

SEXUAL DIFFERENTIATION AND FEEDBACK CONTROL
OF LUTEINIZING HORMONE SECRETION
IN THE RHESUS MONKEY

by

Robert Alan Steiner

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

September 1974

APPROVED:

[REDACTED]

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(Professor in Charge of Thesis)

[REDACTED]

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(Chairman, Graduate Council)

ACKNOWLEDGEMENTS

I wish to thank the staff of the Oregon Regional Primate Research Center for their editorial, surgical, illustrative, and animal handling assistance during the course of this project. Special thanks are offered to Ms. Doris Guenzi for her help with the estrogen radioimmunoassays. I am also indebted to Mr. Don Clifton for his invaluable counsel and encouragement. I gratefully acknowledge the thoughtful advice and guidance of Drs. Harold G. Spies and Reid L. Norman. Particular thanks are extended to Dr. John M. Brookhart for his fostering of a fledgling physiologist. And finally, with love and humility, I tender thanks to my mentor, Dr. John A. Resko, whose scientific excellence and eminent character exemplify the ideal; for his example I will be forever indebted and ever so grateful.

This thesis is dedicated to my beloved mother and father whose nurture and guidance have tendered sustenance and promise to me, their loving son.

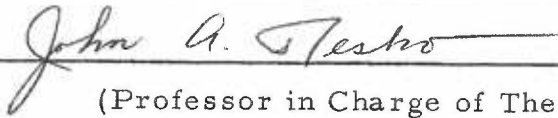
AN ABSTRACT OF THE THESIS OF

Robert Alan Steiner for the Doctor of Philosophy in Physiology
(Name) (Degree) (Major Department)

Date of receiving this degree _____

Title: SEXUAL DIFFERENTIATION AND FEEDBACK CONTROL
OF LUTEINIZING HORMONE SECRETION IN THE
RHESUS MONKEY

Approved _____



(Professor in Charge of Thesis)

The inhibitory and stimulatory effects of estradiol-17 β on systemic luteinizing hormone (LH) levels were studied in gonadectomized female (N = 7), male (N = 7), and prenatally androgenized genetic female (N = 7) rhesus monkeys. The androgenized females, which had been treated in utero with testosterone propionate, showed masculinization of the external genitalia and behavior after birth. To study the long-term inhibitory effects of estradiol, we implanted silastic capsules, 0.5, 1.5, and 3.0 cm in length, of this hormone into the animals for 30 days. LH concentrations in gonadectomized females were significantly suppressed to $47.8 \pm 15, -12$ (SE) % of the initial control levels with the smallest size estradiol capsule, which produced about 22 ± 1.3 pg/ml of serum estrogen. In gonadectomized males and androgenized females,

circulating levels of LH could be significantly suppressed only with the largest (3.0 cm) implant, which produced 106 ± 8 (SE) and 92 ± 4 (SE) pg/ml of serum estrogen in each of the groups, respectively.

These data suggest that females are more sensitive to the long-term negative feedback effects of estradiol than males, and that prenatally androgenized genetic females respond more like males in this regard.

To evaluate the short-term inhibitory effects of estradiol, we implanted 0.5 cm silastic capsules of this hormone into the animals and studied the LH response pattern for the next 72 hours. No significant sex differences in the LH response characteristics could be shown.

To study the positive feedback effects of estrogen on LH release, we injected the groups of animals with estradiol benzoate (50 μ g/kg) and monitored LH and estrogen levels for the next four days. We found that both the male and androgenized female populations showed a significantly larger integrated serum LH response to the treatment than similarly treated females. These data show that unlike the rat both the males and females of this primate species respond to the estrogen-induced mechanism for the LH surge; however, males and females differ in their response characteristics. Moreover, the fact that androgenized females respond like males in this regard, suggests that the prenatal hormonal milieu organizes the neural-pituitary control mechanism for the LH surge.

To explore whether differences in pituitary responsiveness account for the observed sex differences in LH response to negative and positive estrogen feedback, we administered 500 μ g of the synthetic decapeptide, luteinizing hormone-releasing hormone to four monkeys from each of the experimental groups. We observed no sex differences in the LH response of males, females, and androgenized females. These data indicate that the differences in LH response to estrogen between males and females and between prenatally androgenized females and normal females are the function of a mechanism which lies within the central nervous system and which can be influenced by prenatal exposure to androgens.

TABLE OF CONTENTS

<u>CHAPTER</u>	<u>PAGE</u>
I. GENERAL INTRODUCTION AND STATEMENT OF THE PROBLEM	1
II. REVIEW OF SEXUAL DIFFERENTIATION IN MAMMALS	4
Sexual Differentiation of the Reproductive Tract	5
Sexual Differentiation of Systems Tangential to the Reproductive Physiology in Mammals	12
Sexual Differentiation of the Brain: Sexually Dimorphic Behavior in Mammals	22
Sexual Differentiation of the Brain: Gonadotropin Patterns in Subprimates	28
Sexual Differentiation of the Brain: Gonadotropin Patterns in Primates	39
Feedback Control of Gonadotropins in Mammals	43
III. MATERIALS AND METHODS	46
Animals and Animal Care	46
Blood-Handling Techniques	47
Steroid Administration	48
Luteinizing Hormone-Releasing Hormone Administration	49
Experimental Design	50
Long-Term Negative Feedback Effect of Estradiol-17 β on LH Secretion in the Rhesus Monkey	50
Short-Term Negative Feedback Effect of Estradiol-17 β on LH Secretion in the Rhesus Monkey	50
Positive Feedback Effect of Estradiol Benzoate on LH Secretion in the Rhesus Monkey	51
The Release of LH as Effected by the Synthetic Decapeptide, Luteinizing Hormone-Releasing Hormone (LH-RH)	52

<u>CHAPTER</u>	<u>PAGE</u>
Radioimmunoassay for Estrogens and Luteinizing Hormone (LH): General Comments	52
Radioimmunoassay for Estrogens	53
Radioimmunoassay for Luteinizing Hormone (LH)	54
Data Analysis	55
IV. RESULTS	57
Long-Term Negative Feedback Regulation of LH Secretion by Estradiol-17 β	57
Short-Term Negative Feedback Regulation of LH Secretion by Estradiol-17 β	59
Positive Feedback Control of LH Release by Estradiol Benzoate	60
The Release of LH Effected by the Synthetic Decapeptide, Luteinizing Hormone- Releasing Hormone (LH-RH)	62
V. DISCUSSION	64
VI. SUMMARY AND CONCLUSIONS	76
BIBLIOGRAPHY	78
ILLUSTRATIONS	113
APPENDIX A	
I. Estrogen Assay Reagents	134
II. Radioimmunoassay for Testosterone (T)	137
III. Preparation and Handling of LH Radioimmunoassay Reagents	139
IV. Preparation of Column for Iodination of LH	143
V. Procedure for Iodination of LH	144
VI. Half-Life of Exogenously Administered Serum Estradiol-17 β	145

<u>APPENDIX A</u>	<u>PAGE</u>
VII. Long-Term Negative Feedback of LH Secretion by Estradiol-17 β in Female Rhesus Monkeys-Time Course Study	148
VIII. Short-Term Negative Feedback Effects of Estradiol- 17 β on LH Secretion in Ovariectomized Rhesus Monkeys Restricted in Primate Chairs	150
IX. Short-Term Negative Feedback Effects of Estradiol- 17 β on LH Secretion in Caged, Ovariectomized Rhesus Monkeys	152
X. Time Course of LH Release in Ovariectomized Rhesus Monkeys: The Effects of Acute Immobiliza- tion Stress and Sodium Pentobarbital Anesthesia	154
XI. Negative Feedback Effect of Testosterone on LH Secretion in Ovariectomized Rhesus Monkeys	157
APPENDIX ILLUSTRATIONS	158
APPENDIX B	209

LIST OF ILLUSTRATIONS

<u>FIGURE</u>		<u>PAGE</u>
1.	The long-term dose-effect relationship between serum estrogens (E_2) and luteinizing hormone (LH) in the gonadectomized rhesus monkey.	114
2.	The long-term negative feedback effect of silastic capsules containing estradiol- 17β on serum luteinizing hormone (LH) levels in the gonadectomized rhesus monkey.	116
3.	The short-term time-course effects of 0.5 cm silastic capsules containing estradiol- 17β on serum luteinizing hormone (LH) levels in the gonadectomized rhesus monkey.	118
4.	The time-course of serum estrogens (E_2) throughout the short-term exposure to 0.5 cm silastic capsules containing estradiol- 17β in the gonadectomized rhesus monkey.	120
5.	The time-course of serum luteinizing hormone (LH) release after an injection of estradiol benzoate in the gonadectomized rhesus monkey.	122
6.	The time-course of serum estrogens (E_2) after an injection of estradiol benzoate in the gonadectomized rhesus monkey.	124
7.	The time-course of serum luteinizing hormone (LH) after an injection of estradiol benzoate in the gonadectomized rhesus monkey.	126
8.	The time-course of serum estrogens (E_2) after an injection of estradiol benzoate in the gonadectomized rhesus monkey.	128

FIGURE

PAGE

9. The time-course of serum luteinizing hormone (LH) release after an injection of the synthetic decapeptide, luteinizing hormone-releasing hormone (LH-RH) in the gonadectomized rhesus monkey. 130
10. Integrated serum luteinizing hormone (LH) release over a 6-hour period in response to an injection of luteinizing hormone-releasing hormone in the gonadectomized rhesus monkey. 132

LIST OF APPENDIX ILLUSTRATIONS

<u>FIGURE</u>	<u>PAGE</u>
1. Double exponential decay curve (straight lines) of serum estrogens (E_2) after an infusion of 4 μ g of estradiol-17 β (E_2) into ovariectomized rhesus monkey #1419.	160
2. Double exponential decay curve (straight lines) of serum estrogens (E_2) after an infusion of 2 μ g of estradiol-17 β (E_2) into ovariectomized rhesus monkey #3585.	162
3. The time-course of the long-term negative feedback effect of 0.5-cm silastic capsules containing estradiol-17 β (E_2) on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys.	164
4. The time-course of serum estrogens (E_2) throughout the long-term exposure to 0.5-cm silastic capsules containing estradiol-17 β (E_2) in ovariectomized rhesus monkeys.	166
5. The time-course of the short-term negative feedback effects of 1.5-cm silastic capsules containing estradiol-17 β (E_2) on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys.	168
6. The time-course of the short-term negative feedback effects of 3.0-cm silastic capsules containing estradiol-17 β (E_2) on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys restrained in primate chairs.	170
7. The time-course of the short-term negative feedback effects of empty (sham) silastic capsules on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys restrained in primate chairs.	172

<u>FIGURE</u>		<u>PAGE</u>
8.	The time-course of the short-term negative feedback effects of silastic capsules containing estradiol-17 β on serum luteinizing hormone (LH) levels in caged, ovariectomized rhesus monkeys.	174
9.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #669.	176
10.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #669.	178
11.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #1403.	180
12.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #1419.	182
13.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #4034.	184
14.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #5734 under conditions of acute immobilization stress.	186
15.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #3571 under conditions of acute immobilization stress.	188
16.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #4581 under conditions of acute immobilization stress.	190
17.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #5732 under conditions of acute immobilization stress.	192
18.	Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #669 under deep sodium pentobarbital anesthesia.	194

FIGURE

PAGE

19. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #1403 under deep sodium pentobarbital anesthesia. 196
20. The effect of silastic capsules containing testosterone on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkey #2302. 198
21. The effect of silastic capsules containing testosterone on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkey #812. 200
22. The effect of silastic capsules containing testosterone on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkey #5733. 202
23. The concentration of serum testosterone in 3 ovariectomized rhesus monkeys as effected by various sizes of silastic capsules containing testosterone. 204
24. The effect of empty (sham) silastic capsules on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys. 206
25. The concentration of serum testosterone in 3 ovariectomized rhesus monkeys as influenced by empty (sham) silastic capsules. 208

CHAPTER I

GENERAL INTRODUCTION AND STATEMENT OF THE PROBLEM

The question of how males and females differentiate has long intrigued naturalists. In the 18th century, John Hunter described the bovine freemartin and the etiological basis of this intersex female proposed by Lillie (1916, 1917) and Keller and Tandler (1916) represents the birthdate of the hormonal theory of sexual differentiation in the mammalian fetus. Some factors that are thought to control differentiation of the müllerian duct system in the genetic female and the wolffian duct system in the genetic male may also control the differentiation of the central nervous system. Two broad functional criteria can be used to study sexual differentiation of the adult brain: the patterns of gonadotropin secretion and the displays of sexual behavior (Harris, 1964). In the female of most species, the gonadotropin pattern is characteristically cyclic, i. e., it results in a predictable and rhythmic cycle of ovarian function. In the male, however, the pattern is more appropriately described as tonic, i. e., characterized by a relatively constant level of serum gonadotropins and a steady state of testicular function. The second criterion, sexual behavior, is related to those factors which

organize and activate the neural substrate that mediates the sexual behavior of the adult males and females of a species. Since the classical observations of Pfeiffer (1936) many investigators have outlined the fundamental role of fetal hormones in the sexual differentiation of the central nervous system (CNS). The neural mechanisms which regulate the patterns of gonadotropin secretion and those which mediate reproductive behavior are thought to be organized and differentiated by fetal hormones. Recently, Karsch, Dierschke, and Knobil (1973) showed that the castrated male rhesus monkey, like the female, responds to an estrogen challenge with a release of gonadotropins. These data are in striking contrast to those on the rodent whose male member is apparently incapable of such a response to estrogen treatment (Neill, 1972). Karsch and his co-workers concluded that in primates the regulatory system which initiates the LH surge is equally competent in both sexes and that relative to the control of gonadotropin secretion, the schema for the sexual differentiation of the rodent CNS may not apply to primates.

Is the primate CNS sexually differentiated with respect to the control of gonadotropin secretion, or, as some investigators have suggested, is the primate brain pluripotential in this regard? To further explore this problem, we have evaluated the gonadotropin control system in a group of adult, gonadectomized, genetic female

rhesus monkeys which were androgenized in utero by long-term treatment with testosterone propionate. We were interested to see whether these androgenized females, which displayed masculinized external genitalia and masculinized patterns of juvenile and adult behavior (Goy, 1970; Eaton, Goy, and Phoenix, 1973) likewise would manifest a male pattern of gonadotropin secretion as a function of their prenatal exposure to androgens. Our data provide tentative evidence that the sexual differentiation observed in the regulation of gonadotropin secretion by the CNS-pituitary axis of the primate parallels the differentiation in the neural mechanisms which mediate sexual behavior. To put our studies into the perspective of the whole question, we will begin with the early attempts to resolve it, first in the rodent and then in the primate.

CHAPTER II

REVIEW OF SEXUAL DIFFERENTIATION IN MAMMALS

Aristotle (348-222 B.C.) provided some of the earliest descriptions in comparative embryology and anatomy and carefully outlined the breeding habits of a wide variety of species (Thompson, 1910). The association between copulation and pregnancy had been long recognized, but the determinants of fetal sex were unknown. Aristotle believed that male semen provides the vital force which congeals menstrual blood into a viable embryo. He accepted the Homeric notion that fertilization can occur via the wind, and he further suggested that in sheep and goats, north winds preferentially effect male offspring, while south winds generally effect female offspring. Moreover, he noted that castration results in feminization of the male. Aristotle concluded from these observations that certain organs are important in the determination of sex.

Standing on the shoulders of the ancient naturalists, modern biologists continue to search for the physiological mechanisms which underlie sexual differentiation in mammals. Using whatever perspective we have thus gained, we will begin with sexual dimorphism in the mammalian fetus, its control, and its implications for the adult form.

Sexual Differentiation of the Reproductive Tract

Sexual differentiation occurs through a series of successive steps which are ultimately controlled by the individual's chromosomal or genetic sex. These chromosomes and probably a limited number of sex-determining genes strongly determine the predisposition of the gonadal sex (Beatty, 1970; Wolfe, 1971). The testes and ovaries develop from common, undifferentiated gonadal primordia. Witschi (1951) elucidated the concept of gonadal hermaphroditism in his theory of cortico-medullary antagonism. He suggested that the undifferentiated gonad is composed of two anatomically distinct and physiologically competitive parts: the cortex, which is composed of the superficial coelomic epithelium, and the medulla, which develops from the mesonephric blastema. These units compete to organize either a testis or an ovary, depending upon their genetic predisposition. These theories of gonadal hermaphroditism were derived from work performed primarily on amphibians, and their validity for explaining gonadal differentiation in mammals has not been shown. Notwithstanding, it has become clear that, within the context of the hormonal theory of sexual differentiation, these genetically predisposed endocrine glands direct the process of body sex differentiation. The concept that body sex is bipotential during development is widely accepted, and classical embryological methods have revealed

that the developing embryonic sex structures pass through an undifferentiated stage wherein the sexual morphology can be either masculinized or feminized with appropriate hormonal or surgical treatment (Jost, 1970; Neumann, Steinbeck, and Hahn, 1970).

The freemartin is a sexually aberrant, intersex female calf whose twin is a normal male. The word freemartin is probably derived from the scottish words flearr, meaning a dry cow, and mart, which means meat. These dry cows were slaughtered for beef; hence the name for the intersex female calf which did not produce milk. In independent inquiries into the physiological etiology of the freemartin, Keller and Tandler (1916) and Lillie (1916, 1917) observed placental vascular anastomoses, which united the male and female intersex twins, and suggested that hormonal secretions originating from the male twin passed through these anastomoses and influenced his developing partner. More recently, investigators have suggested that germ cells from the male twin migrate through the placental vascular anastomoses and settle on the female gonadal ridge (Moore and Owen, 1965; Short, 1970). Earlier, Bouin and Ancel (1903) had observed activity in the testicular interstitial cells of the pig embryo, and on this basis, had proposed that the reproductive structures are under the differentiating influence of hormones produced by the developing gonads. These early observations implied that the male reproductive tract and its

derivatives are under the differentiating control of the developing testes, and that the female reproductive anlagen develop by default in the absence of testicular secretions (Price, 1970).

In 1968, using pooled samples from 230 one-day-old male rats, Resko, Feder, and Goy showed that testosterone is present in the plasma and testicular tissue during the neonatal period when physiological sexual differentiation is presumed to occur. Resko (1970) further demonstrated that the 45- to 60-day fetal rhesus monkey testis in vitro can convert ^{14}C -pregnenolone to testosterone whereas the ovary is quiescent in this regard. His data also showed more testosterone in the plasma pools of the umbilical artery and gonads of 100- to 150-day fetal male rhesus monkeys than in those of female monkeys and higher concentrations of serum testosterone in rhesus mothers pregnant with male fetuses than in those pregnant with female fetuses. Finally, he showed that during the neonatal period, serum androgens in male rhesus monkeys were high on the first and second postnatal days and subsequently declined with increasing age. In a more recent study, Resko and his co-workers (1973), using a sensitive and specific radioimmunoassay for testosterone, confirmed his earlier observations on the sex-related differences in testosterone levels during gestation in the rhesus monkey. These

studies further revealed that whereas testosterone levels were significantly greater in the umbilical artery of the male fetus than in the vein, no such difference was apparent in the female fetus. Moreover, these investigators found that castrating the male fetus on day 100 of gestation abolished the sex difference in the amounts of testosterone found on day 150 to 156 of gestation. On the basis of these data, the authors concluded that the fetal testis of the monkey is the source of the elevated levels of testosterone found in males and that fetal males develop in a different hormonal milieu than fetal females.

These data in the rhesus monkey differ in some respects from those obtained in the human fetus, which at term, exhibits no apparent sex differences in the quantity of testosterone in cord blood (Rivarola, Forest, and Migeon, 1968; Mizuno et al., 1968). Earlier in gestation, however, human fetal blood samples do apparently show sex differences in testosterone concentration (Murphy and Diez D'Aux, 1972; Reyes, Faiman, and Winter, 1972). In vitro studies in the human fetus, using thin layer and celite columns for quantification of metabolic products after incubation with ^{21}C -steroids, have demonstrated that testosterone is the major androgen formed by the testis at the time of male sex differentiation (Siiteri and Wilson, 1973).

On the basis of experiments on the rabbit, Jost demonstrated

that testicular secretions masculinize the undifferentiated reproductive anlagen, facilitate development of the wolffian ducts, and quash the müllerian duct system, and that testosterone and other androgens masculinize the reproductive tract, but do not cause regression of the müllerian duct system (Jost, 1970, 1972; Jost et al., 1973).

These data suggest that in addition to androgens the fetal testis secretes another substance which causes the müllerian ducts to retrogress. This substance, müllerian-inhibiting factor, has been partially isolated and identified (Josso, 1972, 1973). The müllerian-inhibiting factor or hormone of the human testis is interspecific (Josso, 1971) and nondialysable (Josso, 1972b), and its synthesis is restricted to the fetal and possibly the early postnatal period (Josso, 1972a).

When pregnant guinea pigs are injected with androgens, the genetic female offspring from such pregnancies show a pronounced masculinization of their reproductive anatomy (Dantchokoff, 1936, 1937). Females of other species, including rats (Neumann, Elger, and Steinbeck, 1970), hamsters (Paup, Coniglio, and Clemens, 1972), and rhesus monkeys (Phoenix, Goy, and Young, 1967) exhibit masculinized external genitalia as a function of early androgen treatment. In addition to masculinizing the genitalia of genetic females, early postnatal androgenization decreases the capacity of uterine, anterior pituitary, and hypothalamic tissue to bind

estradiol when the animal becomes an adult (Tuohimaa and Johansson, 1971). Two human syndromes represent clinical counterparts to the artificially induced masculinization observed in lower mammals. In the adrenogenital syndrome, androgens produced from a malfunctioning fetal adrenal gland masculinize the developing female fetus. In another syndrome, progestins, therapeutically administered to gravid women, or androgens from a maternal tumor can masculinize a genetic female child (Money and Ehrhardt, 1972). The degree of masculinization varies considerably within these syndromes, depending upon the timing and concentration of the androgenic substances. Some degree of clitoral hypertrophy and labioscrotal fusion is manifest, and the müllerian duct derivatives are present. These masculinizing effects of androgens apparently can be pharmacologically simulated with early estrogen treatment as well (Greene, Burrill, and Ivy, 1940; Wilson, 1943).

Indirect evidence buttresses the theory that under normal physiological conditions testicular androgens effect the masculinization of the male reproductive tract. Selective inhibition of fetal testicular 17 α -hydroxylase and C-17-20-lyase, which control the metabolic synthesis of testicular androgens, produces hypospadias in the rat (Goldman, 1971). Neumann and his co-workers (1970a) showed that under the influence of the anti-androgen, cyproterone acetate, development of the wolffian ducts of the genetic male

rabbit and dog is arrested, and differentiation of neither the accessory sex glands nor the male external genitalia occurs. Goldfoot, Resko, and Goy (1971) have likewise shown that prenatal cyproterone treatment produced nontubular hypospadiac penes and stunted seminal vesicles and prostate glands and induced androgen-target gland insensitivity to testosterone in adult male guinea pigs. If retrogression of the müllerian ducts in the genetic male were dependent upon androgens, then under the influence of cyproterone acetate they should be preserved; but the müllerian duct system does indeed undergo retrogression in spite of cyproterone acetate treatment (Neumann et al., 1970a), further corroboration of Jost's theory that, in addition to androgens, another factor must be involved in sexual differentiation (Jost, 1965). Injection of antibodies to testosterone-3-bovine serum albumin into the mother effects a blockade of masculine differentiation in male rat fetuses and causes a decrease in the anogenital distance and an inhibition of testicular weight (Goldman et al., 1972a). These data confirm that testicular androgens, particularly testosterone, represent the masculinizing agents that are responsible for effecting the sexual differentiation of the reproductive tract in mammals. Recently, some investigators have suggested that certain circulating hormones, androgens in particular, actually represent "pro-hormones," which are really the metabolic precursors of those substances that are the actual,

molecular active agents at target tissue sites, the sum of whose activities represents the "global effect" of the precursor hormone (Baulieu, 1971). For example, testosterone is thought to be metabolized to its molecular active derivative, dihydrotestosterone, in cells of target tissues like the prostate and other primary sexual organs (Bruchovsky and Wilson, 1968; Wilson and Lasnitzki, 1971; Beyer, Jaffe, and Gay, 1972). It is possible, therefore, that the molecular effects of testosterone upon the differentiating male reproductive tract are mediated via conversion to dihydrotestosterone (Wilson, 1972).

The general conclusion gathered from a vast amount of data on a variety of mammalian forms is that the differentiation of the reproductive tract and external genitalia is under the influence of hormones secreted by the fetal gonads and that the developing testes prevent "an hypothetical state of affairs in which every mammalian embryo would develop into an individual with a female type of reproductive tract" (Wells, 1962).

Sexual Differentiation of Systems Tangential to the Reproductive Physiology in Mammals

Sexual dimorphism of the reproductive morphology in mammals is obvious in most cases, but it has become equally clear that certain more obscure anatomical entities also undergo sexual

differentiation. Sexual dimorphism in the ischium and pubis of three species of South American monkeys has been described (Black, 1970), and Larson and Knapp (1971) showed differences in the appearance of beaver neutrophils as a function of sex.

Brain and pituitary morphology is apparently influenced by sexual determinants. The most obvious sex difference in the brain is that of size. After puberty, the brain weight and the weights of different brain regions are smaller in female rats than in males, and furthermore, the brain weight attains a plateau sooner in females than in males (Donaldson and Hatai, 1931). In utero gonadectomy tends to increase the number of basophilic cells in the anterior pituitary gland of fetal male rats but not in females (Nakai et al., 1972). Raisman and Field (1973) reported that the number of nonamygdaloid synapses on dendritic spines in the preoptic area of normal female rats is higher than in the male and suggested that this difference is related to the ability of the female to maintain a cyclic pattern of gonadotrophin release and/or behavioral estrus. Ratner and Adamo (1971) showed that neonatal androgenization of adult female rats changed the morphology of the nerve terminals on the neurons in the arcuate nucleus. Döcke and Koloczek (1966) also observed sex-linked differences in the preoptic anterior hypothalamic region of rats, the female rats showing a significantly greater nuclear size than the males. In a later study, Dörner and

Staudt (1968) showed that male rats castrated on the first day of life displayed a highly significant increase in the preoptic anterior hypothalamic area, which approximated the size of that in the female animals. Similar observations of sexual dimorphism have been made for the ventromedial nucleus in the hypothalamus of the rat (Dörner and Staudt, 1969a). There is considerable evidence to suggest that these structures are sexually differentiated under the influence of the perinatal hormonal environment (Pfaff, 1966; Raisman and Field, 1971; Dörner and Staudt, 1968, 1969a). Sexual dimorphism has also been reported in the ependymal cells that line the floor of the third ventricle in the anterior tuber cinereum of the rhesus monkey (Kumar, 1969). In the female rhesus monkey, these cells are heavily granulated; in the male, they are only sparsely granulated.

Sex differences in the metabolism of various organ systems have been reported. Denef, Magnus, and McEwen (1974) demonstrated sex-dependent changes in pituitary 5α -androstanediol (DIOL) formation during postnatal development and puberty in the rat. At birth, the in vitro conversion of testosterone-1, 2- H^3 to DHT and DIOL was equal in both sexes. But in females the formation of both metabolites increased abruptly with age to a peak value between 10 and 15 days, then fell precipitously to a nadir at 40 days whereas in males, pituitary DHT formation increased only slightly up to 10

days of life, then decreased until day 25 with a subsequent secondary rise between 30 and 34 days. Their studies also revealed sex-specific changes and responses to gonadectomy and sex hormones in pituitary DHT and DIOL formation during development. Bradlow and Zumoff (1974) showed a sex difference in the systemic formation of androsterone and etiocholanolone from testosterone in adult humans. Sex differences have been demonstrated in the control of adrenal function (Critchlow et al., 1963, Hiroshige et al., 1973) and in the biogenesis of androgens by the adrenal cortex in rats (Askari, 1970). Hagemenas and Kittinger (1972, 1974) studied the influence of fetal sex on progesterone metabolism in the rhesus monkey. The fact that fetal females showed higher concentrations of progesterone than fetal males suggests that placental metabolism is influenced by fetal sex. Numerous investigators have reported sexual differentiation of steroid-metabolizing enzymes in the rat liver (Denef and De Moor, 1969, 1970, 1972; Ota, Sato, and Obara, 1972; Tabei and Heinrichs, 1974a, b; Colby and Kitay, 1972), and studies by Noda and Nakagawa (1972) revealed a sex difference in rat liver histidase. These experiments showed that the activity of entire families of hepatic enzymes and/or enzyme systems can be experimentally manipulated by modulating, either surgically or pharmacologically, the prenatal and early post-natal hormonal environment, and these data tender the conclusion that in normal

physiology these enzyme systems are sexually differentiated as a function of the sex-specific neonatal endocrine milieu. Studies by Gustafsson and Goldman (1972) showed that the hepatic metabolism of steroids in genetic male rat pseudohermaphrodites had a female character. These male pseudohermaphrodites apparently are not sensitive to the masculinizing effects of neonatal androgens and show a female pattern of hepatic metabolism; therefore, we can infer that testicular androgens predestine the character of liver enzymes in the mammalian adult male form. These data, though indirect, support the theory that the fetal endocrine environment organizes the adult metabolic response characteristics of these organ systems.

Certain nonsexual behaviors seem to be organized by the fetal endocrine environment. Normally, female rats are more exploratory and defecate less frequently than do males; however, neonatal treatment of genetic female rats with either androgens or estrogens abolishes these sex differences (Gray, Levine and Broadhurst, 1965). Sex differences have been reported in the fighting behavior of mice (Edwards, 1968, 1971) and in the eating behavior (Bell and Zucker, 1971) and taste preferences (Wade and Zucker, 1969) in the rat. Furthermore, Timiras (1971) and Woolley and Timiras (1962) reported that the seizure threshold to electroshock in the rat differs between the sexes. Moreover, these data implicate fetal

endocrine secretions as organizers of these particular nonsexual behaviors in mammals.

