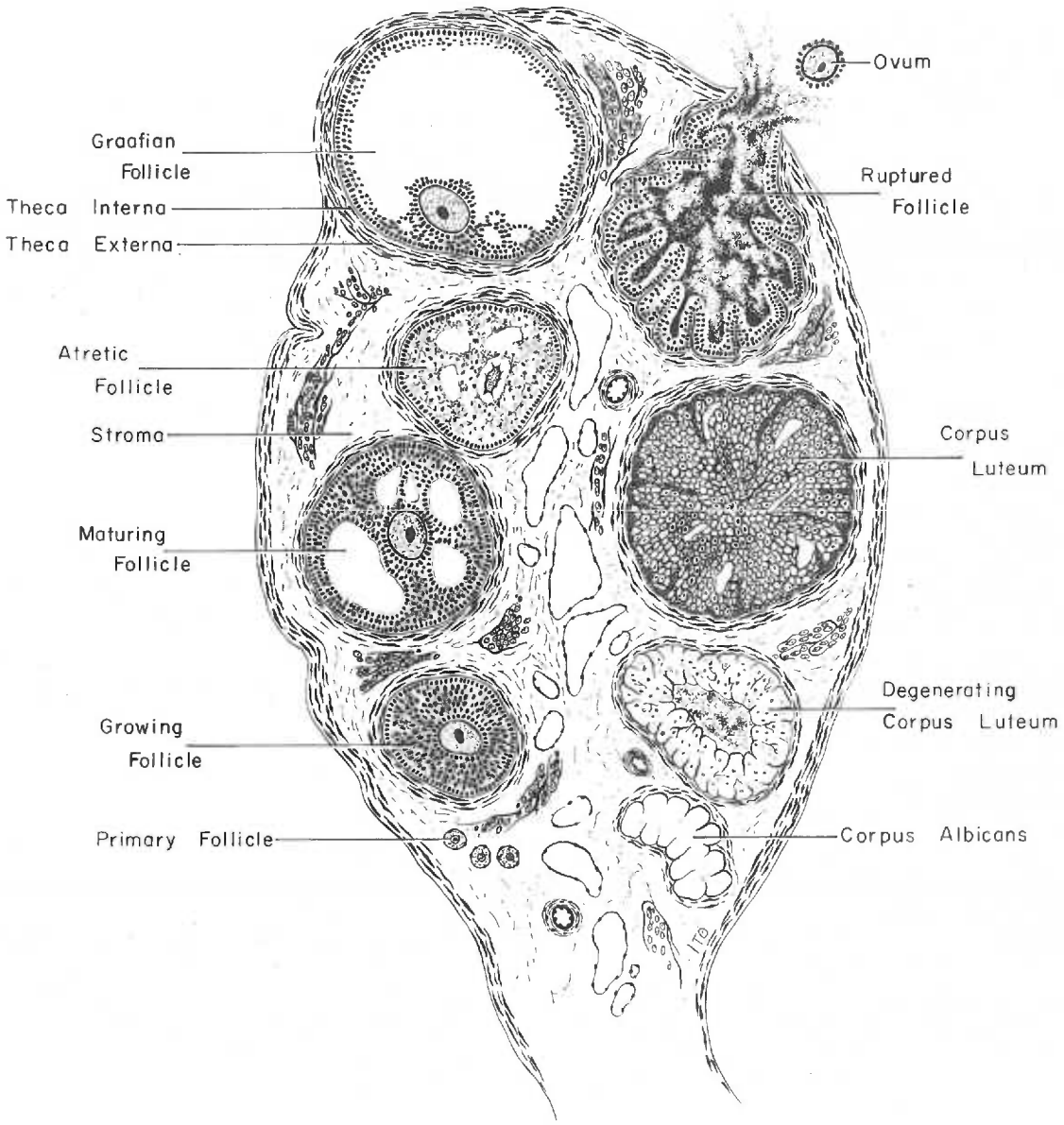
The following review covers four general topics related to the morphology, biochemistry, and physiology of ovarian function: (1) the dynamic morphology of ovarian cell populations; (2) related alterations in biosynthetic capabilities inferred from <u>in vitro</u> and <u>in vivo</u> experiments; (3) the rhythmic patterns of ovarian steroids in the peripheral circulation of the primate; (4) possible regulatory mechanisms for controlling steroid biosynthesis in the ovary.

## Ovarian Cycle: Morphology and Histology of the Ovary

Grossly, the ovaries of sexually mature rhesus monkeys are slightly flattened, paired organs 1 to 2 cm in length, 1 to 1.5 cm in width, and 0.5 to 1.2 cm in thickness. Peritoneal epithelium and the tunica albuginea cover the thick cortex that surrounds the inner or medullary portion. Embedded within the undifferentiated stroma of the cortex are follicles, ranging from extremely small, dormant primordial follicles to large preovulatory structures, as well as corpora lutea in various stages of activity or degeneration (Figure 1). The medulla, consisting of loosely arranged connective tissue, underlies the cortex and a mass of contorted blood vessels enters through the hilus of the ovary.

The changing peripheral patterns of gonadotropins and steroids are both the cause and the result of the dynamic alterations in ovarian morphology during the cycle. Koering (11) recently described the ovarian cycle of the rhesus monkey according to the size of the developing follicles and the functional state of the corpus luteum. Vesicular follicles, 1 mm in diameter or less usually underwent atresia except the one destined to ovulate. This follicle (now referred to as a Graafian follicle) is identifiable by day 9 of the menstrual cycle whereas the others are in various stages of atresia. The initiation of folliculogenesis and continued growth of the follicles probably depend

Figure 1. Schematic illustration of the microscopic anatomy of the ovary (courtesy of Dr. J. A. Resko).


Ovum
Graafian Follicle
Theca Interna
Theca Externa
Ruptured Follicle
Atretic Follicle
Stroma
Corpus Luteum
Maturing Follicle
Degenerating Corpus Luteum
Growing Follicle
Primary Follicle
Corpus Albicans
TB

on the appropriate ratio of gonadotropins (2).

The specialized theca interna cells in the preovulatory follicles are sparse and unevenly distributed but well-vascularized. The interstitial gland tissue surrounding the follicle appears to derive from the theca interna of follicles undergoing atresia and the differentiating perifollicular stromal cells. This tissue is most prominent during the preovulatory period and can scarcely be identified during the rest of the cycle. The interstitial or stromal gland has been described in man (12); it was observed that the number of atretic follicles reached a maximum in the preovulatory period. These authors noted that the formation of vesicular and atretic follicles is a rhythmic process and suggested that the stromal gland is as important for steroid production as the theca interna in the preovulatory state.

Immediately after ovulation, the wall of the follicle collapses and the granulosa cell lining folds into the fluid-filled cavity. The blood vessels supplying the theca interna sprout and invade the granulosa cells as they undergo luteinization and a fully mature corpus luteum is present by day 10 after ovulation. During the entire period of luteal activity, the largest follicle observed was about 1 mm in diameter and attained no further growth until the corpus luteum began to degenerate (11); in all animals degeneration of the corpus luteum began before the onset of menses. These observations confirm and

extend previous reports (13, 14).

Although it is extremely difficult to relate steroidogenic activity directly with cellular morphology, indirect comparisons have been made. In general, steroid production has been related to the appearance of a hypertrophied specialized cell mass that is well-vascularized. Each cell is characterized by diffuse lipoproteins, i.e. large quantities of agranular endoplasmic reticulum, a slight accumulation of lipid or lipid esters, complex tubular mitochondria, and enzyme, activities indicative of steroid hormone synthesis (15, 16). Early studies on the distribution of cholesterol suggested that the theca interna cells of the follicle and the luteinized theca interna and granulosa cells of the corpus luteum were normal sites of hormone synthesis in man (17, 18).

Recent investigators, using more refined histological, histochemical, and electron microscopic techniques, have described in detail the indirect morphological indices of steroid production in the human ovary. Dean, Lobel, and Romney (19), noted that the activity of the 3β-hydroxy steroid dehydrogenase enzyme was highest in the theca interna before ovulation and high also in the luteinized granulosa after ovulation. Confirming previous observations (20), Guraya (21) observed large amounts of agranular endoplasmic reticulum and diffuse lipoproteins in the theca interna with only small amounts of either in the granulosa before ovulation. Similar studies on stromal gland tissue indicated that this cell population is also capable of steroid production (22, 23). The dramatic reversal of the structure

and contents of a particular cell population correlates, within the limits of the various techniques, with the changes in peripheral steroid patterns observed during the cycle (24).

These studies suggest that three morphologically distinct ovarian compartments--the follicle, stroma, and corpora lutea--can be identified in this complex heterogeneous tissue. Judging from histological criteria, these separate cell populations undergo various periods of growth, functional activity, and degeneration that are related to patterns of gonadotropins and steroid hormones in the systemic circulation.

## Ovarian Cycle: Biosynthetic Potential of the Ovary

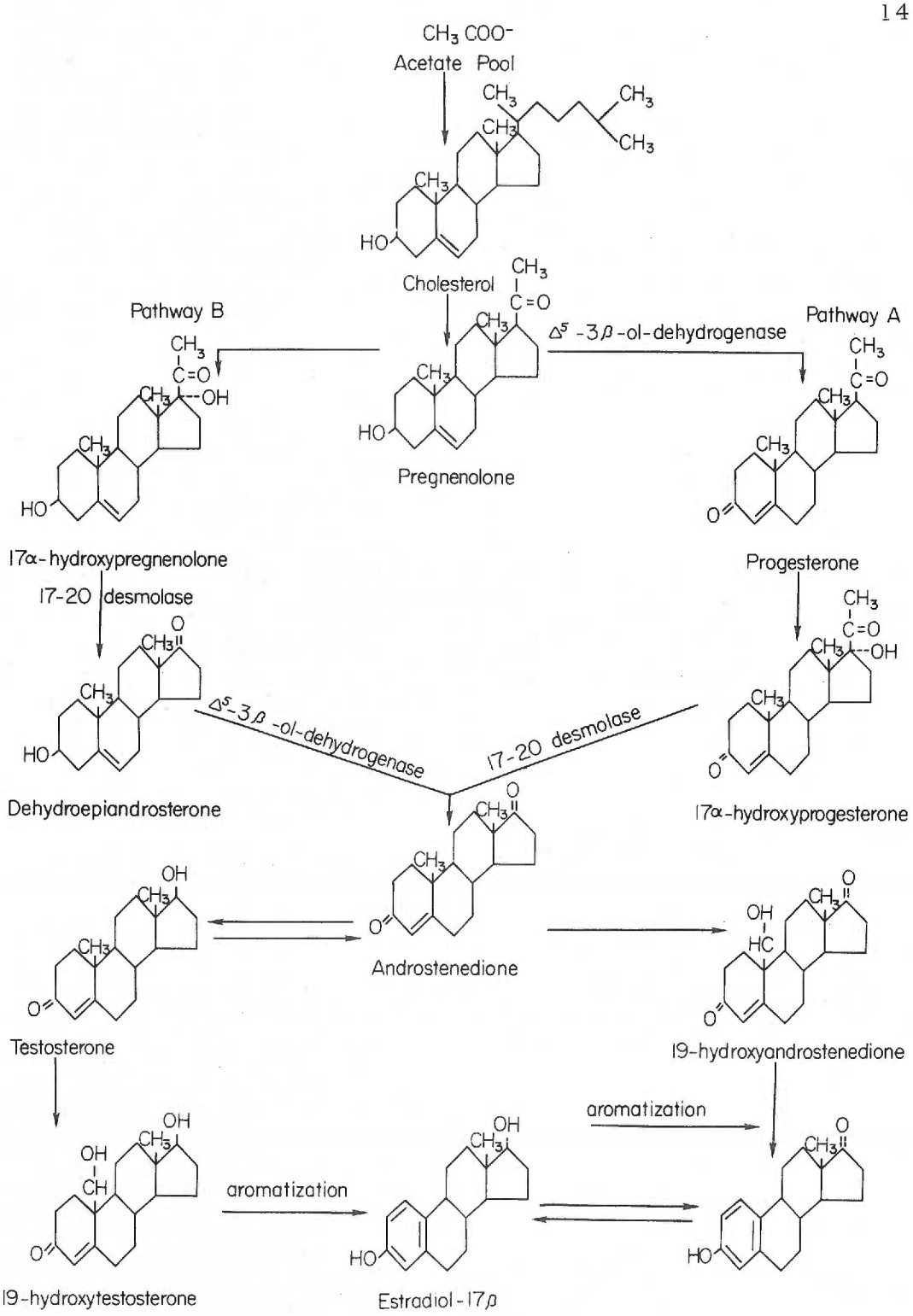
In vivo studies characterizing the concentrations of gonadal and hypophyseal hormones in the systemic plasma are valuable indices of the endocrine state of the female. Similarily, the qualitative functional and structural relationships within the ovary can be inferred by comparing the cellular morphology of the organ with the quantities of circulating steroids. These observations, however, do not directly assess the biosynthetic potential of the anatomical compartments within the ovary or identify the intermediates in the biosynthetic processes leading to the secreted product. These questions are partially answered by investigating the metabolism of various substrates by the ovary in vitro and by identifying the steroid hormones that are secreted into the venous effluent from the gland.

The results obtained from in vitro experiments leave much to be desired. For example, discrimination is lost by the disruption of normal substrate supply routes, the microanatomy of the subcellular organization is drastically altered, and reactions can be inhibited by the abnormal accumulation of metabolites. Despite these limitations, the use of isotopically pure substrates has defined the probable routes of steroid biosynthesis in the primate, especially in the human ovary (Figure 2).

The possible intermediates in pathway A were initially suggested from studies on the bovine ovary in which progesterone was used as the substrate (25). The role of cholesterol in ovarian steroid production was indicated by the identification of labeled pregnanediol and estrone in the urine of pregnant women treated with the labeled sterol (26, 27). Acetate as a precursor was also implicated in man by the identification of radioactive cholesterol and estradiol in incubations with ovarian homogenates (28). The isolation of various progestins and androgens from different parts of the ovary (29) provided the impetus to investigate the biosynthesis of steroids in different morphological compartments.

The earliest studies, which made no attempt to separate follicles from the surrounding stroma, showed that testosterone was metabolized to both estrone and estradiol (30, 31) and that androstenedione was converted to testosterone (32). Other studies indicated

Figure 2. Schematic representation of the metabolic pathways for steroid biosynthesis in the primate ovary. Pathway A is known as the $\Delta^4$-3-keto pathway and predominates in the granulosa. Pathway B is known as the $\Delta^5$-3$\beta$-ol pathway and predominates in the theca and stroma.


CH3 COO-
Acetate Pool
Cholesterol
Pathway B
Pathway A
Δ5 -3β -ol-dehydrogenase
Pregnenolone
17α-hydroxypregnenolone
17-20 desmolase
Progesterone
Δ5-3β -ol-dehydrogenase
17-20 desmolase
Dehydroepiandrosterone
17α-hydroxyprogesterone
Androstenedione
Testosterone
19-hydroxyandrostenedione
aromatization
aromatization
19-hydroxytestosterone
Estradiol-17β

that pregnenolone was converted to progesterone and 17 α-hydroxy progesterone and that a small percentage of 17 α-hydroxyprogesterone could be metabolized to androstenedione (33, 34). In another series of investigations with progesterone, the follicular tissue was more active than luteal tissue in producing 17 α-hydroxyprogesterone and androstenedione although the pattern was similar (35). These authors concluded that their inability to show estrogen formation in either tissue was the result of the low metabolic potential of the preparation. These problems were overcome in multiple experiments in which large cystic follicles were developed by treating women with gonadotropins and separating the follicular linings from the stroma (36-40). The results showed that in the follicle acetate was converted to cholesterol and estrogens; cholesterol and androstenedione were converted to estrogens; and progesterone was converted to testosterone. These observations agreed well with the identification of both testosterone and androstenedione in ovarian homogenates or slices with either 17 α-hydroxyprogesterone or progesterone as substrates (41, 42). Besides confirming the major intermediates in pathway A, they identified pathway B (43). Acetate was incorporated into pregnenolone, 17 α-hydroxypregnenolone, dehydroepiandrosterone, and androstenedione with little incorporation into progesterone under these conditions. This second (B) pathway was confirmed in non-stimulated tissue when both follicles and stroma were present (44-46)

and seemed to be independent of the pathway through progesterone (47, 48). The identification of various 19-oxygenated androgens has been well-documented (49-51), but which of these compounds is the actual intermediate in estrogen biosynthesis is still not known. Previous studies had identified all of the intermediates in separate incubations, but Axelrod and Goldzieher (50) were the first to identify all of the intermediates from a single incubation. These authors cautioned that their results represented only a particular phase of a particular cycle with multiple compartments and that generalizations were difficult. In later studies equimolar amounts of $^{3}$H-pregnenolone and $^{14}$C-progesterone were used to follow the accumulation of products from the two pathways by a comparison of the isotope ratios in tissue incubations from various phases of the cycle (52-55). These authors concluded that if the corpora lutea were excluded, the favored metabolic pathway in normal ovarian tissue was pathway B. The same conclusion was reached in a study of isolated thecal and granulosa cells (56).

Studies on ovarian tissue after the removal of the larger follicles and the corpora lutea showed that this tissue was active in the metabolism of acetate to progesterone (57) and that the major product isolated was either testosterone (58) or androstenedione (59). These last investigators concluded that the stroma is the major source of androgens in the ovary. This ovarian compartment used pregnenolone

at a much higher rate than progesterone in the production of androgens via pathway B (60-61) and could produce estrogens (62). Leymarie and Savard (60) felt that the synthetic activity of the stroma was related to the stage of the cycle since tissue from the follicular phase was much more active than tissue from the luteal phase. The biosynthetic potential of the stroma is probably best evaluated in the postmenopausal ovary since anatomically it consists almost entirely of stromal tissue and is highly stimulated by in vivo gonadotropins (62-65). It is particularly active in the production of androgens, especially of androstenedione from either pregnenolone or progesterone; in most of the incubations, pregnenolone was most actively metabolized (65). Obviously the stroma of the premenopausal and postmenopausal ovary differs since the former produces estrogens whereas the latter does not (64, 65). Nevertheless, the fact that stromal tissue actively produces steroids confirms the morphological observations mentioned above.

The major product isolated from in vitro incubations of corpora lutea was progesterone, but smaller amounts of 17 α-hydroxyprogesterone were also found (66-71). Other intermediates found in pathway A, including estrogens, indicated that luteal tissue can produce estrogens, but at very low concentrations. When pregnenolone was used as a substrate (72), pathway B was almost totally inactive; these results were confirmed by the accumulation of $C_{21}$ steroids in the

absence of dehydroepiandrosterone or other $C_{19}$ products (67, 69). Thus the human corpus luteum can produce estrogen, as is evidenced by its extremely active aromatization of androstenedione (66, 73-75), but to a lesser degree than stromal tissue (73). The fact that corpora lutea aromatize dehydroepiandrosterone to estradiol in slices and estrone in homogenates indicates that if this androgen were formed, estrogens should accumulate (76). Results obtained from human and rhesus monkey granulosa cells in cell culture have confirmed that the pattern of steroids obtained from these cells is different from that of similarly cultured thecal cells (56, 77-79). In all of these studies, progesterone was the major steroid produced by the luteinized granulosa cells; in the last study there was a definite correlation between the age of the corpus luteum and the steroidogenic and morphological characteristics of the cultures. Granulosa cells from the preovulatory ovary and from the early luteal phase produced 10 to 100 times more progesterone than cells from the early follicular phase (79).

Thus we infer that the various anatomical compartments of the ovary have different steroidogenic potentials. Falk (80) in a series of experiments on rats suggested that different steroidal patterns are elaborated by the several compartments of the ovary and that these compartments are somehow interrelated. This theory was further developed in the "two cell" concept (81) which holds that luteal and follicular cells have qualitatively different functional capabilities,

depending on the enzymatic pathways present and the vascular supply of the potentially active cells. Attempts to prove these postulates in vitro (56, 77, 79) have yielded conflicting results, an indication that although the three ovarian compartments are grossly similar qualitatively, the nature of their interrelationships and the fine differences that are probably acting physiologically are still obscure. In a series of in vitro studies on ovarian slices from 19 women in different phases of the cycle, the preovulatory ovary transformed pregnenolone to 17 α-hydroxypregnenolone, dehydroepiandrosterone, androstenedione, and small amounts of estrogen, particularly in the late follicular phase. After ovulation, the pattern was dramatically altered, and the same substrate was converted to progesterone, 17 α-hydroxyprogesterone, and estrogen (82). These results with heterogeneous tissue slices confirm the conclusion by Samuels and Eik-Nes (83), that two separate pathways exist within the ovary and are active at different times in the ovarian cycle concurrent with the presence of different anatomical structures.