In addition to certain enzyme systems in the liver and a variety of nonsexual behaviors, the brain itself seems to undergo a metabolic differentiation as a function of sex. Barry and Leonardelli (1964) reported differences in brain histochemistry between male and female guinea pigs. Hypothalamic structures in female rats concentrate estrogen to a greater degree than those of male rats (McEwen and Pfaff, 1970; McGuire and Lisk, 1969), and neonatal exposure of genetic females to androgens inhibits the ability of these brain regions to bind estrogen in the adult animal (Tuohimaa and Johansson, 1971; McEwen and Pfaff, 1970). Sex differences have been reported in the ability of nervous tissue to accumulate ¹³¹I-1-triiodothyronine (Bleecker, Ford, and Rhines, 1971). Brain serotonin concentrations in the rat vary as a function of sex (Kato, 1960; Giulian, Pohorecky, and McEwen, 1973), and these differences are under the organizational influence of neonatal hormones (Ladosky and Gaziri, 1970). Ladosky and Gaziri (1970), who determined the concentration of serotonin in the brains of male and female rats on the first, fourth, eighth, and twelfth day after birth, observed that the amount of serotonin, which is comparable between the two sexes up to day 8, rises significantly in females on day 12 and that this elevation in serotonin levels can be prevented if the

females are injected with testosterone propionate on the day of birth. In contrast, males castrated at birth had brain serotonin levels comparable to those in intact females and significantly greater than those in intact littermates. These investigators concluded that the serotonin concentration may be related to the process of sexual differentiation of the brain since its concentration is modified by the same procedures that induce androgenization.

The molecular components that synthesize cellular proteins in the brain are also sexually differentiated (Ford, 1973). Sex differences in total RNA content and metabolism in various regions of the brain as a function of the neonatal hormonal environment have been reported for the rat (Novakova et al., 1971; Shimada and Gorbman, 1970; Soriero and Ford, 1971, 1973; Clayton, Kogura, and Kraemer, 1970). For example, Novakova and his co-workers (1971) reported that the RNA content in the neurons of the paraventricular nucleus and in the pyramidal neurons of the hippocampus was higher in estrous than in diestrous females or in male rats. Salaman (1970) found that the rate of ^3H -uridine incorporation in the anterior hypothalamus was higher in estrous than in diestrous and persistent estrous rats. Clayton et al. (1970) showed that testosterone administered to newborn female rats significantly reduced the incorporation of uridine into brain tissue in general. Soriero and Ford (1971, 1973) demonstrated a trend for RNA concentration

to be higher in gray matter from female animals, and a trend for the RNA/protein ratio to be higher in the hypothalamus of female rats. These investigators also showed a trend for the adenine + uracil/guanine+cytosine ratio to be higher in female rats. Within certain brain nuclei, the concentration and incorporation of amino acids, including lysine, arginine, and methionine, into proteins differ between sexes (Block, 1937; Kartzinel, Ford and Rhines, 1971; MacKinnon, 1973; Moguilevsky, Scacchi, and Christot, 1971; Soriero and Ford, 1973; Scacchi et al., 1970). These data suggest a correlation between these sex-specific neurochemical systems and the sexual differentiation of the brain.

In the rat, various brain enzymes, including L-cysteine-aminopeptidase, pineal hydroxyindole-o-methyl transferase, monamine oxidase, 5-hydroxytryptophan decarboxylase, and butylcholinesterase, exhibit sex-specific activity (Bickel et al., 1972; Hyyppä, Cardinali, and Wurtman, 1973; Kamberi and Kobayashi, 1970; Skillen, Thienes, and Strain, 1961; Wooley, 1963; Griffiths and Hooper, 1972; Denef et al., 1974). For example, Hyyppä et al. (1973) showed that male rats treated postnatally with a single intraventricular injection of 10 μ g of 6-hydroxydopamine (6-OHDA) exhibited a significant increase in pineal hydroxyindole-O-methyl transferase (HIOMT) activity on day 30 of life whereas females failed to display any changes in the pineal enzyme. Neonatal

castration abolished the pineal response to postnatal 6-OHDA in males whereas treatment with both testosterone and 6-OHDA caused pineal HIOMT activity to increase in females. These investigators suggested that the sex dependency of the effect of 10 μ g of 6-OHDA on pineal HIOMT is related to the sexual differentiation of the brain which occurs during the critical period in the neonatal rat. Reddy, Naftolin, and Ryan (1973) reported that hypothalamic and limbic system tissue of the male rabbit showed a greater capacity to aromatize androgens to estrogens than that of the female. This observation suggests that the conversion of androgens to estrogens is an important mechanism for the molecular expression of the androgenization phenomenon in males. Components of the oxidative and glycolytic metabolic machinery vary as a function of sex (Campos and Ladosky, 1972; Moguilevsky et al., 1968; Scacchi, Moguilevsky, and Schiaffini, 1973), and these differences are thought to be a function of neonatal hormones (Moguilevsky and Rubinstein, 1967; Moguilevsky et al., 1969). Campos and Ladosky (1972) demonstrated that oxygen consumption by the male hypothalamus is always higher than that of the female and this difference is maintained even when substrates and/or serotonin are added. Moreover, the fact that the differences are not modified by castration in adulthood suggests that these variations are related to the process of sexual differentiation of the nervous system. Moguilevsky et al.,

(1969) found that the oxygen uptake of the anterior hypothalamic area of male prepuberal rats was higher than that of female prepuberal rats and that male castration at birth decreased the value to those found in control females. They also showed that the administration of testosterone to female rats soon after birth increased the oxidative levels of the anterior hypothalamus to values similar to those obtained in control males. The authors postulated the existence of a particular metabolic system within the anterior hypothalamus, which in response to the process of sexual differentiation, might effect the development of either a rhythmic or tonic pattern of gonadotropin release.

Sex differences have been reported in brain cholinergic (Libertun, Timiras, and Kragt, 1973) and monaminergic (Hyypä, 1971; Hyypä and Rinne, 1971) systems in the rat. Libertun et al. (1973) found, for example, that the acetylcholinesterase and choline acetyltransferase activity in the preoptic suprachiasmatic area of prepuberal and adult diestrous and estrous female rats was significantly greater than that in either prepuberal or adult males. They likewise showed that neonatal androgenization of females induced a significant decrease in the activity of both enzymes. These data suggest that cholinergic elements present in this area of the brain are involved in the endocrine regulation of CNS sexual differentiation.

Sexual Differentiation of the Brain: Sexually Dimorphic
Behavior in Mammals

The physiological basis for dimorphic sexual behavior^{*} is not clearly understood, but most experimental models suggest that the mammalian nervous system is inherently bisexual (Beach, Noble, and Orndoff, 1969; Stern, 1969; Whalen, 1968; Whalen and Edwards, 1966; Eaton, 1970; Crossley and Swanson, 1968). The independence of the neural elements that manifest either male or female behavior is also suggested by the works of Gerall and Ward (1966) and Goldfoot et al. (1969). Their investigations showed that male and female behaviors are not mutually exclusive and can be differentially inhibited, augmented, or simultaneously manifested under specific hormonal treatments. Moreover, Dörner, Döcke, and Moustafa (1968) isolated differential brain loci for the independent expression of male and female mating behavior. Their work provided evidence to suggest that male and female mating centers are differentially located in the anterior and central hypothalamic areas, respectively. Goy, Bridson, and Young (1964) suggested that the degree of maleness or femaleness can be represented along a behavioral continuum

^{*} Sexual behavior in this context refers to the entire gamut of behaviors that are elicited during the heterosexual interaction between individual members of a species.

extending from a full complement of female to a full complement of male behavior.

In their classic study, Phoenix, Goy, Gerall, and Young (1959) demonstrated that testosterone propionate administered prenatally to guinea pigs reduced the ability of female offspring to display lordosis and enhanced their propensity to display male-like mounting behavior. They also showed a differential sensitivity to testosterone propionate in the structures that organize mating behavior on the one hand and in those of the external genitalia on the other. These authors concluded that "the prenatal period is a time when fetal morphogenic substances have an organizing or 'differentiating' action on the neural tissues mediating mating behavior. During adulthood the hormones are activational." The masculinizing effects on behavior of androgens administered to genetic females during the period of sexual differentiation have been confirmed in various species including the rat (Gerall and Ward, 1966; Quadagno and Rockwell, 1972), the mouse (Edwards, 1969, 1971; Edwards and Burge, 1971; Gandelman, 1972; Whitsett et al., 1972), the hamster (Carter, Clemens, and Hoekema, 1972; Paup et al., 1972), and the dog (Beach et al., 1972). Testosterone itself secreted from the

fetal testis (Resko et al., 1968) is probably the metabolically active hormone which effects these sex-specific phenomena (Whalen and Luttge, 1971). The antithesis of neonatal androgenization is a fetal hormonal environment that is essentially devoid of physiologically active gonadal steroids. Ovarian secretions apparently contribute to the female sexual differentiation process in an incidental or ancillary fashion, and the adult pattern of female behavior develops by default in the absence of testicular secretions. Paradoxically, estrogens administered neonatally to female rats facilitate the display of male and inhibit the display of female behavior as adults (Wilson, 1943; Levine and Mullins, 1964; Whalen and Nadler, 1965; Dörner, Döcke, and Hinz, 1971).

A critical period for maximum susceptibility of the behavioral substrate to the masculinizing effects of neonatally administered androgens has been demarcated for rats, guinea pigs, and dogs (Ward, 1969; Goy et al., 1964; Beach et al., 1969).

Neonatally castrated male rats treated with exogenous hormones as adults retained the bipotentiality of sexually dimorphic behavior which is more characteristic of the normal female than of the normal male (Whalen, 1964; Grady, Phoenix, and Young, 1965; Whalen and Edwards, 1966; Beach et al., 1969). When Neumann and Elger (1966) administered the antiandrogen cyproterone acetate to newborn male rats, a pharmacological castration resulted, which

prevented the masculinization of the external genitalia and produced a bisexual behavioral potential reminiscent of that in neonatal surgical castrates. These data underscore the implication that the mammalian nervous system is inherently bisexual and that the hormonal environments of the fetus, neonate, and adult predispose the neural substrate to mediate preferentially either male or female behavior patterns.

Goldman and his co-workers (1972b) induced modifications in the sexual behavior of adult rats treated neonatally with gonadotropin antiserum; this suggests that during the critical period pituitary gland secretions contribute to the sexual differentiation of the neural mechanisms which regulate behavior.

Some investigators have suggested that when the developing neural anlagen destined to mediate adult sexual behavior are exposed to androgens during the critical period, the sensitivity of these structures to the activating influence of gonadal steroids decreases (Clemens, 1971; Edwards and Thompson, 1970; Gerall and Kenny, 1970; Goy, Phoenix, and Medinger, 1967).

Brookhart and Dey (1941) demonstrated that lesions made in the anterior hypothalamus of adult male guinea pigs inhibited mating behavior, and Nadler (1968) induced behavioral masculinization in adult female rats by intracranial implantation of androgen during infancy. Together, these observations implicated both the fetal

hormonal environment and the hypothalamus in the manifestation of adult mating behavior in the rodent and implied that perinatal testosterone somehow has an "organizational" effect on the hypothalamic structures that control adult male sexual behavior.

Furthermore, the neural substrate responsible for mediating female sexual behavior can be dissociated by testosterone from that which controls ovulation (Barraclough and Gorski, 1962; Bloch and Davidson, 1971; Thomas and Gerall, 1969).

In the rhesus monkey, the process of sexual differentiation probably begins at the time (40 days) when the gonads can be distinguished as testis and ovary (Van Wagenen and Simpson, 1965). So far it has been technically impossible to show by experimental castration of male fetuses that genetic males deprived of testicular androgens develop feminine behavior patterns. However, testosterone propionate injected into pregnant rhesus monkeys masculinized the external genitalia and the patterns of juvenile and adult behavior displayed by the female offspring (Phoenix, Goy, and Resko, 1968; Goy, 1970; Goy and Resko, 1972; Goy and Phoenix, 1971; Eaton et al., 1973). These sexually dimorphic patterns of behavior in the adult primate depend not only upon the activational effects of gonadal hormones (Michael, 1971) but equally so upon social experience (Harlow and Harlow, 1965). The studies on the rhesus monkeys by Phoenix and his co-workers (1968) suggested

that even in advanced primates prenatal androgens can differentiate the central neural substrates of genetic females so that they preferentially acquire masculine patterns of behavior. Since the external genitalia of these androgenized female rhesus monkeys were also masculinized, it remains a possibility that changes in the neural sensory organization at the periphery could have contributed to the manifestation of the male-like behavior exhibited by these animals as adults. The respective roles of peripheral and central masculinization have yet to be clearly delineated.

Studies of human clinical syndromes indicate a similar influence of prenatal hormones on human behavior (Money and Ehrhardt, 1971; Money and Ehrhardt, 1972a, b; Reinisch, 1974). Genetic females, masculinized in utero by exposure to androgenic actions during progestin therapy to the mother or by the enzymatic defect of the adrenogenital syndrome, displayed patterns of behavior and sex role preferences that were more masculine than those of normal female controls. Genetic males, on the other hand, with the androgen-insensitive, testicular-feminizing syndrome appeared to develop the full complement of feminine behavior patterns and sex role preferences that characterize femininity in our culture (Money, Ehrhardt, and Masica, 1968). Although these studies were seriously complicated by social and environmental interactions and were fraught with clinical subjectivity, they nevertheless suggest

the involvement of the prenatal hormonal environments in the post-natal program of sexual differentiation, only in a more ambiguous and malleable degree than in phylogenetically lower mammalian species.

Although considerable effort has been expended to correlate various endocrine parameters with human homosexuality, little conclusive information on this problem is available (Dörner, Rohde, and Krell, 1972; Gadpaille, 1972; Kolodny et al., 1971; Margolese, 1970; Money, 1970, 1972).

Sexual Differentiation of the Brain: Gonadotropin Patterns in Subprimates

The release of gonadotropic hormone in response to a neuronal "trigger" elicits ovulation in the menstrual or estrous cycle. Goodman (1934) showed that ovarian transplants into male rats castrated as adults failed to form corpora lutea, but ovarian implants into females ovariectomized as adults showed the rhythmic formation of corpora lutea which characterizes normal reproductive cycles. In his classical paper, Pfeiffer (1936) described the effects of neonatal gonadectomy and gonadal transplantation on adult reproductive function in the rat. He showed that 1) male rats castrated at birth and implanted with an ovary as adults formed corpora lutea in the ovarian tissue; 2) female rats ovariectomized

at birth and implanted with an ovary as adults formed corpora lutea; 3) a significant percentage of female rats receiving testicular transplants at birth failed to form corpora lutea when implanted with ovaries as adults. These data led Pfeiffer to conclude that both males and females are born with sexually undifferentiated anterior hypophyses, which if not exposed to testicular secretions, retain the ability to secrete gonadotropins in a cyclic fashion as adults. He suggested that "the sex difference in the hypophysis is not genetic but is secondary to and dependent upon the presence of differentiated sex glands" (Pfeiffer, 1936).

Later investigations by Harris and Jacobsohn (1952) and Martinez and Bittner (1956) showed that the male pituitary transplanted into an hypophysectomized female displays normal rhythmic female cycles. Thus, contrary to Pfeiffer's interpretation, the anterior hypophysis does not appear to become sexually differentiated but rather remains pluripotential. Pfeiffer's data therefore had to be reinterpreted in terms of the CNS. Everett, Sawyer, and Markee, (1949) and Harris (1955) suggested that the brain becomes sexually differentiated under the influence of fetal and neonatal hormone environments.

In 1954, Barraclough and Leathem found that testosterone propionate administered to newborn mice resulted in anovulatory sterility when the animals became adults. These observations have

been confirmed for the rat (Harris, 1964; Ogawa and Nozawa, 1969; Luttge and Whalen, 1970; Fels and Bosch, 1971). Further investigations by Segal and Johnson (1959), Barraclough (1961) and others (Goy et al., 1964; Arai and Gorski, 1968; Napoli and Gerall, 1970; Swanson, 1971) defined a "critical period" of sexual differentiation during which the neural elements that are destined to control adult gonadotropin function may be influenced by fetal hormones. Thus, 1.25 mg of testosterone administered to 5-day-old rats produced anovulatory sterility and lack of corpora lutea and enhanced growth rate. On the other hand, only some 10-day-old and not any 20-day-old rats receiving the same dosage of testosterone exhibited this anovulatory sterility syndrome (Harris, 1964; Barraclough, 1966, 1967; Neumann and Elger, 1966).

Male rats neonatally castrated and implanted with ovaries as adults show evidence of a 5-day rhythmic cycle of ovarian function and female mating behavior at the time of proestrus (Pfeiffer, 1936; Van Der Schoot and Zeilmaker, 1972). The pharmacological castration with the antiandrogen cyproterone acetate effects a similar feminization of adult reproductive function (Elger et al., 1970; Neumann and Elger, 1966; Neumann et al., 1970a; Neumann et al., 1970b). In addition, neonatally administered progesterone can prevent the steroid-induced sterility in male and female rats appropriately treated (Kincl and Maqueo, 1965). Stern and

Eisenfeld (1971) have suggested that this antiandrogenic effect of progesterone is induced by its inhibition of androgen uptake at the cellular level. These data further delineate the role of testicular androgens in the process of sexual differentiation.

Sex differences in gonadotropin patterns have been recognized for many years (Clark, 1935; Harris and Jacobsohn, 1952). The female of most mammalian species has a cyclic pattern of ovarian hormone release and as a consequence, exhibits an estrous or menstrual cycle. This rhythmic ovarian function is controlled by a finely tuned interplay between the gonad and the gonadotropin releasing mechanism in the brain and pituitary gland (Everett, 1969; Davidson, 1969). On the other hand, the male of most mammalian species displays a tonic pattern of gonadotropin release (Harris, 1970). The pituitary content of FSH, LH, and prolactin varies as a function of sex (Labhsetwar, 1970; Dupon and Schwartz, 1971; Neill, 1972; Barraclough, 1971; Parlow, 1964; Lisk, 1968; Kragt and Ganong, 1968a, b; Ramirez and McCann, 1963). Dupon and Schwartz (1971) found, for example, that pituitary LH content increases in both sexes until 26 days of age, but the content of LH is greater in the female than in the male rat. After 26 days, pituitary LH in the female plateaus until the onset of puberty when a significant fall coincides with vaginal opening. In the male, pituitary LH content rises beyond day 26 and surpasses the levels

found in the female rat. Pituitary LH content in the male rat then plateaus at final adult levels by 75 days. Some of these sexually differentiated phenomena can be artificially induced by fetal hormone treatments; those that cannot are thought to be under a more direct kind of genetic influence (Johnson, 1971, 1972; Mallampati and Johnson, 1973a, b).

The adult gonadotropin control system is a sexually differentiated mechanism that is apparently organized by the fetal and early postnatal hormonal environment (Harris, 1964, 1970). Fundamental sex differences also exist in the prolactin control system (Neill, 1972; Taleisnik, Caligaris, and Astrada, 1971; Bishop et al., 1972; Kurcz et al., 1967; Wothius, 1963; Zeilmaker, 1963). Neill (1972) found, for example, that estrogen treatment of female rats ovariectomized when adult or of adult neonatally castrated males effected surges of prolactin and LH secretion on the afternoon of the third day post treatment whereas males castrated as adults or androgen-sterilized females ovariectomized as adults did not elicit this response to estrogen treatment. The prolactin and LH surges elicited in estrogen-treated female rats were prevented with a retrochiasmatic cut separating the anterior from the medial part of the hypothalamus. Neill concluded that neonatal androgens organize the rostral "surge center" in such a way as to suppress the potential to elicit a prolactin and LH surge in response to estrogen treatment.

The time course of the postgonadectomy rise in gonadotropins differs between males and females in a variety of species including the snowshoe hare and the rat (Davis and Meyer, 1973; Gay and Midgley, 1969; Ojeda and Ramirez, 1972; Ramirez and Sawyer, 1974). In both male and female snowshoe hares, pituitary LH and FSH increased progressively between the tenth and sixtieth day after gonadectomy. By day 60, the pituitary LH content in the female had tripled that in the male, but the male pituitary contained more FSH (Davis and Meyer, 1973). Early studies using parabiotic techniques suggested that the female pituitary gland is more sensitive to the negative feedback effects of gonadal steroids than the male gland (Shipley, 1962). Further investigations by Petrusz and Flerkó (1965), Petrusz and Nagy (1967), Barraclough and Haller (1970), Taleisnik et al., 1971 and others (Barraclough, 1971; Bunster and Meyer, 1938; Neill, 1972; Dunlap, Preis, and Gerall, 1972; Kalra et al., 1973; Gans 1959; Gans and deJongh, 1963) showed that the sensitivity of the negative feedback system of gonadal steroids upon gonadotropin secretion may be a function of a sexually differentiated mechanism influenced by fetal and neonatal hormones. Some investigators have found a lower positive feedback and higher negative feedback response to estrogen treatment in male and neonatally androgenized female rats than in normal females (Morrison and Johnson, 1966; Boyd and Johnson, 1968).

Others have reported lower sensitivity in the estrogen-LH negative feedback response system in males and androgenized females than in normal females (Petrusz and Nagy, 1967; Dunlap et al., 1972; Barraclough and Haller, 1970). Boyd and Johnson (1968) reported that male rats are at least as sensitive to the negative feedback effects of testosterone as females, and Miyake (1961) suggested that male mice are less sensitive than females to the negative feedback effects of androgens.

Sex differences in the time-course of barbiturate (Beattie et al., 1973; Tapper, Naftolin, and Brown-Grant, 1972) and atropine (Libertun and McCann, 1973) blockade of LH release in the rat have been described. Libertun and McCann (1973) found, for example, that at 3 to 5 weeks after ovariectomy or orchidectomy, a subcutaneous injection of atropine decreased plasma LH within one hour in females, but the decrease was apparent only in the castrate males after 24 hours. These data imply that cholinergic pathways are involved in the CNS control of gonadotropin secretion and that this control system is differentiated as a function of sex.

Foster and his co-workers (1972b) demonstrated a sex difference in the regulation of LH secretion in the fetal and neonatal lamb. They found that the increase in gonadal weight and pituitary LH concentration was greater for males than for fetal females. They also showed that pituitary and serum levels of LH which

continued to rise in females after birth, tended to plateau in males. Thus, the negative feedback of gonadal steroids on the hypothalamo-hypophyseal axis of the neonate may become established earlier in males than in females.

Using the technique of intrahypothalamic implants of FSH, Ojeda and Ramirez (1972) revealed sex differences in "short loop" FSH feedback control system in the rat. Adult, hemicastrated female rats with hypothalamic FSH implants and treated with estradiol benzoate showed a degree of compensatory ovarian hypertrophy significantly greater than that of the estradiol benzoate alone control group, but adult, hemicastrated male rats treated with FSH implant showed a diminished compensatory testicular hypertrophy. These data suggest that FSH acts as a positive feedback signal in the female but as negative feedback signal in the male.

Estrogen administration to ovariectomized rats results in an LH and prolactin surge at the appropriate time of day; however, this response cannot be elicited in male rats or neonatally androgenized female rats (Caligrais, Astrada, and Taleisnik, 1970; Neill, 1972). Similar results have been reported by Karsch and Foster (1974) for the sheep. The studies of Quinn (1966) and more recently of Terasawa, Kawakami, and Sawyer, (1969) have suggested that neonatal androgens effect an elevation of the thresholds of excitability within the gonadotropin-controlling areas of the hypothalamus.

Exposure of the undifferentiated rat hypothalamus to androgens apparently prevents the LH-prolactin cyclic system from responding to the stimulatory action of estrogens.

Neonatally administered estrogens, as well as androgens, can induce anovulatory sterility and a male growth pattern and can block the estrogen-induced positive feedback mechanism of gonadotropin release in the adult female rat (Arai, 1964, 1971; Gorski, 1963; Dörner et al., 1971; Whalen and Nadler, 1965). These data support the suggestion that testosterone is aromatized to estrogens at neural sites as a mechanism for mediating the molecular action of androgens regarding sexual differentiation (Reddy et al., 1973).

Investigations by Arai and Serisawa (1973) and Goldman and Mahesh (1970), using neonatally administered gonadotropins and gonadotropin antisera, have implicated the pituitary gland, in addition to the fetal gonads, in the process of sexual differentiation.

The mechanism by which androgens effect the process of sexual differentiation in the male is not well understood. However, the work of Gorski and his co-workers (Gorski, 1971; Gorski and Shryne, 1972; Kobayashi and Gorski, 1970) has provided some insight into this problem. They found that the administration of certain antibiotics -- cyclohexamide, puromycin, and actinomycin-D -- during the critical period of sexual differentiation, precluded the development of the androgenization phenomena. Their

data provide tentative evidence that the mechanism of androgen-induced sexual differentiation is effected via a process that involves DNA-dependent RNA and protein synthesis. Other investigators have suggested that the mechanism of androgen-induced sterility is a function of a compromised estrogen-binding network within the brain, caused by the neonatal exposure of this system to gonadal steroids during its early development (Flerkó, Mess, and Illei, Donhoffer, 1969; Flerkó, 1971).

The early studies of Hohlweg and Junkmann (1932) introduced the theory that the gonadotropic functions of the adenohypophysis are regulated by a sexual center in the hypothalamus. Retro-chiasmatic sections abolish spontaneous ovulation (Halász and Pupp, 1965; Halász and Gorski, 1967) and the positive feedback effect of ovarian steroids in the rat (Taleisnik, Caligaris, and Astrada, 1970). Moreover, Blake et al. (1972, 1973) demonstrated a sex difference in the effect of an anterior hypothalamic deafferentation upon LH secretion in the rat. Anterior deafferentation in the female rat resulted in an increase in circulating levels of LH, but in the male, this procedure produced no change in LH concentrations. In progesterone-primed, androgen-sterilized female rats, stimulation of the area just rostral and caudal, but not lateral, to the ventromedial nucleus of the hypothalamus elicits ovulation (Barraclough and Gorski, 1961). Spermatogenic activity is inhibited

after early postnatal hypothalamic lesions in rats (Daikoku, Shimizu, and Ooki, 1970). Neonatal intrahypothalamic implants of testosterone induce a cyclic anovulatory sterility and constant vaginal estrus, suppress adult female mating behavior, and augment male behavior (Lobl and Gorski, 1974; Nadler, 1968). Furthermore, Nadler (1971, 1972a, b, 1973) has provided evidence to suggest that the ventromedial and arcuate nuclei within the hypothalamus represent the primary loci for the androgenization phenomena.

It is notable that the sex differences in the brain-pituitary regulation of gonadotropin secretion are complemented by sex differences in central nervous excitability thresholds (Velasco and Taleisnik, 1969). Electrical stimulation of the amygdaloid nuclei provokes a release of LH in female but not in male rats, and stimulation of the preoptic area provokes an LH release in both male and female rats.

The functional role of fetal and neonatal androgens in organizing the sexual differentiation of the CNS relative to gonadotropin regulation has also been established in the mouse (Barracclough and Leathem, 1954; Barracclough, 1955), the hamster (Swanson, 1970; Alleva, Alleva, and Umberger, 1969), and the guinea pig (Brown-Grant and Sherwood, 1971).

Sexual Differentiation of the Brain:
Gonadotropin Patterns in Primates

Both the reproductive anatomy and the patterns of reproductive and related behaviors are sexually dimorphic in primates as well as in rodents (Money and Ehrhardt, 1972). Sex differences in circulating levels of testosterone have been reported in the rhesus (Resko, 1970; Resko et al., 1973) and human fetus (Reyes, Winter, and Faiman, 1973; Reyes et al., 1974). Testicular androgens are apparently required for the normal physiological differentiation of the epididymus epithelium (Alexander, 1972). Some investigators have suggested that fetal androgens must be "aromatized" to estrogens in order to manifest their physiological functions at target organ sites (Naftolin, Ryan, and Petro, 1971a, b; Ryan et al., 1972); however, this theory is controversial, at best. Sex differences in circulating levels of gonadotropins have been described in the infant chimpanzee (Faiman et al., 1972; Faiman, Winter and Grotts, 1973) and in fetal and young children (Faiman and Winter, 1971; Levina, 1972; Penny, Olambiwonnu, and Frasier, 1974a, b; Reyes et al., 1974), although some of these data are at variance with those reported by Lee, Midgley, and Jaffe (1970).

Whether the obvious sex-specific character of gonadotropin secretion in primates is the result of a hypothalamic

pattern conditioned by sex differences in prenatal exposure to endogenous androgens and whether the sexual differentiation of the primate brain is analagous to that of the rodent brain remain to be demonstrated. Most attempts to demonstrate such an organizing effect of androgens in primates have not been successful. The administration of testosterone to female monkeys on the day of birth failed to modify the cyclic pattern of gonadotropin release after puberty (Treolar, Wolf, and Meyer, 1972); however, the period of androgen sensitivity in the monkey (Goy and Resko, 1972), as in the guinea pig (Phoenix et al., 1959) probably occurs well before birth. However, even repeated administration of testosterone to pregnant monkeys as early as day 40 of gestation did not prevent ovarian cyclicity despite marked anatomical and behavioral masculinization (Goy, 1970; Goy and Phoenix, 1971; Goy and Resko, 1972). Moreover, pregnancies have been reported in human female pseudo-hermaphrodites, masculinized in utero either by the fetal adrenogenital syndrome or by exogenous hormone therapy administered to the gravid mother (Money and Ehrhardt, 1972). Some clinical data, however, suggest that women who have experienced chronic adrenal hyperplasia and its subsequent masculinization from adrenal androgens as children and in utero exhibit marked disruptions of their menstrual cycles as adults (Kirkland et al., 1974). These data, albeit confounded, imply that prenatal and early postnatal

androgen exposure of the developing hypothalamic-pituitary axis compromises its ability to display normal female gonadotropin-ovarian rhythmicity in adulthood. These menstrual irregularities, however, may be a function of a continued adult androgenic hormonal milieu rather than a prenatal "organization" phenomenon. In patients with the androgen-insensitivity, testicular-feminizing syndrome (Polani, 1970; Goebelsmann et al., 1973), the hypothalamic-pituitary axis appears to be less sensitive to the negative feedback effects of testosterone than in normal males, although the feedback loop itself seems to be intact (Judd et al., 1972). These data imply that in individuals who are insensitive to the masculinizing effects of fetal androgens, the adult gonadal steroid negative feedback control system may fail to differentiate as in the normal male. Furthermore, in children, treatment with the synthetic decapeptide, LH-RH, elicits a differential response as a function of sex (Job et al., 1972; Roth, Kaplan, and Grumbach, 1973). Males release more LH and less FSH than comparably treated females. Foster et al. (1972a) demonstrated that fetal male lambs tend to release more LH in response to LH-RH treatment than similarly treated females. In addition, Seyler, Canalis, and Reichlin (1974) reported sexual dimorphism of pituitary responsiveness to estrogen with LH-RH treatment. In men, stilbestrol treatment decreases the LH response to LH-RH but augments it in women. Perhaps, in the

primate, the pituitary itself undergoes a process of sexual differentiation. A recent report by Stearns, Winter, and Faiman (1973) demonstrated positive feedback effects of progestin on the serum concentrations of gonadotropins in estrogen-primed castrate men. These data are at variance with those reported by Odell and Swerdloff (1968) who were unable to demonstrate a positive feedback mechanism in castrate men. In 1971, Yamaji and his co-workers found that on the one hand, gonadectomized female rhesus monkeys released LH in response to an estradiol benzoate injection with a time course that was both qualitatively and quantitatively similar to that observed during the normal, spontaneous menstrual cycle, but that on the other hand, similarly treated gonadectomized males who showed an initial negative feedback response and a subsequent rebound to control levels, failed to demonstrate an LH surge characteristic of the normal female type. None of these animals had received treatment with estradiol before the estradiol benzoate injection. Using a similar design, but preceding the estradiol benzoate injection with estradiol implants to titrate the high postgonadectomy levels of LH to the low levels observed in the intact state, Karsch and his co-workers (1973a) reported that both male and female rhesus monkeys showed estrogen-induced surges in LH secretion similar to those which occur spontaneously during the menstrual cycle with no sex differences apparent. These data suggest that in primates,

unlike rodents, exposure of the hypothalamo-hypophyseal unit to androgens throughout fetal and postnatal development does not prevent the differentiation of the control system that regulates cyclic gonadotropin secretion.

Is the primate brain sexually differentiated with respect to the control of gonadotropin secretion or, as Karsch suggested, is the schema for the sexual differentiation of the nervous system of rodents not applicable to primates?

Feedback Control of Gonadotropins in Mammals

The feedback control of gonadotropin secretion by gonadal steroids in the rodent is well documented (Davidson, 1969; Everett, 1969; Martini, Fraschini, and Motta, 1968; Schwartz, 1969, 1970). Stumpf (1968, 1969, 1970, 1971) has described a complex network of steroid-concentrating neurons throughout the periventricular brain and pituitary of the rat, and various lines of experimental evidence suggest that both the hypothalamus (Piacsek and Meites, 1966; Martini et al., 1968; Davidson, 1969) and the pituitary (Davidson, 1969; Negro-Vilar, Orias, and McCann, 1973; Orias et al., 1974) represent sites for steroid feedback effects upon gonadotropin secretion in the rodent.

The pattern of gonadotropin secretion throughout the menstrual cycle of the female primate have been clearly delineated for both

women (Abraham et al., 1968; Vande Wiele, 1970) and the rhesus monkey (Hotchkiss, Atkinson, and Knobil, 1971; Boorman et al., 1973; Niswender and Spies, 1973; Knobil et al., 1972). Luteinizing hormone levels, which remain relatively constant throughout the interovulatory period of women and female rhesus monkeys, are characterized by a preovulatory surge lasting approximately 30 hours. Follicle-stimulating hormone follows a similar pattern during the cycle. The mid-cycle surge of both LH and FSH are thought to be the result of an estrogen-induced positive feedback mechanism (Yamaji et al., 1971; Yen and Tsai, 1972). In men, serum concentrations of LH and FSH remain relatively constant (Peterson, Midgley, and Jaffe, 1968). There is evidence that estrogen plays the principal role in the negative feedback control of tonic LH secretion in women and in female rhesus monkeys (Bhattacharya et al., 1972; Karsch et al., 1973b, c; Yamaji et al., 1972; Knobil et al., 1972; Yen et al., 1972). Carmel and his co-workers (1972) showed that estradiol infused into the periventricular hypothalamus inhibited LH secretion in the rhesus monkey.

The following studies will outline our attempts to delineate the quantitative aspects of negative and positive feedback control of LH secretion by estradiol in gonadectomized male, female, and androgenized genetic female rhesus monkeys. These comparisons

will constitute the subject matter of this dissertation, which was written with a limited background of information which indicates that primates differ from rodents in the function of prenatal androgens in the differentiation of the hypothalamo-hypophyseal system which regulates gonadotropin secretion.

CHAPTER III

MATERIALS AND METHODS

Animals and Animal Care

The subjects for these studies were mature rhesus monkeys (Macaca mulatta) from the colony at the Oregon Regional Primate Center. In some cases, the exact ages of the animals were unknown since many had been obtained from suppliers who could not provide the information; however, body weights and records from the Oregon Primate Center indicated that they were sexually mature. Groups of 7 ovariectomized female and 7 castrated male monkeys were used in the following studies. In addition, 7 ovariectomized females, androgenized in utero by testosterone propionate treatment, were included in our experimental design. The prenatal androgen treatment regimen for each animal has been published (Phoenix et al., 1968). These genetic female monkeys show pronounced morphological and behavioral masculinization (Phoenix et al., 1968).

Bilateral gonadectomy had been performed 3 to 36 months before the studies commenced. The animals were individually caged (indoors under constant temperature $(20 \pm 2^{\circ}\text{C})$ and maintained on monkey chow (Purina Co., St. Louis, Mo.), fresh fruit,

and water ad libitum. No significant differences in weight (5 to 11 kg) were observed among the experimental groups. Routine health checks were performed by the veterinary staff of the Oregon Primate Center. Blood hematocrit and hemoglobin levels were monitored, and the monkeys were treated with intramuscular injections of 50 mg iron dextran (Inferon, Lakeside Lab., Inc., Milwaukee, Wis.) at the end of each experimental period.

Blood-Handling Techniques

Stainless steel transfer boxes (2-1/2' x 1' x 1') were used to transport the animals from their home cages to a bleeding cage where they could be immobilized against the movable front wall of the cage. The leg was withdrawn and the dorsal surface of the lower leg was swabbed with 70% ethanol. Approximately 3 ml of blood were collected from the saphenous vein or one of its tributaries through a 23-gauge needle into a plastic syringe. After withdrawal of the sample, the leg was bandaged with cotton on tape, and the animal was returned to its home cage. The monkeys were well acquainted with this procedure and usually offered little or no resistance.

The blood was immediately refrigerated and allowed to clot overnight, and then was centrifuged at 2500 rpm for 30 minutes at 4°C. The serum was "quick frozen" in a dry ice-acetone bath and

stored at -20°C . The serum was "quick-thawed" in a warm water bath for gonadotropin and steroid analysis.

Steroid Administration

Estrogens were administered to the experimental subjects either through silastic capsules implanted subcutaneously or through intramuscular injections. Dziuk and Cook (1966) demonstrated that steroids pass easily through silicone rubber. With these silastic implants, estradiol reaches plateau concentrations rapidly and selected serum levels are maintained for many months. The construction, preincubation, method of insertion, and rate of estradiol release from these capsules had been described in detail (Karsch et al., 1973a, b). Silastic tubing (Dow Corning, Midland, Michigan) was sealed at one end with silastic type A cement (approximately 0.5 cm plug) and dried in an oven at 90° for one hour. The empty cylinder was filled with crystalline estradiol (Steraloids, Inc., Pauling, N. Y.), and the open end was sealed with silastic cement. The capsule was allowed to dry at room temperature overnight. The dry implants were then soaked in a beaker containing 0.1% gelatin (Fisher Scientific Co., Fair Lawn, N. J.) in phosphate buffered saline and stirred for at least three hours with frequent changes of the solution.

Just before placement, the implants were soaked for 10

minutes in choroexidine (Nolvasan, Fort Dodge Lab., Fort Dodge, Iowa). Initial blood samples were taken, and the animals were anesthetized with ketamine hydrochloride (Ketaject, 5-10 mg/kg, Bristol Lab., Syracuse, NY). The implant was slipped subcutaneously into a 1-2 cm incision in the mid-scapular region of the back, which was then closed with a few stitches. A prophylactic dose of antibiotics was given IM (penicillin, 600,000, Bicillin, Wyeth Lab., Philadelphia). Long-term treatment with relatively high physiological levels of estradiol caused marked swelling and irritation of the sex skin and interfered with their eating behavior.

For injection procedures, estradiol benzoate (Sigma, St. Louis, Mo.) was suspended in sesame oil and administered subcutaneously with a tuberculin syringe through a 22-gauge needle.

Luteinizing Hormone-Releasing Hormone Administration

A solution of 14% gelatin was prepared, and 3 mg of luteinizing hormone-releasing hormone (LH-RH) (LH-RH/FSH-RH, N.I.A.M.D.D., Abbott Lot #19-192 AL) were added to 120 ml of the solution for a final LH-RH concentration of 25 μ g/ml. Each animal was injected subcutaneously with 500 μ g of LH-RH (20 ml) in a 20 cc syringe through a 20-gauge needle. This procedure has been shown by Krey et al. (1973) to cause a significant LH release in the rhesus monkey.

Experimental Design

Long-Term Negative Feedback Effect of Estradiol-17 β on LH Secretion in the Rhesus Monkey

This study was designed to evaluate the long-term (about 30 days) effects of estrogen on LH secretion in gonadectomized male, female, and androgenized genetic female rhesus monkeys. After three initial blood samples, at 20-minute intervals, we implanted silastic capsules containing crystalline estradiol-17 β into each of the 21 monkeys. Each capsule was 4.0 cm in total length with 0.5 cm plugs sealing each end. After approximately 30 days, another blood sample was taken from each monkey, and the 3.0 cm (steroid-containing length) implants were removed and replaced with 1.5 cm capsules. These and subsequent implant sizes refer to the steroid-containing length rather than the absolute capsule length. Again, after approximately one month, another blood sample was taken from each animal. Finally, we implanted 0.5 cm capsules into each animal and bled them at the end of one month.

Short-Term Negative Feedback Effect of Estradiol-17 β on LH Secretion in the Rhesus Monkey

To study the short-term effects of estrogen on the release of LH in gonadectomized male, female, and androgenized genetic

female rhesus monkeys, we implanted them with 0.5 cm-long silastic capsules containing estradiol-17 β and bled them over a 72-hour period. The animals and methods were the same as in the long-term study. After two initial blood samplings, 20 minutes apart, the animals were again bled at 6, 24, 48, and 72 hours after implantation of estradiol.

Positive Feedback Effect of Estradiol Benzoate on LH Secretion in the Rhesus Monkey

In order to evaluate the positive feedback effects of estrogen on LH release, we injected the groups of gonadectomized male, female, and androgenized genetic female rhesus monkeys with estradiol benzoate. The animals had been pretreated with a 1.5 cm estradiol implant for about 1 month before the estradiol benzoate injection. Just before being injected with estradiol benzoate (50 μ g/kg) and for 84 hours thereafter, the animals were bled at 12-hour intervals. This study was then repeated using the same treatment regimen, except that the bleeding schedule was changed to alternating 6- and 18-hour intervals for 102 hours. In this study the animals had been pretreated with an 0.5 cm estradiol implant for approximately one week before the estradiol benzoate injection.

The Release of LH Effected by the Synthetic Decapeptide,
Luteinizing Hormone-Releasing Hormone (LH-RH)

To evaluate the effects of LH-RH on the release of LH in gonadectomized male, female, and androgenized genetic female rhesus monkeys, we injected 500 μ g of the LH-releasing hormone into four monkeys from each of the three groups. The monkeys were pretreated with 0.5 cm silastic capsules containing estradiol for approximately 3 weeks before the LH-RH injection.

Radioimmunoassay for Estrogen and Luteinizing
Hormone (LH): General Comments

The procedures for preparing and purifying the solvents, reagents, and labeled and unlabeled hormone standards are given in Appendix sections I, II, III, IV, and V. Total estrogen values were determined by the "rapid" method described by Hotchkiss et al. (1971) and verified for use in our laboratory (Resko, 1971). This method obviates the use of chromatography and provides a rapid and accurate measure of total serum estrogen. Double antibody radioimmunoassay techniques were used to determine the concentration of LH according to the method developed by Niswender et al. (1971) and modified for use in our laboratory.

Radioimmunoassay for Estrogens

Estrogens were extracted from 50-250 μ l of serum with 7 ml of fresh anhydrous diethyl ether (Mallinckrodt AR) with 2% distilled ethanol. The extract was dried under air. Standard curves with duplicate points (0, 5, 10, 20, 30, 50, 75, 100, and 150 pg) of estradiol were included for each assay. For each standard curve, four zeros were carried through the procedure. Antisera for the estrogen assays were derived from two different sources. For the long-term negative feedback study and those experiments included in the appendix, we used antisera developed in sheep immunized with estradiol-17 β -succinyl-bovine serum albumin (1:15,000 dilution). Assays for the remaining studies used antisera developed against estradiol-17 β -6-oxime-BSA in a rabbit and diluted 1:5,000. In either case, the antisera (0.1 ml at room temperature) were added to each tube, vortexed 10 seconds, and allowed to incubate for 30 minutes at room temperature. Approximately 4000 cpms of $^3\text{H-E}_2$ (100 Ci/mM, New England Nuclear) in 0.1 ml phosphate buffered saline (PBS) containing 0.1% gelatin were added to each tube, vortexed 10 seconds, and incubated overnight at 4 $^{\circ}$ C. For the duration of the assay procedure, all tubes were kept on ice. 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 vortexed

10 seconds. The tubes were incubated on ice for 15 minutes and then centrifuged at 2500 g at 4° C for 10 minutes on a Sorval RC2-B using a GSA head at 4000 rpm or on a Sorval RC-3 using the HG-4L swinging bucket at 2000 rpm. A 1.0 ml aliquot of the supernatant was removed and added to 10 ml of Triton-N101 liquid scintillation fluid and counted for 10 minutes. Independent blank samples of equal volumes of deionized water were carried throughout the procedure, and individual blanks were averaged and subtracted in each assay. The counts per minute from the scintillation counter of the standard curve and samples were then computed for results in picograms per ml of serum.

Radioimmunoassay for Luteinizing Hormone (LH)

Luteinizing hormone was measured by a double antibody radioimmunoassay method with ovine LH antiserum, ovine ¹²⁵I-LH, and rhesus monkey LH standard as previously described by Niswender and his co-workers (1971). Dr. Leo E. Reichert, Jr., provided the partially purified pituitary preparation from rhesus monkeys (LER-M907-D) with an LH biological activity of 0.025 NIH-LH-S1 units per mg for standard reference and the ovine LH (LER-1056-C2) for iodination. Ovine LH-antibody was diluted 1:40,000 for use. The second antibody, developed in sheep against rabbit gamma globulin, was supplied by Dr. Douglas Bolt; it was diluted 1:80 for

use. For the second positive feedback study and the LH-RH challenge study, a different second antibody was used. This antibody was developed by Dr. Kaleem Quadri in pigmy goats against rabbit gamma globulin and diluted 1:40 for use. Assay protocol and the analysis of the data were computerized according to the "RIANAL" program (Duddleson, Midgley, and Niswender, 1972). The standard curve was based on triplicate estimates at each of 11 points, and all samples were assayed in duplicate. The limit of sensitivity of the LH assay was 80 ng LER-M-907 D per ml serum. Two hundred μ l from a pool of serum from ovariectomized monkeys were run in duplicate in each assay, and the coefficient of variation between LH assays was approximately 12%.

Data Analysis

For the long-term negative feedback study, within-group comparisons of time versus concentration of estrogen were made by 2-way analysis of variance and the Duncan's multiple range test for significance (Fryer, 1966). All data were analyzed on a Hewlett Packard programable calculator Model #9810A. In the long-term study, LH values between groups of males, females, and androgenized females were compared with the dose of estradiol by means of the 2-way analysis of variance (ANOVA) with the Duncan's multiple range (DMR) test. Analysis of the data for the short-term

negative feedback study was also made by ANOV and DMR. Estrogen and LH data in the two positive feedback studies were integrated for the area under the concentration versus time curve. A one-way analysis of variance followed with a Duncan's multiple range test for significance was used to test between groups. Data in the LH-RH challenge study were handled similarly. All data points were converted to log-normal values for analysis (Kletzky et al., 1974), in order to correct for the non-normal distribution pattern of the hormonal data.

CHAPTER IV

RESULTS

Long-Term Negative Feedback Regulation of LH Secretion by Estradiol-17 β

The long term negative feedback effects of various doses of estradiol on LH secretion in the gonadectomized female, male, and prenatally androgenized genetic female rhesus monkey are illustrated in Figure 1. The abscissa represents serum estrogen concentrations, and the ordinate represents LH concentrations plotted as the percent of the initial, pre-implant level. Control LH levels were not significantly different between groups, and in each animal, control blood samples exhibited only minor fluctuations in LH levels over the one hour of sampling time. With increasing concentrations of serum estrogens, LH levels were inhibited in all groups. The arrows in Figure 1 denote the point at which the first significant suppression in LH concentrations occurred within each group as a function of increasing levels of serum estradiol. LH concentrations in gonadectomized females were significantly suppressed to $47.8 \pm 15, -12$ (SE) % of the initial control levels with the smallest size estradiol capsule, which produced 22 ± 1.3 (SE) pg/ml of serum estradiol. Implant sizes of 1.5 and 3.0 cm effected serum estradiol

levels of 50 ± 8 , -7 (SE) and 99 ± 10 , -9 (SE) pg/ml, respectively. These implants significantly suppressed LH concentrations to 28 ± 9 , -7 (SE) % and 21 ± 5 , -4 (SE) % of the initial control levels, respectively. In all groups, the control values for serum estrogen were extraordinarily high (> 200 pg/ml) and probably reflected contamination.

In gonadectomized males, LH levels could be significantly suppressed only with the largest (3.0 cm) estradiol implant, which effected 106 ± 8 (SE) pg/ml of serum estrogen. Therefore, something in excess of 68 ± 4 (SE) pg/ml of serum estrogen achieved with the 1.5 cm implant and less than the levels achieved with the largest implant were required to initiate a significant reduction in circulating LH levels in these males. With the 3.0 cm estradiol implant, LH levels in the males were suppressed to 43.5 ± 12 , -9 (SE) % of their initial control values.

In androgenized females, as in males, circulating levels of LH could be significantly suppressed only with the largest (3.0 cm) implant, which effected 92 ± 4 (SE) pg/ml of serum estrogen. Therefore, something greater than 51 ± 4 (SE) pg/ml of serum estrogen produced by the 1.5 cm implant and less than the levels achieved by the largest implant were required to initiate a significant reduction in circulating LH levels in these masculinized females.

These data suggest that females are more sensitive to the long-term negative feedback effects of estradiol than males and that prenatally androgenized females respond more like males in this regard.

Figure 2 illustrates the data presented in Figure 1 in histogram form. No significant differences between the groups of males, females, and androgenized females could be shown at any of the various implant sizes, except for androgenized females versus normal females at the 1.5 cm implant size ($p < 0.05$). These data, however, do not militate against the fact that in males and androgenized females estrogen levels nearly four times greater than those for normal females are required to significantly suppress circulating LH levels below initial control values.

Short-Term Negative Feedback Regulation of LH Secretion by Estradiol-17 β

The short-term negative feedback effects of estradiol on LH secretion in the gonadectomized female, male, and prenatally androgenized genetic female rhesus monkey are illustrated in Figure 3. The abscissa represents time after implantation of the estradiol capsule, and the ordinate represents LH concentrations normalized to preimplant levels. No significant differences in the time course of LH inhibition in response to estradiol could be shown

between the experimental groups at any of the post implant times. Six hours after implantation serum estrogen values peaked at levels ranging from 97 to 117 pg/ml and gradually declined in all groups to between 67 and 83 pg/ml (Figure 4); no differences in estrogen levels between groups could be seen at any of the post implant times. At 6 hours, LH levels were suppressed in all groups to approximately 60% of their initial preimplant values. It should be noted that whereas a rebound in serum levels of LH occurred in the group of females at 24 hours and persisted through 72 hours, no such rebound was apparent in the population of males. Furthermore, an equivocal rebound, with LH levels intermediate between those found in the males and females, was observed in the group of androgenized females. These data suggest that on a short-term basis, the ability of estradiol to inhibit postgonadectomy levels of LH does not differ between sexes.

Positive Feedback Control of LH Release by Estradiol Benzoate

The 4-day time course of the characteristic response of the estrogen-induced facilitation of LH release in gonadectomized male, female, and androgenized female rhesus monkey is illustrated in Figure 5. Both the male and androgenized female populations showed a markedly larger integrated serum LH response to the

treatment than similarly treated females. The integrated serum LH response in the groups of males and androgenized females ranged from 271 to 1095 and from 250 to 1627 $\mu\text{g} \cdot \text{hours}/\text{ml}$, respectively, but from 169 to 338 $\mu\text{g} \cdot \text{hours}/\text{ml}$ in the group of normal females. However, there were no statistically significant differences between any of the groups. Individual animals which did not show an LH surge in response to the estrogen treatment (2 females, 1 male) were not included in either the overall analysis or the Figure 5 illustration. Figure 6 illustrates the time course of serum estrogen concentrations effected in response to the estradiol benzoate injection in the three groups of monkeys. These data revealed no differences between the groups so treated.

The time course of the LH response to the second estradiol benzoate challenge study is shown in Figure 7 for each of the three groups of monkeys. In this study, the integrated serum LH response of males and androgenized females was significantly greater ($P < 0.05$) than that of normal females. The integrated serum LH response in the groups of males and androgenized females ranged from 107 to 616 and from 181 to 950 $\mu\text{g} \cdot \text{hours}/\text{ml}$, respectively, but from 89 to 167 $\mu\text{g} \cdot \text{hours}/\text{ml}$ in the group of normal females. In this study, all 21 of the monkeys treated with the estradiol benzoate responded with an LH surge. Figure 8 illustrates the time course of serum estrogen concentrations for

females to androgens predisposes their gonadotropin control system to acquire a male-like response pattern as adults. From these data we conclude that the adult pattern of LH release is, at least in part, a function of an in utero hormonal preconditioning that organizes the neural-pituitary elements which are responsible for effecting this LH release in the rhesus monkey.

The influence of stress on LH secretion and its potential interference with the experimental design is discussed separately in Appendix B.

the three groups effected in response to the estradiol benzoate injection. As in the first positive feedback study, a visual inspection of these data reveals no differences between the groups so treated. The LH data show that unlike the rat, both the males and females of this primate species respond to the estrogen-induced mechanism for the LH surge; however, the response characteristics differ between males and females. Moreover, prenatal exposure of genetic females to androgens predisposes their CNS-pituitary axis to acquire a male pattern of LH release in response to estrogen treatment as adults.

The Release of LH Effected by the Synthetic Decapeptide,
Luteinizing Hormone-Releasing Hormone (LH-RH)

The time course of the LH response effected by LH-RH in the three groups of monkeys is illustrated in Figure 9. The peak LH responses in the groups of males, females, and androgenized females were 5.29 ± 1.0 , -0.8 (SE), 4.10 ± 1.9 , -1.3 (SE), and 4.26 ± 0.98 , -0.80 $\mu\text{g/ml}$, respectively, which represent increments of 1 to 3 $\mu\text{g/ml}$ of LH over a baseline of 1 to 7 $\mu\text{g/ml}$ and a statistically significant release of LH by all groups. Figure 10 shows the integrated serum LH release in response to the LH-RH injection by each group over the 6-hour period. No significant differences in pituitary responsiveness to LH-RH could be shown

between any of the groups. Interpretation of these data is equivocal at best because of the small amount of LH released by the monkey pituitary in response to LH-RH. Nevertheless, these data suggest that the different responses of LH to estrogen observed in males and females and in prenatally androgenized females and normal females are a function of a mechanism which lies within the central nervous system and that this mechanism can be influenced by pre-natal exposure to androgens.

CHAPTER V

DISCUSSION

There is considerable evidence that sex differences in the primate CNS-pituitary axis may exist and that they may be a function at least in part, of a hormonal preconditioning during fetal life. In the Introduction, I have reviewed the experimental basis for this theory as it has been developed in the rodent and other subprimate species. I have also described the few reported attempts to delineate and manipulate sexually dimorphic phenomena in primates. These data provided the conceptual background for my efforts to show that the primate brain is sexually differentiated in its patterns of gonadotropic hormone secretion. These efforts were enhanced by the fortuitous opportunity of studying a particular group of genetic female rhesus monkeys that had been masculinized in utero by maternal treatment with testosterone propionate. Our finding that the response characteristics of the CNS-pituitary axis in these androgenized females more closely resembled those of males than of normal females corroborates the earlier theory that these sex differences are organized by the prenatal hormonal milieu.

This theory, that certain cells in the body undergo a kind of preconditioning for their adult performance as a function of their

particular in utero hormonal environment, has widespread implications for drug therapy during pregnancy (Bern, Gorski, and Kawashima, 1973). For example, Herbst, Ulfelder, and Poskanzer (1971) and Herbst et al. (1972) reported that the female offspring of women treated during pregnancy with the synthetic estrogen diethylstilbestrol develop adult-onset vaginal adenocarcinoma at an alarming rate. These data, though tangential to the issue of sexual differentiation, emphasize the continuum of the cell organization and function processes from fetal into adult life and serve notice of its fragile integrity.

It is apparent from the literature that estradiol-17 β represents the major element in the long-term negative feedback control of LH secretion in the female rhesus monkey (Karsch et al., 1973a, c). In our experiments on the long-term negative feedback control of LH secretion by estradiol-17 β , we found that in the normal female, postgonadectomy levels of LH were significantly suppressed below their initial, preimplant levels with the smallest sized estradiol implant, whereas in males and androgenized females, these levels were significantly suppressed only with the largest sized estradiol implant. Moreover, the 0.5- and 1.5-cm estradiol capsules produced greater mean (though not statistically significant) concentrations of circulating estrogens in males and androgenized females than in similarly treated normal females. Nevertheless,

at both these points this latter group showed a greater degree of LH suppression than either the males or androgenized females. We infer, therefore, that male and androgenized female rhesus monkeys are less sensitive to the long-term feedback inhibition of LH secretion by estradiol than similarly treated females. We also infer that this feedback control mechanism is differentiated as a function of a hormonal preconditioning in fetal life.

Our data for the rhesus monkey are congruent with those reported by Petrusz and Nagy (1967) for hemispayed rats which showed that compensatory ovarian hypertrophy was significantly inhibited by estradiol in doses of 0.1 μ g/day administered for a period of one month whereas the inhibitory effect of the same dose of estradiol was less marked and not significant in hemispayed rats which had received a single injection of 1.0 mg testosterone one day after birth. An earlier study by Petrusz and Flerkó (1965) had shown that the secretion of gonadotropic hormones was more markedly inhibited by exogenous estrogen in male rats castrated on the second day of life than in those gonadectomized 60 days after birth. These authors concluded that androgen exposure during the critical period of sexual differentiation desensitizes the estrogen-sensitive hypothalamic structures that are instrumental in the negative feedback action of estradiol on LH secretion.

The mechanism whereby this apparent desensitization is

effected remains obscure. However, as Gorski (1971) suggested, the process may involve an inhibition of a particular DNA-dependent RNA and protein synthesis mechanism, or perhaps this phenomenon is a function of a compromised estrogen-binding network within the brain which results from androgen exposure during the critical period (Flerkó et al., 1969; Flerkó, 1971). These hypotheses are not mutually exclusive; a complex sequence of events must occur to program the response characteristics of this sexually differentiated negative feedback system.

The neuroanatomical or pituitary loci involved in the sexual differentiation of the estrogen-LH negative feedback control system have not been identified; however, on the basis of work done on rats and monkeys, it is probable that both hypothalamic and pituitary sites are involved (Davidson, 1969; Negro-Vilar et al., 1973; Orias et al., 1974; Carmel et al., 1972).

The work of Yamaji et al. (1972) and Yen et al. (1972b) has suggested that estradiol-17 β represents the major element in the short-term negative feedback regulation of LH secretion in both the rhesus and human female. We observed that on a short-term basis (i.e., within 72 hours), the time course of the estradiol-induced negative feedback inhibition of LH secretion did not differ significantly between sexes. We did, however, observe a pronounced rebound in serum LH values in the group of females, which was less

obvious in both the males and androgenized females. The significance, if any, of this observation remains a mystery. The levels of estrogen effected by the implants did not differ remarkably between groups; hence, the differences in treatment could not account for the post-implant rebound differential between males and females. The rapid onset (within 6 hours) of the negative feedback inhibition of LH secretion by estradiol suggests that this estrogen-mediated phenomenon does not require the biosynthesis of additional inhibiting factors for its action. The mechanism of this short-term suppression of LH probably involves either a direct inhibition of LH release at the pituitary level or an indirect inhibition of LH-RH release from hypothalamic-median eminence stores. The works of Krey et al. (1973) and Spies and Norman (1974) suggest that estrogen acts directly on the pituitary gland to inhibit the LH-RH induced release of LH in the rhesus monkey, but hypothalamic sites for estrogen feedback inhibition cannot be ruled out (Carmel et al., 1972). Our study, however, does not provide definitive data for either hypothesis.

The etiological basis for the rebound phenomenon observed in the group of females warrants consideration. Perhaps the peak estrogen levels at 6 hours were sufficient to suppress LH equally in all groups at that time, but the differential sensitivities found in the long-term negative feedback study suggest that females are more

sensitive to the subsequent decline in serum estrogen levels and hence display a more pronounced rebound from their 6-hour nadir than either males or androgenized females during the final 48 hours of the short-term study.