Perhaps the best indicator of glandular secretory activity is the measurement of steroids in the venous effluent draining the ovary. In the postovulatory ovary, the largest amounts of progesterone are present in blood from the ovary with the corpus luteum (84). On the other hand, in one subject, much larger quantities of 17 α-hydroxyprogesterone and androstenedione were found in the effluent plasma

from the ovary with the ripe follicle (85). However, no progestins were isolated in venous blood from monkeys whose ovaries were in the follicular phase (86). Estradiol concentrations were found to be highest in plasma from the preovulatory ovary with the Graafian follicle, and in some cases progesterone has also been identified (85, 87). The amounts of 17-keto steroids in ovarian venous blood (88), the reduced concentration of androgen in ovarian venous plasma by the suppression of gonadotropins (89), and the identification of an a-v difference in hormone concentration across the ovary indicate that dehydroepiandrosterone, androstenedione, estradiol, and testosterone are secreted during the ovarian cycle (90-97). The latest attempt to correlate steroid levels with a phase of the ovarian cycle was relatively unsuccessful because any single steroid varied widely between subjects, whether at the same phase or at different phases of the cycle (97). However, these results showed that the major steroids secreted by the ovary with the active follicle and corpus luteum were estradiol, androstenedione, and progesterone and that the levels of these steroids tended to be higher in the luteal than in the follicular phase. According to the estimation of steroids in ovarian venous plasma, the ovary produces a variety of androgens, estrogens, and progestins; but without sequential sampling from a single subject during the cycle, few concrete conclusions about the steady or variable rates of steroid secretion are possible. Except for two

studies on the postovulatory ovary (98, 99), little is known about the concentration of steroids in the ovarian venous plasma of subhuman primates. However, the low levels of estrogens measured peripherally during the luteal phase indicates that the luteal tissue of the monkey probably secretes only minute quantities of these steroids.

In summary, the systemic patterns of steroid hormones observed during the normal ovarian cycle reflect dynamic alterations in the structural and functional anatomy of the ovary. The active follicles and surrounding stroma release estrogens and androgens in the follicular phase, and the corpus luteum produces mostly progesterone, although some overlap tempers this strict compartmentalization.

## Ovarian Cycle: Pattern of Steroids and Gonadotropins in Systemic Plasma

Until recently, it was impossible to measure the actual amounts of hormone produced by the ovaries and found in the systemic circulation. Instead, ovarian function was determined by chemical estimates or bioassay of hormone quantities in pooled 24-hour collections of urine.

Brown, Klopper, and Lorain (100) not only defined the patterns of urinary estrogens, gonadotropins, and progestins, but reviewed the earlier studies in man. They described the characteristic

excretion of estrogen beginning on Day 8 of the menstrual cycle and increasing to a well-defined peak on Day 12. The gonadotropin peak that follows about a day later is succeeded by the excretion of pregnanediol, a metabolite of progesterone. These temporal sequences are similar in man (101, 102), the baboon (103), and the rhesus monkey (104). In this last study, the peak of urinary estrogen occurred two days before ovulation as shown by laparoscopic inspection of the ovary.

Initial efforts to estimate the quantities of estrogens in peripheral blood were unsuccessful except in a few isolated cases near the time of ovulation (105-107). Other studies using similar methods resulted in conflicting data (108-111). In 1969, numerous reports established that estradiol and estrone peaks are present at midcycle (112-114). These results were substantiated in later studies on man (3, 115-118) and the rhesus monkey (4). The increasing level of estrogen in plasma at midcycle indicates the greater secretory capacity of the maturing follicles and the estrogen peak reflects maximum secretion by the preovulatory follicle. Moreover, the observation that larger quantities of estradiol than estrone are present peripherally is compatible with the hypothesis that estradiol is the major estrogenic steroid released by the follicle. This view is further confirmed by the fact that that follicular fluid contains more estradiol than estrone (119) and that much larger amounts of estradiol

are found in ovarian venous plasma (85, 87, 97). However, during the luteal phase the concentrations of estrogens in the rhesus monkey do not increase as they do in the human female.

The urinary excretion of pregnanediol is greatest during the second half of the human menstrual cycle (100, 120). Earlier bioassay measurements of progestin in the plasma of man (121, 122) and the rhesus monkey (123, 124) supported this finding although early chemical measurements were only partially successful. An early study found the steroid present throughout the cycle (125), and Short and Levett reported that luteal concentrations were greater than follicular levels (126). Lack of specificity in the analytical methods probably accounts for such divergent results. However, in a study of progesterone concentrations in daily plasma samples (127), Woolever confirmed the findings of Short and Levett. Despite later confirmation, the results were still ambiguous and more qualitative than quantitative (128, 129). Questions about the periovulatory levels of progesterone, the exact peripheral concentrations, and the temporal relationships to ovulation and other physiological parameters during the cycle remained unanswered.

With the advent of sensitive radioimmunoassays and competitive protein binding assays as well as refined separation techniques, the simultaneous quantitative determination of steroids and gonadotropins in systemic plasma became possible (130, 131). Consequently,

the subtle and rapid alterations in hormonal patterns during the cycle could be detected daily and the quantity of progestins in the general circulation was found to increase either at or immediately after the midcycle surge of LH in man (1, 2, 118, 132-136), the monkey (130, 137-139), and the baboon (103). The measurement of a serum gonadotropin peak in subhuman primates confirmed the previous observation of an increase in urinary gonadotropin at midcycle (140).

The principal progestin to appear with the increased quantities of LH is 17 α-hydroxyprogesterone (2, 18, 141, 142). These observations established that 17 α-hydroxyprogesterone is present near the time of ovulation (143) and others identified it in ovarian venous blood (84). However, progesterone is also present in the preovulatory follicle (144) and in venous blood from the ovary with the preovulatory follicle (98). In a study based on laparoscopic examination, significant amounts of progesterone appeared in the peripheral plasma of the rhesus monkey before ovulation (137); however, the reliability of the morphological criteria used to determine the time of ovulation has been questioned (145). In earlier studies neither progesterone nor its 17 α-hydroxy metabolite could be identified in the ovaries or in the ovarian venous plasma of the monkey (86). The mean concentration of progesterone in women increased significantly (1-2 ng/ml) on the day of the LH surge but not in every cycle (118). Preovulatory progesterone is consistent with the qualitative morphological changes

in the human endometrium (146) and with the observation that the synthesis of progesterone probably depends on LH activity (147).

Progesterone, the major progestin in blood during the luteal phase, is found in quantities two to seven times greater in man (1) than in the subhuman primate (103, 130). These results confirm the observation that the amount of progesterone in ovarian venous blood is related to the relative size and maturity of the corpus luteum (84, 99).

Some of the studies mentioned previously have characterized the temporal relationship between the estrogens and the gonadotropins in peripheral plasma (2-4, 113, 115, 118). Apparently both LH and follicle stimulating hormone (FSH) are necessary for the appropriate growth and maturation of the follicles during the early and middle periods of the follicular phase (2). In man, the midcycle surge of LH follows the peak of estradiol by at least 24 hours (118). According to some interpretations, the rapid rise in LH is the result of a positive feedback of estradiol on the neural substrate mediating gonadotropin release (148-150). During the remainder of the cycle, the negative feedback effect of the peripheral steroids directs the release of the pituitary hormones.

The two feedback relationships explain the changes in systemic patterns of steroids found throughout the menstrual cycle except during the brief period after the estradiol peak and during the LH surge.

At this time, peripheral estrogen concentration is falling whereas the gonadotropins are still increasing. In the rhesus female, the estradiol and gonadotropin peaks occur on the same day when evaluated on a 24-hour basis (4); however, further investigation has shown that estradiol precedes the burst of gonadotropin in the monkey as it does in women.[1] Although the precise interval between the LH surge and ovulation in the primate is unknown, it is generally assumed to be at least one day later or perhaps even longer (137, 151). Therefore, this rapid decrease in systemic estrogen during the release of LH is most probably not a consequence of the postovulatory physical disruption of the follicle. The temporal relationships observed in the peripheral patterns of estrogens, progestins, and gonadotropins are summarized in Figure 3.

For the most part, the temporal patterns of androgens in the systemic plasma during the ovarian cycle are unknown. In the rhesus monkey, the earliest report indicated that androgens in the urine increased near the time of ovulation, then decreased, and increased again at menstruation (152). After reviewing the scanty data available, the same author concluded that there is no correlation between the excretion of androgens or 17-ketosteroids and the stage of the menstrual cycle (153). Later investigators evaluated the excretion

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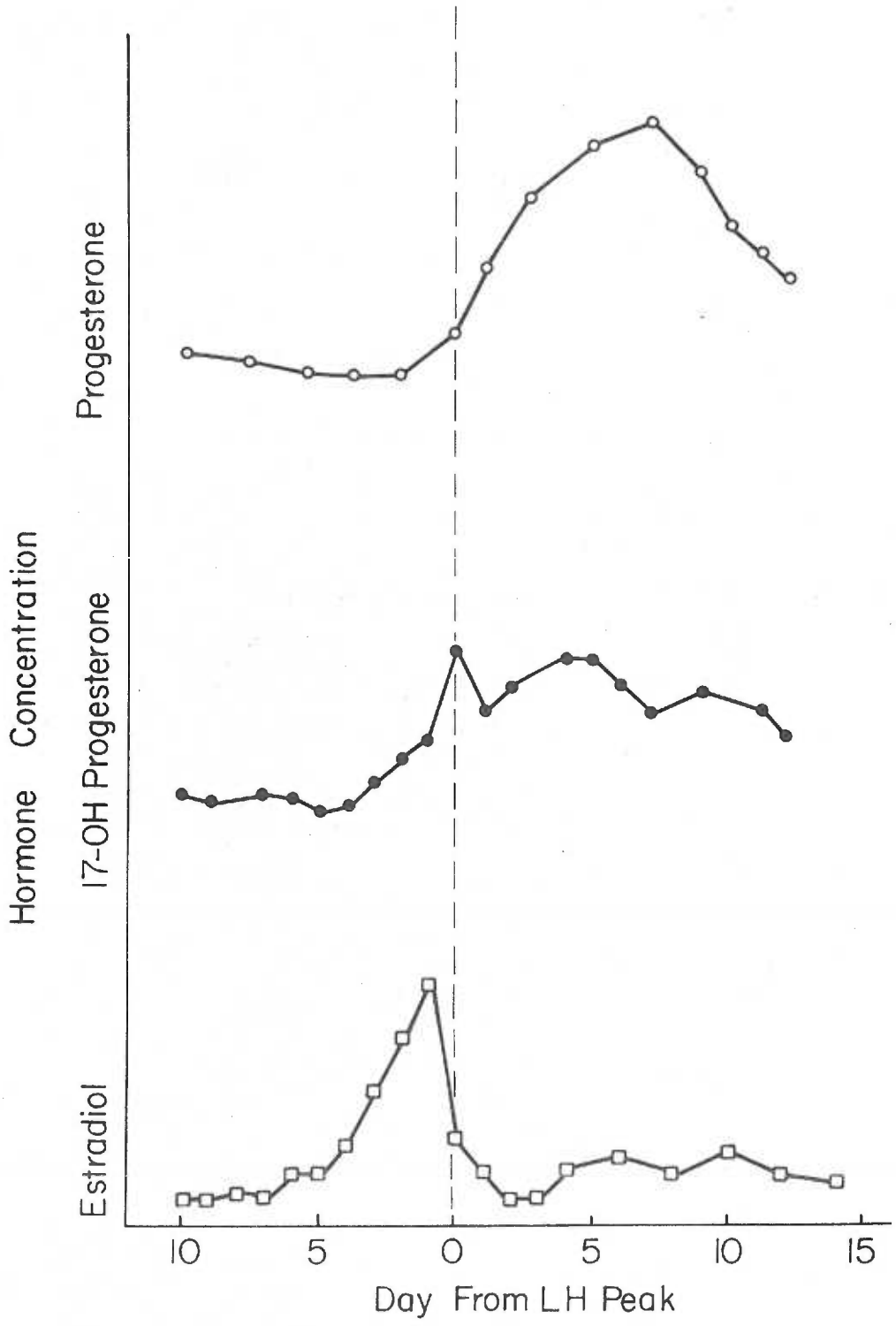
[1] Dr. R. Wieck, personal communication.

Figure 3. Schematic representation of the ovarian hormones in the systemic plasma of the primate during the intermenstrual period. Day 0 is the day of the preovulatory surge of LH (118).


Hormone Concentration
Progesterone
17-OH Progesterone
Estradiol
10
5
0
5
10
15
Day From LH Peak

of testosterone in man and observed a midcycle peak in eight of the ten cycles studied (154-156). These results were not supported in four cycles studied by other workers (157). Such inconsistencies are not surprising since each plasma androgen does not have a unique excretory metabolite and thus may contribute to the total excretion of testosterone (158); therefore, testosterone concentrations in the urine are not related to concentrations in the peripheral plasma (159).

The development of a spectrofluorometric assay for testosterone in plasma made it possible to estimate the quantity of this hormone circulating in plasma (160, 161). During the subsequent ten years various techniques--including double isotope derivatives, gas-liquid chromatography, and competitive protein binding--were developed to reduce the quantities of plasma as well as the time and expense involved in completing the analysis. But even the most sensitive of these techniques required at least 2 ml of plasma because of the small amounts of testosterone in female blood. Consequently detailed studies of androgen pattern during the cycle were impossible in subhuman primates and difficult in man. These studies (94, 162-190) identified both testosterone and androstenedione in the general circulation of the female, although the ranges varied considerably. The average (± SD) concentration in systemic plasma without reference to the stage of the cycle was 0.062 ± 0.029 μg/100 ml (testosterone) and 0.154 ± 0.028 μg/100 ml (androstenedione).

Both the ovary and the adrenal contribute significantly to the pool of androgen in the systemic circulation (162, 191, 192). This conclusion was based on decreased concentration of the steroids in peripheral plasma with ovariectomy and adrenalectomy (162, 166, 190). The efforts to indirectly identify the source of these androgens by simple stimulation or repression of the organs were inconclusive. According to several workers, ACTH stimulation increased the peripheral levels of testosterone (176, 179, 193) and urinary testosterone excretion (194), but others could not detect any change in plasma under similar circumstances (85, 175). Dexamethasone treatment reduced the plasma levels of both testosterone and androstenedione (171, 177) but again these results were not obtained by other investigators (85, 166, 175). Similar attempts to increase ovarian androgen secretion with placental gonadotropins were unsuccessful (171, 175). Ovarian production of testosterone, rather than the diurnal activity of the adrenal was thought to result in the steady plasma level of this steroid in females. Moreover, the observation that peripheral testosterone levels are at least two times greater in adults than in prepubertal females was considered additional evidence that androgen is released by the active ovary (94, 195). These results suggest both ovarian and adrenal production of androgen, but the most conclusive evidence was the significant differences in the concentrations of both androstenedione and testosterone across the circulation

of the ovary (84, 85, 91, 94, 169, 190).

Since the ovarian cycle is known to influence almost every other physiological variable (196), numerous attempts have been made to relate the plasma levels of testosterone with the phases of the menstrual cycle (90, 159, 163, 165-167, 172, 176, 179, 187, 188). Several of these investigators reported that peripheral testosterone or androstenedione levels increased significantly near midcycle (163, 176, 188). During the luteal phase of the cycle in women, plasma androgens show a secondary rise and then decrease again before menstruation (159, 187, 188). Most of these investigators, however, concluded that there are no significant alterations in the plasma levels of either androgen during the menstrual cycle.

In summary, the patterns of estrogens and progestins in the systemic circulation are well-defined during the ovarian cycle, and their appearance is related to the cyclical appearance of gonadotropins. Androstenedione and testosterone are present in the peripheral plasma of the female, but technical limitations have made it impossible to characterize the secretory activity of the ovary by this criterion.

## Ovarian Cycle: Regulatory Mechanisms for Steroidogenesis

Gonadotropins of either pituitary or placental origin were implicated in the regulation of steroid biosynthesis by the fact that they

alter the morphology of the ovary. The increased excretion of urinary estrogens that resulted from the administration of a hormone pituitary extract to amenorrheic women was consistent with the stimulatory effect of gonadotropins on follicular growth (197). Similar increases in the production rate of estrogen in plasma in women (3, 198) and in the excretion of estrogens in the urine of monkeys have been observed with either gonadotropic preparation (199). When monkeys in the late follicular phase were treated with human chorionic gonadotropin (HCG), progestins appeared in the ovarian venous plasma before the rputure of the follicle and further elevated the venous levels of progesterone in the ovary containing the corpus luteum (86). The systemic level of progesterone can be augmented by the administration of LH or HCG during the luteal phase in either man (141, 200) or the rhesus monkey (201). Antisera to LH administered to monkeys before ovulation blocked the secretion of progesterone by the corpus luteum and resulted in premature luteal regression (202). These results are direct evidence for the stimulatory effects of gonadotropins in vivo.

Ovaries treated in vivo with ovine FSH or human menopausal gonadotropin (HMG) before the removal of the tissue during the follicular phase (56, 77, 203) were highly active in steroid biosynthesis in vitro. Other investigators added HCG to in vitro incubations of ovarian tissue to obtain measurable quantities of metabolites (33, 34,

42). In all primate tissues studied, the addition of LH or HCG to *in vitro* incubations of ovarian slices (48), follicular tissue (141), stroma (58, 59, 61), luteal tissue (61, 68, 71) or isolated granulosa cells (78, 79), resulted in 2 to 20-fold increase in all of the steroidal products isolated relative to control incubations. In studies with double-labeled substrates (71, 91), the isotopic ratios of the purified products were not affected, and in luteal tissue, both the absolute quantities and the activity (dpm) of the *de novo* progesterone were increased (70). The formation of androgens by the postmenopausal ovary was stimulated by treatment with HCG *in vivo* (204). These results indicate that LH has a stimulatory effect on the *in vitro* biosynthesis of steroids when acetate, cholesterol, or pregnenolone are used as substrates. However, the increase in the activity of the aromatizing enzyme system by either FSH or LH was blocked by antigonadotropins (205).

To identify the site of gonadotropin action, Savard, Marsh and Rice (206) added LH and FSH to *in vitro* preparations of bovine luteal tissue. They concluded that FSH is ineffective in increasing steroid synthesis in this system and that the stimulatory effect of LH probably occurs between cholesterol and pregnenolone. However, they did not rule out the possibility that all steps in the biosynthetic pathway can be accelerated. The general conclusion from the *in vivo* and *in vitro* studies is that the function of gonadotropins is stimulatory.

Their effect on the ovary in vivo differs from the dramatic general increase in steroidogenesis in both pathways in vitro, but the fact remains that no inhibitory effects have yet been demonstrated for the pituitary tropic hormones.

Other possible sites for regulation have been proposed despite the paucity of definitive experimental work. The production of different steroids by different cell types has been suggested (80, 81), but a simple change in vascularity or diffusion parameters even in the presence of the third or stromal compartment does not sufficiently explain the large quantitative alterations in steroid release throughout the menstrual cycle. The most obvious mechanism identified by the in vitro studies is a differential change in the activity of the two enzymatic pathways, i.e., the $\Delta^5$-3β-ol pathway in the follicle (Path B) and the $\Delta^4$-3-keto pathway in luteal tissue (Path A). This may be one of the ways of chemically defining the luteinizing process, although there is currently no evidence for a differential effect of LH on this process. Another factor, described by Savard, et al. (206) is the availability of NADPH. This may be important, but it is unlikely that it is a major limiting factor in the control of steroid biosynthesis.

This present study was developed to explore another possibility, viz., the direct local control of steroidogenesis by a secretory product of the ovary. The traditional concepts of enzymatic induction,

activation, or inhibition have not been extensively studied in ovarian cell population because in vitro studies have not produced sufficient evidence for such regulatory mechanisms. The observation that the systemic levels of estradiol are decreasing whereas the cellular populations presumably responsible for their release, that is, the theca interna of the active follicles, are still intact and under intense LH stimulation, suggests such an internal regulatory mechanism. The concurrence of progestins with the initial release of pituitary LH provides a logical substrate for inhibiting some critical site(s) in estrogen synthesis. With reference to Figure 2, such inhibition could affect the biosynthesis of $C_{18}$ steroids at the aromatization step or the formation of $C_{19}$ steroids by inhibiting the 17-20 desmolase (lyase) complex with a subsequent depression in estrogen formation. In homogenates of rat testis, progesterone was an effective competitive inhibitor of the desmolase enzyme responsible for the conversion of 17 α-hydroxyprogesterone to androstenedione (207). In a study that used pregnenolone as the substrate in human corpora lutea, progesterone and 17 α-hydroxyprogesterone accumulated with only a slight concurrent formation of androstenedione (69). In the same study, neither progestin was directly converted to androstenedione. These findings complement those in the testis. Similarly, the active cleavage of 17 α-hydroxyprogesterone by the microsomal fraction from rat testis was completely inhibited by the progesterone metabolite 17 α,

20α-dihydroxypregn-4-en-3-one (208). We propose here that progesterone or a progestin acts in a similar fashion in vivo and we will produce some evidence for this regulatory action.

# CHAPTER III

# MATERIAL AND METHODS

## Animals and Animal Care

The subjects used for these studies were mature females (Macaca mulatta) from the rhesus colony of the Oregon Regional Primate Research Center. In most cases, the exact ages of the subjects were unknown since many had been obtained as juveniles or adults of indeterminate age. However, body weights and reproductive performance before these studies indicated that they were mature females.

The animals were individually caged indoors under constant temperature (20 ± 2°C) and were maintained on monkey chow (Purina Co.) ad libitum and fresh fruit. The average weight of the subjects was 7.8 kg with a range from 5.5 to 11 kg. The animals were checked each morning for menses, and routine health checks for tuberculosis, weight loss, or other abnormalities were carried out by the veterinary staff of the Primate Center. Bimonthly during an experimental period, the animals were treated with intramuscular injections of 50 mg iron dextran (Inferon, Lakeside Lab., Inc., Milwaukee, Wis.), and blood hematocrit and hemoglobin levels were monitored as an

additional precautionary measure.

Individual menstrual records from twenty normal females were examined from January, 1970, to July, 1971, and ten subjects were chosen from this group on the basis of menstrual regularity during these 19 months. The mean menstrual period (± SD) was 28.7 ± 3.0 days with a range of 26.5 to 31.1 days.