To attribute LH rebound phenomenon entirely to the declining levels of serum estrogen is probably not adequate, however. Notwithstanding the decline in estrogen levels during the LH rebound, they are nevertheless in excess of 75 pg/ml throughout most of this period and thus should be fully adequate to suppress LH values and to prevent this putative LH rebound. It may be that a dual system, like that suggested for the adrenal-corticotropin system (Jones et al., 1972), is operating for the negative feedback regulation of LH. One element might be a fast-adapting, rate-sensitive component such that $LH \propto d[E_2]/dt$ which could account for the initial 6-hour inhibition, during which the estrogen-sensitive aspect of this negative feedback element is responding to the changing levels of estrogen, i.e., $d[E_2]/dt$, rather than its absolute serum concentration. This element, which adapts rapidly during the next 66 hours, could account for the subsequent LH rebound. Sex differences may exist during the adapting phase of this rate-sensitive element. Such a rate-sensitive component would be important for the minute-to-minute dynamics of the estradiol-LH negative feedback system. Superimposed on this rate-sensitive element might be

another slow-adapting, level-sensitive component such that LH α [E_2]. This more sluggish element would act over the following days to restrain LH levels within normal physiological bounds. Our long-term negative feedback studies have suggested that sex differences reside within the level-sensitive component of this system. The existence of such putative rate- and level-sensitive elements remains highly speculative, but our data do not militate against such a dual control system.

Having demonstrated a sex-specific negative feedback control system in the rhesus monkey, we focused our efforts on the estrogen-induced positive feedback mechanism in order to determine possible sex differences in this system. In these experiments, we observed that gonadectomized male and genetic female rhesus monkeys that had been treated with testosterone propionate in utero released significantly larger quantities of LH in response to an injection of estradiol benzoate than normal female rhesus monkeys. These data are at variance with those of Karsch et al. (1973a) who failed to establish any sex differences in the quantity of LH released in response to the estradiol benzoate challenge. Two differences between the experimental design used by Karsch and co-workers and us are apparent. Our challenging dose of estradiol benzoate was 50 μ g/kg, that of Karsch was 42 μ g/kg. According to their and our data, however, this difference produced only negligible

differences in the serum levels of estrogen during the time course of the experiment; peak values for serum estrogens ranged from 500 to 1300 pg/ml during our experiment and from 600 to 1000 pg/ml during theirs. Another difference in method between our design and that of Karsch was the estrogen pretreatment regimen. Whereas Karsch rigorously titrated with estradiol implants the high postgonadectomy levels of LH to the low values observed in intact monkeys, our pretreatment regimen with estradiol did not fully suppress LH levels to those observed in the intact female. In our first and subsequent positive feedback studies, mean preinjection estrogen levels were 45 and 38 pg/ml for the combined groups, respectively, whereas in Karsch's study, the initial levels of estrogen ranged from 60 to 150 pg/ml. It might be argued that compared with normal, intact, preovulatory estrogen environment (Hotchkiss et al., 1971) our estradiol pretreatment regimen effected a more physiological estrogen milieu than Karsch's. But it might also be asked of what physiological importance is an estrogen milieu, regardless of the concentration, in a male rhesus monkey? Nevertheless, this discrepancy in the pretreatment paradigm might account for Karsch's failure to demonstrate sex differences in the LH response effected by the estradiol benzoate injection. The work of Yamaji et al. (1971) supports this contention. They found sex differences in the estrogen-induced LH response in gonadectomized

rhesus monkeys which, in contradistinction to Karsch's animals, had received no estrogen treatment before the injection of estradiol benzoate. The pretreatment regimen, therefore, appears to be of critical importance in establishing the subtle differences in the positive feedback mechanism between sexes in the primate.

It should be noted that the purified standard reference for the LH radioimmunoassay used in our laboratory differs from that used by Karsch in the laboratory of Dr. Ernst Knobil.

Our data show that the controlling mechanism for the LH surge is competent in both male and female rhesus monkeys, if not in rats; however, the response characteristics differ between males and females. Moreover, prenatal exposure of genetic females to androgens predisposes their CNS-pituitary axis to acquire a male pattern of LH release in response to estrogen treatment as adults. From these data we conclude that the adult pattern of LH release in response to estrogen challenge is, at least in part, a function of an in utero hormonal preconditioning which organizes the neural-pituitary elements responsible for effecting this LH release in the rhesus monkey.

The sexually dimorphic response characteristics of the CNS-pituitary axis may be a function of either a sexually differentiated CNS or a pituitary gland. To explore whether differences in pituitary responsiveness account for the observed sex differences in LH

response to negative and positive estrogen feedback, we administered the synthetic decapeptide LH-RH to 4 monkeys from each of the three experimental groups. We observed, however, no differences in the LH response of males, females, and androgenized females. Although the LH response was significant in all groups, it was so small as to make any interpretation of the data equivocal at best. Nevertheless, these data indicate that the differences between males and females and between prenatally androgenized females and normal females in their LH response to estrogen are the function of a mechanism which lies within the central nervous system and which can be influenced by prenatal exposure to androgens.

These negative data certainly do not rule out the possibility that sex differences in pituitary responsiveness to LH-RH may exist in the primate, but under our experimental conditions we were unable to demonstrate them in the rhesus monkey. In fact, a sexually dimorphic pituitary response to stilbestrol with LH-RH has been reported in adult men and women (Seyler et al., 1974). Moreover, in children, treatment with LH-RH apparently elicits a differential LH response as a function of sex (Job et al., 1972). LH-RH seems to effect a greater release of LH in male than in similarly treated female children. These data suggest that either the male pituitary gland is more sensitive to LH-RH or that the male pituitary gland has a larger releasable store of LH than the female gland. Either

explanation could account for our observation that the male and androgenized female release more LH in response to an estrogen challenge than similarly treated, normal female rhesus monkeys. These data in adults and children imply that the primate pituitary may be sexually differentiated. If these data for adults and children are valid, our data for the rhesus monkey implying brain differentiation may be reinterpreted to mean pituitary differentiation. In this case, we would conclude that the sex differences in the response characteristics of the primate CNS-pituitary axis are a function of a pituitary gland that differentiates under the influence of the prenatal and early neonatal hormonal environment. This conclusion is at variance with the currently held theory regarding rodent sexual differentiation, which is thought to be a function of a central nervous system mechanism (Harris, 1964). Whether or not the primate central nervous system and/or the pituitary gland are sexually differentiated with respect to the patterns of gonadotropin secretion remains at issue; unfortunately, our data do not fully resolve the question.

This work provides a physiological basis for confirming and understanding sexually dimorphic phenomena, including behavior, in primates. Moreover, these data can serve as a framework for further investigations into the mechanism whereby the primate CNS-pituitary axis becomes sexually differentiated under the influence

of the fetal hormonal environment. Finally, our study has suggested some clues for further research into the biological basis of certain human sexual syndromes such as the testicular feminizing syndrome, the adrenogenital syndrome, and perhaps homosexuality.

CHAPTER VI

SUMMARY AND CONCLUSIONS

We have shown that the long-term negative feedback effect of estradiol on LH secretion differs in male and female rhesus monkeys and that the ovariectomized female appears to be more sensitive to these effects than the castrate male. Genetic females, masculinized in utero, respond to this treatment more like males. In the short-term regulation of LH secretion by estradiol, however, no differences between males and females could be shown. The positive feedback effect of estradiol benzoate on LH secretion delineated a significant difference in the response characteristics of the CNS-pituitary axis between males and females similarly treated. Again, females masculinized in utero responded more like males than their normal female counterparts. No sex differences in the LH-RH-induced LH response pattern were shown.

The fact that the male primate, unlike the male rodent, responds to the positive feedback effects of estrogens makes it difficult to establish a direct analogy between the process of brain differentiation in the primate and in the rodent. However, in the rhesus monkey quantitative aspects of the CNS-pituitary axis do apparently differ as a function of sex. Moreover, prenatal exposure of genetic

females to androgens predisposes their gonadotropin control system to acquire a male-like response pattern as adults. From these data we conclude that the adult pattern of LH release is, at least in part, a function of an in utero hormonal preconditioning that organizes the neural-pituitary elements which are responsible for effecting this LH release in the rhesus monkey.

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Figure 1. The long-term dose-effect relationship between serum estrogens (E_2) and luteinizing hormone (LH) levels in the gonadectomized rhesus monkey. LH levels are plotted as the percent of the initial preimplant values. The arrows denote the point at which the first statistically significant ($p < 0.05$) suppression of LH levels occurred as a function of increasing levels of estrogen. The lines connect means $\pm$ standard errors for each group.

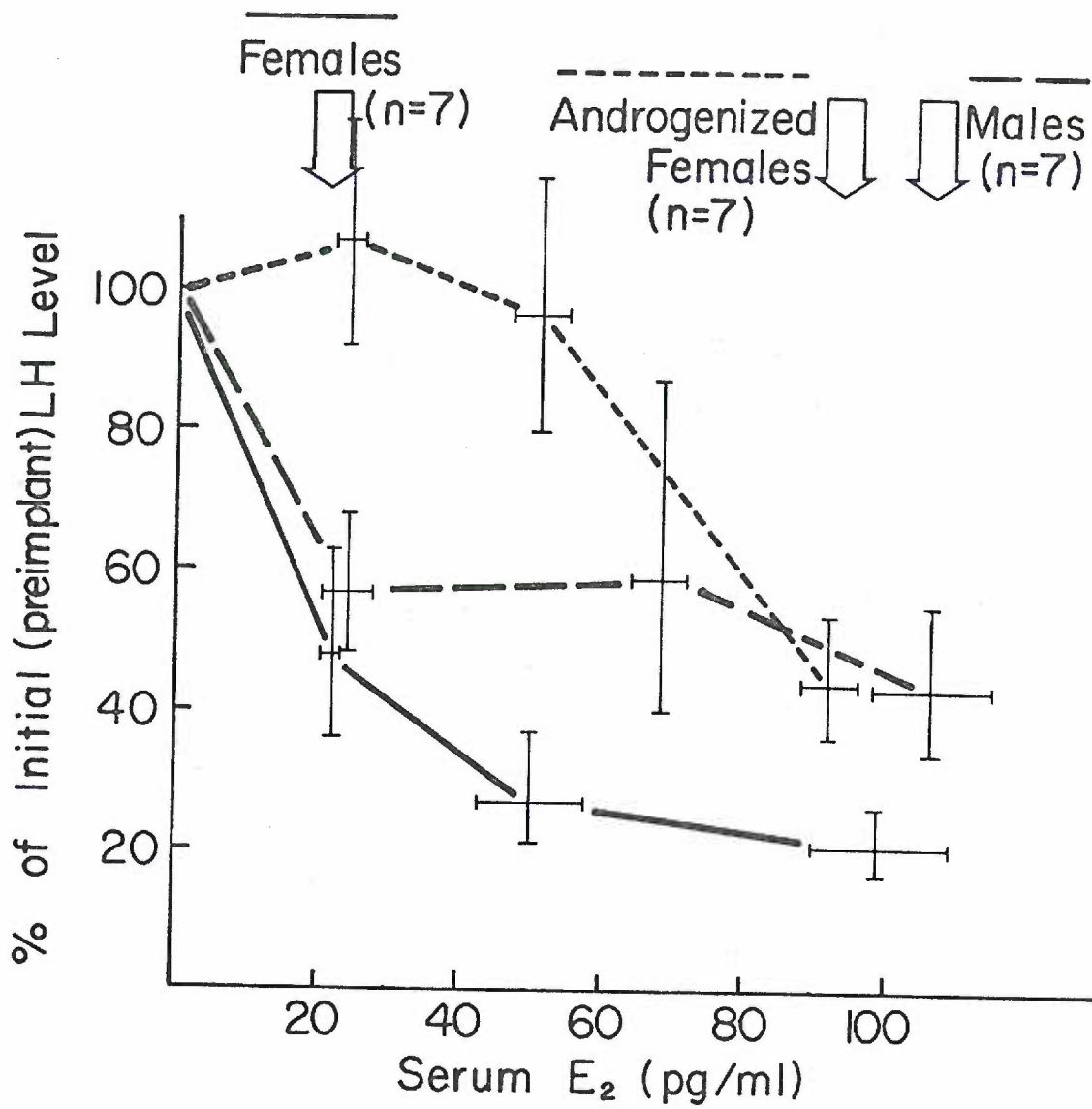


Figure 1

Figure 2. The long-term negative feedback effect of silastic capsules containing estradiol-17 β on serum luteinizing hormone (LH) levels in the gonadectomized rhesus monkey. LH levels are plotted as the percent of the initial preimplant values. Mean estrogen (E_2) levels are denoted below each respective bar. Lines connecting the top of bars denote a statistically significant ($p < 0.05$) suppression of LH levels under preimplant levels. Data are presented as means + standard errors for each bar.

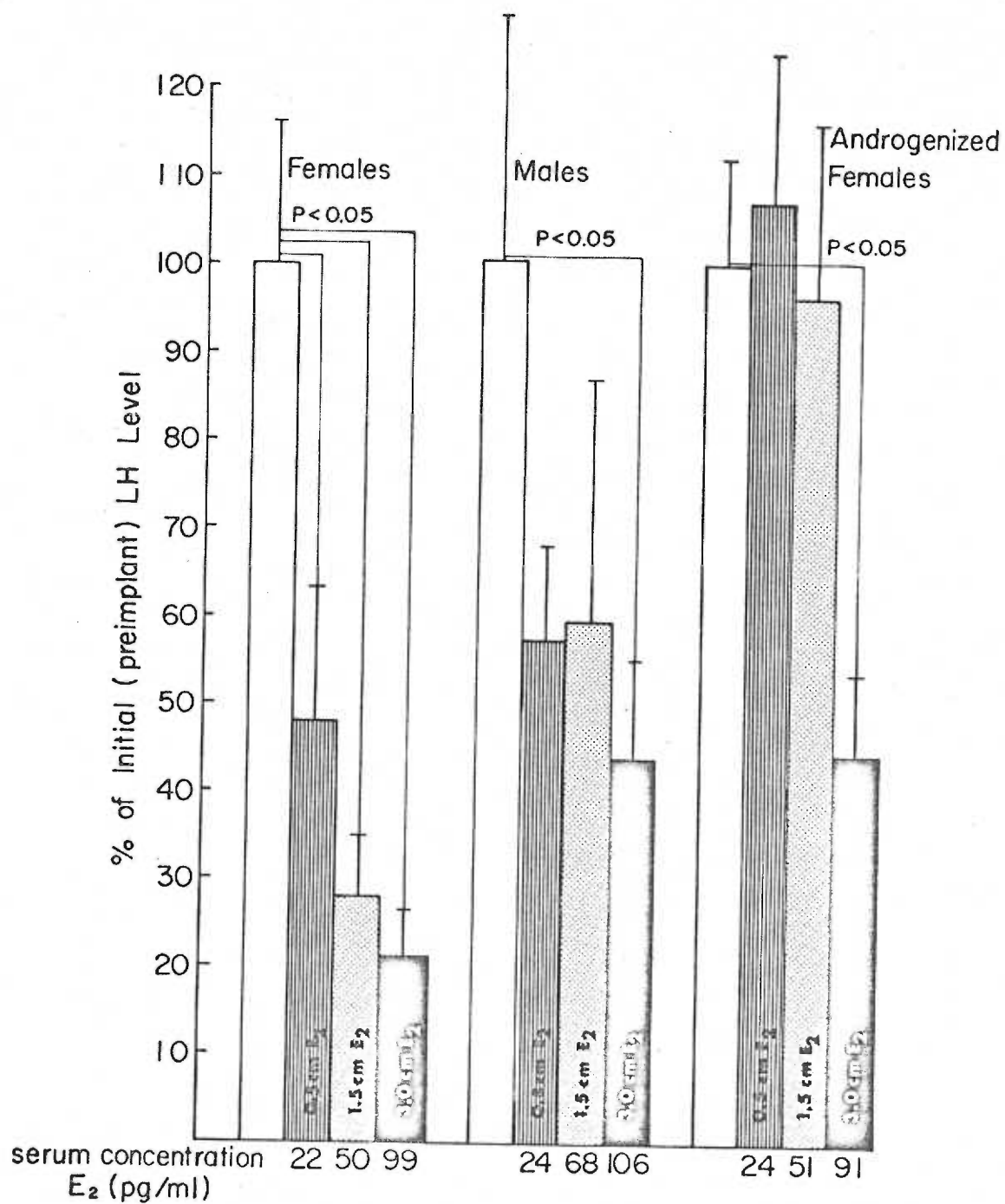


Figure 2

Figure 3. The short-term time-course effects of 0.5-cm silastic capsules containing estradiol-17 β on serum luteinizing hormone (LH) levels in the gonadectomized rhesus monkey. LH concentrations are plotted as values normalized to preimplant levels. The lines connect the means $\pm$ standard errors for each group.

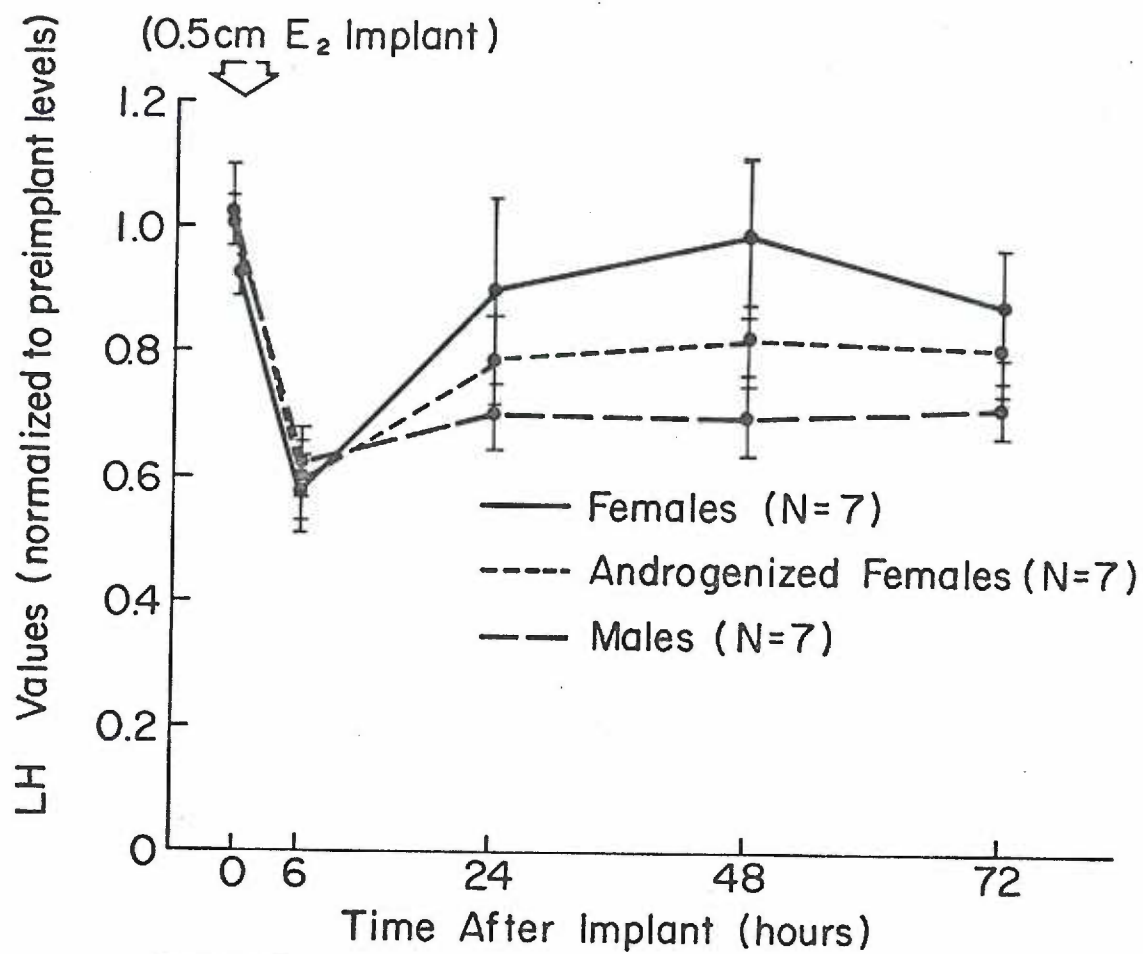


Figure 3

Figure 4. The time-course of serum estrogens (E_2) throughout the short-term exposure to 0.5-cm silastic capsules containing estradiol- 17β in the gonadectomized rhesus monkey. The data are presented as means $\pm$ standard errors for each group.

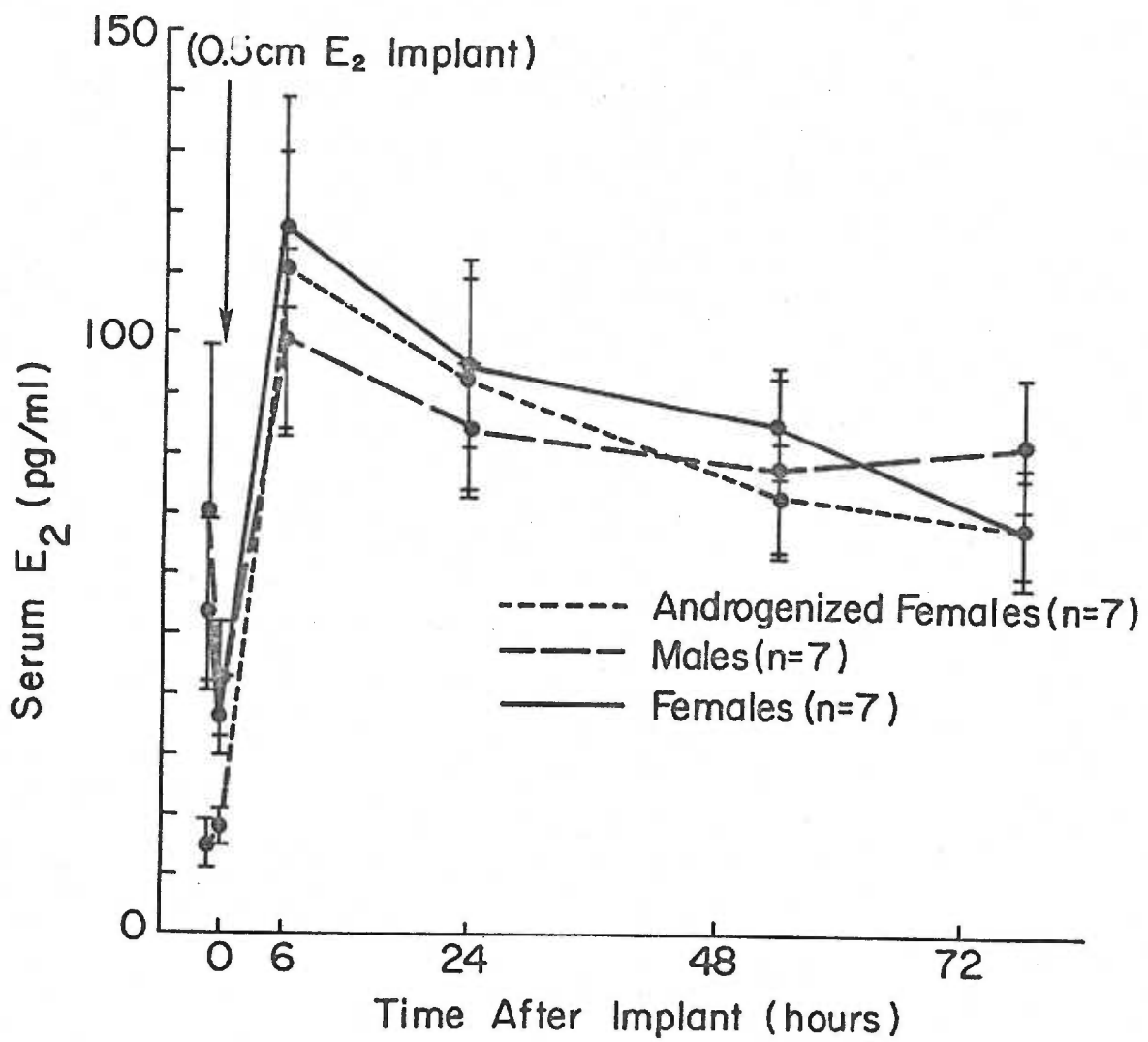


Figure 4

Figure 5. The time-course of serum luteinizing hormone (LH) release after an injection of estradiol benzoate in the gonadectomized rhesus monkey. Each animal had been pretreated with a 1.5-cm estradiol implant for about one month before the estradiol benzoate injection. The data are presented as means $\pm$ standard errors for each group.

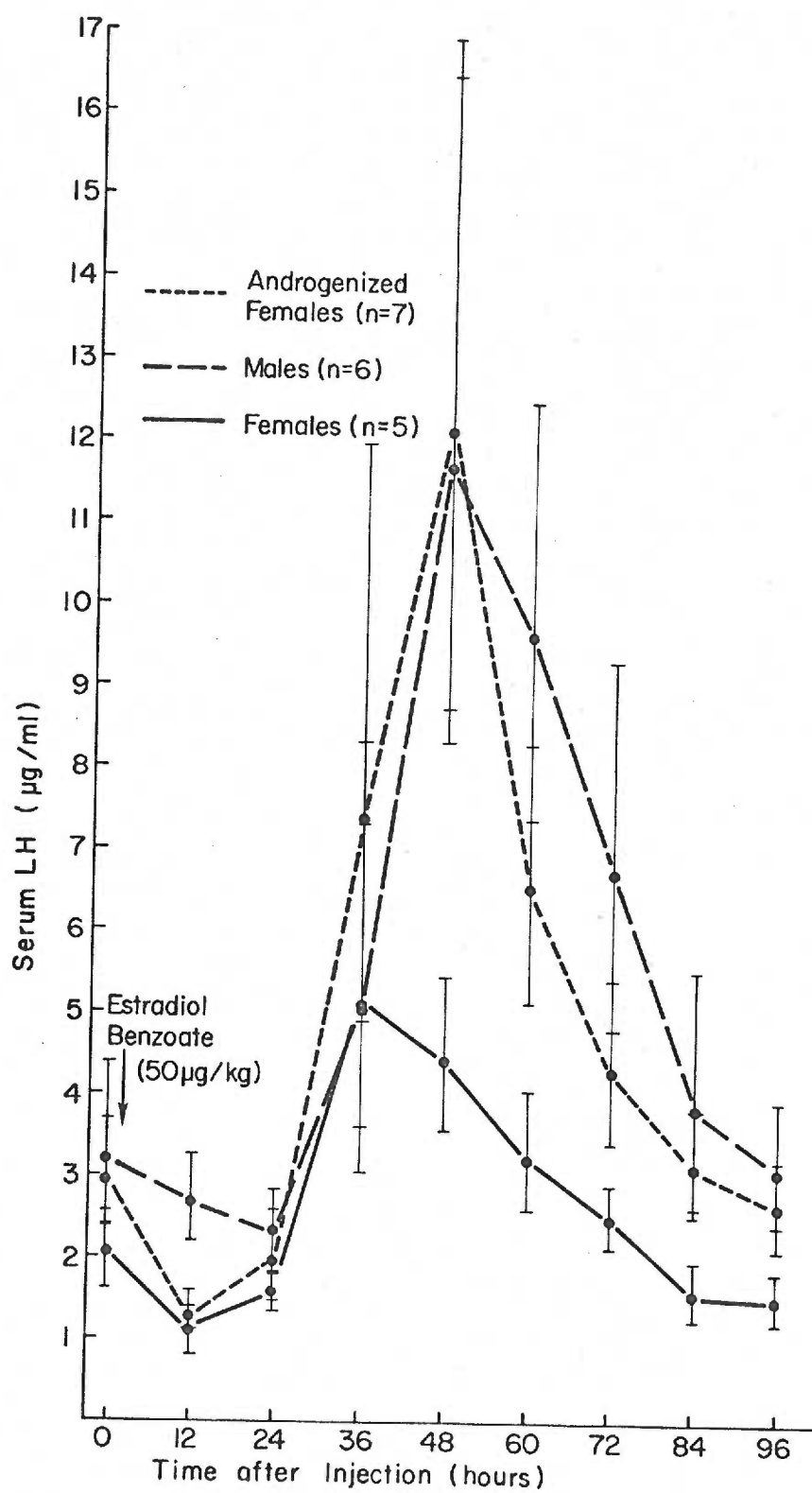


Figure 5

Figure 6. The time-course of serum estrogens (E_2) after an injection of estradiol benzoate in the gonadectomized rhesus monkey. The data are presented as means $\pm$ standard errors for each group.

Estradiol Benzoate (50 $\mu\text{g/kg}$, s.c.)