## Blood-Handling Techniques

Animals were individually transported from their home cage to the bleeding cage in a catch box. The bleeding cage was designed so that the monkey could be immobilized without trauma against one wall of the cage. The end of the cage at the rear of the animal was then opened, the desired leg was withdrawn and stabilized, and 5 ml of blood were collected from the saphenous vein. The animals were well-acquainted with the procedure and in some cases would extend a leg without assistance. Samples were removed from alternate legs on alternate days between 0830 and 0900 hours. The caudal surface of the leg was washed with 70% ethanol and blood collected through a 23-gauge needle into an heparinized plastic syringe. After the withdrawal of the sample, a cotton ball was firmly taped over the venipuncture site and the monkey was returned to her home cage.

The blood was kept on ice until centrifuged at 1500 rpm for 20 minutes at 4°C. Special care was taken to begin plasma separation

within 20 minutes after the first sample was drawn; typically only 5 to 10 minutes elapsed before centrifugation. Aliquots of one half ml of plasma were stored at -20°C until used for steroid assay.

## Steroid Administration

It was desirable to use a technique for steroid administration whereby plasma progesterone levels typical of the luteal phase could be rapidly produced and maintained during the follicular phase of the cycle. Since steroids easily pass through silastic sheets (209), silastic implants containing progesterone were the method of choice.

Six females (from 7 to 11.5 kg) that had been ovariectomized for at least two years were used to determine the dimensions of a silastic implant that would release physiological amounts of progesterone. The criterion for normal luteal function in our laboratory is arbitrarily defined as the production of concentrations of systemic progesterone greater than 2 ng/ml; blood levels in the treated animals had to be above this concentration throughout the treatment period.

The implant used in the rest of the experiments was a 4 cm length of silastic tubing (3.35 mm ID X 4.65 mm OD), (Dow Corning, Midland, Michigan) sealed at one end with silastic type A cement (plug thickness = wall thickness) and cured for two to three hours at 90°C. This cylinder was filled with either crystalline progesterone or cholesterol (Steroloids, Inc., Pawling, N. Y.) and the open end washed

carefully with methanol to remove any adhering steroid before being sealed with cement. The completed implant was allowed to cure overnight at room temperature and just before placement was sterilized by being soaked for 5 to 10 minutes in chlorhexidine (Nolvasan, Fort Dodge Lab., Fort Dodge, Iowa).

On the day of surgery (Day 0), the ovariectomized females were fasted and a 5 ml blood sample was drawn before they were anesthetized with ketamine hydrochloride (Ketaject, 5-10 mg/kg, Bristol Lab., Syracuse, N. Y.). A 4 to 5 cm incision was made under aseptic conditions 1 to 2 cm lateral to the linea alba on the left side at the level of the umbilicus. The implant was placed between the top layer of fascia and the underlying mass of the rectus abdominus, every precaution being made to avoid disturbing the muscle. A few stitches were taken in the fascia, the rest of the incision was closed, and antibiotics (penicillin, 600,000 units, Bicillian, Wyeth Lab., Philadelphia) were given IM. Peripheral blood was collected on the afternoon of Day 0 and twice daily at 0830 and 1530 hours through Day 8 except on the weekend when only the morning samples were drawn. The implant was removed on the morning of Day 6 after the usual blood collection. A photographic record of the implant placement and removal procedure is given in Appendix B. The animals ate normally on the afternoon of surgery and no complications were observed. A check for menstrual flow was made by inserting a

cotton-tipped swab into the vagina daily for three days after the removal of the implant; no vaginal bleeding was ever noted.

## Normal Cycle

The ten normal subjects previously described were bled daily during a menstrual cycle where Day 1 was designated as the first day of the onset of menses. Except for bleeding and iron replacement therapy, no experimental or other manipulations were permitted during this cycle.

## Cholesterol and Progesterone Treatment

Daily collections of peripheral blood were continued during the subsequent menstrual cycle. In addition, the animals were treated with either a progesterone (N = 5) or a cholesterol (N = 5) implant from Day 6 to Day 15. Preparation of the implants and surgical placement were the same as previously described, and the usual blood samples were drawn before surgery on both Day 6 and Day 15. Animals were assigned to either treatment group according to the order in which the last digit of the individual identification number appeared in a random number table. Gross examination of the implant sites showed no signs of connective tissue incapsulation when the implants were removed (Appendix B). All animals were checked for menstrual flow for two to three days after Day 15, and peripheral samples were

collected until the onset of menstrual flow in both treatment groups. These studies were conducted from August to November, 1971.

## Laparotomy Cycle

A four-month interim period was allowed to elapse before the final phase of these studies began. Daily 3 ml peripheral blood samples were drawn at 1300 hours and ovarian venous samples were obtained by three sequential laparotomies on Days 7, 9, and 11 of the menstrual cycle. A progesterone (N = 2) or cholesterol (N = 2) implant was placed on Day 7 after the collection of the ovarian vein samples and removed on Day 15, four to five hours before the collection of the peripheral blood sample.

The following surgical procedure was used. Animals were brought to surgery at 1245 hours, a 17-gauge polyethylene catheter (Tomac Intrafusor) was placed in the saphenous vein, and the infusion of 5% dextrose in normal saline began. Atropine (0.2 mg, Eli Lilly Co.) and succinyl choline chloride (20 to 40 mg, Quelicin, Abbot Lab.) was administered to each animal and an endotracheal tube was inserted. Surgical anesthesia was maintained with vaporized halothane (Fluothane, 1 to 1.5%, Ayerst Lab, Inc., N. Y., N. Y.) in 50% oxygen and 20% nitrous oxide and a 3 ml sample of systemic blood was obtained from the venous catheter. The abdomen was opened with a midline incision and the ovaries were exposed and examined

grossly for evidence of follicles. Physical disturbance of the ovaries was kept at a minimum during these observations. At a site 2 to 4 cm distal to the ovary, one ovarian vein was located by blunt dissection and 2 to 3 ml of blood were drawn through a 25-gauge needle bent to a 30-45° angle and attached to a heparinized plastic syringe. After withdrawal of the needle, hemostasis was achieved with surgical gauze before a similar sample was collected from the contralateral ovarian vein. Before the incision was closed, a final check was made for hemostasis. On Day 7, in two animals, the implant was placed in the rectus abdominus muscle through the initial incision. In the other two animals, a second incision was made for the implant. On Days 9 and 11, the same midline incision was used and ovarian vein blood collected as above. In addition, on Day 11, the laparotomy incision was completely debrided to obtain a primary closure. The implant was removed on Day 15 under local anesthesia (1% lidocaine, Xylocaine, McKesson and Robbins) to prevent further surgical stress and blood loss. After surgery, the animals were treated with antibiotics, and after the last laparotomy, an intravenous vitamin preparation was given (Solu-B-Forte, Upjohn Lab.). In a single animal (#3594) methyl prednisolone sodium succinate (Solu-Medrol, Upjohn Lab.) was administered. The total time course for drug action was about 36 hours. The effects of this treatment on the experimental protocol seemed negligible because of the

time of injection (after the main effects had been observed) and because steroid levels in ovarian venous plasma were low in three of the animals, even those without prednisolone. Peripheral samples of blood were collected until the onset of the first menses after surgery. The animals recovered well from these procedures and required no special care.

## Steroid Assays

Preparation and purification procedures for solvents, reagents, labeled and unlabeled standards, and the chromatographic plates used in the steroid assays are given in Appendix C. In general, steroid levels were estimated in duplicate by radioimmunoassay on samples drawn at daily intervals until the pattern from five normal cycles was obtained. After this period, single determinations were carried out with occasional duplicates from the same and previous assays as a method check. Initially, blank samples consisted of plasma from ovariectomized monkeys for the progesterone and estrogen assays and plasma from ovariectomized, adrenalectomized monkeys for the androgen assay. When it became apparent that the blank values obtained from equal volumes of deionized water were the same as those from plasma, water blanks were routinely used thereafter.

Competitive Protein Binding Assay for Progesterone (P)

The competitive protein assay for P is a modification of the method recently reported (1). P was extracted from 500 µl of systemic plasma with 2.5 ml (X3) of fresh anhydrous ether (Mallinckrodt AR). The combined ether extracts were dried and concentrated under nitrogen. P was separated from other substrates by thin-layer chromatography in benzene:ethyl acetate (2:1) on silica gel G with 1,4-diaminoanthroquinone dye (K and K Laboratories, Inc., Hollywood, Calif.) as a marker. The steroid was eluted with 4% methanol-methylene chloride from the silica gel scrapings that were placed on an aluminum oxide column. P runs 1.7 cm above the dye, and a 2.5 cm wide area was removed for elution. The column extracts were taken to dryness under purified nitrogen. The binding protein was obtained from dog plasma diluted to 2.5% with distilled water and saturated with 250 µl of $^{3}$H-corticosterone (5.5 µCi/ml, 50 Ci/mM, New England Nuclear Corp., Boston, Mass.). Standard amounts of P (0.3, 1.2, 2.1, 4.2, 8.4, 9.9 ng) run in triplicate were included with each assay. One ml of the labeled binding protein was added to each tube, shaken, and allowed to incubate at 38$^{\circ}$C for 5 minutes. After incubation for an additional 10 minutes on ice, 80 mg of florisil were added to the tube, shaken for 30 seconds, then allowed to settle for 30 seconds before a 500 µl aliquot was removed and added

to a counting vial containing 0.5 ml water and 10 ml Triton-N101 scintillation fluid (2 liters xylene, 1 liter Triton-N101, Rohm-Haas Co. and 10 g Omnifluor, New England Nuclear Corp.). All samples in this and the following assays were counted for 20 minutes or to an accumulation of 20,000 cpm in a liquid scintillation spectrometer (Packard Model 3375 with automatic external standardization). The maximum relative statistical error was less than 2% under these conditions. Independent samples of $^{3}$H-P (20 Ci/mM, New England Nuclear Corp.) were carried throughout the extraction and chromatography procedure to correct for procedural losses. Individual blanks in a given assay were averaged and subtracted from plasma values before being corrected for procedural losses (210). The average (mean ± SD) blank and recovery for P in this assay were 0.31 ± 0.19 ng, N = 66 and 69.8 ± 12.8%, N = 66 respectively.

Radioimmunoassay for Estradiol-17 β ($E_2$) and Estrone ($E_1$)

Steroids were extracted from 250 µl of systemic plasma or 25 to 50 µl of ovarian vein plasma with 6 ml of fresh anhydrous ether (Mallinckrodt AR). The ether extract was taken to dryness under nitrogen. The two estrogens were separated by chromatography on Eastman silica gel thin-layer sheets (#6060 with fluorescent indicator) in chloroform:acetone (95:5). Before use, the sheets were washed (X2) with redistilled methanol. Isatin dye (J. T. Baker Chemical Co.)

was used to locate $E_2$ since the $R_f$ for $E_2$ and the dye are similar and $E_1$ runs 2.5 cm above the dye in this system. $E_1$ and $E_2$ areas were eluted with 6 ml of fresh ether into assay tubes and dried under purified nitrogen. Duplicate standard curves (0, 5, 10, 20, 30, 50, 75, 100 and 150 pg) of respective steroids were included in each assay. Antisera (0.1 ml) obtained from sheep immunized with estradiol-17 β-succinyl bovine serum albumin (1:15,000 dilution) were added to each tube, shaken, and allowed to incubate for 0.5 hour at room temperature. Approximately 4000 cpm of $^3$H-$E_2$ (100 Ci/mM, Amersham/Searle) in 0.1 ml phosphate buffered saline (PBS) containing 0.1% gelatin were added to each tube, shaken, and incubated overnight at 4°C. The next morning, 0.1 ml of a 0.5% gelatin-PBS solution and 1.0 ml of dextran-coated charcoal in PBS were added to each tube and shaken. After incubation for 15 minutes on ice, the tubes were centrifuged at 2500 rpm at 4°C and a 1.0 ml aliquot of the supernatant was removed and added to 10 ml of the Triton-N101 scintillation fluid. Independent samples of $^3$H-$E_2$ and $^3$H-$E_1$ (46.6 Ci/mM; 40.0 Ci/mM respectively, New England Nuclear Corp.) were carried throughout the procedure to correct for procedural losses, and individual blanks were averaged and subtracted in each assay as mentioned above. The sensitivity, precision, accuracy, and specificity of this method have been previously reported (211). The averages (mean ± SD) for $E_2$ and $E_1$ blanks and recovery values in this assay were 7.5 ± 2.2 pg,

N = 73; 77.7 ± 8.7%, N = 78, and 7.6 ± 2.2 pg, N = 26; 78.6 ± 9.5%, N = 33 respectively.

Radioimmunoassay for Testosterone (T)

Testosterone was extracted from 100 µl of systemic plasma or 50 µl of ovarian vein plasma with 6 ml of fresh anhydrous ether (Mallinckrodt AR), and the extract was taken to dryness with nitrogen. T was isolated by chromatography on Mallinckrodt ChromAR 1000 fiberglass sheets impregnated with silica gel in a chloroform:acetone (90:10) system. Before use, the sheets were washed (X2) with redistilled methanol. The area corresponding to T was located 1 cm below the isatin dye (J. T. Baker Chemical Co.), and this area was eluted with 6 ml of fresh ether into assay tubes and dried with purified nitrogen. Duplicate standard curves (5 to 150 pg) were included in each assay. Antisera (0.1 ml) obtained from rabbits immunized with testosterone-3-oxime bovine serum albumin (1:3000 dilution) were added to assay tubes. The remainder of the assay procedure was exactly as described for the estrogen radioimmunoassay except that approximately 4000 cpm of $^{3}$H-T (91 Ci/mM, New England Nuclear Corp.) were added to assay tubes after the initial 15 minutes incubation with the antibody. In Appendix D, data are presented that document the sensitivity, precision, accuracy, and specificity of this method. Independent $^{3}$H-T (45 Ci/mM, New England Nuclear Corp.)

samples were used to measure procedural losses, and the average blank in each assay was calculated and subtracted from the individual values determined from the standard curve. The average (mean ± SD) blank and recovery values for T in this assay were 11.6 ± 4.6 pg, N = 78; 79.2 ± 6.1%, N = 79 respectively.

Radioimmunoassay for Androstenedione (A)

In samples from normal and experimental cycles, A was estimated by difference, i.e., by subtracting the previously measured T from a determination of total androgen in systemic plasma after A had been reduced to T with sodium borohydride. In the samples taken during laparotomy, the hormone was quantified by the reduction of A after chromatographic purification and radioimmunoassay of the resulting T.

In the first method, 20 µl of systemic plasma were extracted with ether as before and the dried extract was redissolved in 0.5 ml of redistilled methanol. The A was reduced to T by the addition of 50 µl of aqueous $NaBH_4$ (10 mg/ml, Matheson, Coleman and Bell, Norwood, Ohio) to the methanolic solution. The reaction mixture was diluted after 20 seconds with 2 ml of glass distilled water and extracted with 6 ml of fresh ether. The dried extract was chromatographed on ChromAR sheets in a chloroform:acetone (90:10) system. The T area was eluted into assay tubes and measured by radioim-

munoassay. Blanks were averaged as before and procedural losses were corrected by carrying independent $^{3}$H-A (48 Ci/mM, New England Nuclear Corp.) samples through the procedure. Testosterone was reported as pg/ml and A was calculated by subtracting the values of T previously measured.

In the second method, 100 µl of systemic plasma or 50 µl of ovarian vein plasma were extracted with fresh ether and chromatographed in the chloroform:acetone system. The A area which runs 1.5 cm above the isatin dye marker, was eluted with 6 ml of fresh ether. The dried extract was dissolved in 0.5 ml methanol and reduced as described above. Testosterone was removed from the unreduced A by an additional chromatography in the usual system and the T area eluted from the ChromAR sheets by 6 ml of fresh ether into assay tubes. Since the quantity of reduced A exceeded the upper limits of the T assay, the dried eluate was dissolved in 1 ml of redistilled ethanol and a 200 to 500 µl aliquot removed, dried under purified nitrogen, and assayed. Appendix D presents experimental data on the accuracy, precision, and sensitivity of this analytical method for A. The average (mean ± SD) A blanks and recoveries measured as T were 12.7 ± 5.1 pg, N = 25 and 64.0 ± 10.8%, N = 30 respectively.

## Data Analysis

Because the follicular phase varies widely in primates, the menstrual cycle is a poor index of ovarian function. Therefore, the data were combined and plotted by designating Day 0 as the day of the preovulatory surge of $E_2$ in each animal. The measurements of steroids made on the days before the surge were designated -1, -2, -3, etc. and those after the surge as 1, 2, 3, etc.

A one-way analysis of variance was computed to test for general effects (212). After this, comparisons between concentration of steroids in the follicular and luteal phase of the cycle were made by a paired $t$ test for correlated samples (213). Similar tests were used to compare the mean concentrations of steroids in the systemic plasma of animals before and after treatment with P and the concentrations of $E_2$ and A in ovarian venous plasma on Days 7, 9, and 11 of the cycle. A Pearson's coefficient of correlation (r) was calculated to test the correlation between the concentrations of steroid in the ovarian venous effluent and the systemic circulation as well as the possible correlations between the levels of steroid in plasma flowing through the two ovarian veins (214).

# CHAPTER IV

# RESULTS

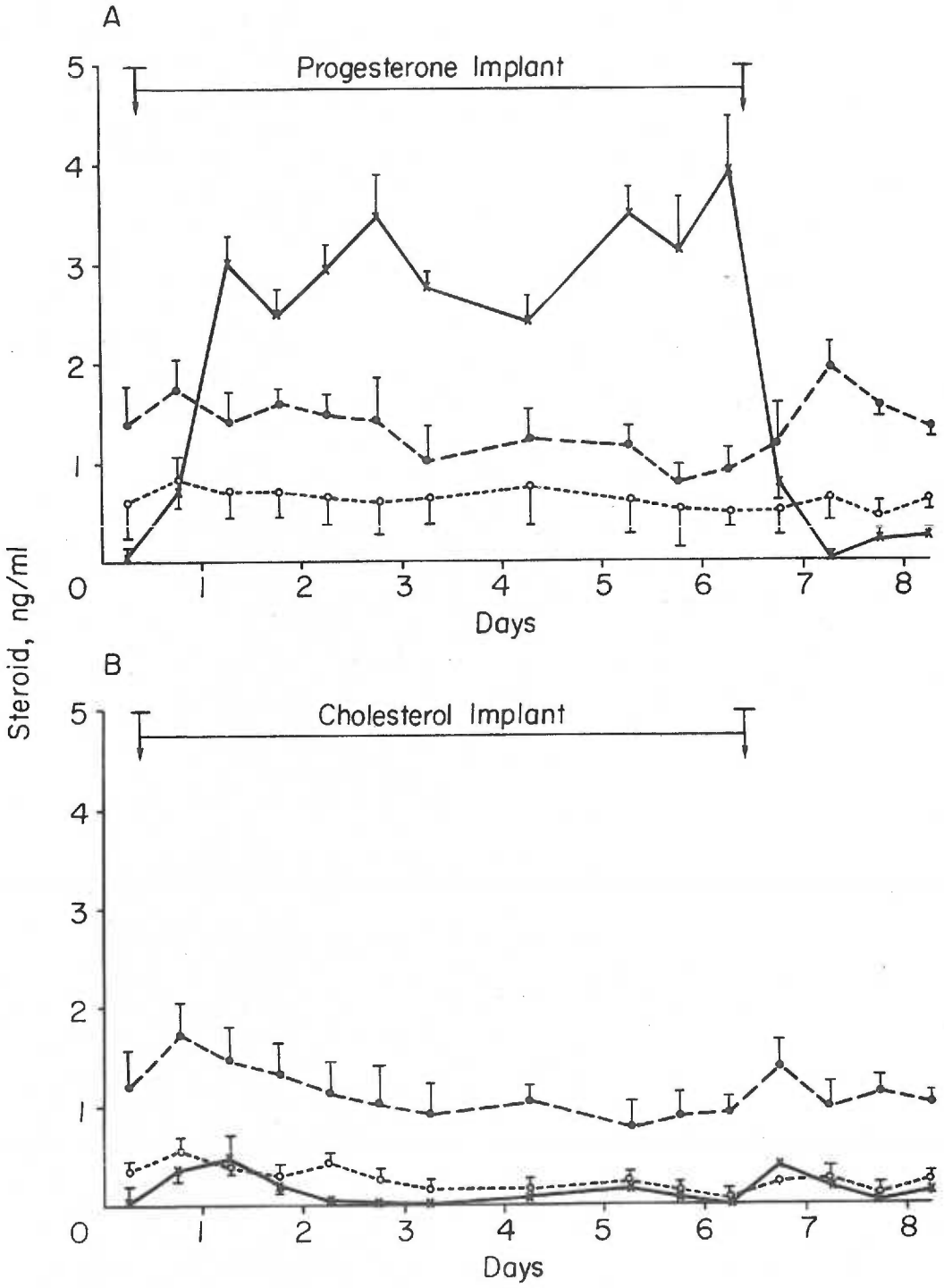
## Patterns of Steroids in Systemic Plasma of the Ovariectomized Female Rhesus Monkey; Treatment with Progesterone

The concentrations of progesterone (P), testosterone (T), and androstenedione (A) in the plasma of ovariectomized rhesus monkeys before, during, and after the removal of a silastic implant containing either P or cholesterol are shown in Figure 4.

During the period of the P implant (A), the plasma concentration of the hormone was 2.81 ± 0.26 ng/ml, N = 3 (mean ± SE), and ranged from 0.74 ± 0.17 ng/ml on the afternoon of Day 0 to 3.96 ± 0.46 ng/ml on the morning of Day 6 before removal of the implant. Androstenedione was present in greater quantities than T and the amount of A on the morning of Day 7 (1.96 ± 0.25 ng/ml) was significantly different from that on the morning of Day 6 (0.93 ± 0.21 ng/ml, $t = 4.15$, 2df, $p < 0.05$), but not significantly different from the quantity present before surgery on Day 0. The quantities of T found in the systemic plasma did not differ throughout the course of the treatment with P.

In animals treated with cholesterol (B), the concentration of P

Figure 4. The concentrations of progesterone (x——x), androstenedione (· — — ·), and testosterone (o----o) in the systemic plasma of six ovariectomized rhesus monkeys treated either with progesterone (A, N = 3) or cholesterol (B, N = 3). Data are plotted as means ± standard error (vertical bars).


A
Progesterone Implant
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Days
Steroid, ng/ml
B
Cholesterol Implant
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Days

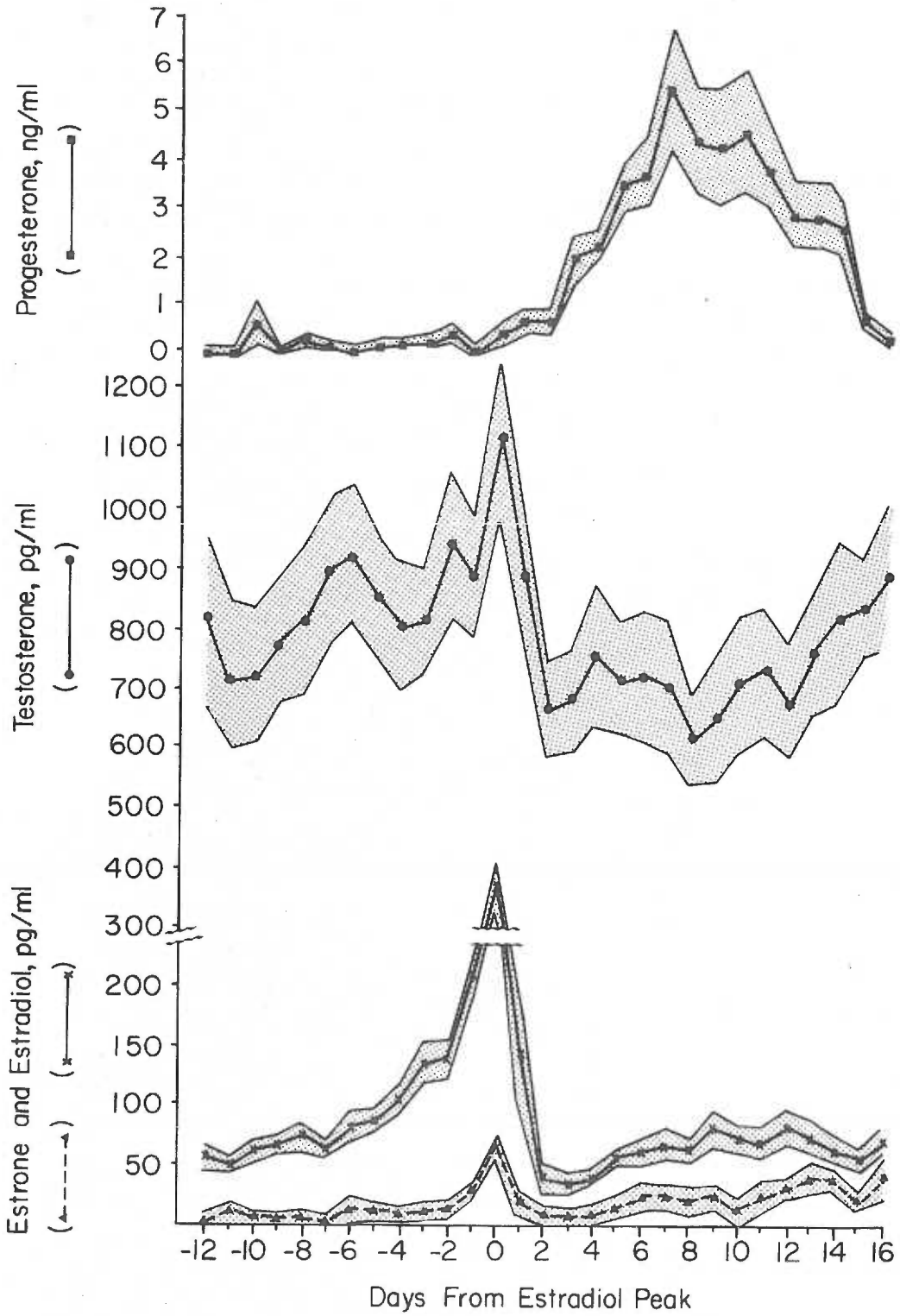
did not exceed 0.5 ng/ml during the treatment period. The mean level of P on the afternoon of Day 6 (0.40 ± 0.01 ng/ml, N = 3) was significantly greater than that on the morning of Day 0 (0.07 ± 0.07, $t = 19.0$, 2df, $p < 0.01$) or that on the morning of Day 6 (0.02 ± 0.01 ng/ml). Except for a suggestion of elevated concentration presumably caused by stress, there was no significant effect on A and T levels. The plasma levels of both androgens were relatively constant.

Before surgery, the concentrations of A and T were 1.31 ± 0.29 ng/ml and 486 ± 178 pg/ml respectively. On the morning of Day 8, the concentration of the two steroids was 1.20 ± 0.10 ng/ml and 461 ± 105 pg/ml respectively. These results were not significantly different.

## Patterns of Steroids in Systemic Plasma During the Normal Cycle

The concentrations of estrone ($E_1$), estradiol ($E_2$), T, and P throughout the menstrual cycle of nine females are shown in Figure 5. Coinciding with the midcycle surge of $E_2$ is a surge of $E_1$. A 5-fold difference, however, was observed in the concentration of $E_2$ (365 ± 34 pg/ml, mean ± SE) and $E_1$ (65 ± 6 pg/ml) on the day of the pre-ovulatory surge. Testosterone peaked (1123 ± 140 pg/ml) on the same day as the estrogens, and the systemic concentrations of these steroids began falling when the systemic level of P was increasing. In the individual animals, T reached maximum concentrations on the

Figure 5. Cyclical variations in the concentrations of steroids during the intermenstrual period of rhesus monkeys (N = 9). Day 0 is the day of the preovulatory surge of estradiol. Data are plotted as means ± standard errors (shaded areas).


Progesterone, ng/ml
Testosterone, pg/ml
Estrone and Estradiol, pg/ml
7
6
5
4
3
2
1
0
1200
1100
1000
900
800
700
600
500
400
300
200
150
100
50
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16
Days From Estradiol Peak

same day as $E_2$ in seven of nine cycles. In the other two cycles, the maximum level was seen on the day after the peak, although $E_2$ levels were still high.

The average concentration of T during the follicular and luteal phases of the cycle are shown in Table 1. The amount of T in the systemic plasma after the preovulatory surge differed significantly from that before the preovulatory surge. The mean concentration of this hormone 12 days before (836 ± 99 pg/ml) and 12 days after the estrogen peak (707 ± 89 pg/ml) were compared by means of a $t$ test ($t = 4.57$, 8df, $p < 0.01$).

The concentrations of A and T during the menstrual cycles of four females are shown in Figure 6. A preovulatory increase in A was not seen and the short-term decrease in systemic levels appears at least one day after the initial decrease in T. In addition, the luteal concentrations of A did not differ significantly from that in the follicular phase. The systemic alterations of this androgen did not parallel those of $E_2$ at any time of the cycle, and further analysis was deemed unnecessary.

The tenth subject in this group is not included because of a long 48-day anovulatory cycle as judged by the absence of the preovulatory surge of $E_2$ and by concentrations of P that were less than 2 ng/ml during the entire cycle. This animal also had a prolonged second

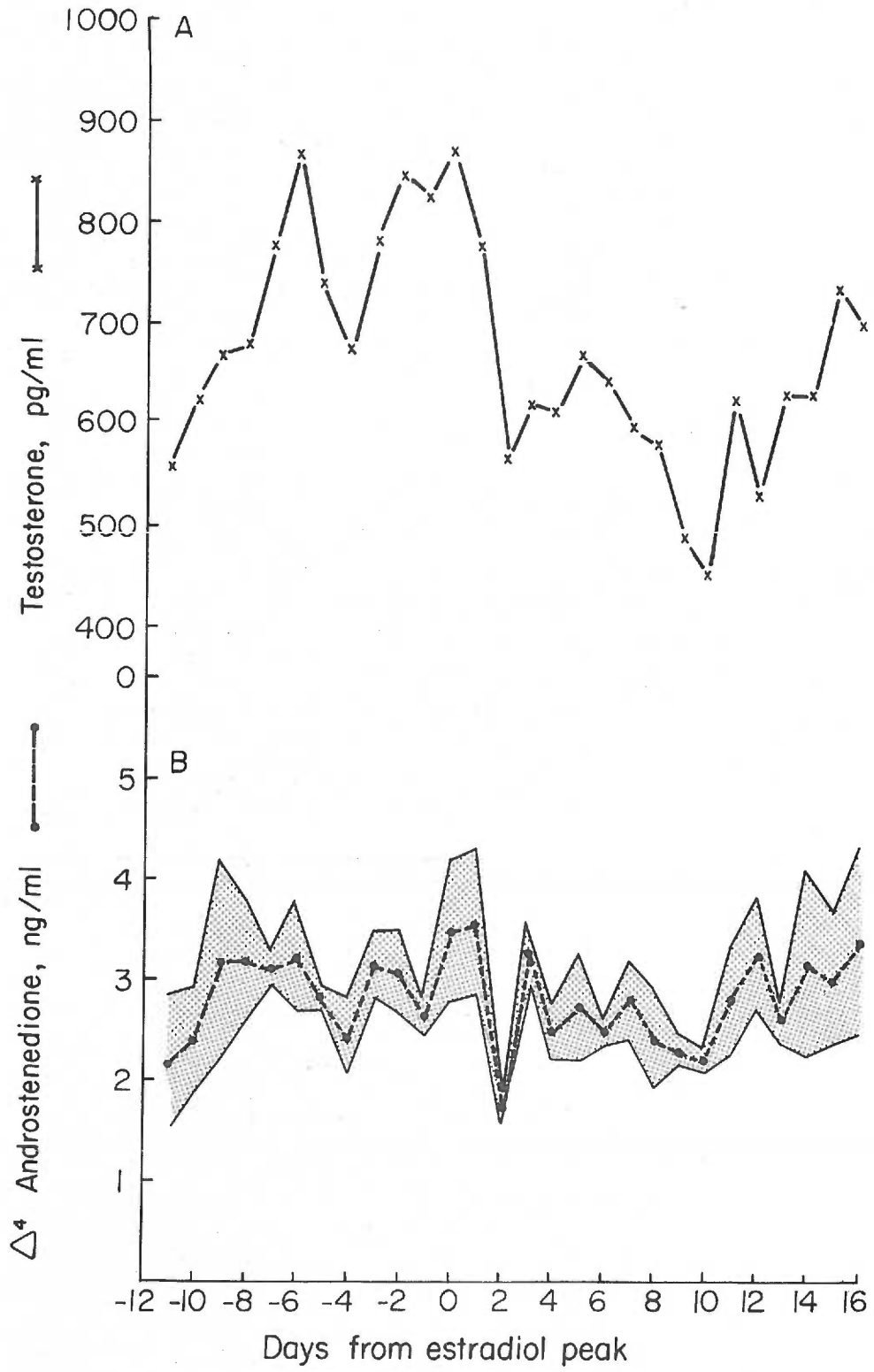
Table 1. The concentration of testosterone in the systemic plasma of rhesus monkeys during the follicular (Day -12 to Day -1) and the luteal (Day 1 to Day 12) phases of the menstrual cycle.

| Animal Number | Testosterone (mean pg/ml ± SE) Follicular | Luteal |
|---|---|---|
| 807 | 489 ± 35 (10)[a] | 358 ± 28 (12)[a] |
| 833 | 842 ± 81 (10) | 712 ± 63 (12) |
| 1252 | 957 ± 48 (11) | 716 ± 57 (12) |
| 3585 | 705 ± 14 (12) | 576 ± 20 (12) |
| 3594 | 1411 ± 33 (10) | 1267 ± 81 (12) |
| 3598 | 745 ± 49 (11) | 721 ± 34 (12) |
| 3599 | 1116 ± 64 (12) | 935 ± 50 (12) |
| 3601 | 452 ± 24 (12) | 480 ± 26 (12) |
| 3609 | 807 ± 34 (12) | 601 ± 53 (12) |
| Mean ± SE | 836 ± 99[b] | 707 ± 89[b] |

[a]Values in parenthesis indicate the number of sample days.

[b]Mean compared by a t test for correlated samples; $t = 4.57$, 8 df, $p < 0.01$.

Figure 6. Cyclical variations in the concentrations of androstenedione and testosterone from the same monkeys during the intermenstrual period (N = 4). Data are plotted as means and the standard error of the androstenedione estimation is the shaded area.


A
B
1000
900
800
700
600
500
400
0
5
4
3
2
1
Testosterone, pg/ml
Δ⁴ Androstenedione, ng/ml
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16
Days from estradiol peak

cycle of 43 days with similar steroid patterns and therefore is not included in the results reported for the implant study.

## Patterns of Steroids in Systemic Plasma During the Experimental Cycle

### Cholesterol Implant (Control)

Figure 7 demonstrates that the surgical manipulations required to place a silastic implant containing cholesterol did not alter the patterns of peripheral steroids in the subsequent intermenstrual period of four animals. The usual midcycle increase in T (983 ± 289 pg/ml, mean ± SE) and $E_2$ (249 ± 42 pg/ml) was found and the quantities of P in the systemic plasma indicated a functional corpus luteum. The concentrations of both $E_2$ and T declined concurrently with the initial rise in P. As in the previous cycle, the mean concentration of T in the follicular phase (803 ± 109 pg/ml) was significantly greater than that in the luteal phase (579 ± 161 pg/ml, Table 2). The concentration of $E_2$ in the luteal phase of the cholesterol treatment cycle (31 ± 11 pg/ml) was not significantly different from that of the normal cycle (61 ± 5 pg/ml, $t = 2.74$, 3df, $p > 0.05$). The duration of the menstrual cycle in the untreated and cholesterol-treated females was 28.7 ± 1.7 and 27.8 ± 1.7 days (mean ± SD) respectively. Similarly, the length of the luteal phase was 16.1 ± 0.78 and 16 ± 1.1 days respectively, an indication that neither the stress of daily bleeding nor the implantation

Figure 7. Cyclical variations in the concentrations of steroids in the systemic plasma of animals treated with a silastic implant that contained cholesterol from days 6 to 15 of the menstrual cycle (N = 4). Data are plotted as means ± standard errors (shaded areas).

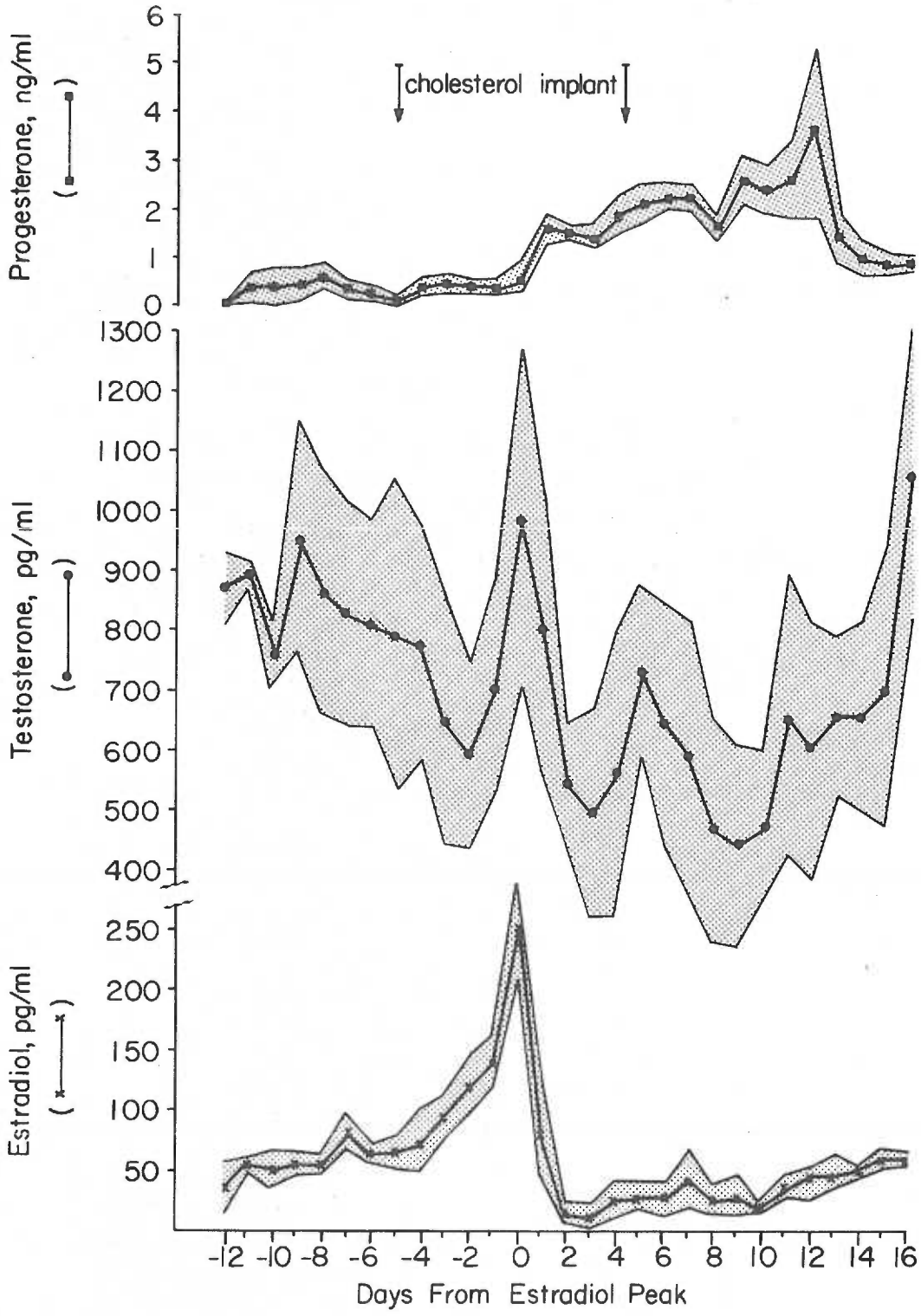

Progesterone, ng/ml
cholesterol implant
Testosterone, pg/ml
Estradiol, pg/ml
Days From Estradiol Peak

Table 2. The concentration of testosterone in the systemic plasma of rhesus monkeys (treated with cholesterol from Day 6 to Day 15) during the follicular and luteal phases of the menstrual cycle.

| Animal Number | Testosterone (mean pg/m/± SE) | |
|---|---|---|
| | Follicular | Luteal |
| 3594 | 1342 ± 50 (9)[a] | 1117 ± 60 (12)[a] |
| 3398 | 686 ± 46 (12) | 519 ± 39 (12) |
| 3601 | 514 ± 34 (10) | 270 ± 29 (12) |
| 3609 | 670 ± 48 (12) | 409 ± 47 (12) |
| Mean ± SE | 803 ± 109[b] | 579 ± 161[b] |

[a] Values in parenthesis indicate the number of sample days.

[b] Means compared by a t test for correlated samples: $t = 11.0$, 3df, $p < 0.01$.

procedure had significantly altered the duration of the menstrual cycle or the length of the luteal phase.

Progesterone Implant (Experimental)

The treatment of five animals with P from Day 6 to Day 15 of the menstrual cycle blocked the midcycle increase in T and $E_2$ (Figure 8). The initial rise in P was accompanied by a rapid and dramatic decrease in the circulating levels of $E_2$, which persisted throughout the entire treatment period. The average concentration of this hormone in the peripheral plasma during the treatment (39 ± 6 pg/ml, mean ± SE) was significantly less than that before placement of the implant (71 ± 4 pg/ml) and during the previous control luteal phase (71 ± 14 pg/ml, Table 3). After removal of the implant, the level of $E_2$ gradually increased to pretreatment concentration.

Although the quantity of T in systemic plasma, like that of $E_2$ appeared to decline, the apparent reduction in the concentration of T for the first six days of P administration was not significantly different from the pretreatment level. However, the usual peak of this steroid that should appear on the day of the $E_2$ surge was absent. Two days before the implant was removed, the systemic concentration of T seemed elevated. The levels of A in two of these animals also showed no significant alterations during the treatment with P (Figure 9). However, the patterns of the two androgens may be related.

Figure 8. Cyclical variations in the concentrations of steroids in the systemic plasma of animals treated with a silastic implant that contained progesterone from days 6 to 15 of the menstrual cycle (N = 5). Data are plotted as means ± standard error (shaded area) and the presumptive day of the estradiol surge is the modal day of the surge in the cholesterol treated cycle.