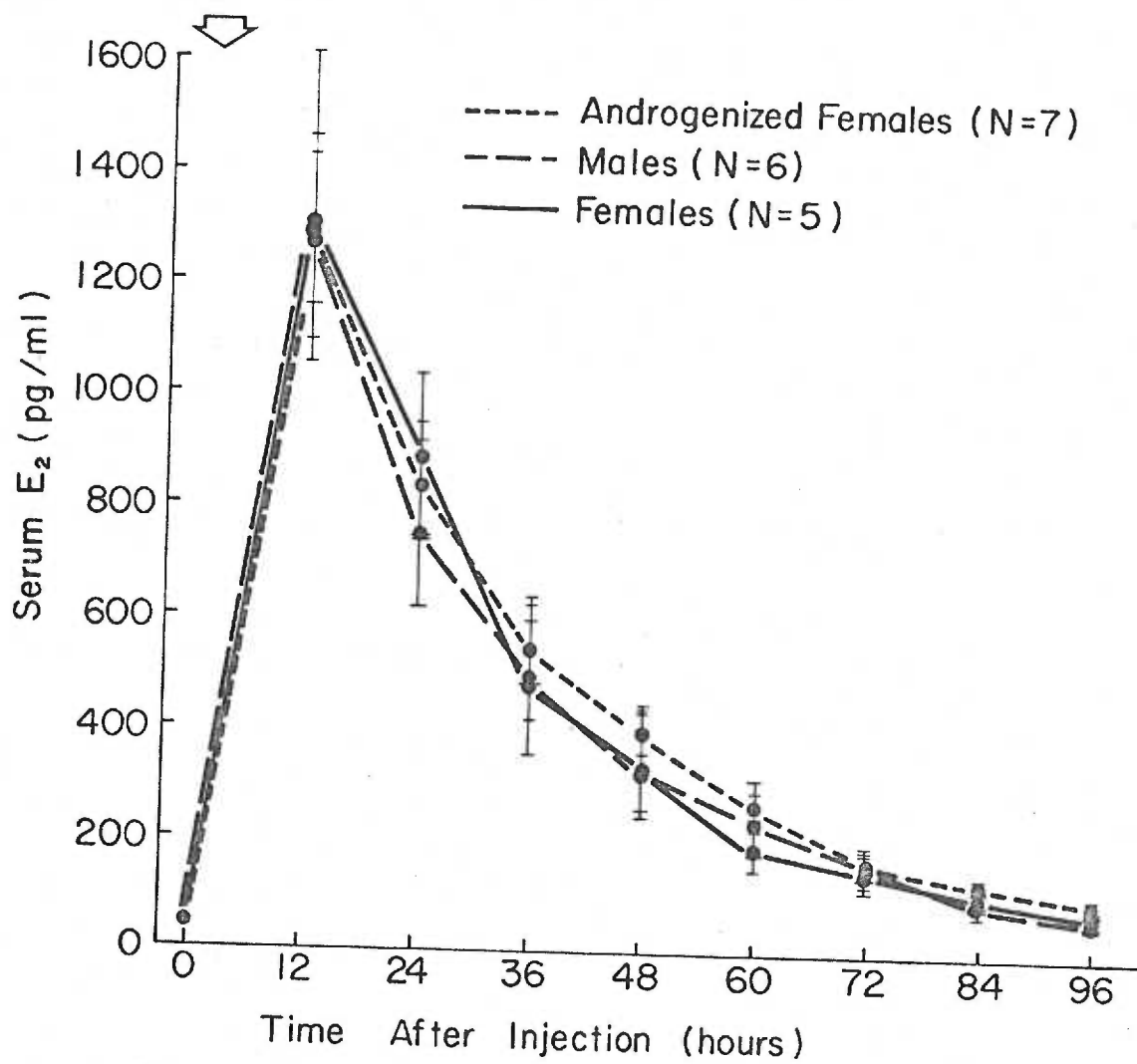


Figure 6

Figure 7. The time-course of serum luteinizing hormone (LH) release after an injection of estradiol benzoate in the gonadectomized rhesus monkey. Each animal had been pretreated with an 0.5 cm estradiol implant for approximately one week before the estradiol benzoate injection. The data are presented as means $\pm$ standard errors for each group. Analysis of variance (ANOV) describes the statistical evaluation of integrated serum LH levels for all groups.

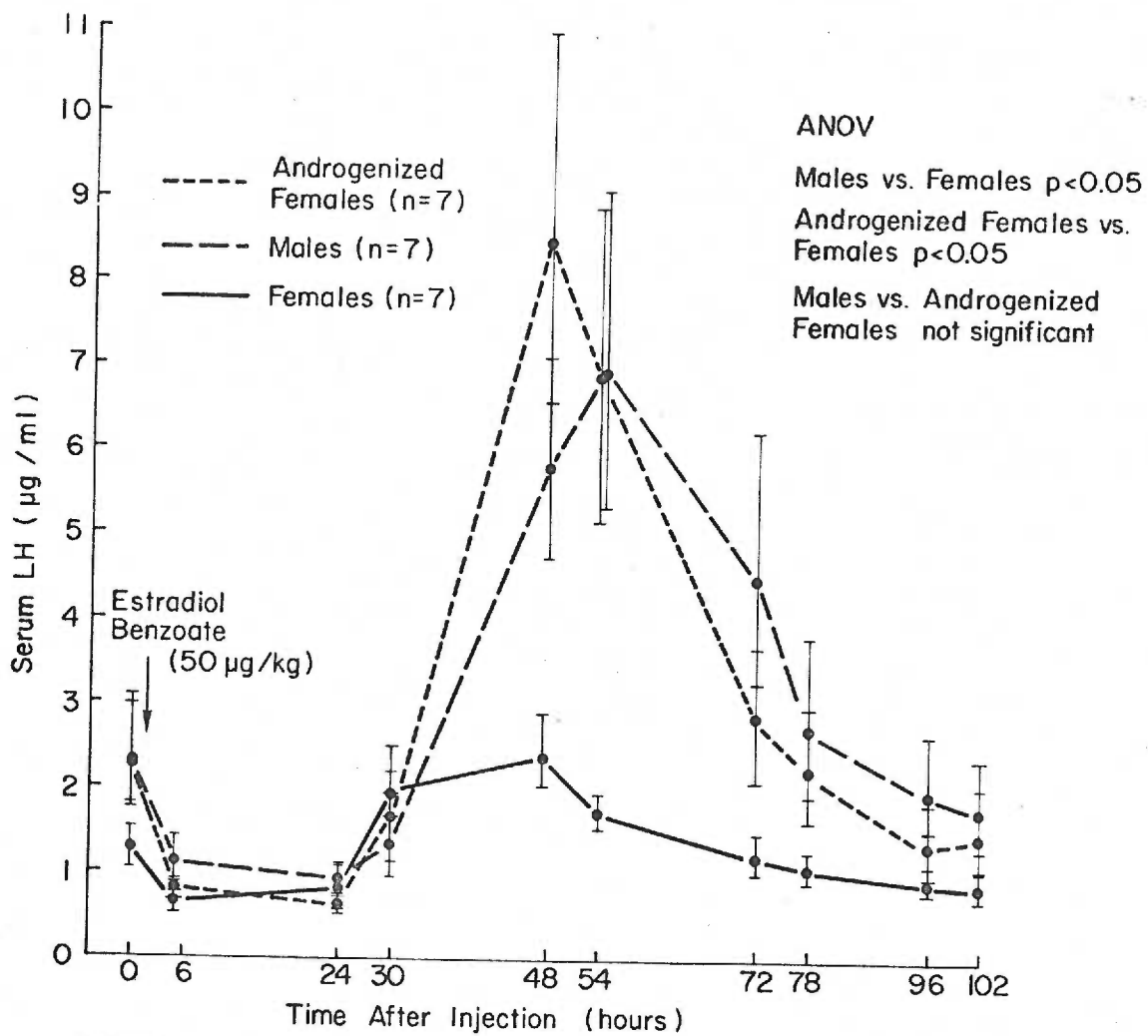


Figure 7

Figure 8. The time-course of serum estrogens (E_2) after an injection of estradiol benzoate in the gonadectomized rhesus monkey. The data are presented as means $\pm$ standard errors for each group.

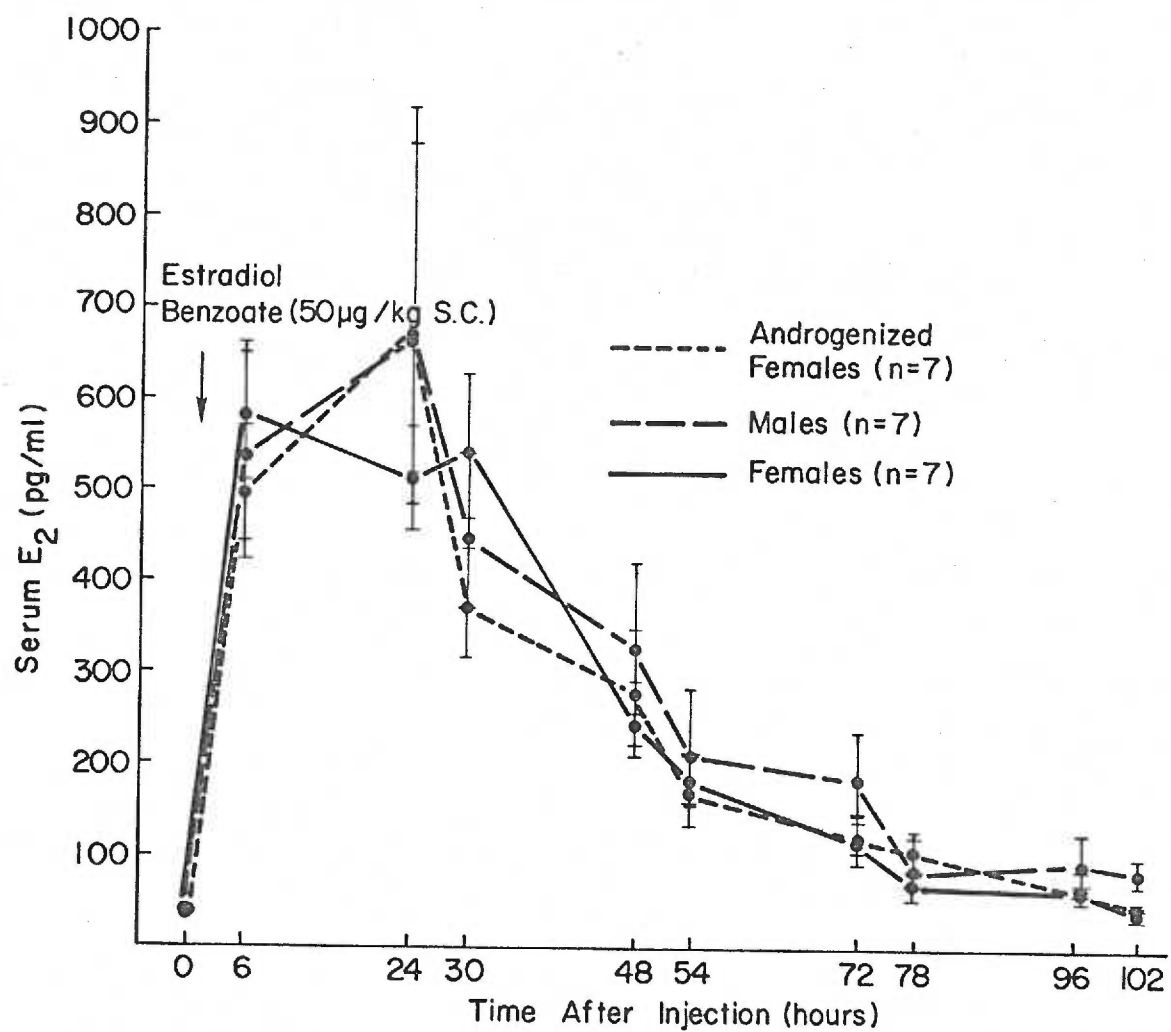


Figure 8

Figure 9. The time-course of serum luteinizing hormone (LH) release after an injection of the synthetic decapeptide, luteinizing hormone-releasing hormone (LH-RH) in the gonadectomized rhesus monkey. The data are presented as means $\pm$ standard errors for the group of androgenized females, + standard errors for the males, - standard errors for the normal females.

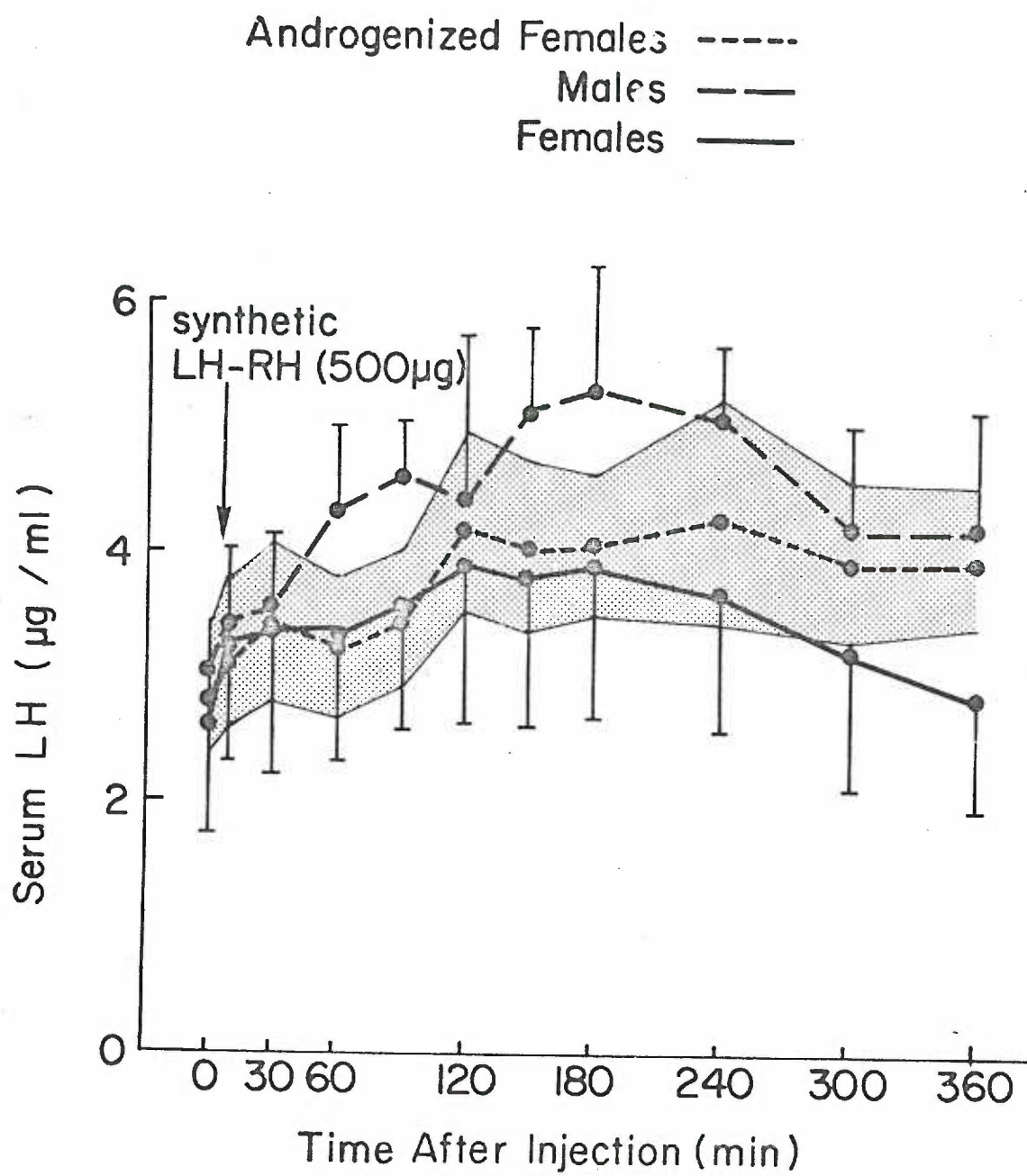


Figure 9

Figure 10. Integrated serum luteinizing hormone (LH) release over a 6-hour period in response to an injection of luteinizing hormone-releasing hormone in the gonadectomized rhesus monkey. The data are presented as means + standard errors for each group.

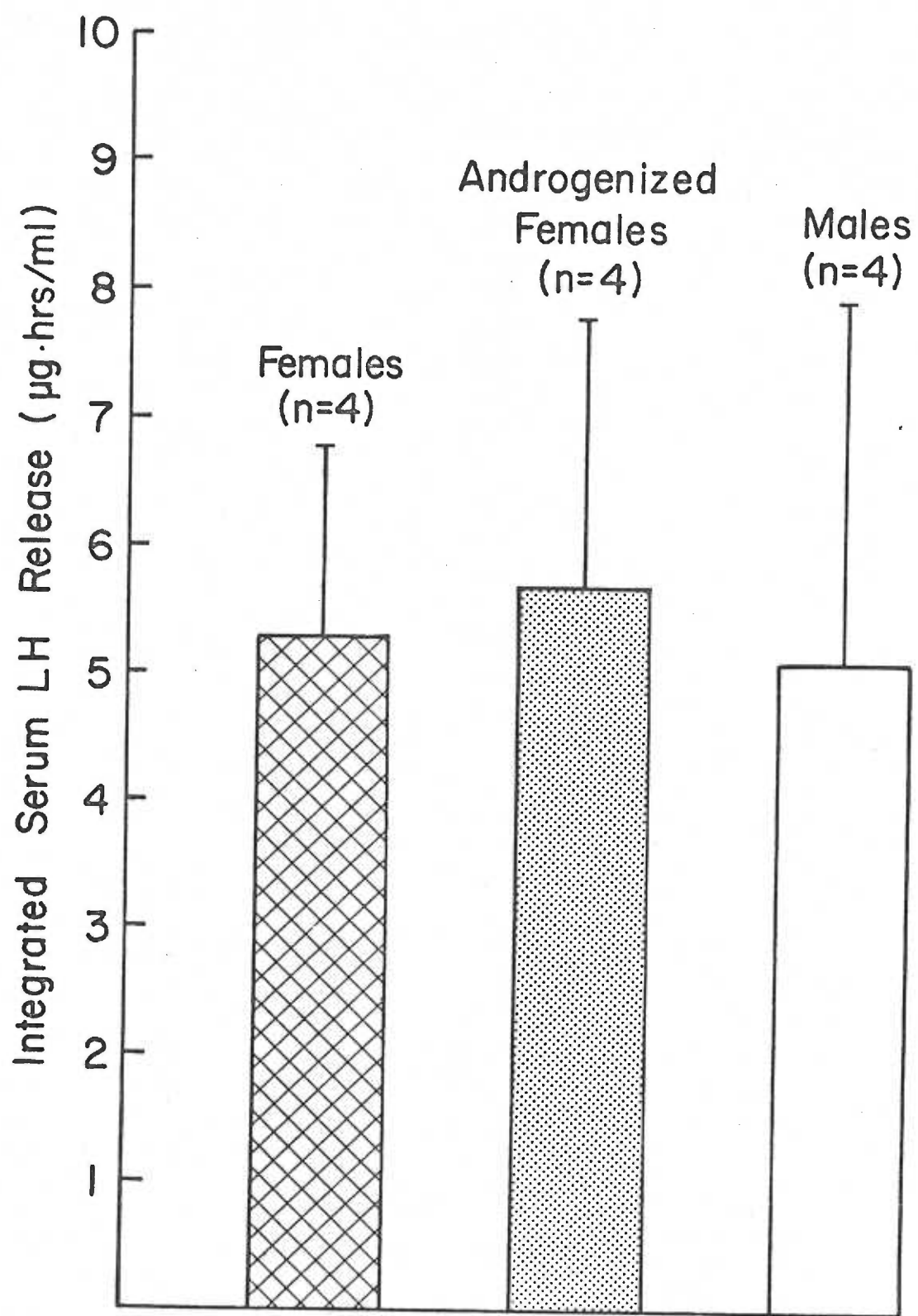


Figure 10

APPENDICES

These appendices include a detailed description of certain techniques and methodology used in the preparation and operation of the estrogen and luteinizing hormone radioimmunoassay. They also include a brief outline of the testosterone radioimmunoassay. Various experiments performed on the ovariectomized rhesus monkey which were either preliminary or ancillary to the main thesis are also described. In many cases, these data merely corroborate those in the literature or represent incomplete studies. But since they provide an empirical basis on which to test my thesis, I am briefly describing them here. Appendix B contains a discussion of the influence of stress on LH secretion and its possible interference with the experimental design.

APPENDIX I

Estrogen 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, NY) to PBS and stored at 4° C.
3. The estradiol-17 β antisera stock solution was made by diluting the concentrated antibody 1:300 with 0.1% gel-PBS. Approximately 100 ml of working solution were prepared by diluting the stock solution 1:50 with 0.1% gel-PBS. The diluted antibody (1:3000) was stored at 4° C. The estradiol-17 β antisera that were developed in the rabbit against estradiol-17 β -6-oxime-BSA were diluted similarly to a final working concentration of 1:5000.
4. Dextran-coated charcoal solution: 0.25% charcoal (Norit A, Fisher Scientific Co., Fairlawn, NJ) and 0.025% dextran T-70 (Pharmacia, Uppsala, Sweden) were suspended in PBS. This solution was made up in 1-liter volumes 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. Eleven or 12 lanes were etched in the Eastman #6060 plates

with fluorescent indicator (Sargent-Welch Scientific Co., Skokie, Ill.) before being washed twice by ascending chromatography with redistilled methanol. After air drying, plates were stored over Drierite (W. E. Drierite Co., Xenia, Ohio) in a desiccator.

7. Standard estradiol-17 β stock solutions were made by weighing 10 mg on a Mettler M-5 microbalance and dissolving the steroid in 10 ml of ice-cold absolute ethanol. Further dilutions of the stock solution were made with ice-cold ethanol to achieve a final working concentration of 1 pg per μ l. This solution was stored in a glass-stoppered volumetric flask at 4^o C, and all aliquots were removed from the volumetric flask while it was immersed in an ice bath.

8. Ether used throughout the complete procedure was freshly distilled 2% ethanol (ETOH)/ether (not over 5 days old). ETOH is absolute glass distilled. The ether is collected over the ETOH. The collecting flask is shaken periodically so the ETOH is well mixed in the ether during distillation. Ether is anhydrous absolute from Mallinckrodt in 35 lb cans.

9. ³H labeled estradiol (2, 4, 6, 7 - ³H) (Amersham Searle, 250 μ c) was made up in 0.1% gelatin-PBS as follows:

A) 250 μ c/250 λ when received was transferred to a 50 ml amber glass stoppered bottle and dried down. 50 ml of distilled ice-cold ethanol were placed into the bottle, which is called the stock solution. This is 5 μ c/1 ml of the label ³H; it is stored

in the freezer at -16°C .

- B) A working solution made up in 0.1% gel/PBS should contain about 4000 cpm for $^3\text{HE}_2$. 4000 cpm at 20% counting efficiency with Triton N-101 was made up by placing a certain volume of stock solution into a 100 ml volumetric flask. The ^3H is dried down in ethanol with air. Refrigerated 0.1% gel/PBS is added to the 100 ml volume and this working solution is stored in the refrigerator. This working solution should be checked before use the first time. Each new batch ordered will be different and needs to be checked out.

10. Triton solution (water solubilizer) used in the counting vials for assay is made up as follows:

Omniflor - 5 grams (New England Nuclear)

Xylene - 100 ml (Mallinckrodt)

Triton N-101 - 500 ml (Rohm and Haas - Philadelphia, Pa.)

11. Filtered air was used throughout the complete methodology for drying down the extracts in a 37 to 40°C water bath.

APPENDIX II

Radioimmunoassay for Testosterone (T)

The method for the radioimmunoassay for testosterone has been previously described by Resko et al. (1973). Testosterone was extracted from 10-200 μ l of serum 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 ChromAF 1000 fiberglass sheets (Mallinckrodt Chemical Works, St. Louis, Mo.) impregnated with silica gel in a chloroform:acetone (90:10) system. Before use, the sheets were washed twice by ascending chromatography with redistilled methanol. The area corresponding to T was located 1 cm below the isatin dye (J. T. Baker Chemical Co.); 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 were developed in rabbits immunized with testosterone-3-oxime bovine serum albumin. The antisera stock solution was made by diluting the concentrated antibody 1:50 with 0.1% gel-PBS. A working solution of 100 ml was prepared by diluting the stock solution 1:60 with 0.01% gel-PBS. The diluted antibody was stored at 4^o C until used. The antisera (0.1 ml) were then added to each tube. The procedure for the

testosterone assay continues exactly as that described for the estrogen assay except that approximately 4000 cpms of ^3H -T(91 Ci/mM, New England Nuclear Corp.) were added to each assay tube after the initial 15 minutes of incubation with the antibody. Independent ^3H -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 sensitivity, precision, accuracy, and specificity of this method have been reported by Resko and his co-workers (1973).

APPENDIX III

Preparation and Handling of LH Radioimmunoassay Reagents*

*Only double-distilled H_2O should be used.

Add merthiolate (1/10,000) to the H_2O stock on arrival

(1) 0.5 M sodium phosphate (monobasic)

- A Weigh 69.0 gm $NaH_2PO_4 \cdot H_2O$
- B Dissolve in 500 cc distilled H_2O
- C Bring to one liter volume
- D Store at room temperature up to 6 months

(2) 0.5 M sodium phosphate (dibasic)

- A Weigh 134 gm $Na_2HPO_4 \cdot 7 H_2O$
- B Dissolve in 500 cc distilled H_2O
- C Bring to one liter volume
- D Store at room temperature up to 6 months

(3) Serum Pools

- A Collect about 40 ml of monkey serum (OVX)
Mix thoroughly
- B Aliquot 0.8 ml volume into serum vials
Store at $-20^{\circ}C$

(4) 0.5 M sodium phosphate buffer pH 7.5

- A Add 100 ml of #1 ($0.5 M NaH_2PO_4$) to two liter bottle
- B Add #2 ($0.5 M Na_2HPO_4$) until pH reaches 7.5
- C Store at room temperature up until 6 months

(5) 0.05 M sodium phosphate buffer pH 7.5

- A Place 50 ml of #4 (0.5 M sodium phosphate buffer pH 7.5) in 1/2 liter bottle
- B Add 450 ml of distilled H₂O
- C Store at room temperature up to six months

(6) Phosphate buffered saline (PBS) pH 7.0

- A Place 143 gm NaCl in 500 ml distilled H₂O in 17.5 liter bottle
- B Add 130 ml of #1 (0.5 sodium phosphate monobasic)
- C Add 260 ml of #2 (0.5 M sodium phosphate dibasic)
- D Add 1.75 g Merthiolate or Thimersal (Sigma T-5125)
- E Bring to 17.5 liters with distilled H₂O
- F Store at room temperature up to four months
- G The pH should be 7.0

(7) LH for iodination

- A Thaw the frozen reagent containing 100 µg/ml or add #5 (0.05 M sodium phosphate buffer pH 7.5) to the powder to give 100 µg/ml
- B Transfer 25 µl quantities to 1 ml serum vials
- C Apply stoppers and snap freeze
- D Store frozen at -20°C

(8) PBS 0.1% Gelatin

- A Place 1 gm powdered gelatin in liter bottle
- B Add 1000 ml #6 (PBS pH 7.0)
- C Dissolve on hot plate
- D Store frozen in 500 ml aliquots up to 1 year or at 0-4°C up to 4 months
- E Make sure thawed solution is at pH 7.0

(9) 0.05 M EDTA - PBS pH 7.0

- A Place 18.6 gm EDTA in 800 ml #6 (PBS pH 7.0)
- B Dissolve on hot plate
- C Bring volume to 1 liter
- D Add 9.5 N NaOH until pH reaches 7.0
- E Store in refrigerator up to six months

(10) Chloramine-T (Eastman Kodak Corp., Rochester, NY)

- A Weigh out 24 mg on day of use
- B Just before use add 4 cc of #5 (0.05 M sodium phosphate pH 7.5) to give 30 ug/10 ul
- C Upon arrival of new bottle of Chloramine-T aliquot all in small light insensitive bottles, pack well.
- D Store in desiccator in dark

(11) Sodium Metabisulfite

- A Weigh out 24 mg on day of use
- B Just before use add 4 cc of #5 (0.05 M sodium phosphate buffer pH 7.5) to give 150 ug/25 ul

(12) Gels

- A Slowly add 20 gm of gel particles P-60:100-200 mesh to 700 ml of #5 (0.05 M NaPO_4 pH 7.5) and stir for 30 minutes
- B Allow to stand 24 hours at room temperature
- C Store in refrigerator up to four months
- D Place at room temperature 24 hours before use

(13) ARGG (Anti-Rabbit Gamma Globulin)

- A Upon arrival keep at $0-4^{\circ}\text{C}$ (up to eight months)
- B Any time up to one week before use dilute to proper concentration with #6 (PBS pH 7.0).

(14) Preparation of LH standard(LEP-M907-D)

- A Weigh out 300-800 ug of standard on Cahn electrobalance
- B With #8 (PBS-0.1% gel pH 7.0) dilute to 300 ug/ml
- C Pipette accurately 100 ul of solution into 4 ml shell vials
Snap freeze
Store up to 4-6 months at -20°C

(15) Anti-Ovine-LH Antibody

- A Dilute the antibody with #17 (1/200 NRS-0.05 M EDTA-PBS) 1/400 and snap freeze in 400 ul volumes.
- B Store frozen at -20°C up to one year
- C Dilute with above buffer up to one week before use
- D The excess should be refrozen for later use since this antibody is valuable

(16) 1/200 NRS - 0.05 M EDTA - PBS

- A Take 2.0 ml #18 (NRS) and add 398 ml of #9 (0.05 M EDTA-PBS pH 7.0)
- B Store at $0-4^{\circ}\text{C}$ up to two months

(17) NRS (normal rabbit serum)

- A Bleed a normal rabbit
Collect serum by allowing blood
to clot at room temperature for
30 minutes
- B Store frozen at -20°C in 1 ml aliquots

(18) 0.05 M EDTA - PBS pH 7.5 - 1% BSA

- A Take 100 ml of #9 (0.05 M EDTA-PBS pH 7.0)
- B Add 1 gm BSA
- C Bring pH to 7.5 with 0.5 M NaOH

APPENDIX IV

Preparation of Column for Iodination of LH

1. Gel has been at room temperature for 24 hours
Use Bio Gel P-60; 600-200 mesh
2. Place under vacuum for 30 minutes to remove bubbles;
heat if necessary
3. Place small glass bead in a disposable 10 cc pipette
4. Attach rubber tubing about 1.5" at bottom of pipette
5. Wash pipette 2x with #5 (0.05 M sodium phosphate buffer
pH 7.5)
6. Fill with above buffer to 7 cc mark
Clamp tubing with alligator clip
7. Pipette gel from bottom of flask to fill 10 ml pipette to
zero cc level
8. Remove clip
9. Add more gel if necessary to have packed gel reach 5 cc mark
10. Run 4 cc of buffer #5 (0.05 M sodium phosphate buffer pH 7.5)
through after settling
11. Add 3 cc of #19 (0.05 M EDTA-PBS pH 7.5-1% BSA)
12. Clamp off until use
13. Column can be kept at room temperature for a week
14. The 3 cc of #19 (0.05 M EDTA-PBS pH 7.5-1% BSA) should
be run through within 4 hours of use of column
15. Never let the top of the gel be exposed to the air for more
than 5 seconds

APPENDIX V

Procedure for Iodination of LH

1. Have column ready and under hood
2. Add 25 ul of #4 (0.5 M buffer) to vessel containing hormone
3. Add 1.0 mC of ^{131}I or 0.5 mC of ^{125}I to vessel
4. Make up #10 (Chloramine-T)
5. Add 10 ul #10 (Chloramine-T)
Time
Flick reaction vessel continuously
6. After 2 minutes transfer reaction mixture to column and let it soak in
7. Add 3 drops of #19 (0.05 M EDTA-PBS pH 7.5) and let it soak in before gently filling the column with this solution
8. Collect drops in culture tubes containing 100 ul of #8 (0.1% PBS-gel pH 7.0)
9. Count 10 ul aliquots
10. Take peak tube
Dilute it with 50 ml of #8 (0.1% PBS-gel)
Tube 3 contains the hormone and tube 7 contains the iodide peak
11. Keep at $0-4^{\circ}\text{C}$ up to 1 and 1/2 weeks before use
12. Alternatively dilute to 15,000 counts per 0.1 ml and use immediately [dilute with #8 (0.1% PBS-gel pH 7.0)]

APPENDIX VI

Half-Life of Exogenously Administered Serum Estradiol-17 β

The objective of this investigation was to study the half-life of serum estrogens after an intravenous injection of estradiol-17 β into two ovariectomized rhesus monkeys.