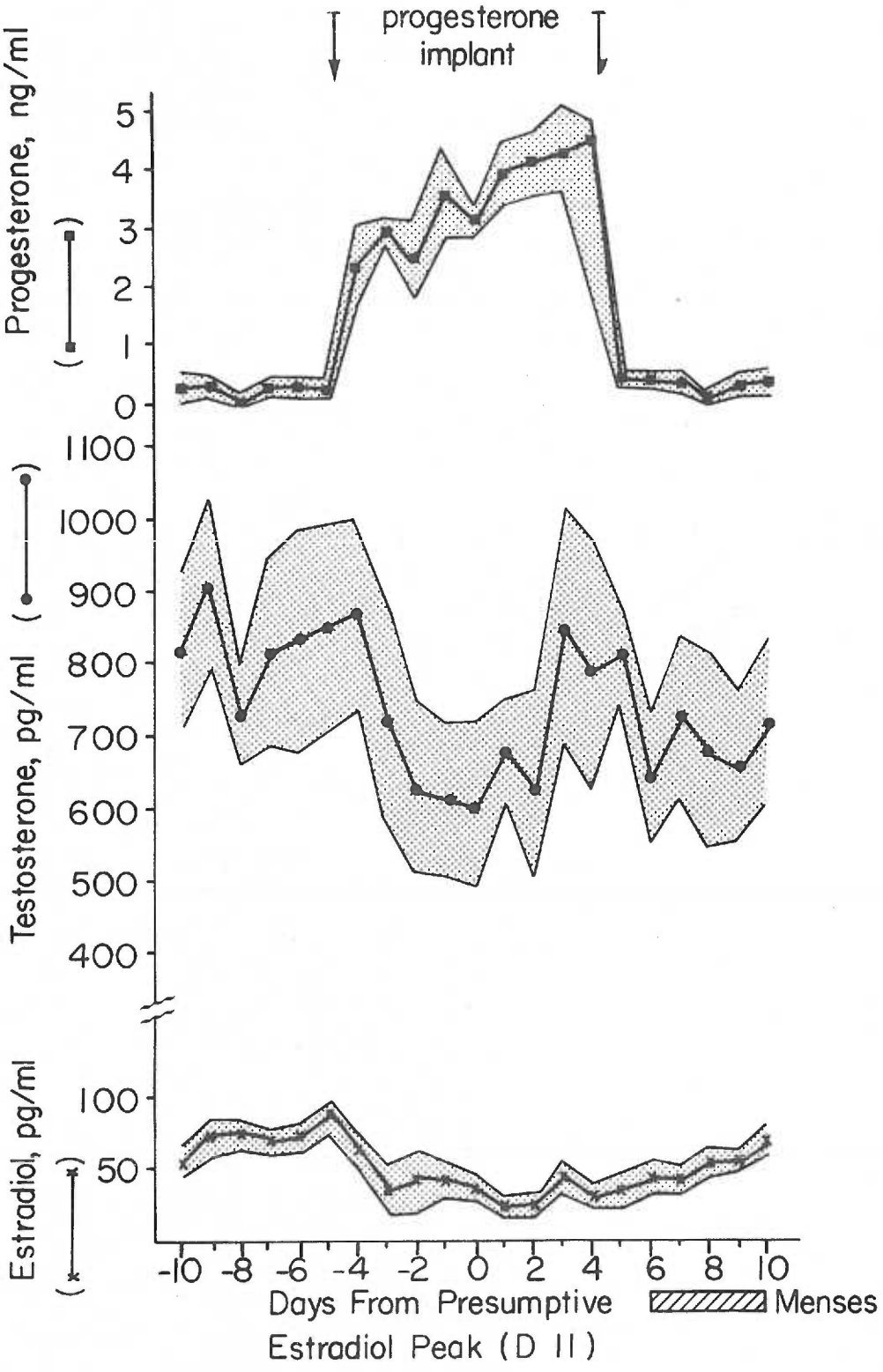

progesterone
implant
Progesterone, ng/ml
5
4
3
2
1
0
1100
1000
900
800
700
600
500
400
Testosterone, pg/ml
Estradiol, pg/ml
100
50
-10 -8 -6 -4 -2 0 2 4 6 8 10
Days From Presumptive
Estradiol Peak (D 11)
Menses

Table 3. The concentration of estradiol in the systemic plasma of rhesus monkeys during the luteal phase of the control cycle and in the next cycle before and during the administration of progesterone.

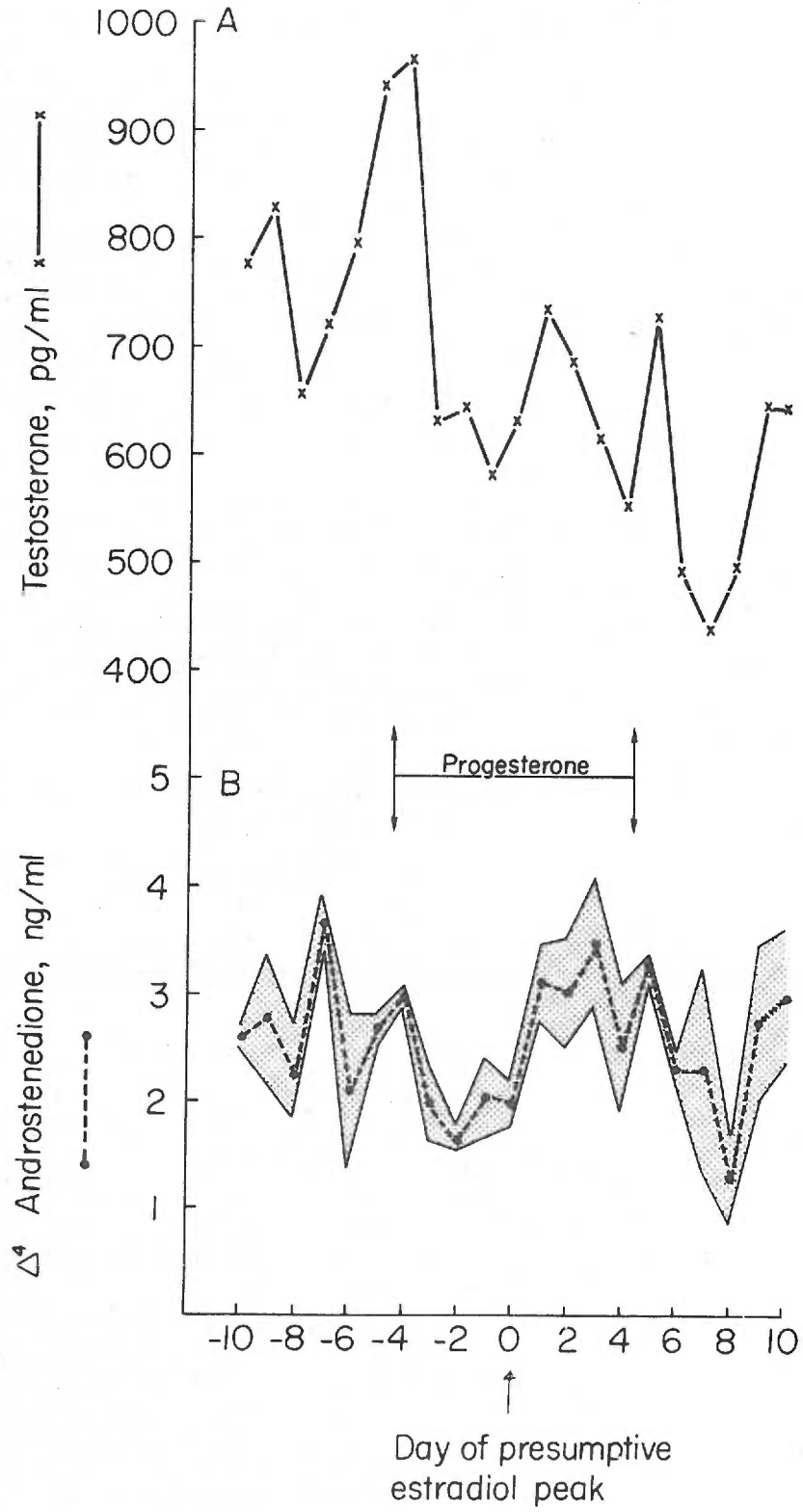
| Animal Number | Estradiol (mean pg/m/ ± SE) | | |
|---|---|---|---|
| | Normal Cycle | Treatment Cycle | |
| | Luteal Phase | Before Treatment | During Treatment |
| 807 | 34 ± 5 (12)[a] | 58 ± 10 (6)[a] | 15 ± 4 (9)[a] |
| 833 | 119 ± 20 (12) | 83 ± 12 (7) | 56 ± 12 (8) |
| 1252 | 49 ± 12 (12) | 75 ± 11 (6) | 33 ± 24 (9) |
| 3585 | 54 ± 6 (12) | 69 ± 7 (6) | 43 ± 8 (9) |
| 3599 | 99 ± 8 (12) | 72 ± 7 (6) | 47 ± 4 (9) |
| Mean ± SE | 71 ± 14[b] | 71 ± 4[c] | 39 ± 6[b,c] |

[a]Values in parenthesis indicate the number of sample days.

[b]Mean compared by a t test for correlated samples: $t = 3.05$, 4df, $p < 0.05$.

[c]Mean compared by a t test for correlated samples: $t = 8.04$, 4df, $p < 0.01$.

Figure 9. Cyclical variations in the concentrations of androstenedione and testosterone in the systemic plasma of animals treated with a silastic implant that contained progesterone from days 6 to 15 of the menstrual cycle (N = 2). Data are plotted as means and the standard error of the androstenedione estimation is the shaded area.


1000
900
800
700
600
500
400
A
Testosterone, pg/ml
5
4
3
2
1
B
Progesterone
Δ⁴ Androstenedione, ng/ml
-10 -8 -6 -4 -2 0 2 4 6 8 10
Day of presumptive
estradiol peak

at least immediately after placement and removal of the implant.

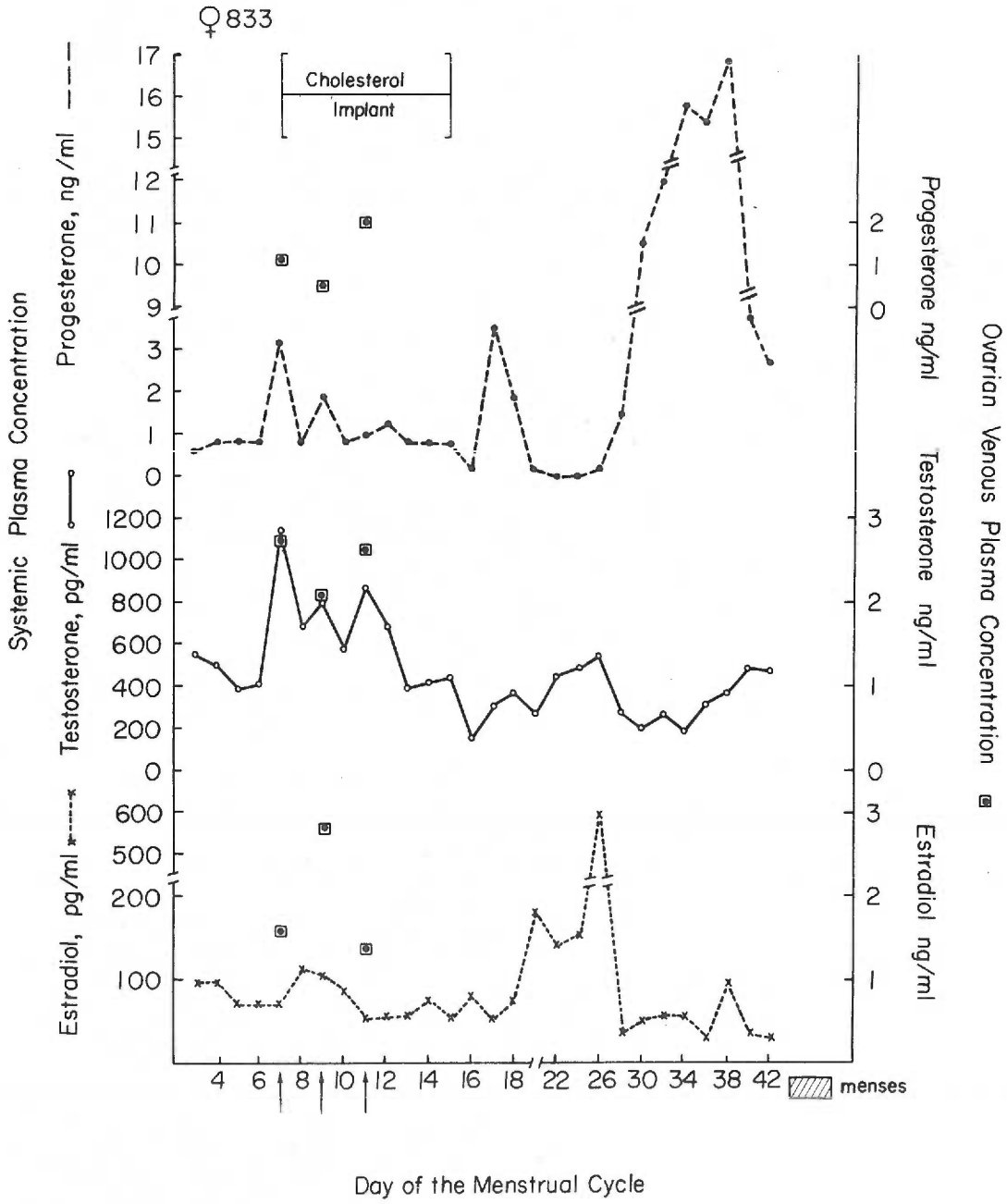
Progesterone treatment had profound effects on the length of the menstrual cycle, and the onset of menstrual flow began within two days after the removal of the implant; moreover, the cycle length was reduced to 15.8 ± 0.5 days (mean ± SD). These results were also observed in two other animals similarly treated with P.

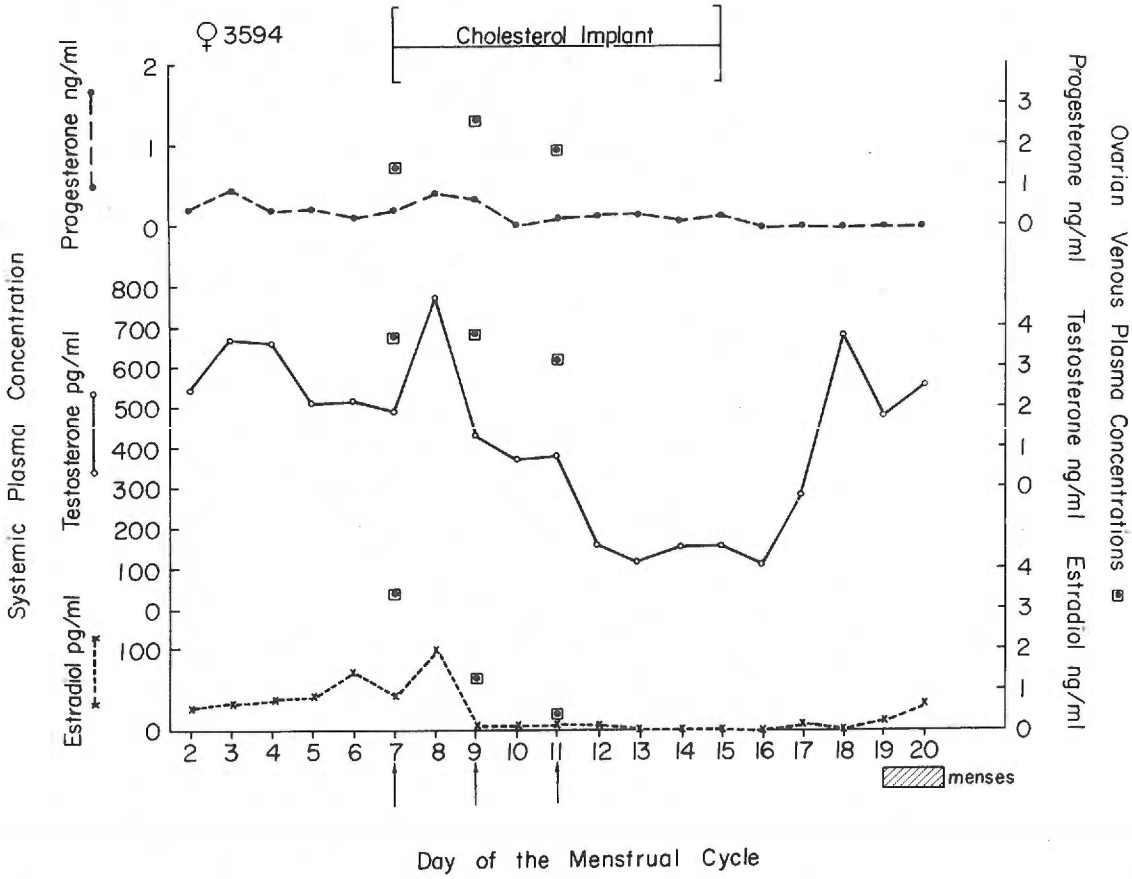
## Effects of Progesterone on the Steroid Concentrations in Ovarian Venous Effluent

In view of the limited number of animals in the control (N = 2) and the experimental (N = 2) groups, each animal will be briefly described individually before the combined results are discussed.

As illustrated in Figures 10 and 11, serial laparotomies are associated with gross changes in ovarian function in those animals treated with cholesterol. These results are in sharp contrast with other reports that simple serial laparotomies for observational purposes before, during, and after ovulation do not affect the length of subsequent luteal phases (215). The delayed appearance of a preovulatory surge of $E_2$ and a functional corpus luteum indicated that the cycle length of animal #833 was abnormally prolonged and ovulation was postponed. On the day after the first laparotomy, the peripheral levels of $E_2$ began to increase, but the rise was only transitory and after the final laparotomy had returned to baseline levels. In the

Figures 10 and 11. The concentrations of steroids in the systemic and ovarian venous plasma of rhesus monkeys #833 and 3594 treated with a silastic implant that contained cholesterol from days 7 to 15 of the menstrual cycle. The arrows indicate serial laparotomies performed on days 7, 9, and 11 of the cycle. The paired steroid measurements in the ovarian venous plasma from the ovary with and without the follicle were combined for the sake of clarity.

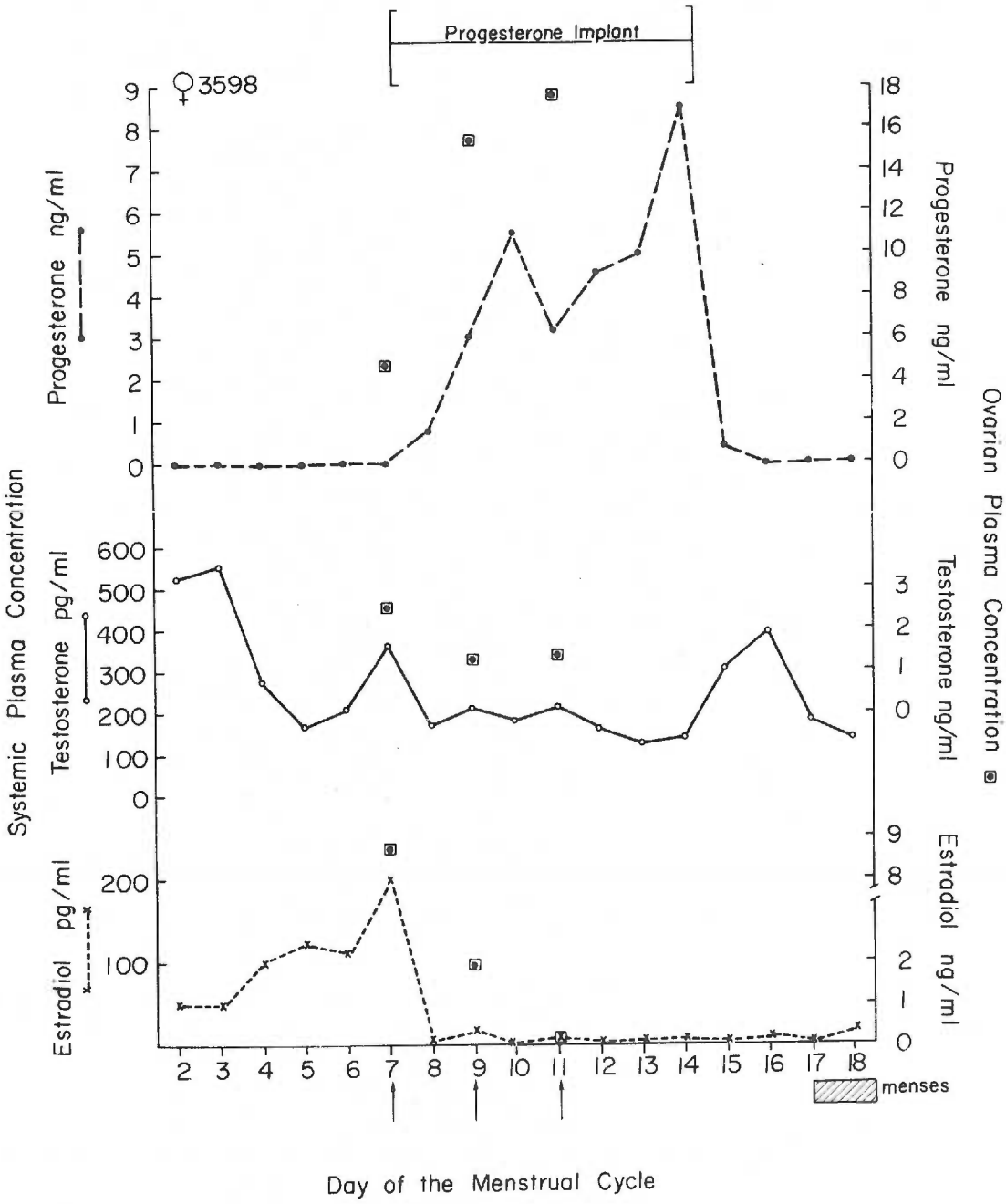

♀833
Cholesterol
Implant
Systemic Plasma Concentration
Progesterone, ng/ml
Testosterone, pg/ml
Estradiol, pg/ml
Ovarian Venous Plasma Concentration
Progesterone ng/ml
Testosterone ng/ml
Estradiol ng/ml
menses
Day of the Menstrual Cycle