Approximately 5 ml of blood were drawn from the two monkeys to be used in the study; plasma was drawn off and stored at 4⁰ C. Approximately 2 mg of estradiol-17 β were diluted to 10 ml with absolute ethanol; this constituted the working standard. Volumes of fifteen (3 ug) and thirty ul (6 ug) of this standard were added to individual quantities of 10 mg each of Celite powder (Johns-Manville Co., New York, N. Y.) and dried under air. One and one-half ml aliquots of plasma from animals #1419 and #3585 were added to the 6 and 3 ug E₂-Celite mixture, respectively, sealed, and put on a shaker overnight at room temperature. This suspension was centrifuged at 2500 rpms for ten minutes, and the plasma which contained the estradiol in solution was drawn off. Animal #1419 received 1 ml of its plasma solution (4 ug E₂), and animal #3585 received 1 ml (2 ug E₂) of its own plasma solution. The solutions were injected intravenously through an indwelling venous catheter (20 gauge, 2 inch, Becton-Dickinson and Co., Rutherford, N. J.)

which was inserted into the saphenous vein in the early morning, about one-half hour before the experiment began. The animals were bled through the catheter at the following intervals with reference to the time of injection: -10 minutes, -5, 30 seconds, 1 minute, 1.5, 2, 3, 4, 5, 8, 10, 15, 20, 25, 30, 40, 50, and 60 minutes. Approximately 2 ml of blood were drawn at these intervals from each animal. Serum estrogens were assayed by the "rapid" method, and LH values were also determined; both methods are described in the body of the thesis. The decay time course of serum estrogens and the concentrations of serum LH are illustrated for each animal in Appendix Figures 1 and 2. The composite estrogen decay curves appear to be at least double exponential functions. Individual regression lines were fit to the initial 10 and the last 45-minute periods after the injection of estradiol. The "slow phase" decay functions were essentially parallel between animals, although absolute estrogen levels in animal #1419 were shifted higher as a result of its larger initial dose. The calculated estrogen half-life for the initial "fast phase" was 5.78 minutes for animal #1419 and 1.93 minutes for #3585, while for the "slow phase" it was 26.9 minutes and 30.8 minutes for each animal, respectively. The initial "fast phase" may represent an initial loading and saturating of target tissue receptors, whereas the "slow phase" probably represents the

real metabolic half-life for the estrogen. Despite a slight tendency toward decline, LH levels remained essentially unchanged throughout the one hour of post injection measurement.

APPENDIX VII

Long-Term Negative Feedback of LH Secretion by Estradiol-17 β
in Female Rhesus Monkeys-Time Course Study

This study was designed to characterize the long-term effects of estradiol-17 β on LH release in the ovariectomized rhesus monkey. Five female rhesus monkeys were each implanted with a 0.5 cm silastic capsule containing crystalline estradiol-17 β . The implant procedure is outlined in the body of the thesis. Blood samples were taken just before implantation of E₂ and every monday, wednesday, and friday morning for one month thereafter. The time course of the inhibition of circulating LH levels for the five animals is illustrated in Appendix Figure 3. Methods for "rapid" estrogen and LH radioimmunoassay are described in the body of the thesis. LH levels declined over a period of 7 to 10 days to approximately 30% of their initial control values. In 4 of the 5 animals, this declining phase was followed by a tendency to "rebound" toward preimplant levels; this trend was not significant, however. Estrogen levels averaged less than 10 pg/ml before implantation of the estradiol capsule. These values climbed to 64 pg/ml at 48 hours after implantation, and gradually declined to approximately 31 pg/ml by the end of the month (Appendix Figure 4). Forty-eight hours after

the implantation of the estradiol, the mean LH level for the five animals was suppressed to about 71% of its initial control value. In the short-term negative feedback studies in similarly treated females included in Appendix sections VIII and IX (Appendix Figures 5, 6 and 8), LH levels were suppressed at 6 hours to approximately 50% of their initial control values. These data suggest that after an estrogen implant, LH levels reach an initial nadir at 6 hours and rebound at 48 hours. This rebound phenomenon could be a function of the declining tide in estrogen levels which occurs during this period. Alternatively, perhaps the early 6-hour decline reflects the initial activity of a putative "rate"-sensitive LH control system and its response to the changing levels of estrogen, i. e. $LH \propto d[E_2]/dt$, followed by a shift to a predominantly "level"-sensitive LH control system, in which $LH \propto [E_2]$. Some precedents for this dual type control system have been established in the adrenal-CNS-pituitary axis (Jones et al., 1972), but our data do not elucidate this phenomenon beyond speculation.

APPENDIX VIII

Short-Term Negative Feedback Effects of Estradiol-17 β
on LH Secretion in Ovariectomized Rhesus Monkeys
Restricted in Primate Chairs

This study was designed to characterize the short-term negative feedback effects of estradiol-17 β on LH secretion in ovariectomized rhesus monkeys restricted to primate chairs two days before and during the experimental period. Each of three ovariectomized rhesus monkeys received either an empty sham capsule, a 1.5 cm, or a 3.0 cm capsule containing estradiol-17 β . The implant procedure is outlined in the body of the thesis. All animals received each treatment on an alternating basis, with a four-day period between experiments, and all animals received a transfusion of 50 ml of whole blood after each bleeding period. The animals were bled through an indwelling venous catheter (20 gauge, 2 inch, Becton-Dickinson and Co., Rutherford, N.J.) which was inserted into the saphenous vein about half an hour before the experiment began. The animals were bled at the following intervals with reference to the time of the estrogen implant: -40 minutes, -20, 0, 20, each subsequent 20-minute interval until 4 hours, and at 5, 6, 8, 10, 12, 16, 20, and 24 hours thereafter. Approximately 2 ml of blood was drawn for each sample. Estrogen values could not be determined in

this experiment because of a contamination problem; LH values were determined by the method described in the body of the thesis. The LH data for the sham, 1.5, and 3.0 cm estrogen implants are shown in Appendix Figures 5 and 6. In the sham implant group, LH levels were slightly inhibited during the first 6 hours after the implant (Appendix Figure 7). This initial suppression was followed by a pronounced "rebound" which continued for the remaining hours of the study. This phenomenon may reflect the stresses of being bound to a chair and the implantation procedure. The estrogen implant groups also showed an initial suppression over the first 6-hour period which was maintained for the duration of the experimental period in these groups, however. No differences were evident between the degree or time course of suppression effected by the 1.5 cm or the 3.0 cm implant. These data suggest that certain environmental stresses, such as those involved in the chaired implant-bleeding situation, affect a stress-labile LH control system.

APPENDIX IX

Short-Term Negative Feedback Effects of Estradiol-17 β
on LH Secretion in Caged, Ovariectomized
Rhesus Monkeys

This study was designed to evaluate the short-term negative feedback effects of estradiol-17 β on LH secretion in caged, ovariectomized rhesus monkeys. Each of five ovariectomized rhesus monkeys received either an empty sham capsule, a 2.0, 1.0 or an 0.5 cm estradiol capsule containing estradiol-17 β . The implant procedure is outlined in the body of the thesis. All animals received each treatment on an alternating basis with a 24- to 72-hour respite between experimental periods. All animals were transferred to a small "squeeze cage" for bleeding, and samples were drawn by saphenous venapuncture at the following intervals with reference to the time of the implant: -40, -20, 0 minutes, 6, 24, and at 30 hours. Approximately 2.5 ml of blood were drawn at each sampling period. Estrogen levels were determined by the "rapid" method described in the body of the thesis; the method for LH analysis is described elsewhere in the thesis. Mean estrogen levels for the three control samplings, sham implants, 0.5, 1.0, and 2.0 cm estradiol implants were 24 ± 2 (SE), 19 ± 3 (SE), 98 ± 10 (SE), 103 ± 3 (SE), and 145 ± 5 (SE) pg/ml, respectively. Unfortunately, these various

sized implants did not produce well-demarcated estrogen values because apparently they were not adequately incubated to effect smaller and more discrete circulating levels of estrogen on the short-term basis. The time course of LH secretion during the experimental period is illustrated in Appendix Figure 8. The sham implant group showed no inhibition as a result of the handling procedure. These data are contrary to those described in Appendix, Section VIII which showed a pronounced inhibition followed by a rebound in the sham-treated "chaired" primate. These collective data suggest that the chaired-bleeding procedure is stressful, at least for certain animals. The 2.0, 1.0, and 0.5 cm implant sizes effected a marked inhibition to approximately 50% of the initial control LH values in all groups. No differences could be seen between these groups at this or any subsequent time period. The "rebound" phenomenon which appeared at 24 hours in the chaired animal experiment (Appendix, Section VIII, Appendix Figures 5 and 6) was not apparent here. This suggests that the chair-induced stress, along with the implantation procedure, results in an "oversuppression" of the normal estrogen-induced LH inhibition phenomenon followed by a recovery with a slight "overshoot." Our data do not elucidate this issue.

APPENDIX X

Time Course of LH Release in Ovariectomized Rhesus Monkeys:
The Effects of Acute Immobilization Stress and
Sodium Pentobarbital Anesthesia

The objective of this study was to characterize the time-course of LH release in the ovariectomized rhesus monkey. LH values were determined by the method described in the body of the thesis. In the first experiment, four adult, ovariectomized rhesus monkeys were placed in primate chairs and bled at 20-minute intervals for 8 hours through an indwelling saphenous vein catheter. The data from the individual animals are illustrated in Appendix Figures 9-13. Animals number 669 and 1403 showed evidence of a pulsatile oscillation of serum LH levels over time whereas the other two animals showed markedly suppressed LH levels and no apparent tendency toward an oscillatory pattern. Either the pulsatile release of LH described in the rhesus monkey by Dierschke et al. (1970) and in women by Yen et al. (1972a, b) is not characteristic of every gonadectomized individual or perhaps the stress of the bleeding procedure differentially affects certain members of the rhesus female population. Although we do not have sufficient data for a complete analysis, the pulsatile release of LH in these ovariectomized monkeys occurred within a period of about an hour.

In another experiment, four ovariectomized rhesus monkeys were immobilized on primate crosses and an indwelling femoral vein catheter was inserted under aseptic conditions. The animals were then bled at 10-minute intervals for 3 hours. The time course of LH release under these conditions is illustrated in Appendix Figures 14-17. The initial LH samples were taken within 10 minutes after the insertion of the venous catheter. The initial samples revealed relatively high levels of LH which seemed to decline gradually over the three-hour time course of the experiment. These data suggest that an initial release followed by a more prolonged inhibition of LH release prevails under conditions of acute immobilization stress. The absence of a pulsatile pattern of LH release under these experimental conditions suggests that certain stresses interfere with the mechanisms that are responsible for these rhythmic oscillations.

In a final experiment, two ovariectomized rhesus monkeys were anesthetized with ketamine hydrochloride and maintained under deep general anesthesia by sodium pentobarbital (Nembutal, Abbott Labs, North Chicago, Ill.) for 4 hours. During this period, the animals were bled at 20-minute intervals through an indwelling saphenous vein catheter inserted within 20 minutes of the initial ketamine injection. A relatively constant level of anesthesia was maintained with periodic injections of 50 mg sodium pentobarbital. The sodium pentobarbital had no discernible effect on the pulsatile

release of LH in either of the two animals tested (Appendix Figures 18 and 19). These data are in agreement with those of Bhattacharya et al. (1972) who could show no consistent effect of sodium pentobarbital on the circadian mode of LH secretion.

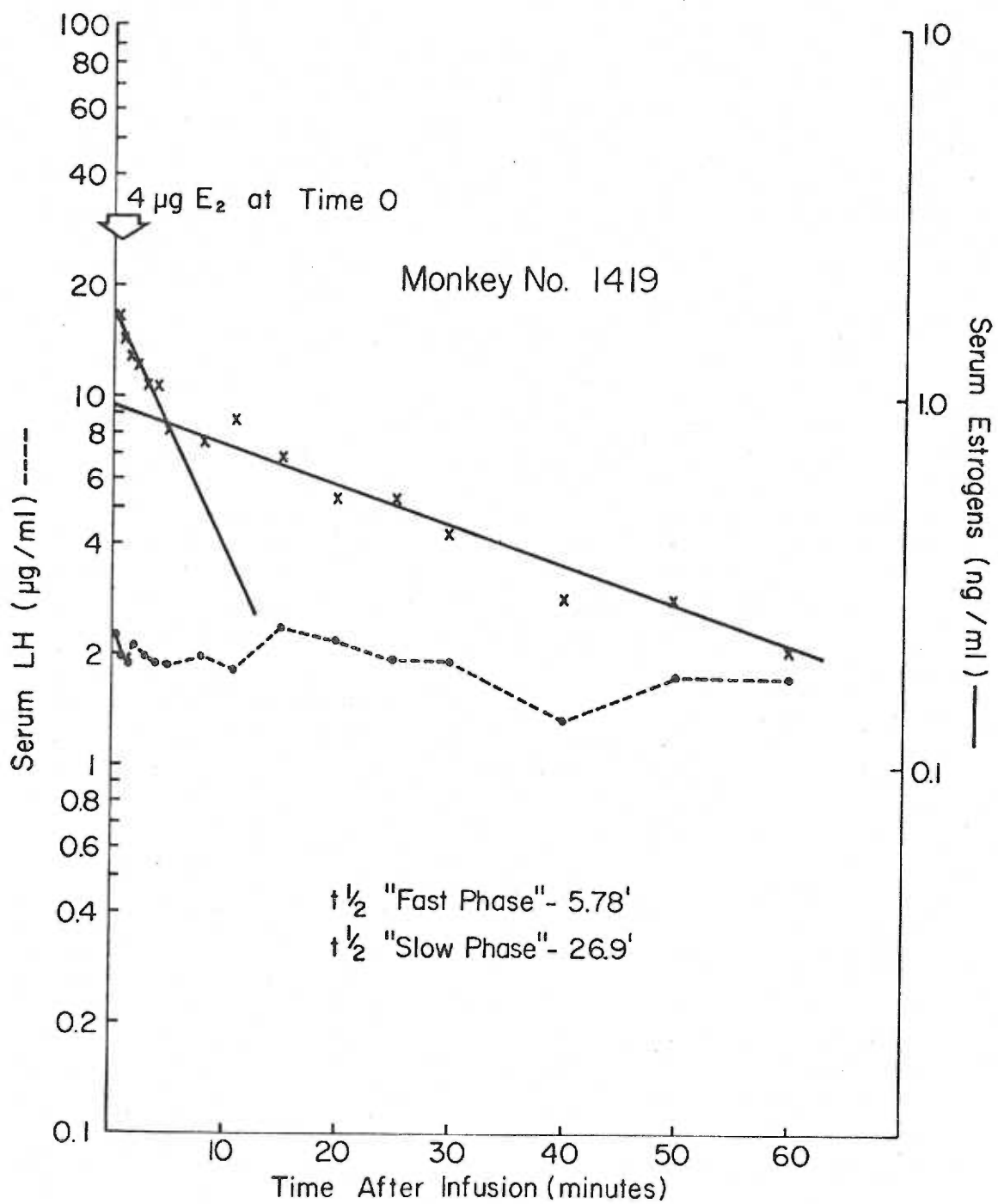
APPENDIX XI

Negative Feedback Effect of Testosterone on LH Secretion
in Ovariectomized Rhesus Monkeys

This study was designed to evaluate the negative feedback effects of testosterone on LH secretion in the ovariectomized rhesus monkey. Each of three female rhesus monkeys was implanted with a 4 cm silastic capsule containing testosterone (Steraloids Inc., Pauling, N. Y.). On each of the following two days, one additional implant of the same size was added. Multiple blood samples at 20-minute intervals were taken just before the first implant and each day for three days thereafter. The effect of these implants on circulating LH levels in each animal is illustrated in Appendix Figures 20, 21, and 22. LH levels were determined by the method described in the body of the thesis. In animals number 2302 and 812, testosterone effected either no inhibition or only marginal suppression of LH levels, whereas in animal number 5733 the initial levels were gradually inhibited to 43% over the course of the three-day experimental period. Testosterone levels produced by the implants in each animal are illustrated in Appendix Figure 23. Before insertion of the implants, testosterone levels were below 1 ng/ml in all animals. With the addition of one, two, and finally three 4-cm

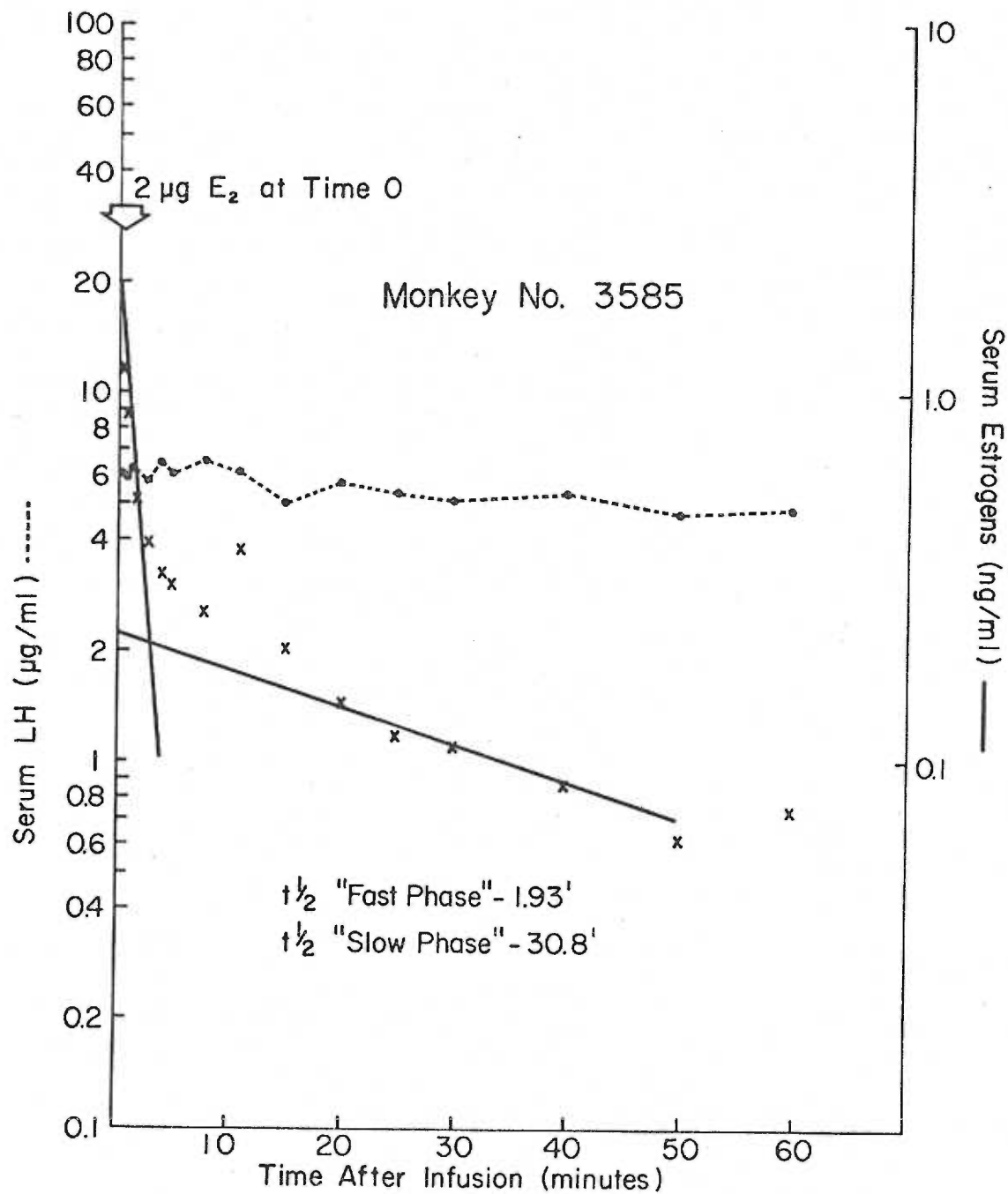
implants, testosterone levels increased sharply from 12 to more than 17 ng/ml of serum testosterone. Since serum testosterone levels in normal intact female rhesus monkeys rarely exceed 1.2 ng/ml (Hess and Resko, 1973), the fact that those in excess of 12 ng/ml were inadequate to notably suppress serum LH levels in two of the three animals treated implies that the normal physiological role of the testosterone negative feedback regulation of LH secretion in the female rhesus monkey is at best ancillary to the negative feedback function of estradiol. In many cases, a statistically significant suppression or augmentation appeared, but these differences are probably statistical artifacts which occurred as a result of the extremely tight multiple LH measurements within each given day. Therefore, we believe that in this particular case, physiological significance is best determined by an educated look and interpretation of the data. The effect of sham implant manipulations on serum LH levels in three monkeys is illustrated in Appendix Figure 24. No remarkable suppression of LH levels was evident as a result of the sham procedure. Levels of testosterone in these three sham implant monkeys are shown in Appendix Figure 25. Mean levels did not appreciably exceed 1.0 ng/ml at any point.

Appendix Figure 1. Double exponential decay curve (straight lines) of serum estrogens (E_2) after an infusion of 4 μ g of estradiol-17 β (E_2) into ovariectomized rhesus monkey #1419. The dotted line shows the serum luteinizing hormone (LH) levels throughout the postinfusion period.



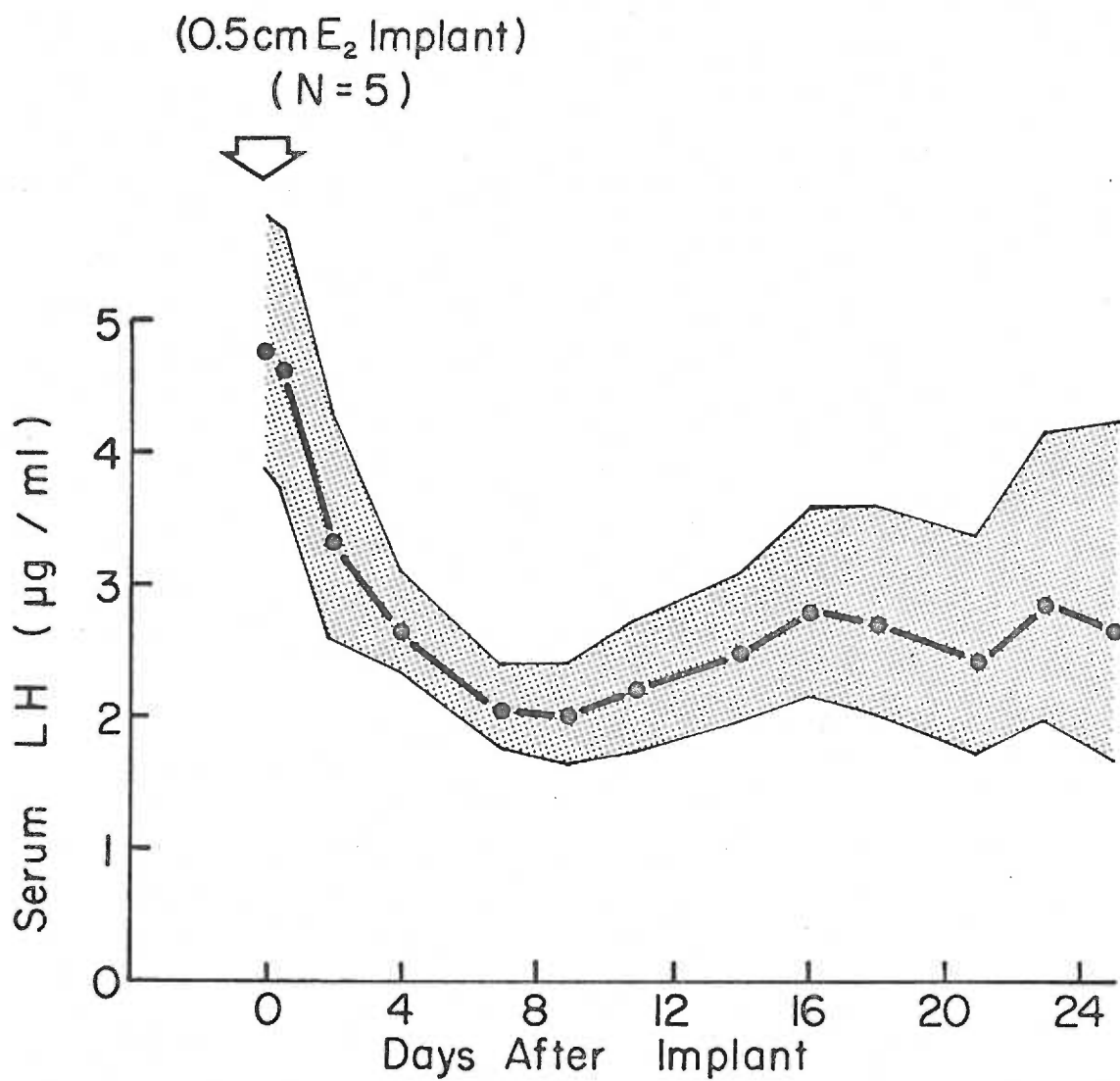
Appendix Figure 1

Appendix Figure 2. Double exponential decay curve (straight lines) of serum estrogens (E_2) after an infusion of 2 μ g of estradiol-17 β (E_2) into ovariectomized rhesus monkey #3585. The dotted line shows the serum luteinizing hormone (LH) levels throughout the postinfusion period.



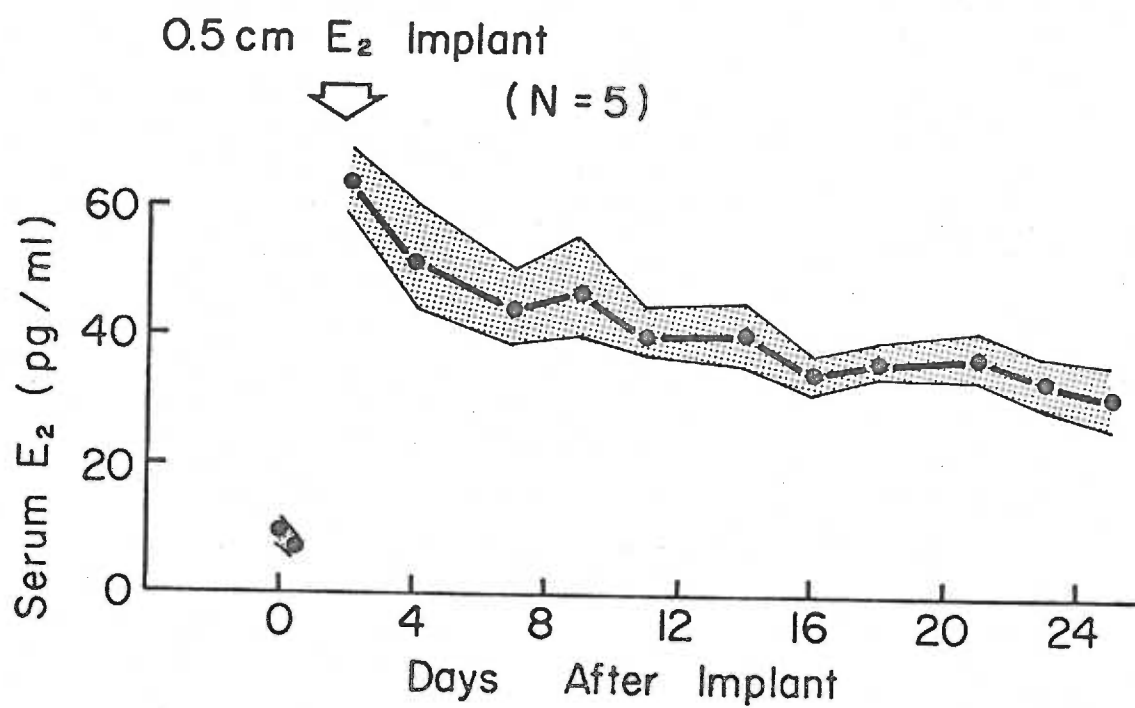
Appendix Figure 2

Appendix Figure 3. The time-course of the long-term negative feedback effect of 0.5-cm silastic capsules containing estradiol-17 β (E_2) on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys. The shaded area delimits the standard error of the data.



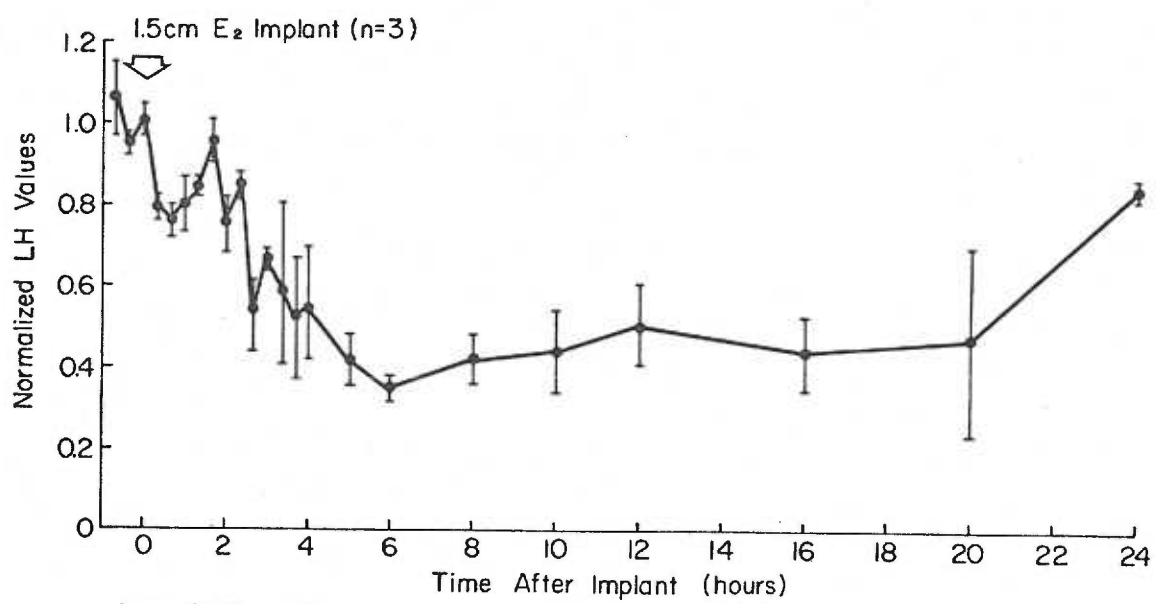
Appendix Figure 3

Appendix Figure 4. The time-course of serum estrogens (E_2) throughout the long-term exposure to 0.5-cm silastic capsules containing estradiol-17 β (E_2) in ovariectomized rhesus monkeys. The shaded area delimits the standard error of the data.