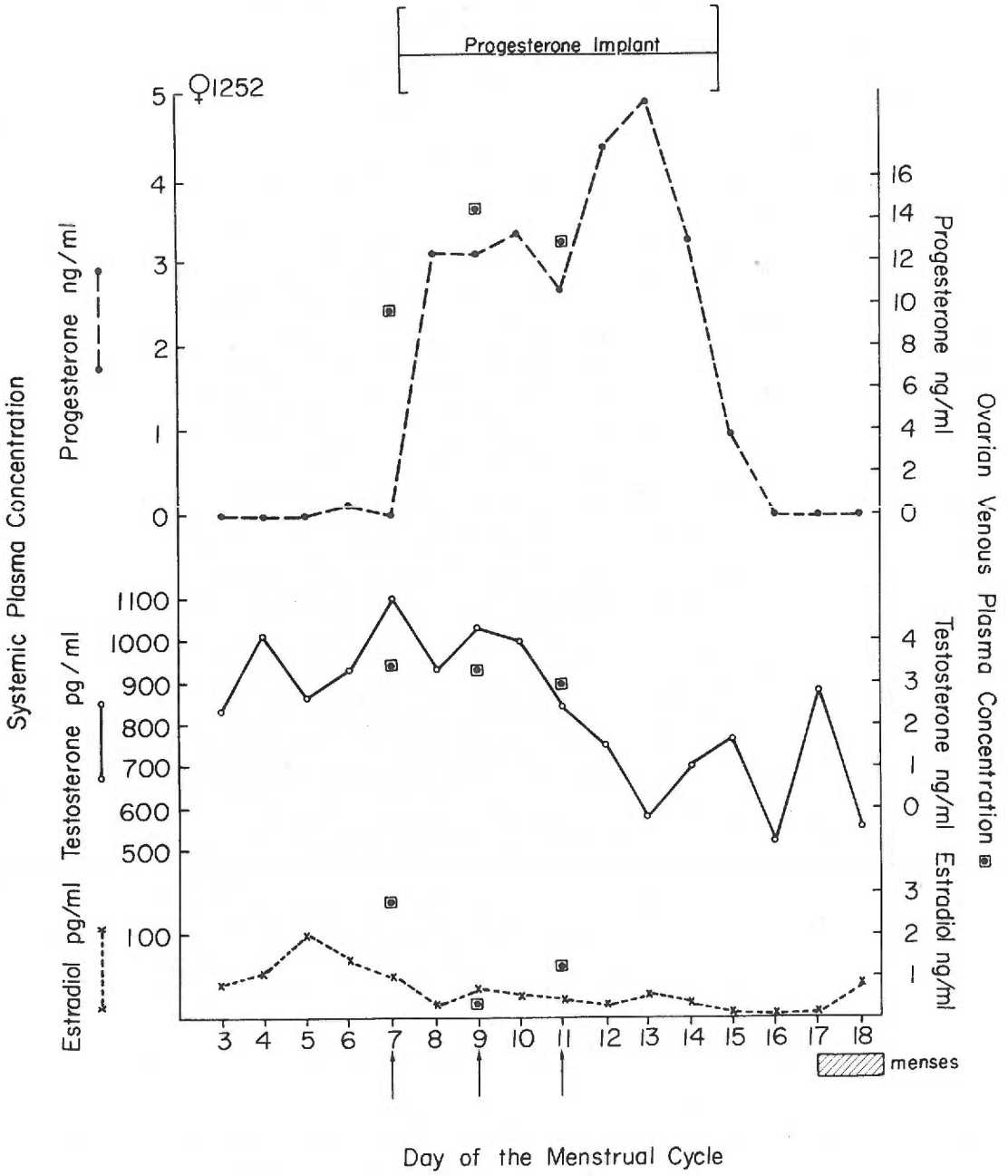

♀3594
Cholesterol Implant
Systemic Plasma Concentration
Progesterone ng/ml
Testosterone pg/ml
Estradiol pg/ml
Ovarian Venous Plasma Concentrations
Progesterone ng/ml
Testosterone ng/ml
Estradiol ng/ml
menses
Day of the Menstrual Cycle

same animal T showed three peaks that coincided with the surgical procedure and a much smaller increase was seen on the day of the $E_2$ peak. The concentration of P during the follicular phase was also unusually erratic, and beginning about day 32 of the cycle, there was evidence of a functional corpus luteum.

The second cholesterol-treated animal, #3594, had an abnormally short cycle which was judged to be anovulatory in the absence of a preovulatory surge of $E_2$ and minimal quantities of circulating P. Even though the concentration of $E_2$ in the systemic circulation was extremely low after the second laparotomy on Day 9, the concentration increased on Day 8 relative to Day 7. An increase in the concentration of T was observed on Day 8, and the systemic plasma level decreased thereafter on a time course similar to that of $E_2$. The quantities of $E_2$, T, and, as will be shown later, A were greater in the blood draining the ovary than in the systemic circulation.

The systemic patterns of steroids in the two animals treated with P (Figures 12 and 13) were similar to those seen previously. The gradual increase in circulating P in #3598 resembled that seen in the preovulatory period of the normal cycle. The rise in $E_2$ in the early follicular period was completely inhibited on Day 8 and the level remained near zero for the rest of the cycle. This effect was probably the result of the dramatic 60-fold decrease in the quantity of $E_2$ coming from the ovary (8200 pg/ml, Day 7) with the beginning


Progesterone Implant
♀3598
Systemic Plasma Concentration
Progesterone ng/ml
Testosterone pg/ml
Estradiol pg/ml
Ovarian Plasma Concentration ◙
Progesterone ng/ml
Testosterone ng/ml
Estradiol ng/ml
menses
Day of the Menstrual Cycle


Progesterone Implant
♀1252
Systemic Plasma Concentration
Progesterone ng/ml
Testosterone pg/ml
Estradiol pg/ml
Ovarian Venous Plasma Concentration
Progesterone ng/ml
Testosterone ng/ml
Estradiol ng/ml
menses
Day of the Menstrual Cycle

of P treatment (125 pg/ml, Day 9). A rapid reduction in the systemic concentration of T between Day 7 and Day 8 was also found, but the amount of the reduction was limited. Similarly, a fall in the peripheral level of $E_2$ was observed in female #1252, but the degree of inhibition was not as large as in the first animal. The systemic pattern of T showed a delayed response to P administration. The ovarian concentration of these steroids was, in all cases, greater than that in the general circulation.

The principal source of the $E_2$ found in the systemic plasma during the follicular phase of the cycle is the ovary. Concentrations of $E_2$ in the ovarian venous plasma with and without the growing follicle are shown in Table 4. A significant correlation was found between the quantities of $E_2$ in the ovarian venous plasma and those in the systemic circulation (with the largest follicle, $r = 0.91$, $p < 0.001$; contralateral ovarian vein, $r = 0.85$, $p < 0.001$). Both ovaries released T (Table 5) as well as A (Table 6). The ovarian samples showed higher levels of the former hormone than the systemic samples even though the concentrations of T in either ovarian vein were not correlated with systemic levels (with follicle, $r = 0.48$, $p > 0.05$, without follicle, $r = 0.18$, $p > 0.05$) nor with the concentrations of $E_2$ in the ovarian vein (with follicle, $r = 0.24$, $p > 0.05$, without follicle, $r = 0.04$, $p > 0.05$). The ovarian samples also showed higher levels of A than the systemic samples, and these were not correlated with the

Table 4. Correlation of the concentrations of estradiol in ovarian venous and systemic plasma during the follicular phase of the cycle.

| Sample No. | Estradiol (pg/ml plasma) | | |
|---|---|---|---|
| | (1) Ovarian Vein (with follicle)[a] | (2) Ovarian Vein (without follicle)[b] | (3) Systemic plasma[a,b] |
| 1 | 8200 | 3550 | 198 |
| 2 | 1980 | 836 | 104 |
| 3 | 1491 | 87 | 71 |
| 4 | 1140 | 230 | 53 |
| 5 | 2634 | 157 | 50 |
| 6 | 2980 | 425 | 44 |
| 7 | 352 | 28 | 37 |
| 8 | 736 | 513 | 20 |
| 9 | 125 | 73 | 14 |
| 10 | 553 | 752 | 7 |
| 11 | 26 | 57 | 7 |
| 12 | 286 | 181 | 3 |
| Mean ± SE | 1709 + 624[c] | 574 + 280[c] | |

[a] Ovarian vein and systemic concentrations are significantly correlated. Classification of ovaries with (defined by the largest follicle seen macroscopically) and without follicle was made by inspection after laparotomy on Days 7, 9, and 11 of the cycle, $r_{1-3} = 0.91$, $p < 0.001$.

[b] Ovarian vein and systemic concentrations are significantly correlated, $r_{2-3} = 0.85$, $p < 0.001$.

[c] Means are significantly different, $t = 2.71$, 11df, $p < 0.05$.

Table 5. Correlation of the concentrations of testosterone in ovarian venous and systemic plasma during the follicular phase of the cycle.

| Sample No. | Testosterone (pg/ml plasma) | | |
|---|---|---|---|
| | (1) Ovarian Vein (with follicle)[a] | (2) Ovarian Vein (without follicle)[a] | (3) Systemic plasma[a] |
| 1 | 1471 | 1257 | 1145 |
| 2 | 1985 | 1445 | 1098 |
| 3 | 1873 | 1501 | 1029 |
| 4 | 1328 | 1293 | 865 |
| 5 | 1462 | 1538 | 841 |
| 6 | 1126 | 938 | 798 |
| 7 | 2160 | 1736 | 487 |
| 8 | 1747 | 2186 | 429 |
| 9 | 1531 | 1583 | 390 |
| 10 | 1426 | 1147 | 365 |
| 11 | 658 | 800 | 215 |
| 12 | 589 | 713 | 211 |
| Mean ± SE | 1446 ± 133[b] | 1345 ± 115[b] | |

[a] Ovarian vein and systemic concentrations are not significantly correlated, $r_{1-3} = 0.48$, $p > 0.05$ and $r_{2-3} = 0.18$, $p > 0.05$.

[b] Means are not significantly different; $t = 1.24$, 11df, $p > 0.05$.

Table 6. Correlation of the concentrations of androstenedione in ovarian venous and systemic plasma during the follicular phase of the cycle.

| Sample No. | Androstenedione (ng/ml plasma) | | |
|---|---|---|---|
| | (1) Ovarian Vein (with follicle)[a] | (2) Ovarian Vein (without follicle)[a] | (3) Systemic plasma[a] |
| 1 | 6.4 | 6.0 | 3.6 |
| 2 | 16.3 | 12.5 | 3.5 |
| 3 | 17.5 | 19.3 | 3.5 |
| 4 | 12.2 | 11.6 | 3.3 |
| 5 | 11.0 | 7.7 | 3.0 |
| 6 | 14.3 | 8.3 | 2.8 |
| 7 | 10.7 | 4.7 | 2.4 |
| 8 | 4.6 | 5.0 | 2.4 |
| 9 | 12.6 | 5.5 | 2.3 |
| 10 | 11.5 | 7.3 | 2.1 |
| 11 | 6.4 | 7.1 | 2.0 |
| 12 | 5.7 | 6.5 | 1.9 |
| Mean ± SE | 10.8 ± 1.2[b] | 8.4 ± 1.2[b] | |

[a]Ovarian vein and systemic concentrations are not significantly correlated, $r_{1-3} = 0.51$, $p > 0.05$, and $r_{2-3} = 0.26$, $p > 0.05$.

[b]Means are significantly different, $t = 2.57$, 11df, $p < 0.05$.

systemic levels (with follicle, $r = 0.51$, $p > 0.05$, without follicle, $r = 0.26$, $p > 0.05$), the ovarian effluent concentrations of $E_2$ (with follicle, $r = 0.48$, $p > 0.05$, without follicle, $r = 0.06$, $p > 0.05$) nor the ovarian levels of T (with follicle, $r = 0.20$, $p > 0.05$, without follicle, $r = 0.33$, $p > 0.05$).

# CHAPTER V

# DISCUSSION

## An Evaluation of the Implant Technique

Although steroids are known to pass through silastic membranes (209), the amounts released and present in the general circulation have not been measured. Radioactivity in the plasma (216) and the inhibition of reproductive function in rats (217), sheep (218), women (219, 220) and monkeys (217) treated with progestin implants indicate that such implants release biologically active steroid. By assaying the compound isolated from the systemic plasma of normal and ovariectomized female rhesus monkeys, the present investigation has confirmed that this treatment is highly successful in producing physiological concentrations of P. The method avoids the difficulties inherent in subcutaneously injecting steroid solutions or suspensions in oil and the necessity of using synthetic progestins for oral administration. In addition, the localization of the implant at the same relative anatomical site in each animal reduces the absorption differences among subjects. The release of the steroid from the implant resulted in a physiological plasma concentration within 24 hours, and

the plasma values fell to low or nondetectable concentrations within 24 hours after the removal of the implant. A true steady state equilibrium was not achieved, however, since the quantity of P in the plasma gradually increased over the 9-day treatment period. The increments were small and the amounts were never pharmacological. In fact, two animals treated with similar implants for three weeks had concentrations of P in the systemic circulation of 6 and 9 ng/ml on the morning before the implant was removed.[1] Therefore, in spite of the empirical testing required to estimate the proper implant dimensions for a given weight range, this treatment regimen is the method of choice for short-term steroid administration, i.e. for less than one month. It is simple and rapid and both the stress of daily or more frequent injections and the irregular plasma concentrations associated with such injections are avoided.

## The Source of the Systemic Steroids During the Ovarian Cycle

Both the ovary and the adrenal contribute to the systemic levels of steroids during the ovarian cycle. It is therefore imperative to consider the possibility that the results reported here reflect alterations in adrenal function. The adrenal gland of the monkey secretes A, T, and $E_2$ (211). However, $E_2$ is secreted in such small

[1] Dr. R. Brenner, personal communication.

quantities that only a minute part of that amount assayed in the systemic plasma is adrenal in origin. In contrast, the current measurements of A and T in the ovariectomized monkey agree with the previous observations that the adrenal produces significant amounts of these androgens in man (191, 194) and the monkey (211). The systemic concentration of these steroids showed no gross alterations over the 8-day observation period except for those probably associated with the surgical manipulations involved in placing the implant and the gradual adaption of these inexperienced animals to the bleeding schedule. It is generally accepted that ACTH stimulates A production in man (193, 221) and these results tend to support the argument that the short-term increases observed in these animals are stress-related. The gradual decline in A during the bleeding period between surgical procedures is most probably due to the adaptation of the animals to the stress of bleeding. A similar adrenal adaptation was made by naive subjects unaccustomed to experimental manipulations and handling (222). Furthermore, the insignificant effects of P on the circulating level of A in both the ovariectomized and intact monkey also supports the concept that the adrenal is the major source of this androgen. The relatively constant level of T in systemic plasma indicates that stress does not alter the concentration of this hormone in any well-defined way. However, the magnitude of the variation between animals for either A or T strongly supports the

view that the steroidogenic activity of the adrenal is highly variable in each animal, even without stress. The measurement of P in the systemic plasma of ovariectomized females treated with cholesterol confirms the previous observation that this steroid is present in the plasma of spayed monkeys (211) and the observations here support the earlier findings that treatment with ACTH increases the plasma level of this hormone in man (223, 224).

The analysis of the systemic androgen concentration in the intact monkey further substantiates the concept of a dual source of A and T. Androstenedione is found in large quantities in the general circulation of the monkey and in agreement with earlier predictions (3) appears to have no obvious relationship to the ovarian cycle. The large variance in the estimation of T in the systemic plasma compared to the smaller variances in the E and P estimations supports the previous conclusion that some of the T is adrenal in origin, either directly as a secretory product or indirectly as a result of the peripheral conversion of A, or both. Such conversions in the female have recently been reviewed (225). These data, therefore, indicate that the adrenal gland produces significant amounts of the two androgens, as well as much smaller quantities of P and $E_2$ and that any ovarian secretion of these compounds is superimposed on the direct or indirect contribution of the adrenal.

Our attempts to relate ovarian production of these hormones

with systemic levels were only partially successful. Regardless of the treatment group, the secretion of $E_2$ in the ovarian veins from both ovaries was correlated with the hormone level in the systemic circulation although the major source of $E_2$ for the preovulatory surge is probably the ovary containing the largest developing follicle. This conclusion is based on the fact that a 3-fold greater concentration of $E_2$ was found in the venous plasma from the ovary with the largest follicle than from the contralateral side. In the rhesus monkey, the two ovarian veins are interconnected via the uterine circulation and as described by Riesen, Koering, Meyer, and Wolf (99) the P in the venous effluent from the ovary without the corpus luteum was derived mainly from the contralateral ovary. Vascular channels through the uterus could explain the quantity of $E_2$ found in the present studies in the ovary without the largest follicle, since these channels were intact. The stress involved in obtaining the ovarian vein samples in all probability increased the production of adrenal androgens and this may explain the lack of a significant correlation between the concentrations of A and T in the ovarian venous plasma and the systemic circulation. The ovarian-peripheral concentration gradient in the monkey proves that androgens, estrogens, and progesterone are secreted by the ovary of subhuman primates as well as by the ovary of man (97). The slight, but significant, increase in the mean level of A in the plasma draining the ovary with the largest

follicle suggests differences in the steroidogenic capacities of the two ovaries. The precise nature of this difference is obscure since the concentrations of A and T in ovarian venous plasma are not correlated with those of $E_2$. These results could indicate a different source of these hormones in the preovulatory ovary or a multiple compartment source for one hormone and not the other.

The coincidence of the midcycle surge of T with the preovulatory surge of $E_2$ is additional evidence for the ovarian secretion of this androgen. The close congruence of these temporal relationships in the midfollicular phase and the periovulatory period, as well as the significant reduction in the plasma level of T in the luteal phase suggests the ovary as the source of the cyclical release of this androgen. Therefore, the preovulatory ovary releases sufficient T to influence the plasma concentration of T in spite of adrenal production and peripheral conversion of prehormones. These results do not support the previous contention that direct or indirect production of T from non-ovarian sources would mask the cyclic ovarian contribution to peripheral blood levels (3).

## A Comparison of the Levels of Testosterone in Systemic Plasma in Man with Those in the Monkey

In relation to the question of the general application of these results to animals of the primate series, a comparison of data from

the monkey with those from systemic plasma of women shows them to be similar, but not entirely alike. In the studies that have shown the cyclical appearance of androgens in systemic plasma throughout the menstrual cycle of women, relatively insensitive techniques have been used for steroid estimation and the identification of ovulation. Lobotsky, Wyss, Serge, and Lloyd (163) using the thermal nadir as a reference point, found a midcycle peak of T similar to that observed in the monkey. Others, however, noted a brief increase in plasma T slightly after the thermal nadir (176). Mikhail (85) observed that three of four subjects showed higher systemic levels of androgens during the preovulatory period than in the postovulatory period. Other estimations of androgens, estrogens, and progestins in the ovarian vein blood of women showed no consistent relationship between the patterns when one hormone was compared with another at various stages of ovarian development (97). However, ovaries taken from the late follicular phase synthesized androgens and estrogens more efficiently in vitro than the ovaries from the early follicular phase (82). Unlike the decline in peripheral T levels seen in previous studies during the luteal phase, a secondary elevation in the concentration of T has been reported in human subjects (159, 187, 188). Similarly, the in vitro results from human corpora lutea have shown that this tissue produces both androgens and estrogens from appropriate precursors (66, 73-76). Unlike those in women, the systemic concentrations

of neither $E_2$ nor T were elevated in the luteal phase of the rhesus monkey. These contradictory observations are compatible with the hypothesis that the steroidogenic capability of the monkey corpus luteum is different from that of man. Certainly other species such as the cow (226), mare (81), sow (227), and ewe (228), differ from man in that the corpus luteum does not produce estrogens. Additional work is needed, however, before these conclusions can be applied to the rhesus monkey.

## The Secondary Effects of Progesterone Administration

An unusually high concentration of P in the peripheral circulation during the follicular phase alters the usual concentrations of other ovarian steroids during this time. It is, therefore, necessary to consider the reasons for these changes.

In the cholesterol treatment cycle, unlike the P treatment cycle, the steroid patterns in the systemic circulation were identical with those observed in the normal cycle; hence the effects of the P implant were obviously not due to the surgical procedure.

The midcycle surge of both T and $E_2$ was inhibited by quantities of P similar to those found in the early luteal period (2 to 5 ng/ml of plasma). Note that the baseline level of $E_2$ was significantly lower in the P treatment period than that in the follicular phase before the administration of the steroid. Although the alterations in

the peripheral levels of T would suggest that P influences the systemic concentrations of this hormone, the wide variation in levels among the experimental subjects prevents any firm conclusions about the effects of P during the initial 6-day period or the apparent early return to pretreatment levels. However, the rapid reduction of the systemic level of $E_2$ concurrently with the appearance of exogenous circulating P closely resembles the temporal relationship between the two steroids observed in the normal cycle. Several explanations of how P affects the peripheral concentrations of androgens and estrogens can be given. (1) It may affect the clearance of $E_2$ and T from the circulation or alter the plasma protein binding of these steroids. (2) It may alter the rate of gonadotropin release. (3) It may act directly upon the ovary, inhibiting androgen and estrogen biosynthesis.