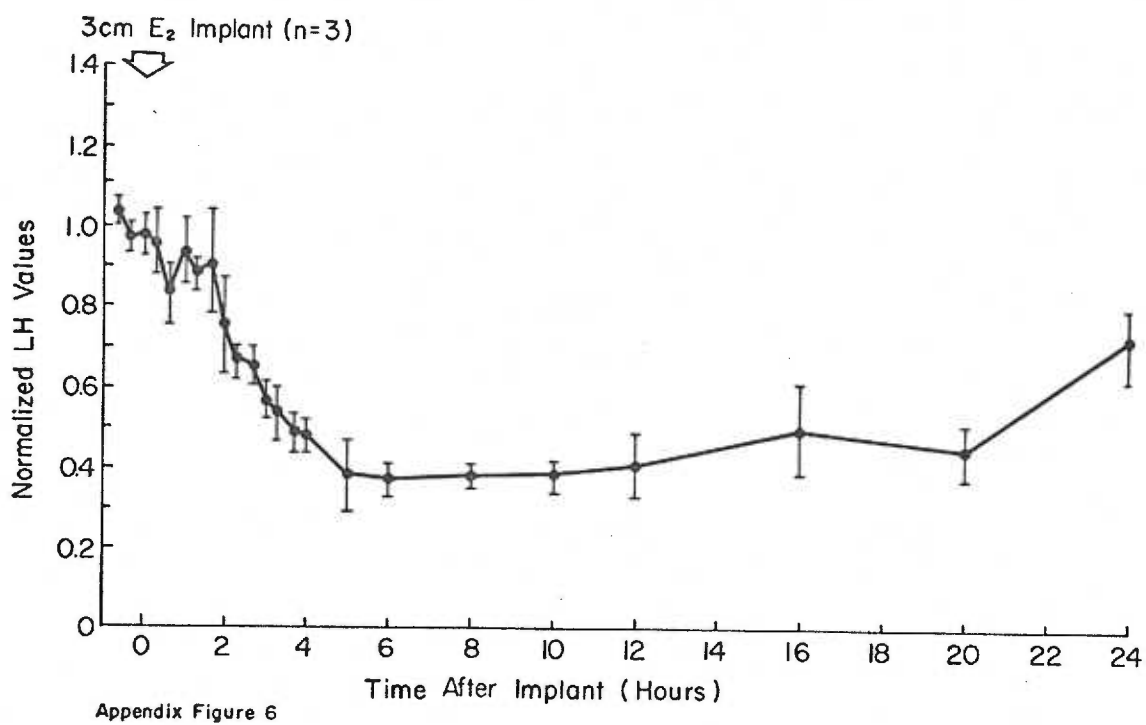
Appendix Figure 4

Appendix Figure 5. The time-course of the short-term negative feedback effects of 1.5-cm silastic capsules containing estradiol-17 β (E_2) on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys restrained in primate chairs. LH data are normalized to preimplant values. The data are presented as means $\pm$ standard errors.

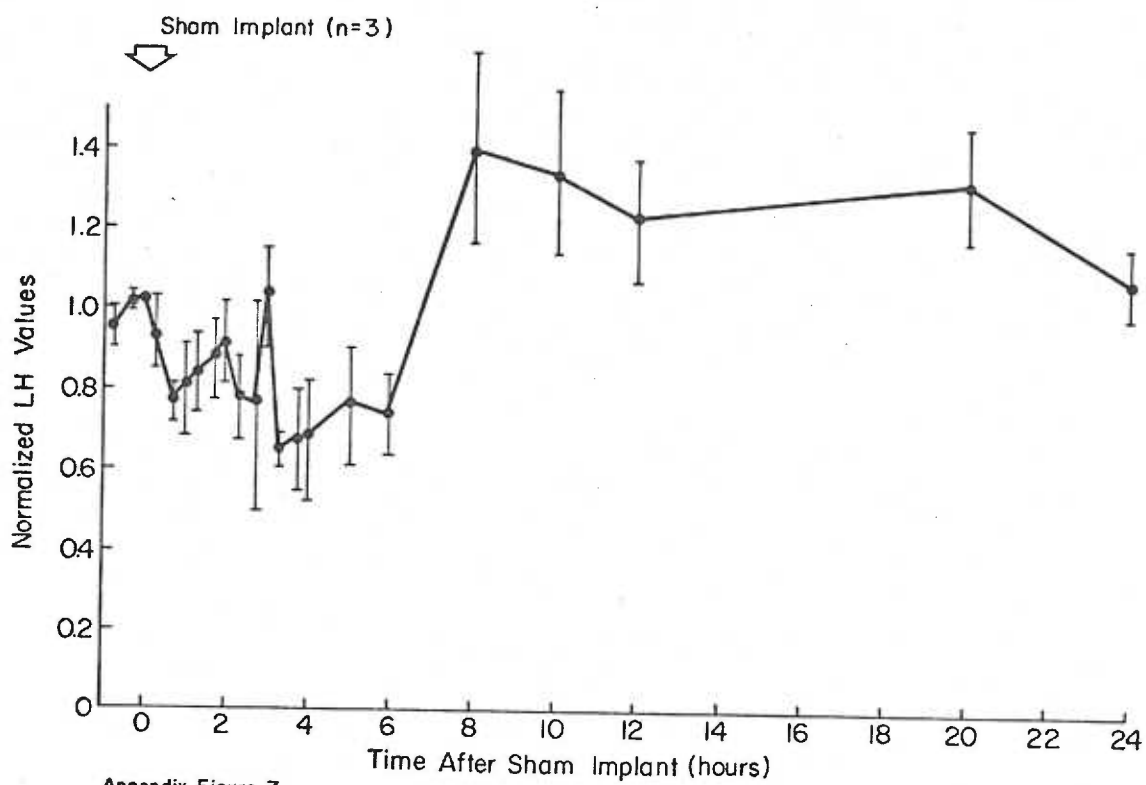


Appendix Figure 5

Appendix Figure 6. The time-course of the short-term negative feedback effects of 3.0-cm silastic capsules containing estradiol-17 β (E_2) on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys restrained in primate chairs. LH data are normalized to preimplant values. The data are presented as means $\pm$ standard errors.

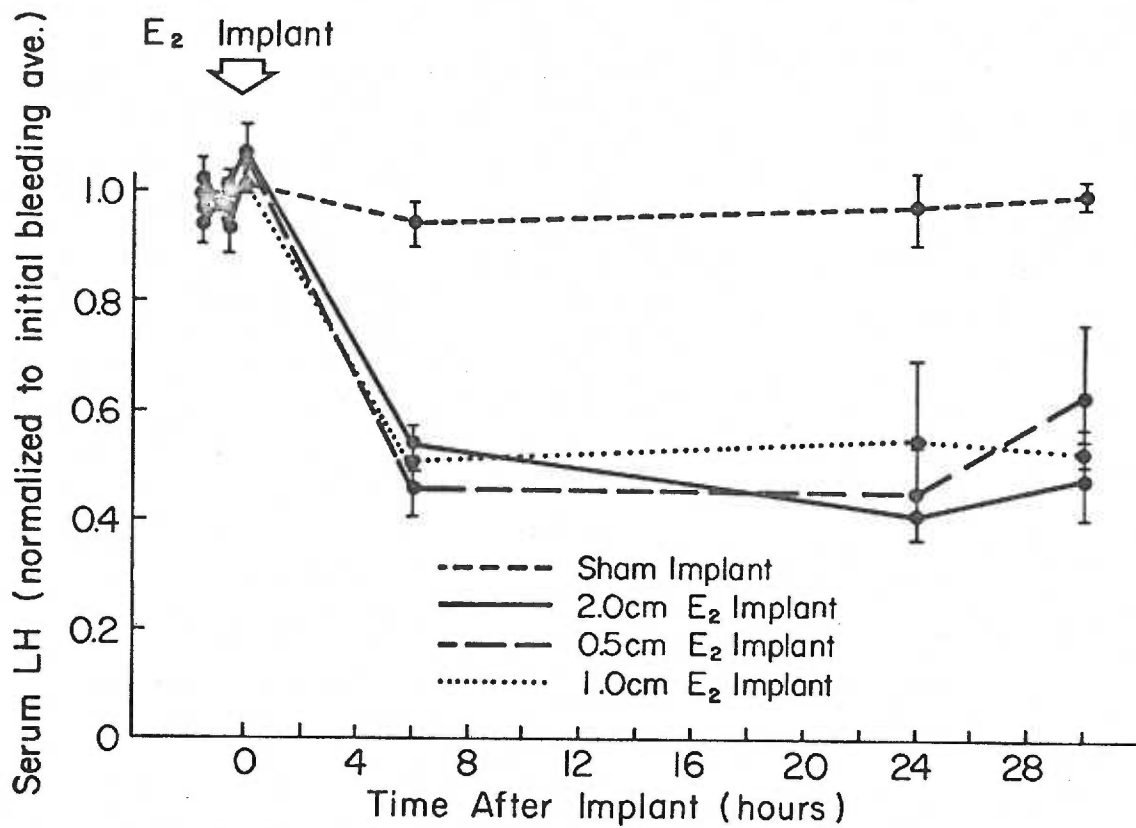


Appendix Figure 7. The time-course of the short-term negative feedback effects of empty (sham) silastic capsules on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys restrained in primate chairs. LH data are normalized to preimplant values. The data are presented as means $\pm$ standard errors.



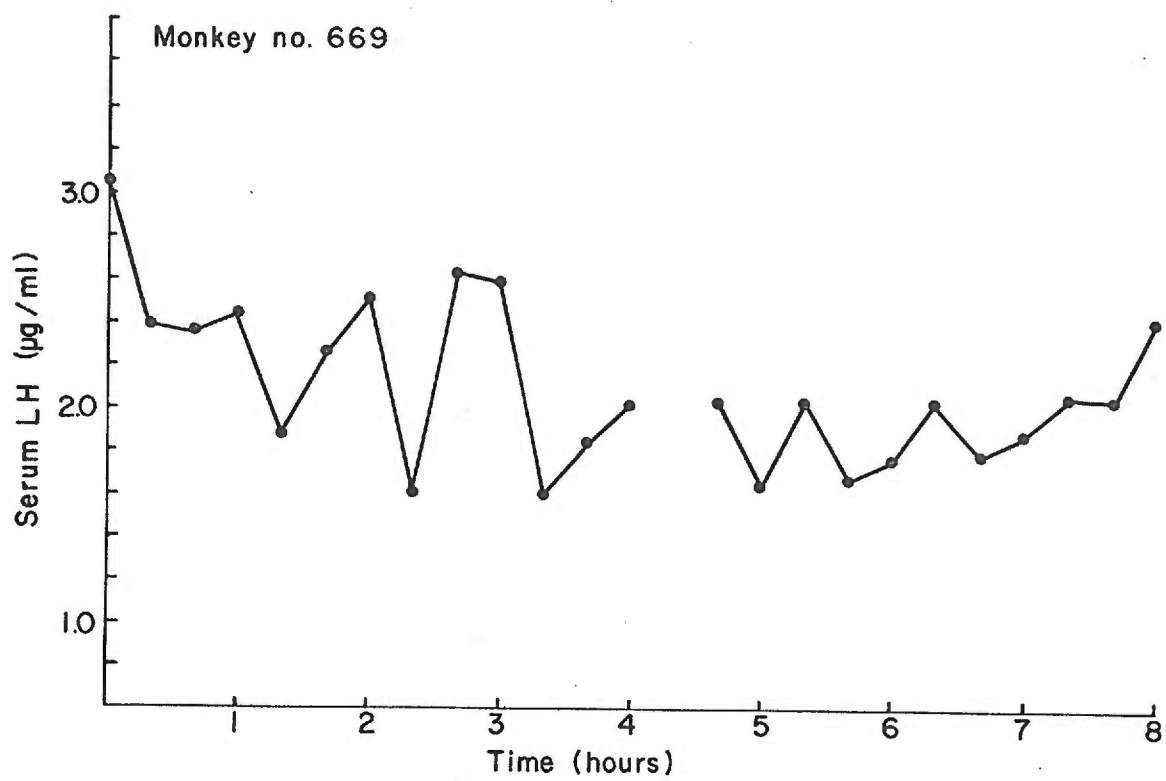
Appendix Figure 7

Appendix Figure 8. The time-course of the short-term negative feedback effects of silastic capsules containing estradiol-17 β on serum luteinizing hormone (LH) levels in caged, ovariectomized rhesus monkeys. The LH data are normalized to preimplant values. The data are presented as means $\pm$ standard errors.



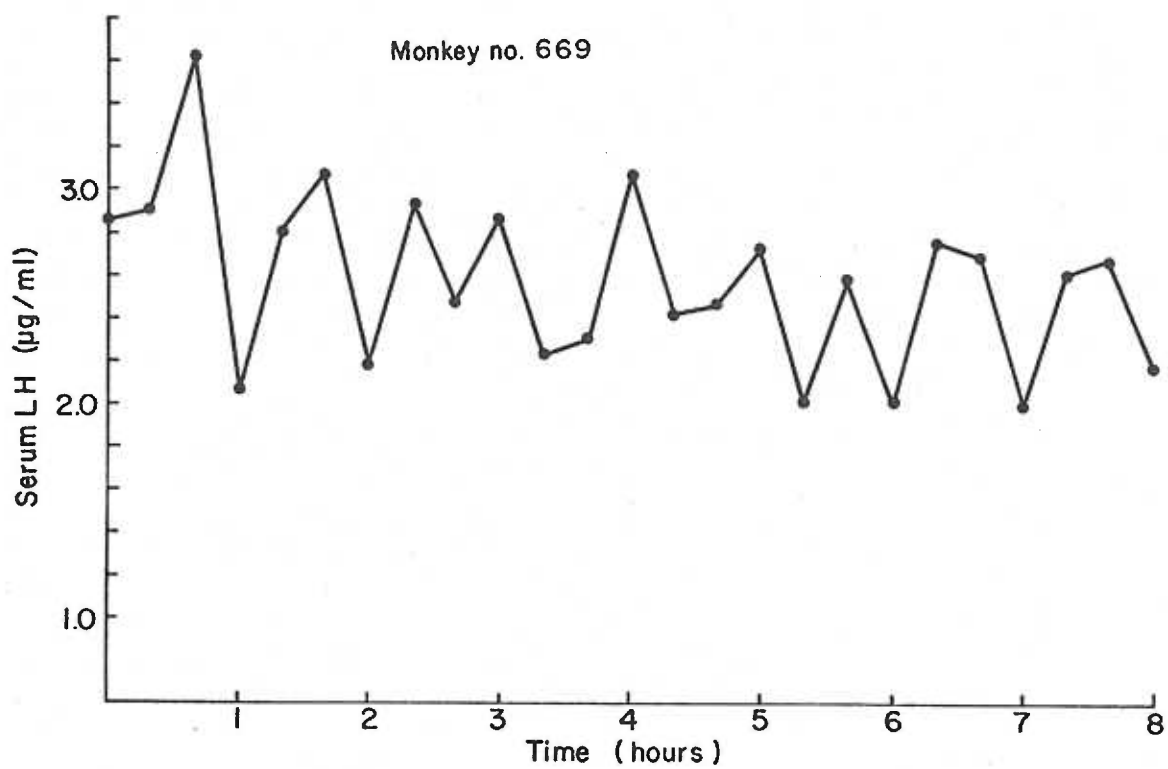
Appendix Figure 8

Appendix Figure 9. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #669. Each data point represents a single determination run in duplicate in the LH assay.



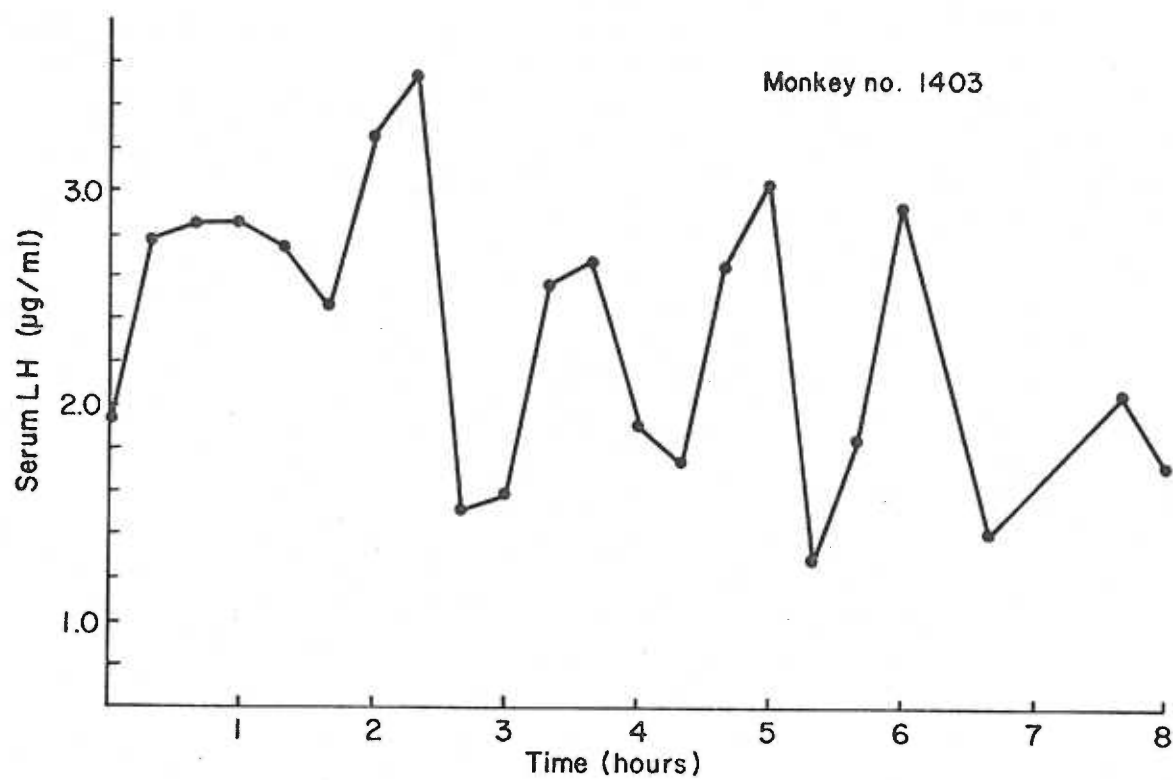
Appendix Figure 9

Appendix Figure 10. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #669. Each data point represents a single determination run in duplicate in the LH assay.



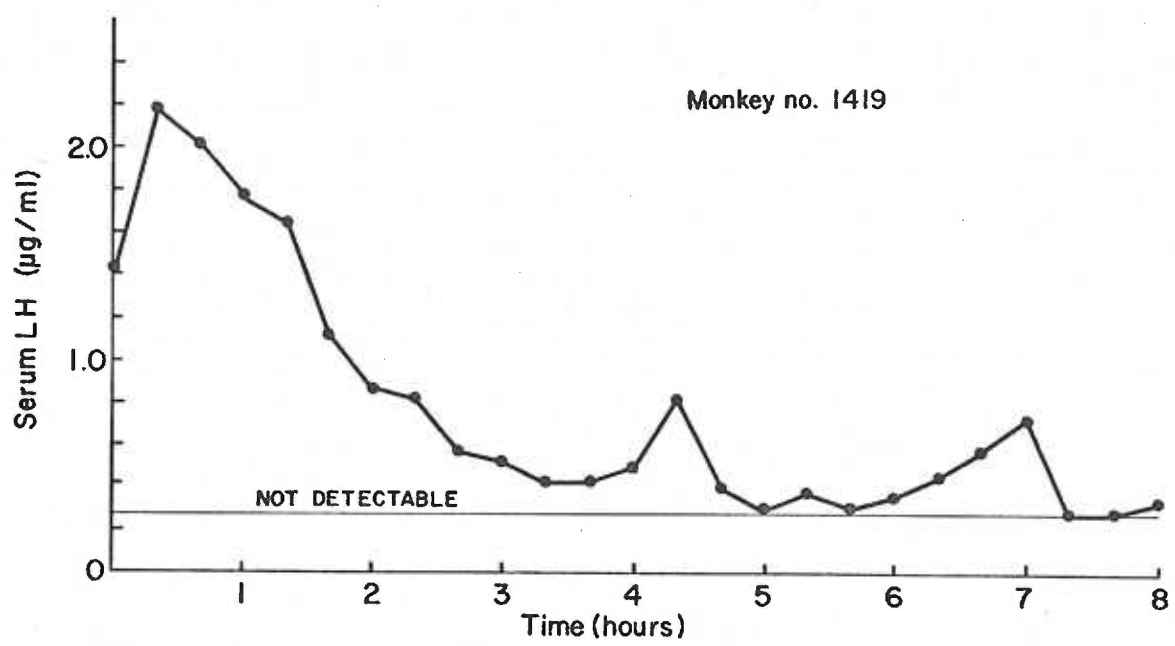
Appendix Figure 10

Appendix Figure 11. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #1403. Each data point represents a single determination run in duplicate in the LH assay.



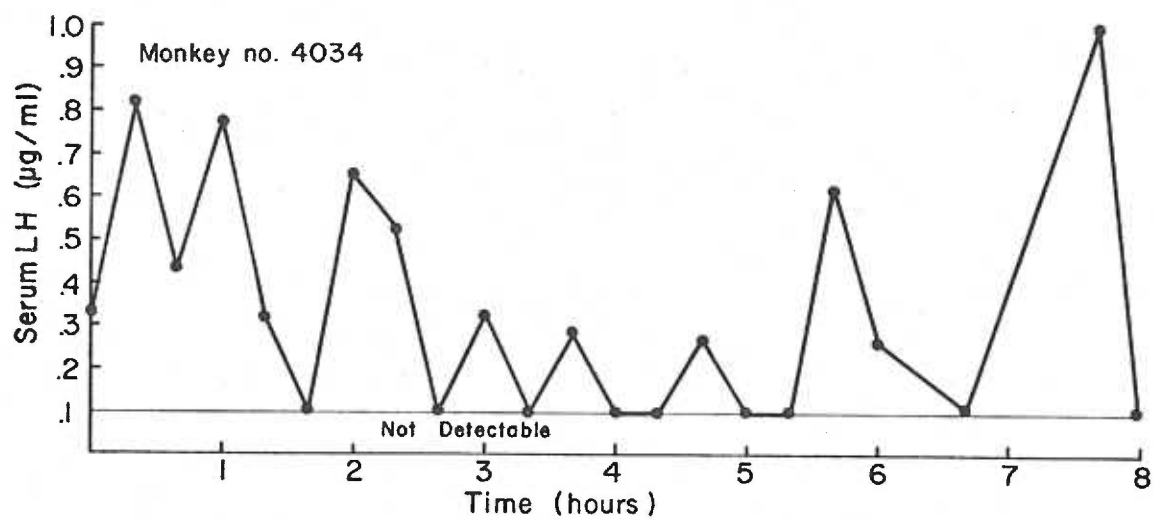
Appendix Figure II

Appendix Figure 12. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #1419. Each data point represents a single determination run in duplicate in the LH assay.



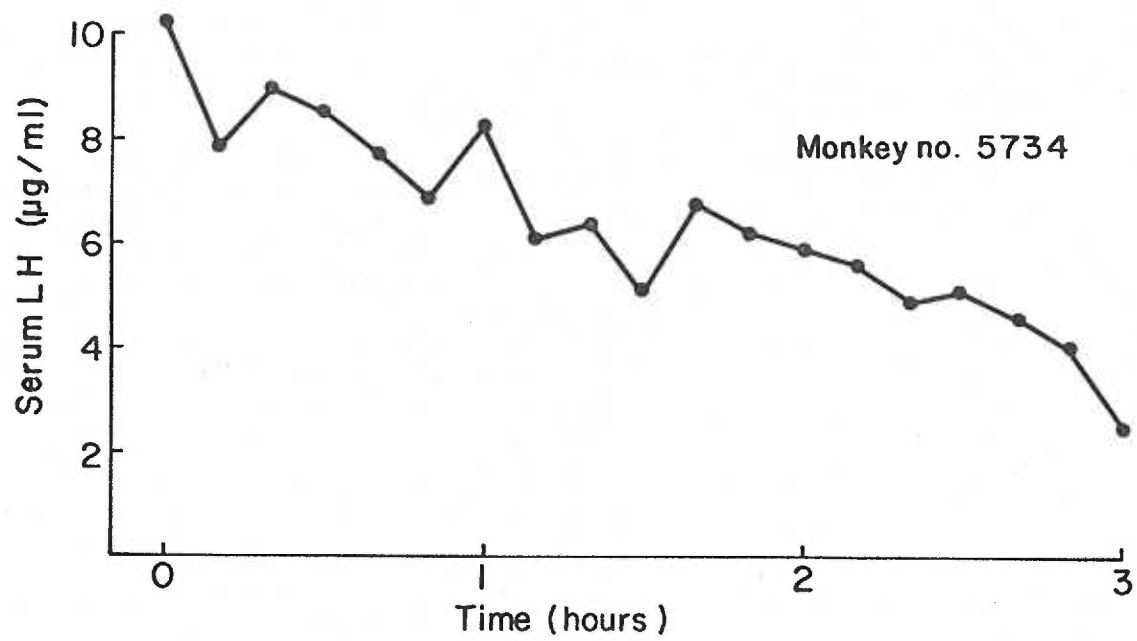
Appendix Figure 12

Appendix Figure 13. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #4034. Each data point represents a single determination run in duplicate in the LH assay.



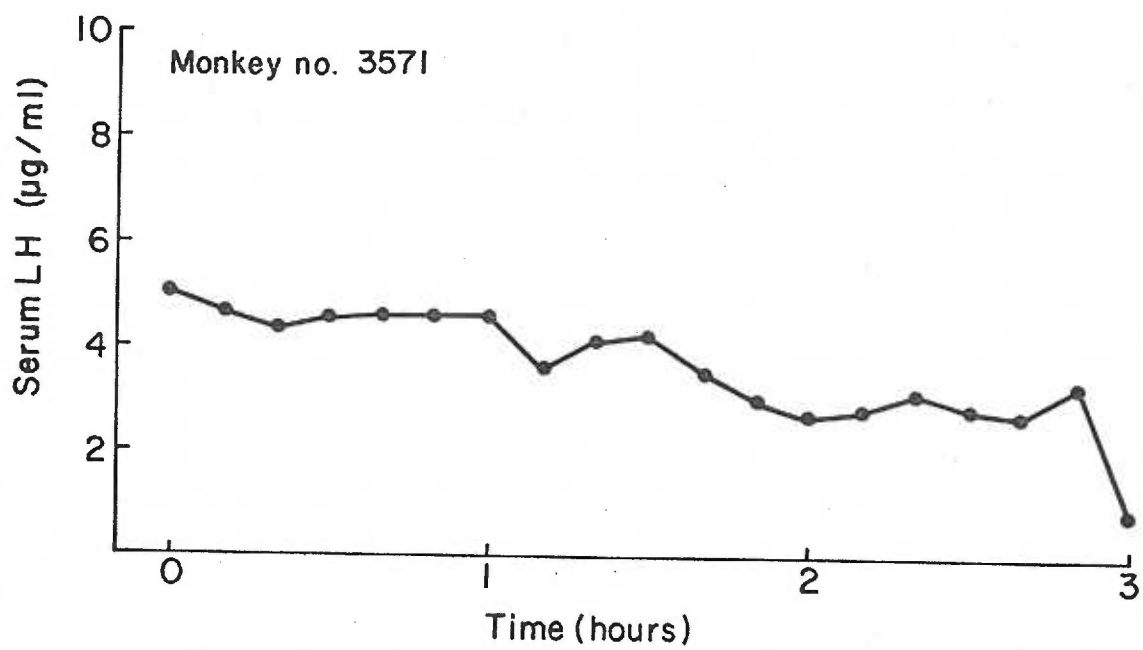
Appendix Figure 13

Appendix Figure 14. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #5734 under conditions of acute immobilization stress. Each data point represents a single determination run in duplicate in the LH assay.