Very little evidence can be found to support the hypothesis that P functions by affecting the clearance of either T or $E_2$. The possible alterations in steroid metabolism in response to changing physiological conditions during the menstrual cycle have not been closely examined. Recent studies in man have shown that the metabolic clearance rate (MCR) of A is the same in both sexes whereas that of T is greater in males than in females. Furthermore, the metabolic clearance rates are unchanged during the menstrual cycle (181, 229-231). However, it has been reported that an increase in MCR of

T in females can be induced by a large increase in the plasma level of this hormone (181). Similarly, an increase in T MCR was observed after treatment with the artificial progestin, medroxyprogesterone acetate, for four weeks (232). However, both of these results may be pharmacological rather than physiological responses. The metabolism of $E_2$ is similar in the follicular and luteal phases and apparently is not affected by P (187, 233, 234). Peripheral conversion of androgens to estrogens is known to occur, but this slight interconversion (about 1% in females) is not important in determining the systemic levels of these steroids (231, 235). These results indicate that steroid clearance rates remain relatively constant and therefore, alterations in systemic concentrations reflect changes in secretory activity.

Alterations in the plasma protein concentration brought about by changing hormonal conditions may alter the amounts of steroid available for metabolism, and therefore delay or reduce the clearance rate. The level of plasma proteins responsible for the binding of steroids is greater in human females than in males (236, 237), and long-term administration of $E_2$ elevates the concentration of the steroid-binding β-globulin (238). However, the binding of androgens and other steroids to this protein fraction is unchanged during the cycle (239, 240). Although the free circulating levels of steroid may be affected by hormonally induced changes in plasma proteins, the

scanty data available suggest that the slow response time precludes the involvement of this mechanism in the rapid changes of systemic steroid levels in the ovarian cycle.

As described in the introduction, the normal maturation and consequently the steroid production of the follicle is probably directed by the proper ratio of circulating gonadotropins. Therefore, it is conceivable that any alterations in the baseline quantity of these hormones would alter the systemic levels of ovarian hormones. Recently subcutaneous injections of P in monkeys did not reduce baseline levels of LH although the midcycle surge of this hormone was inhibited (241, 242). Moreover, similar injections of P were also ineffective in reducing the high postcastration levels of LH in female rhesus monkeys (148). The effects of P on baseline concentrations of FSH and prolactin have not been reported. Treatment of women with oral contraceptives inhibited the midcycle surge of both FSH and LH, and it was suggested that these compounds reduced the basal levels during the treatment cycles below those observed in the control follicular phase (243, 244). Since the level of LH during the follicular phase are near the limits of assay sensitivity, the complete resolution of this problem is not yet possible.

If it is assumed that basal levels of gonadotropins are not reduced, then P could be acting directly on the ovary. The steroid levels in the systemic and ovarian venous plasma of animals treated

with cholesterol and P tend to support this conclusion. In the control pair of laparotomized animals, the systemic level of $E_2$ was higher on the day after the implant was placed than on the day before, whereas in the P-treated pair, the peripheral $E_2$ concentrations fell immediately on the day after implant placement. This finding is especially noteworthy because of the high correlation between the quantities of $E_2$ in ovarian venous plasma and in systemic plasma. However, any conclusions based on later time periods in the laparotomized animals are not justified in view of the obvious surgical effects on ovarian function in the control animals.

## Role of Progesterone in the Biochemical Regulation of the Ovary

The hypothesis that a progestin serves a regulatory function in steroid biosynthesis has been suggested for the testis (207), and the human ovary (69). The rapid decline in the quantities of $E_2$ and T in the systemic blood in the periovular condition may be a response to the increased synthesis of P or a progestin as a consequence of the rising levels of gonadotropins. Both 17 α-hydroxyprogesterone and P (2, 142) are elevated in the systemic plasma during this time in primates. As the quantity of progestin increases, the 17-20 desmolase enzyme may be inhibited and estrogen and androgen biosynthesis may be decreased. It is known that 17α, 20α-dihydroxypregn-4-ene-3-one is a competitive inhibitor of desmolase activity

in the biosynthesis of androgens by the rat testis (208). Consistent with this observation is the finding that the concentration of 20α-hydroxysteroid dehydrogenase, which is relatively high in the immature rat ovary (245), can be elevated by gonadotropins. Follicular development in the prepubertal monkey treated with P or 17α-hydroxyprogesterone and PMS (247) indicate that the ovary is still responsive to gonadotropins in the presence of progestins. Certainly the hypothesis that P serves a regulatory function in the biosynthesis of estrogens by the preovulatory ovary in the primate is more tenable if the current results are compared with the recent data in man showing that estrogen levels decline at a time when serum gonadotropins are still increasing (118). Under these circumstances, the mechanism that turns off estrogen production in the preovulatory ovary can hardly be a deficiency in trophic stimulation. This hypothesis, however, does not concur with the observations of increased estrogen biosynthesis by the human ovary during the luteal phase; these differences require further investigation.

The rapid and continued suppression of follicular estrogen synthesis, indicated by a reduction in peripheral concentrations during P treatment, supports the hypothesis that P directly affects estrogen biosynthesis at an ovarian site. Although additional definitive work is needed to study this proposed control directly, as well as its

relationship to other factors affecting ovarian steroidogenesis, this mechanism would assign a key regulatory role to progesterone in the events that surround ovulation in the primate.

# CHAPTER VI

## SUMMARY AND CONCLUSIONS

A midcycle peak of T was seen to occur simultaneously with that of $E_2$ in the systemic plasma. The close temporal relationship of the two steroid hormones and the identification of an a-v difference in the concentrations of these hormones across the ovary indicates that the ovary is responsible for this cyclical pattern. Progesterone levels typical of the luteal phase could be obtained during the follicular phase by treatment with silastic implants containing the hormone. This treatment blocked the preovulatory increase of both $E_2$ and T and in addition, for the duration of the treatment, reduced the levels of $E_2$ below the pretreatment concentration and the concentrations found in the previous luteal phase. Additional attempts to identify the site of P action on the ovary by examining the steroid content in sequential samples from both ovarian veins were confounded by surgical effects on ovarian function. However, the rapid alterations in the systemic patterns of $E_2$ and T during the periovulatory period and the effects of exogenous P on the patterns of these steroids in systemic plasma during the follicular phase of the cycle are

compatible with the hypothesis that P acts directly on the ovary to inhibit the production of androgen, estrogens, or both.

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# APPENDICES

# APPENDIX A

## SYSTEMATIC CHEMICAL NOMENCLATURE OF STEROIDS

| Trivial Name | Systematic Name |
|---|---|
| Androstenedione | Androst-4-ene-3, 17-dione |
| Cholesterol | Cholest-5-en-3β-ol |
| Dehydroepiandrosterone | 3β-hydroxyandrost-5-en-17-one |
| Estradiol | Estra-1, 3, 5(10)-triene-3, 17β-diol |
| Estrone | 3-hydroxyestra-1,3,5(10)-triene-17-one |
| 17α-hydroxypregnenolone | 3β, 17α-dihydroxypregn-5-en-20-one |
| 17α-hydroxyprogesterone | 17α-hydroxypregn-4-ene-3, 20-dione |
| Pregnanediol | 5β-pregnane-3α, 20α-diol |
| Pregnenolone | 3β-hydroxypregn-5-en-20-one |
| Progesterone | Pregn-4-ene-3, 20-dione |
| Testosterone | 17β-hydroxyandrost-4-en-3-one |

# APPENDIX B

## PHOTOGRAPHIC RECORD OF THE IMPLANT PROCEDURE

Plate 1. A lateral view of the incision site (blue line). The umbilicus is 1 cm above the line.

Plate 2. The fascia covering the rectus abdominus muscle was exposed by an incision through the dermal and subcutaneous adipose tissue.

Plate 3. The implant was slipped through a 1.2 to 2 cm incision in the muscle fascia with minimum trauma to the underlying tissue.

Plate 4. The fascia was closed with three or four stitches.

Plate 5. The wound after closure.

Plate 6. The wound after the 9-day treatment period.

Plate 7. The implant within the muscle bed. Note how the implant has "settled" into the muscle mass.

Plate 8. The muscle bed after removal of the implant. Note the vascular appearance of the implant site and the absence of fiberous or necrotic tissue.

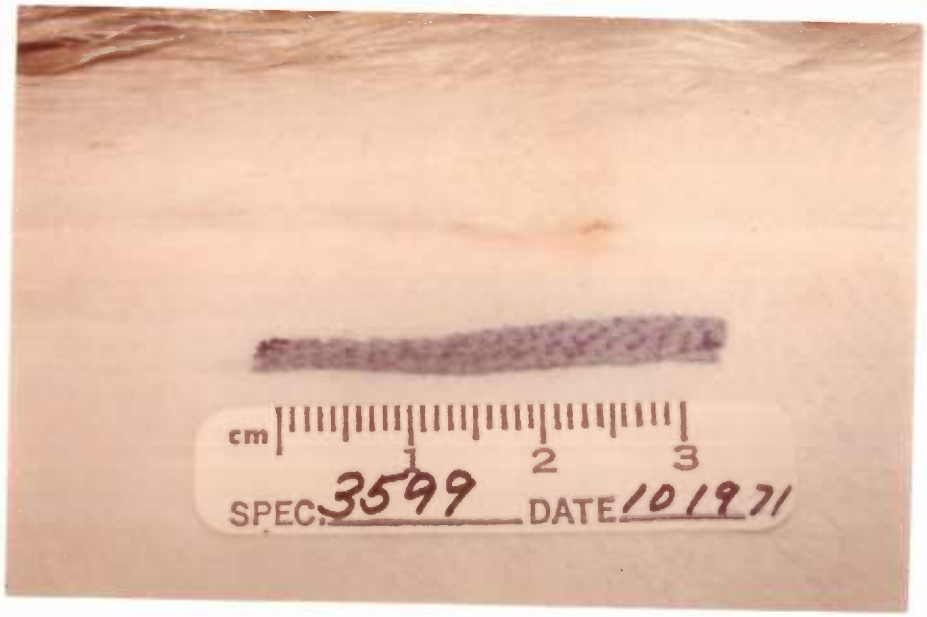
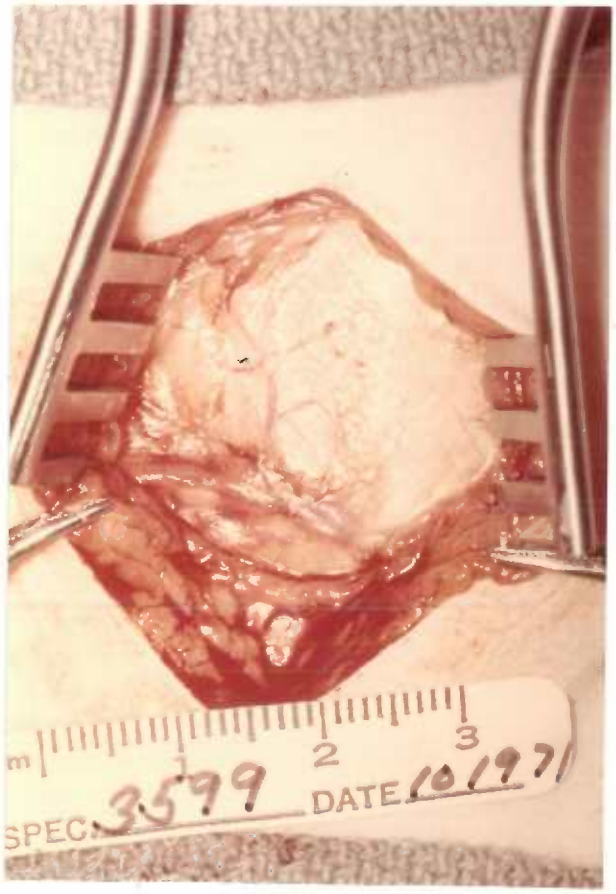



plate 1

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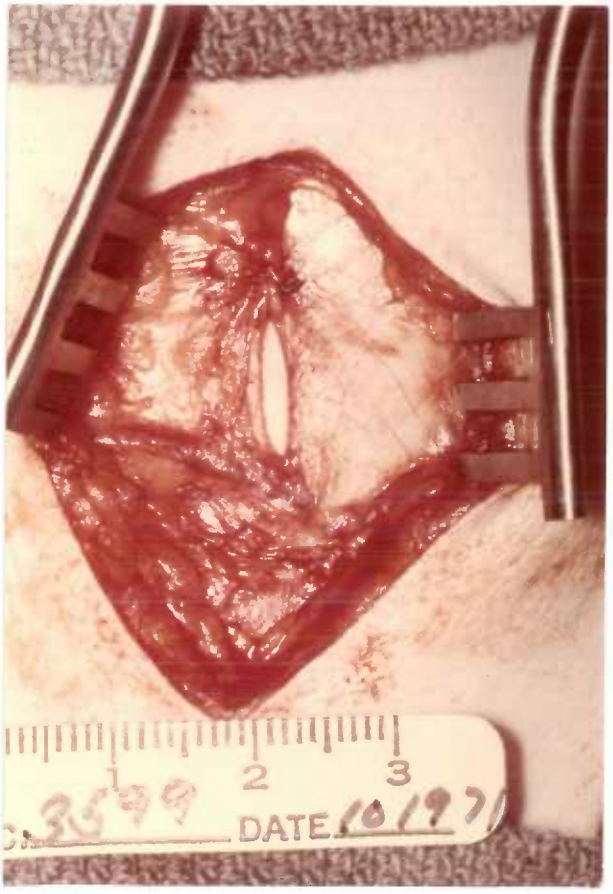


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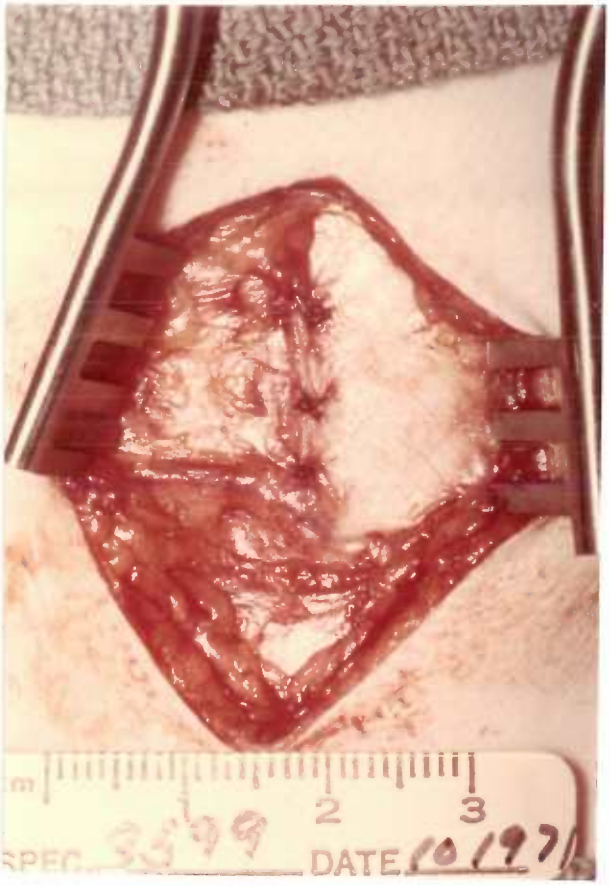



PLATE 4

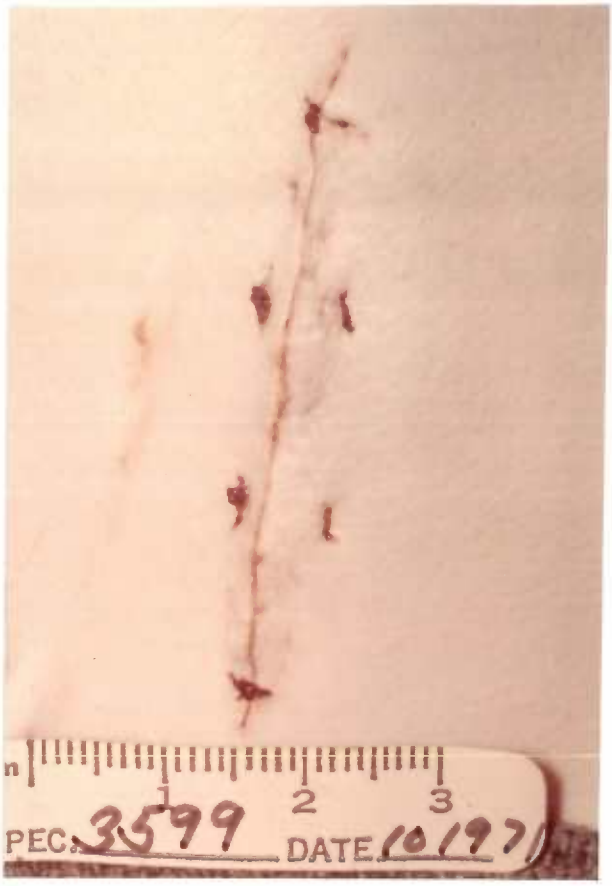



PLATE 5

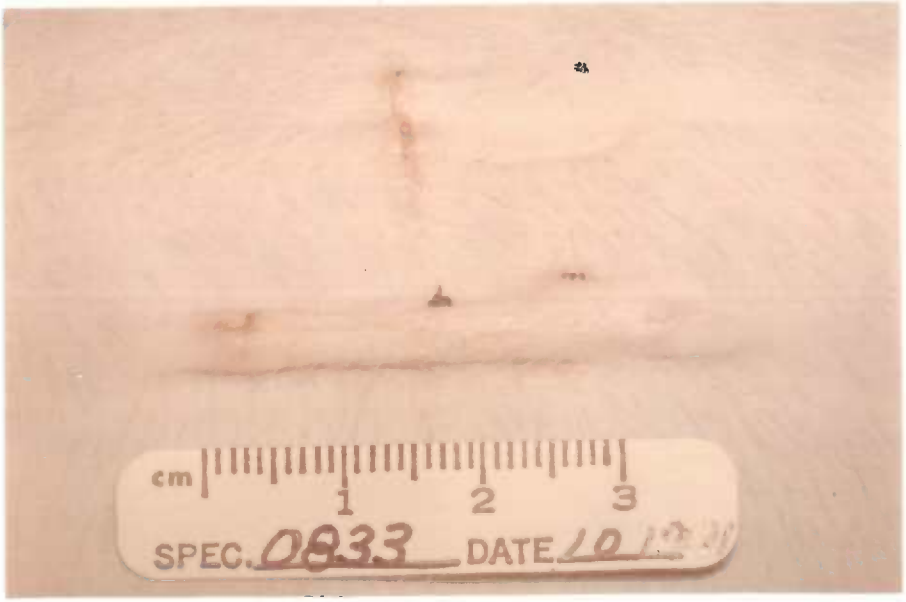



PLATE 6

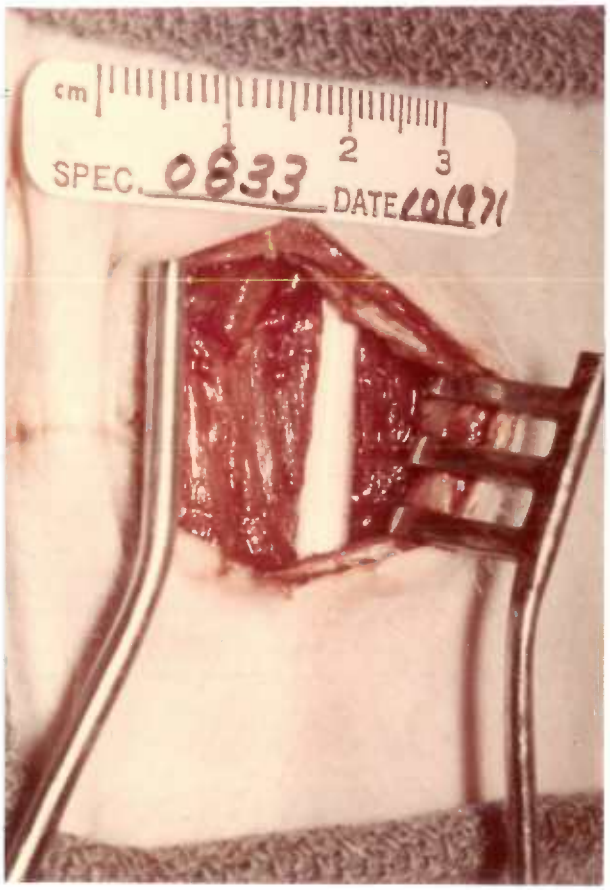



plate 7

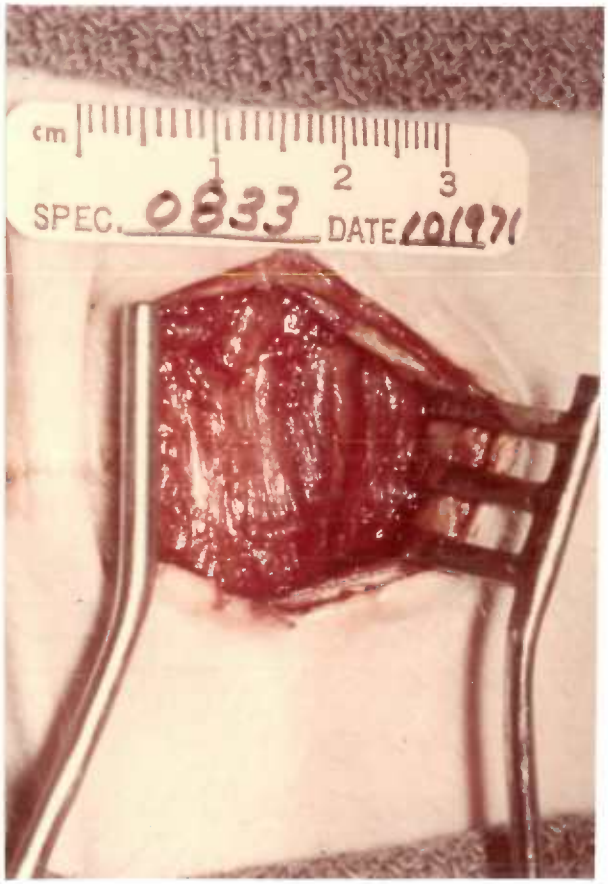



plate 8

# APPENDIX C

## PREPARATION OF REAGENTS

### Purification of Solvents

All solvents were routinely distilled in acid-washed glassware, and chromatography solvents were redistilled every three weeks. In addition, the chloroform and acetone used to separate the androgens were further purified according to the methods suggested by Bush (248). Solvents were stored in amber bottles and protected from light as much as possible when in use.

### Progesterone Assay Reagents

1. Heparinized dog plasma was obtained by bleeding male dogs and centrifuging the blood at 1500 rpm for 30 minutes. Two and one-half ml aliquots of plasma were stored at -20°C until used.

2. Tritiated corticosterone (3300 cpm, 50 Ci/mM, New England Nuclear Corp.) in ethanol was added to a 100 ml volumetric flask and dried under purified nitrogen. 2.5 ml of dog plasma were added and diluted to volume with deionized distilled water.

3. Florisil (J. T. Baker Chemical Co.) was purified by

repeated washings with deionized distilled water until the washings were clear. After drying for 12 to 16 hours at 100°C, 80 ± 1 mg per sample were weighed and stored in dessicated gelatin capsules until used.

4. Silica gel plates were prepared by spreading a mixture of 1 part purified silica gel G (Brinkman Inst. Inc., Westbury, N. Y.) to 2.5 parts deionized distilled water over washed glass plates (the layer is about 250 μ thick). The air-dried plates were stored in a 100°C oven until used. The silica gel was purified as follows: 120 grams of gel were mixed with water, and the pH of this slurry was adjusted to 2 with glacial acetic acid. After filtration through a scintered glass funnel, the gel was washed four times with hot water and filtered after each wash. Finally the gel was boiled three times with methanol, filtered after each wash, and stored in a desiccator after it had been dried at 100°C.

5. Aluminum oxide columns were prepared as follows: 5-3/4 inch disposable pipettes were washed with methylene chloride and a small wad of glass wool (washed with methanol) was placed in the tip. 2.5 cm of aluminum oxide (M. Woelm, Eschwege, Germany) were added to the pipettes and the columns were activated at 100°C overnight. The columns were washed with 100% methylene chloride after the silica gel scrapings from the thin layer plates had been added.

6. 1,4-diaminoanthroquinone dye: 50 mg of the dye were

dissolved in 100 ml of methanol, filtered, and stored at 4°C.

## Estrogen and Androgen Assay Reagents

1. Phosphate buffered saline (PBS):0.01 M sodium phosphate monobasic; 0.14 M sodium chloride and 0.01% sodium azide in distilled water, adjusted to pH 7.0 with HCl and stored at 4°C.

2. Gel solutions were prepared by adding 0.1% or 0.5% gelatin (Knox Gelatin, Inc., Johnston, N. Y.) to PBS and stored at 4°C.

3. Antisera Solutions

a. The estradiol-17β antisera stock solution was made by diluting the concentrated antibody 1:300 with 0.1% gel-PBS. 100 ml of a working solution were prepared by diluting the previous solution 1:50 with 0.1% gel-PBS. The diluted antibody (1:15000) was stored at 4°C until used.

b. The testosterone-3-oxime antisera solution was made by diluting the concentrated antibody 1:50 with 0.1% gel-PBS. 100 ml of a working solution were prepared by diluting the previous solution 1:60 with 0.01% gel-PBS. The diluted antibody (1:3000) was stored at 4°C until used.

4. Dextran-charcoal solution: 0.25% charcoal (Norit A, Fisher Scientific Co., Fairlawn, N. Y.) and 0.025% dextran T-70 (Pharmacia, Uppsala, Sweden) were suspended in PBS. The above solution was made up in one liter batches and stored at 4°C.

5. Isatin dye: 15 mg of the dye were dissolved in 20 ml of redistilled ethanol and stored at 4°C.

6. ChromAR 1000 sheets (Mallinckrodt Chemical Works, St. Louis, Mo.) were cut into 21x18 cm plates with 11 or 12 individual 14 cm lanes separated by 1 to 3mm spaces. The plates were washed twice by ascending chromatography with distilled methanol and stored over Drierite (W. E. Hammond Drierite Co., Xenia, Ohio) in a desiccator after air drying.

7. Eleven or 12 lanes were etched in the Eastman #6060 plates with fluorescent indicator (Sargent-Welch Scientific Co., Skokie, Ill.) before they were washed twice by ascending chromatography with redistilled methanol. After air drying, plates were stored over Drierite in a desiccator.

8. Standard steroid solutions were made by weighing 10 mg of pure steroid on a Mettler M-5 microbalance and dissolving the steroid in 10 ml of ice-cold absolute ethanol. Suitable aliquots and further dilutions with ice-cold absolute ethanol were made to arrive at a final concentration of 1 ng/ml (1 pg/μl). The working standard was stored in a glass-stoppered volumetric flask at 4°C and all aliquots were removed from the volumetric flask while it was immersed in an ice bath.

9. The ether used for extraction and elution of chromatrograms

was Mallinckrodt anhydrous reagent, grade AR. This ether gave the best blank values of any tested including redistilled ether stored over sodium.

# APPENDIX D

## CHARACTERISTICS OF THE ANDROGEN RADIOIMMUNOASSAY

### Sensitivity

As used here, sensitivity is the minimum levels that are detectable by a given analytical technique. This detection limit was defined mathematically by Kaiser and Specker (249) as $X = k\sqrt{2S_b} + \bar{x}_b$ where $X$ is the theoretical sensitivity, $\bar{x}_b$ is the average blank reading, $S_b$ is the standard deviation of the blank and $k$ is a constant equal to the cumulative probability at the confidence level specified by the investigator. Table 7 lists the results obtained from a series of determinations on 100 µl of plasma from ovariectomized and adrenalectomized monkeys and the same plasma with 10 pg of testosterone added. A students *t* test shows that these results are significantly different at the $p < 0.001$ level.

If the above equation and the average blank from Table 7 are used and $k$ is 1.65 as defined by the 95% confidence level, the theoretical sensitivity is calculated to be 19 pg which is in good agreement with the empirically determined value of 17.5 pg. The theoretical sensitivity of the assay as defined by the average blank from the

Table 7. Testosterone concentration (pg) estimated in the absence of the hormone in ovariectomized, adrenalectomized rhesus plasma and the same plasma with 10 pg of testosterone added.

| Sample No. | Plasma Alone, pg | Plasma + 10 pg Testosterone |
|---|---|---|
| 1 | 19.5 | 21.5 |
| 2 | 7.5 | 31.0 |
| 3 | 12.3 | 15.2 |
| 4 | 8.7 | 16.6 |
| 5 | 12.0 | 14.6 |
| 6 | 11.0 | 18.0 |
| 7 | 12.1 | 18.4 |
| 8 | 14.6 | 19.5 |
| 9 | 12.0 | 16.5 |
| 10 | 9.5 | 17.7 |
| 11 | 5.8 | 21.0 |
| 12 | 4.9 | 12.3 |
| 13 | 4.8 | 12.4 |
| 14 | 8.8 | 13.5 |
| 15 | 8.6 | 21.3 |
| 16 | 9.2 | 10.6 |
| Mean ± SD | 10.1 ± 3.6[a] | 17.5 ± 4.8[a] |

[a]Means are significantly different, $t = 6.16$, $df = 30$, $p > 0.001$.

testosterone and androstenedione reduction methods was 22.3 and 24.6 pg respectively. The actual sensitivity of the method probably lies between the two extremes.

## Precision

Precision as used here represents the reproducibility of an analytical measure and is usually reported as the standard error of the estimate. Since the assay was used to measure quantities spanning the total range of the standard curve, a simple standard error calculated for a single point on the curve is an inadequate measure of the precision. Therefore, the precision was calculated as the standard deviation of the differences between duplicate estimations, and the following formula was used: $S = (\Sigma d_i^2/2N)^{\frac{1}{2}}$ where S is the standard deviation, $d_i$ is the difference between duplicates and N is the number of duplicate estimations (250). The precision was calculated from duplicate measures over a range from 17 to 134 pg after the subtraction of the blank and correction for procedural losses. The within assay variation was ± 6.3 pg (SD); N = 90 and the between assay variation was ± 9.4 pg (SD); N = 66. The within assay variation before correction for procedural losses for the reduction of A to T over a range from 24 to 118 pg was ± 5.0 pg (SD); N = 38 and the between assay variation was not calculated because of the small N.

## Accuracy

The term accuracy is restricted to describe the agreement between the estimated amount and the actual amount present in the sample. Therefore, a measure of the accuracy was obtained by adding known amounts of T, A, and a combination of both steroids to 100 μl of plasma from ovariectomized, adrenalectomized animals. These data are found in Table 8 and indicate that acceptable accuracy was obtained. In addition, the effects of volumes of plasma on quantities of steroid estimated by the radioimmunoassay are given in Figure 14. The values from 10, 20, and 30 μl of male plasma represent a straight line that intersects the ordinate at approximately 10 pg, the blank value previously noted in Table 7.

## Specificity

The specificity of the radioimmunoassay for steroids is directly related to the specificity of the individual steps used for isolation, purification, and detection. A detailed analysis of the steroids that have similar chromatographic $R_f$ values to T and their ability to interact with the antibody from a particular rabbit are presented in Table 9. Of the compounds that interact with the antibody, only 5α-$\Delta^4$-androstenediol, 5α-androstane-3α,17β-diol, and 11β-hydroxy-$\Delta^4$-androstenediol are eluted with T from the chromatogram. These

Table 8. Recovery of known amounts of testosterone (T), androstenedione (A), and a combination of the two steroids added to 100 μl of ovariectomized, adrenalectomized rhesus plasma. Average blank values were subtracted and corrections were made for procedural losses. The combination data reported include both A + T and T + A at the concentrations indicated. Final figure in each column is mean ± SD in pg.

| | T | | A | | A + T or T + A | |
|---|---|---|---|---|---|---|
| | 10 pg | 30 pg | 50 pg | 100 pg | 20 pg + 50 pg | 75 pg + 50 pg |
| 1 | 8.6 | 36.8 | 55.2 | 116.7 | 62.6 | 114.9 |
| 2 | 8.7 | 36.1 | 37.0 | 88.9 | 69.5 | 135.9 |
| 3 | 10.0 | 40.4 | 39.5 | 90.0 | 61.1 | 152.0 |
| 4 | 2.6 | 27.0 | 45.9 | 86.3 | 64.2 | 83.0 |
| 5 | 14.8 | 28.3 | 49.0 | 84.9 | 66.6 | 84.0 |
| 6 | 10.0 | 27.7 | 51.8 | 83.6 | 58.7 | 114.0 ± 30.7 |
| 7 | 6.9 | 34.0 | 48.3 | 104.3 | 63.1 | |
| 8 | 8.7 | 30.5 | 46.7 ± 6.5 | 84.0 | 70.4 | |
| 9 | 11.1 | 38.8 | | 93.0 | 65.4 | |
| 10 | 6.2 | 33.1 | | 92.4 ± 11.1 | 73.5 | |
| 11 | 7.0 | 27.4 | | | 84.0 | |
| 12 | 7.7 | 33.1 | | | 67.2 ± 7.0 | |
| 13 | 5.6 | 25.0 | | | | |
| 14 | 8.2 | 31.9 | | | | |
| 15 | 6.7 | 43.0 | | | | |
| 16 | 9.6 | 33.1 | | | | |
| 17 | 5.3 | 32.9 ± 5.0 | | | | |
| 18 | 8.0 | | | | | |
| | 8.1 ± 2.6 | | | | | |

Figure 14. The estimation of testosterone (in triplicate) in increasing volumes of plasma from an adult male rhesus monkey by radioimmunoassay. Data are plotted as means ± standard deviation (vertical bars).

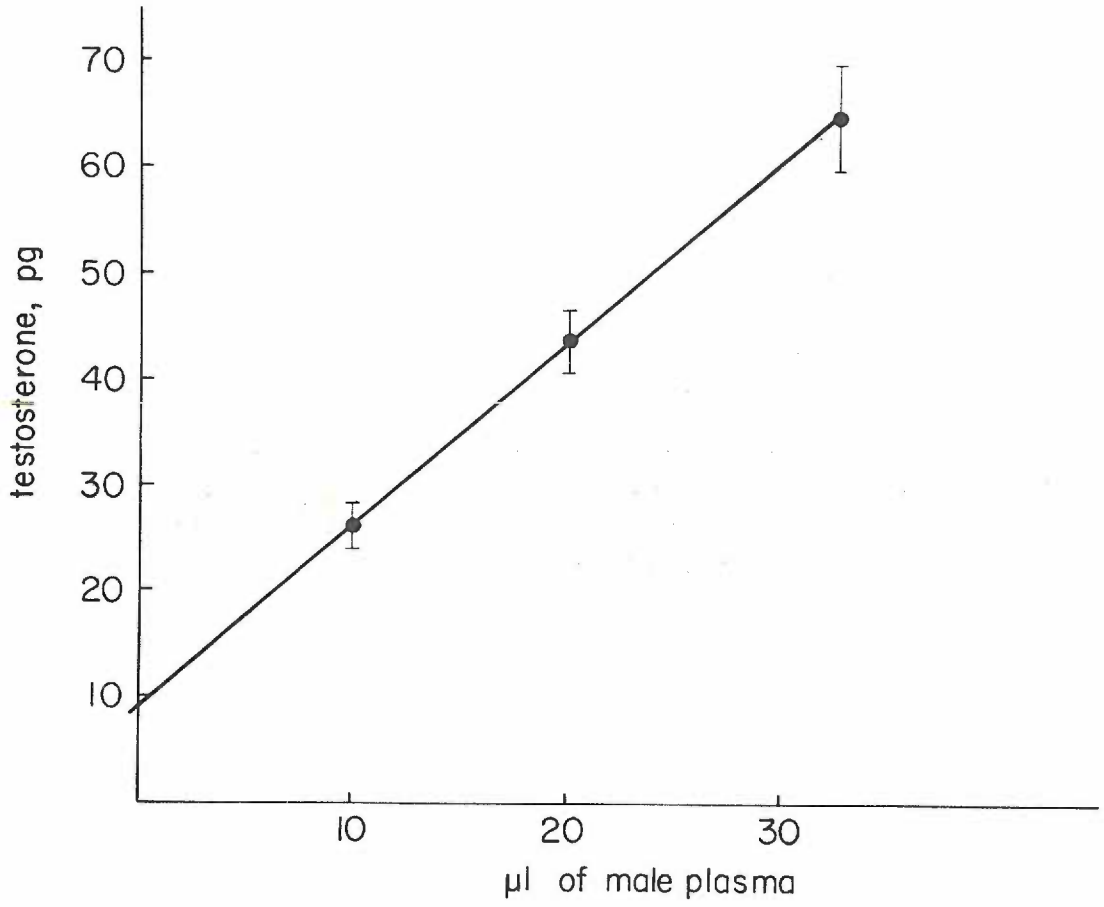

70
60
50
40
30
20
10
testosterone, pg
10
20
30
µl of male plasma

Table 9. $R_f$ values and specificity of the antibody[a] in the radioimmunoassay for testosterone.

| Compound | $R_f$[c] | % Displacement of $^3$H-Testosterone 100[b] | 500[b] | 1000[b] |
|---|---|---|---|---|
| Cortisol | 0.08 | --[d] | -- | -- |
| Corticosterone | 0.20 | -- | -- | -- |
| Estriol | 0.26 | -- | -- | -- |
| 11-deoxycortisol | 0.33 | -- | -- | -- |
| 5α-$\Delta^4$-androstenediol | 0.44 | 27 | 34 | 35 |
| 5α-androstane-3α,17β-diol | 0.45 | 10 | 34 | 35 |
| 11β-hydroxy-$\Delta^4$-androstenediol | 0.48 | 3 | 5 | -- |
| Testosterone | 0.52 | 55 | 90 | 92 |
| Estradiol | 0.53 | 0 | 4 | 6 |
| Epi-testosterone | 0.55 | 0 | 16 | 9 |
| Etiocholanolone | 0.56 | 0 | 0 | 5 |
| 17α-hydroxyprogesterone | 0.58 | 0 | 0 | 8 |
| 17α-hydroxypregnenolone | 0.59 | -- | -- | -- |
| 20α-hydroxypregn-4-en-3-one | 0.60 | 0 | 0 | 1 |
| Dehydroepiandrosterone | 0.62 | 0 | 19 | 30 |
| 5α-androstane-3,17-dione | 0.65 | 5 | 19 | 27 |
| Desoxycorticosterone | 0.65 | 7 | 9 | 0 |
| Androsterone | 0.65 | 2 | 4 | 0 |
| Dihydrotestosterone | 0.66 | 54 | 76 | 80 |
| Pregnenolone | 0.69 | -- | -- | -- |
| Estrone | 0.75 | 0 | 29 | 36 |
| $\Delta^4$ androstenedione | 0.77 | -- | -- | -- |
| Progesterone | 0.80 | -- | -- | -- |

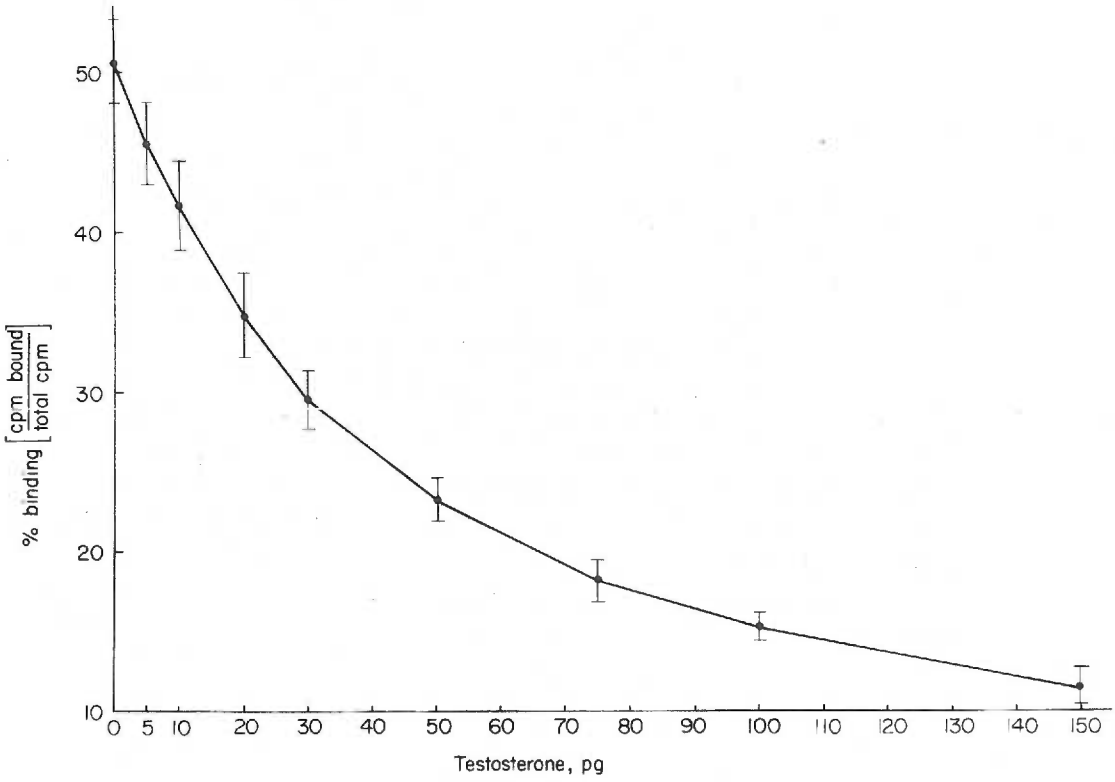
[a] Rivanol treated antisera diluted to 1:3000 in PBS.
[b] Picograms of steroid added to assay tube.
[c] Chromatography on chromAR sheets in chloroform:acetone (90:10).
[d] Dash--not tested.

compounds were tested in the radioimmunoassay and large quantities (500 to 1000 pg) gave only minimal interference in terms of their estimation as testosterone. Therefore, the combination of purification and detection techniques used in this study strengthens the conclusion that only testosterone was measured.

## Standard Curve

Figure 15 represents the composite of 18 standard curves used to calculate the results reported in this thesis. The average coefficient of variation at a given point was 6.7 ± 1.5% (SD). These results indicate that the standard curve was reproducible from assay to assay and that the relative precision of the curve is independent of the quantity measured.

Figure 15. Composite standard curve for testosterone determined from 18 individual assays. The % binding was calculated from the total radioactivity added and the radioactivity bound with varying amounts of cold testosterone after treatment with charcoal to remove the free label. Data are plotted as means ± standard deviation (vertical bars).


50
40
30
20
10
% binding [cpm bound / total cpm]
0 5 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
Testosterone, pg