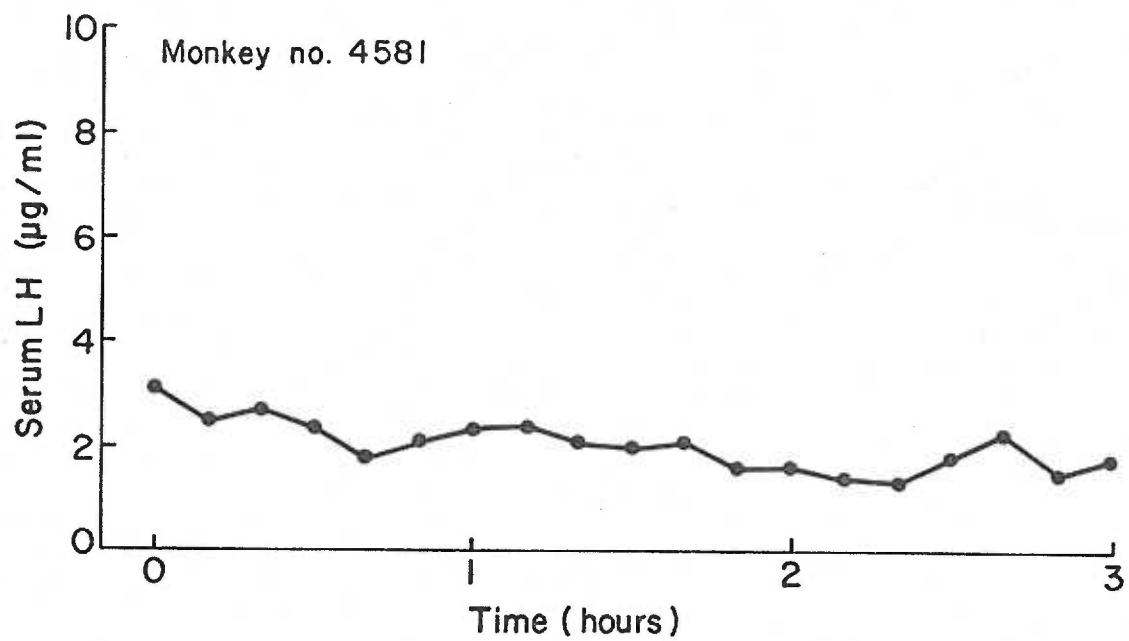
Appendix Figure 14

Appendix Figure 15. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #3571 under conditions of acute immobilization stress. Each data point represents a single determination run in duplicate in the LH assay.



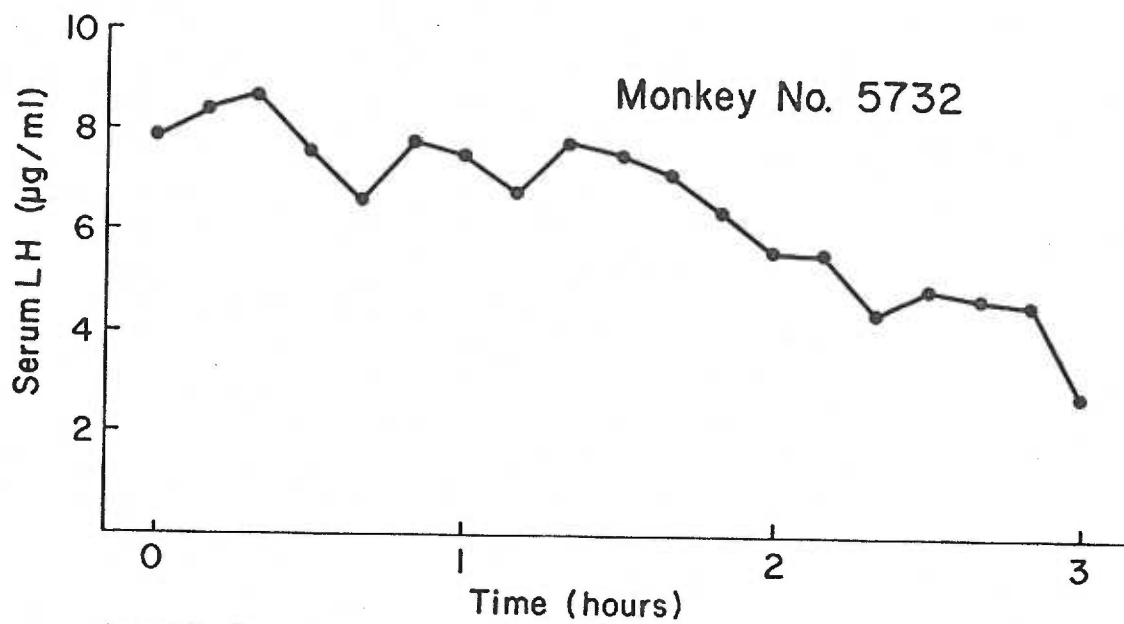
Appendix Figure 15

Appendix Figure 16. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #4581 under conditions of acute immobilization stress. Each data point represents a single determination run in duplicate in the LH assay.



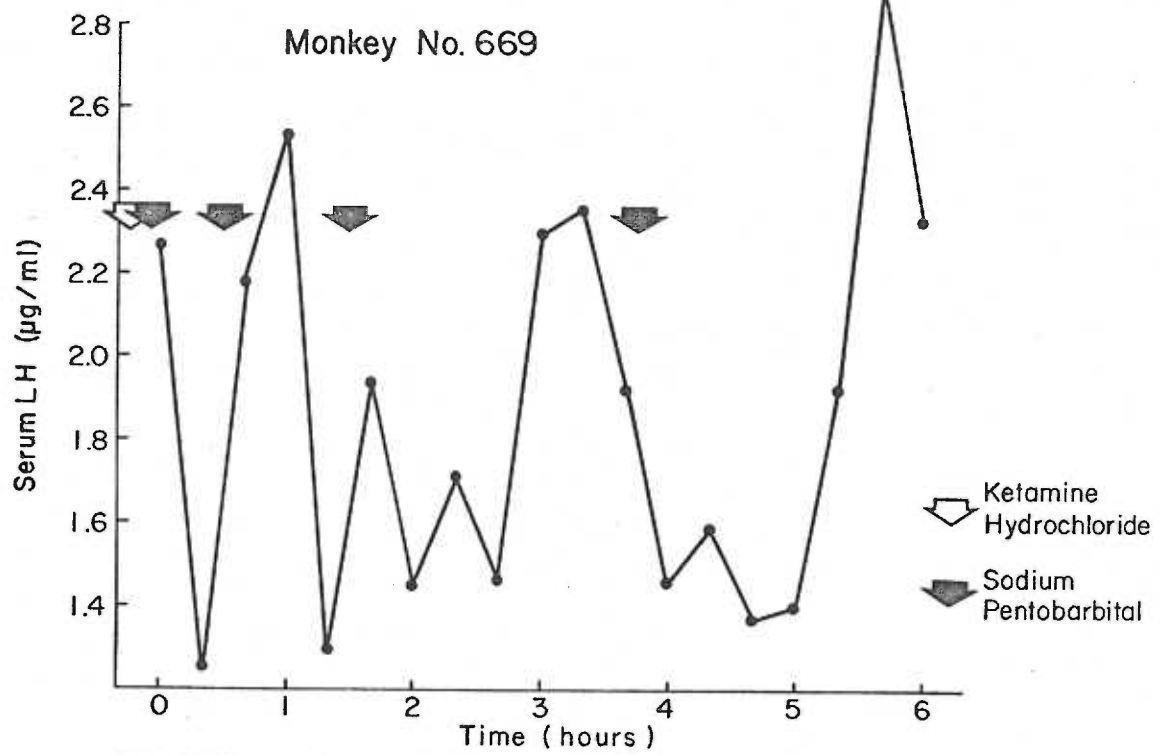
Appendix Figure 16

Appendix Figure 17. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #5732 under conditions of acute immobilization stress. Each data point represents a single determination run in duplicate in the LH assay.



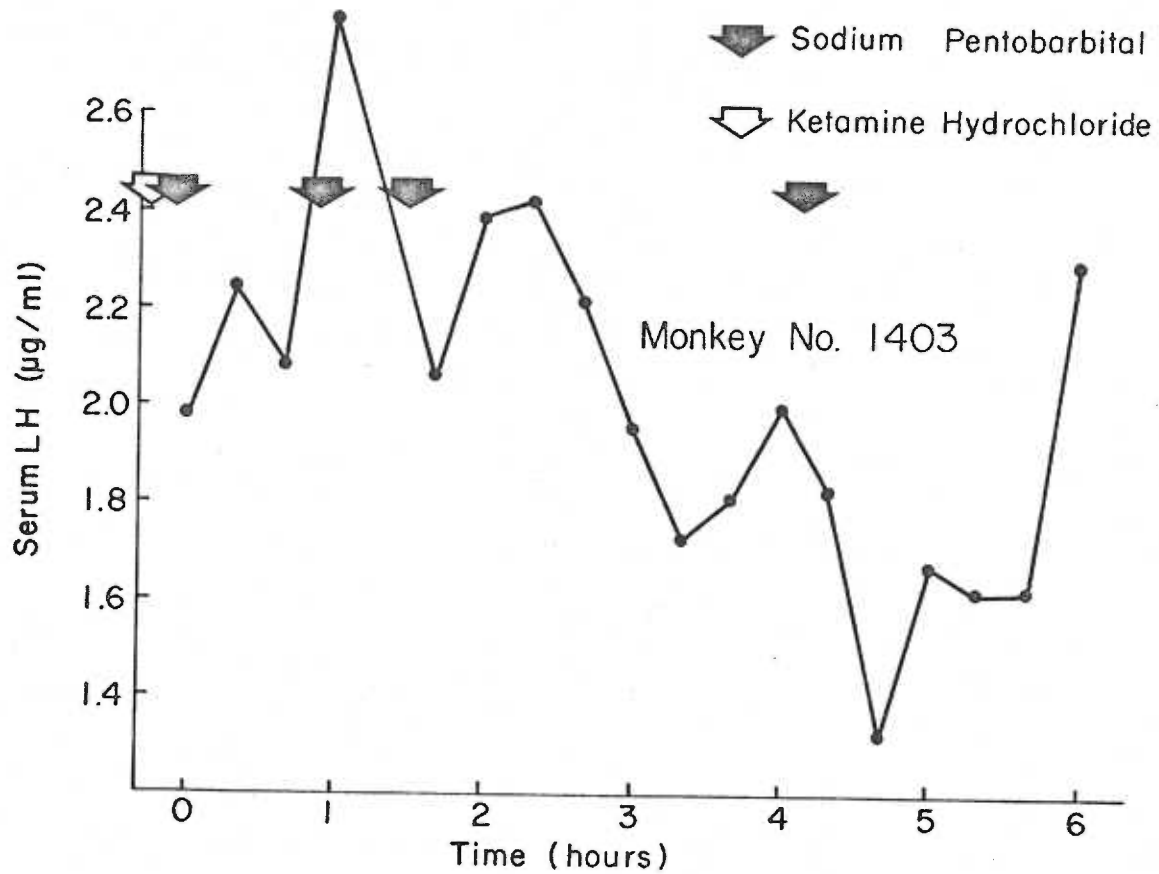
Appendix Figure 17

Appendix Figure 18. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #669 under deep sodium pentobarbital anesthesia. Each data point represents a single determination run in duplicate in the LH assay.



Appendix Figure 18

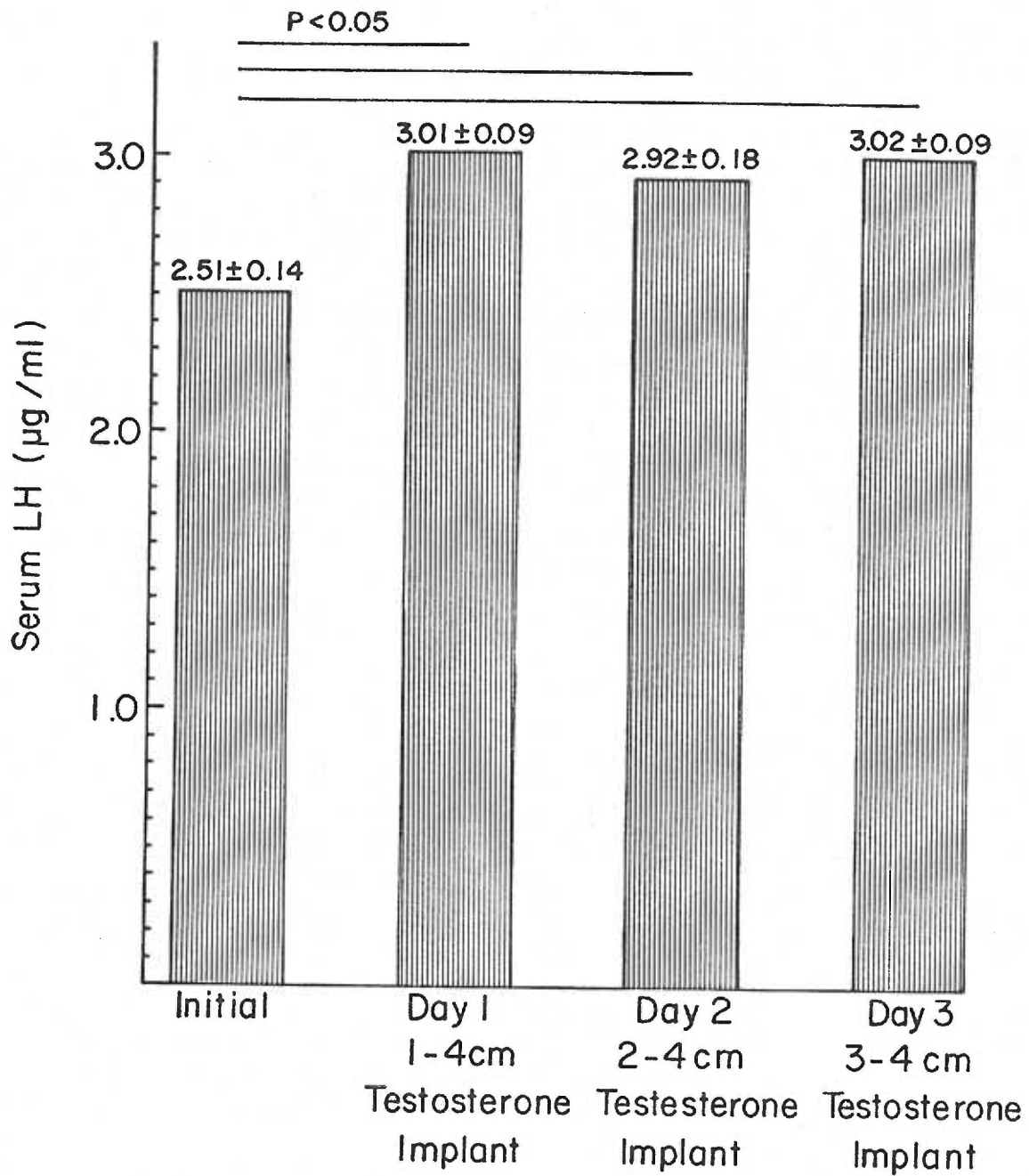
Appendix Figure 19. Pattern of serum luteinizing hormone (LH) concentration in ovariectomized rhesus monkey #1403 under deep sodium pentobarbital anesthesia. Each data point represents a single determination run in duplicate in the LH assay.



Appendix Figure 19

Appendix Figure 20. The effect of silastic capsules containing testosterone on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkey #2302. Lines above the top of bars denote statistically significant differences by analysis of variance. The data are presented as means $\pm$ standard errors.

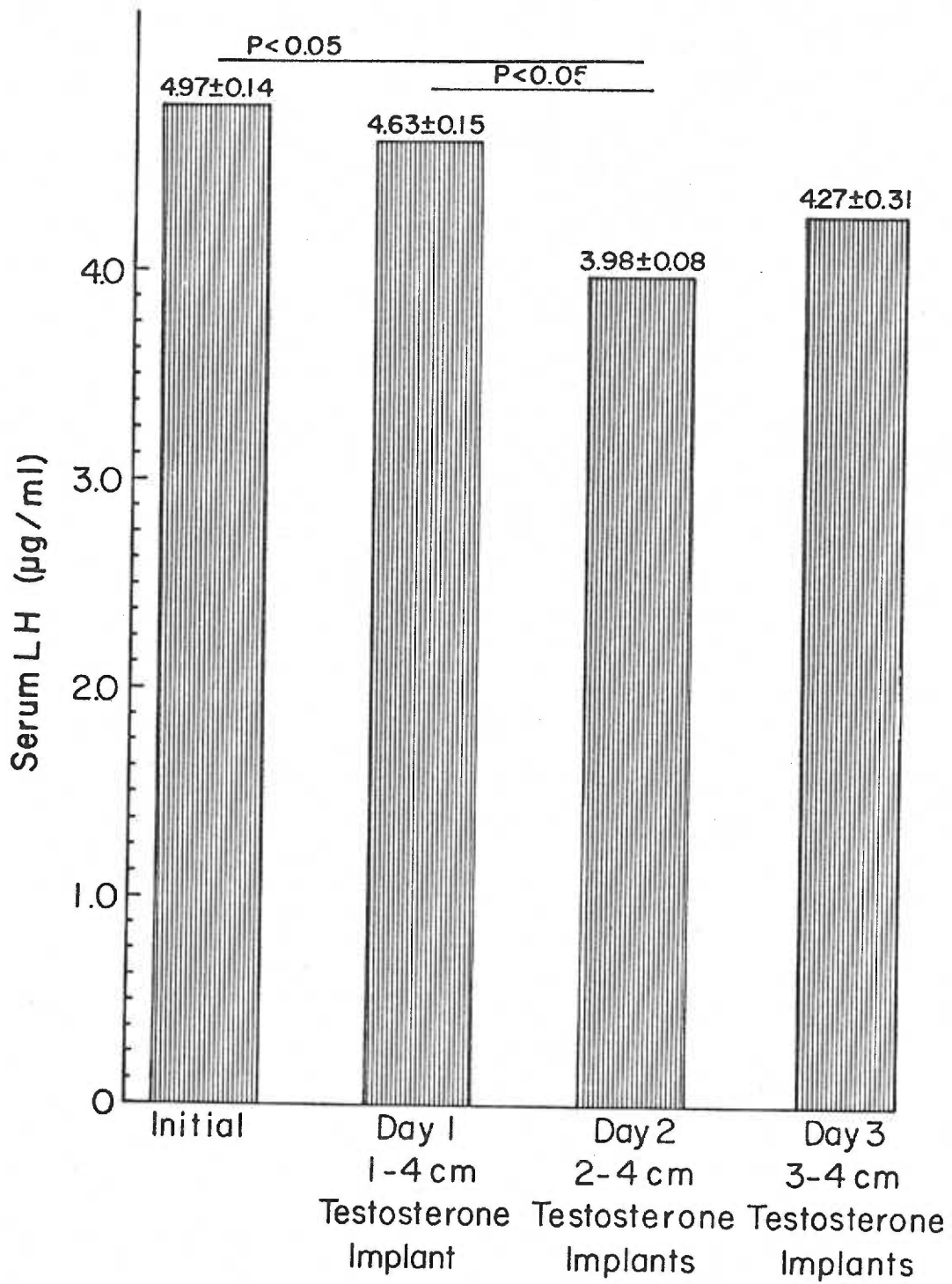
Monkey No. 2302



Appendix Figure 20

Appendix Figure 21. The effect of silastic capsules containing testosterone on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkey #812. Lines above the top of bars denote statistically significant differences by analysis of variance. The data are presented as means $\pm$ standard errors.

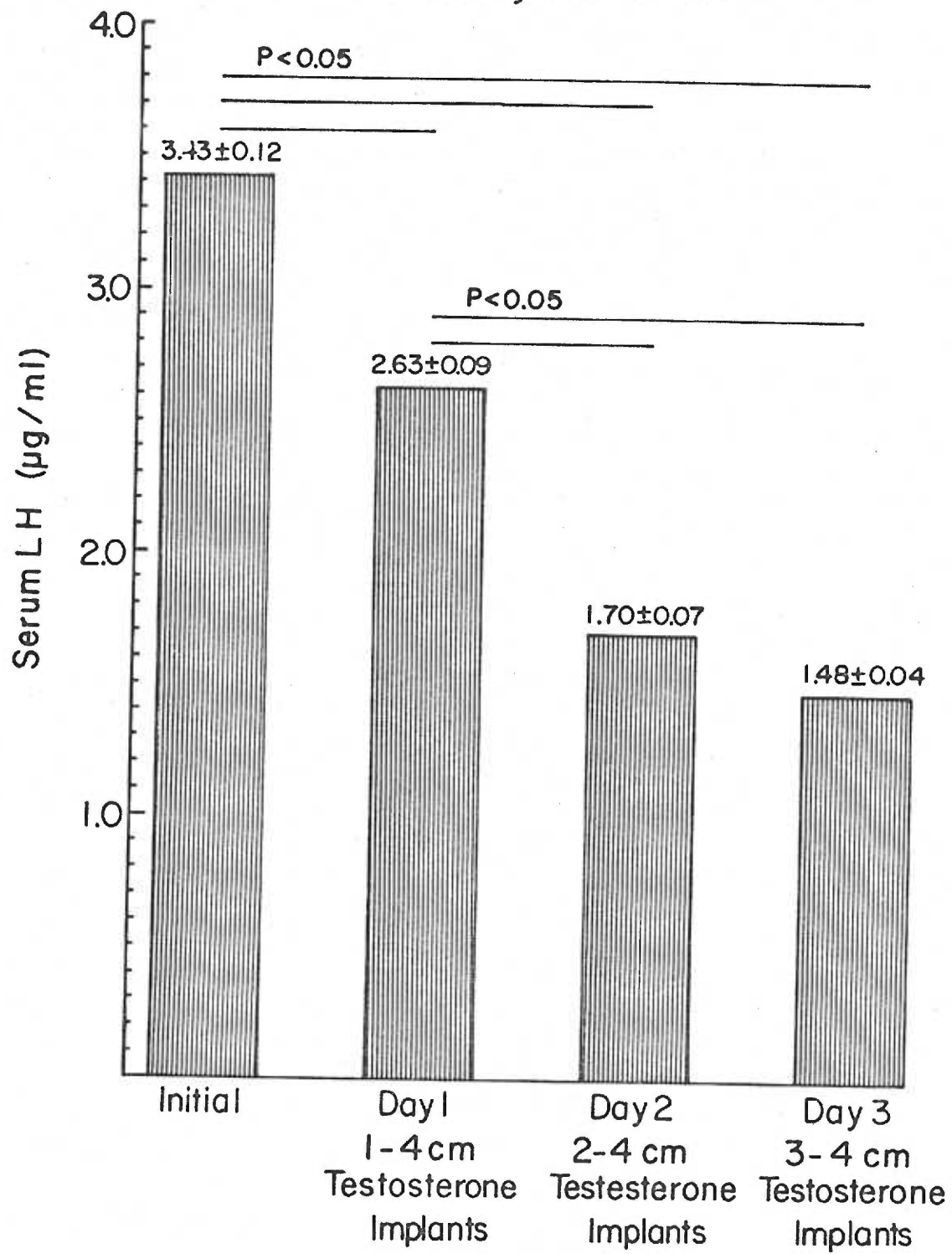
Monkey No. 812



Appendix Figure 21

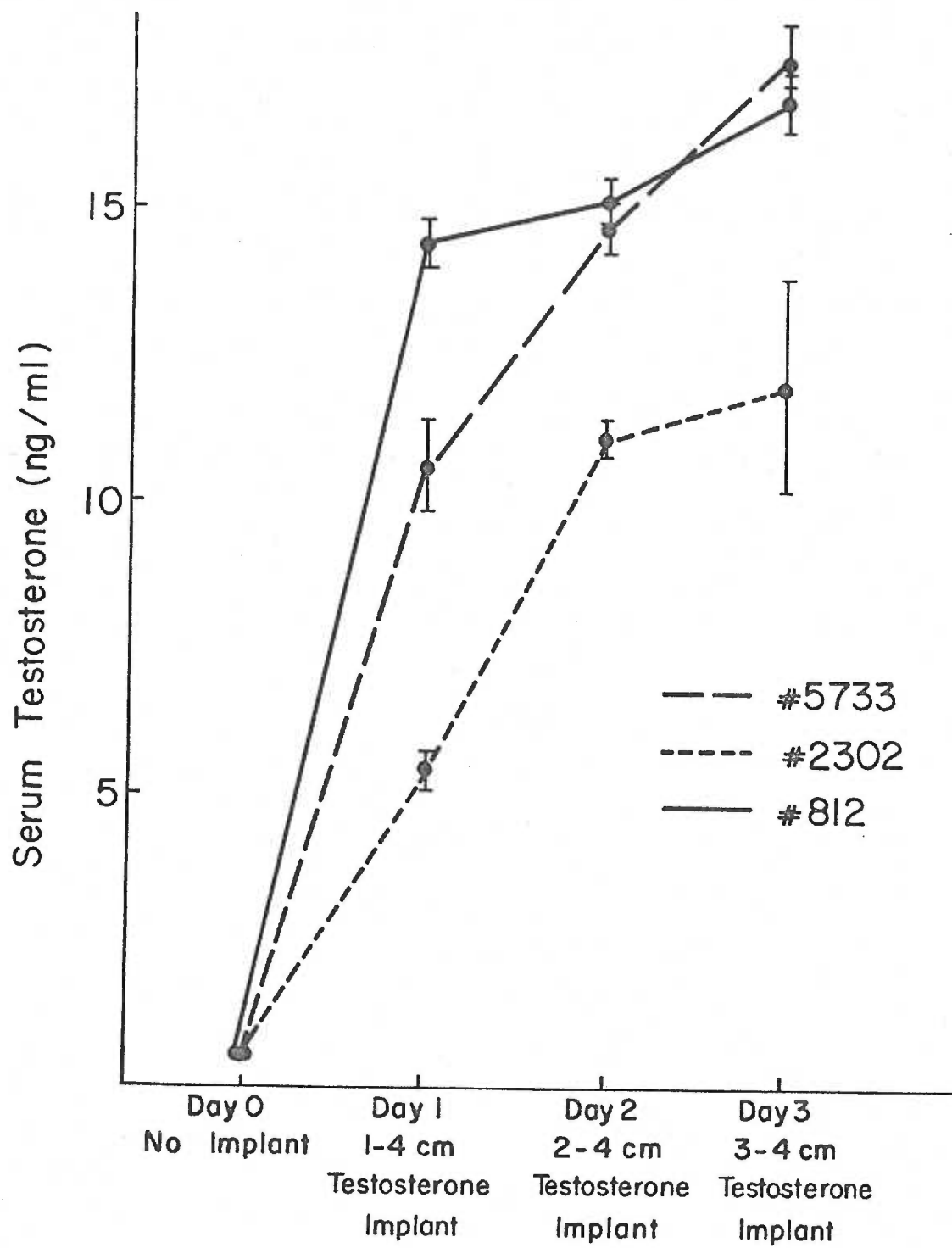
Appendix Figure 22. The effect of silastic capsules containing testosterone on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkey #5733. Lines above the top of bars denote statistically significant differences by analysis of variance. The data are presented as means $\pm$ standard errors.

Monkey No. 5733



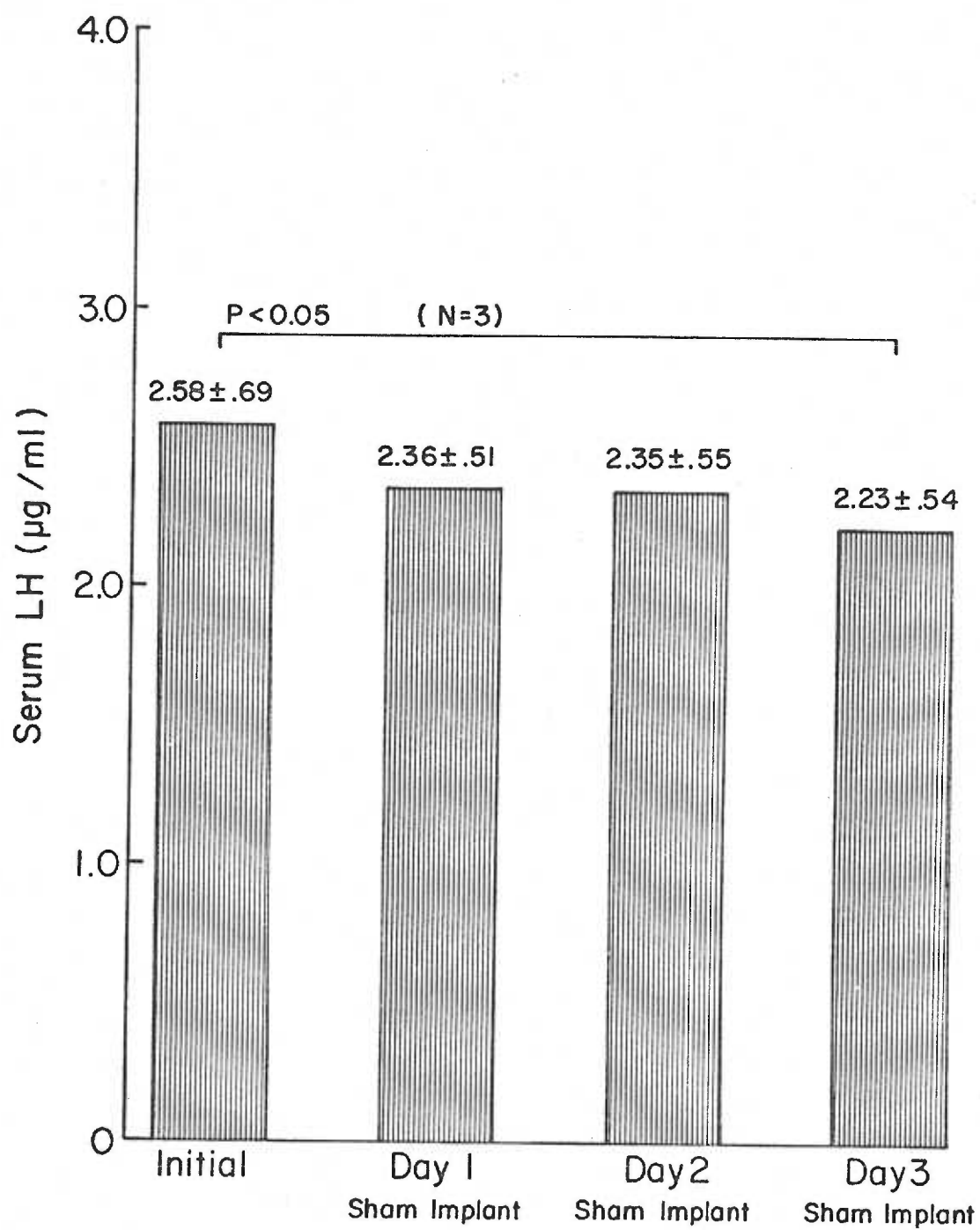
Appendix Figure 22

Appendix Figure 23. The concentration in serum testosterone in 3 ovariectomized rhesus monkeys effected by various sizes of silastic capsules containing testosterone. Each data point represents a mean $\pm$ the standard error of 3 determinations taken about 20 minutes apart.



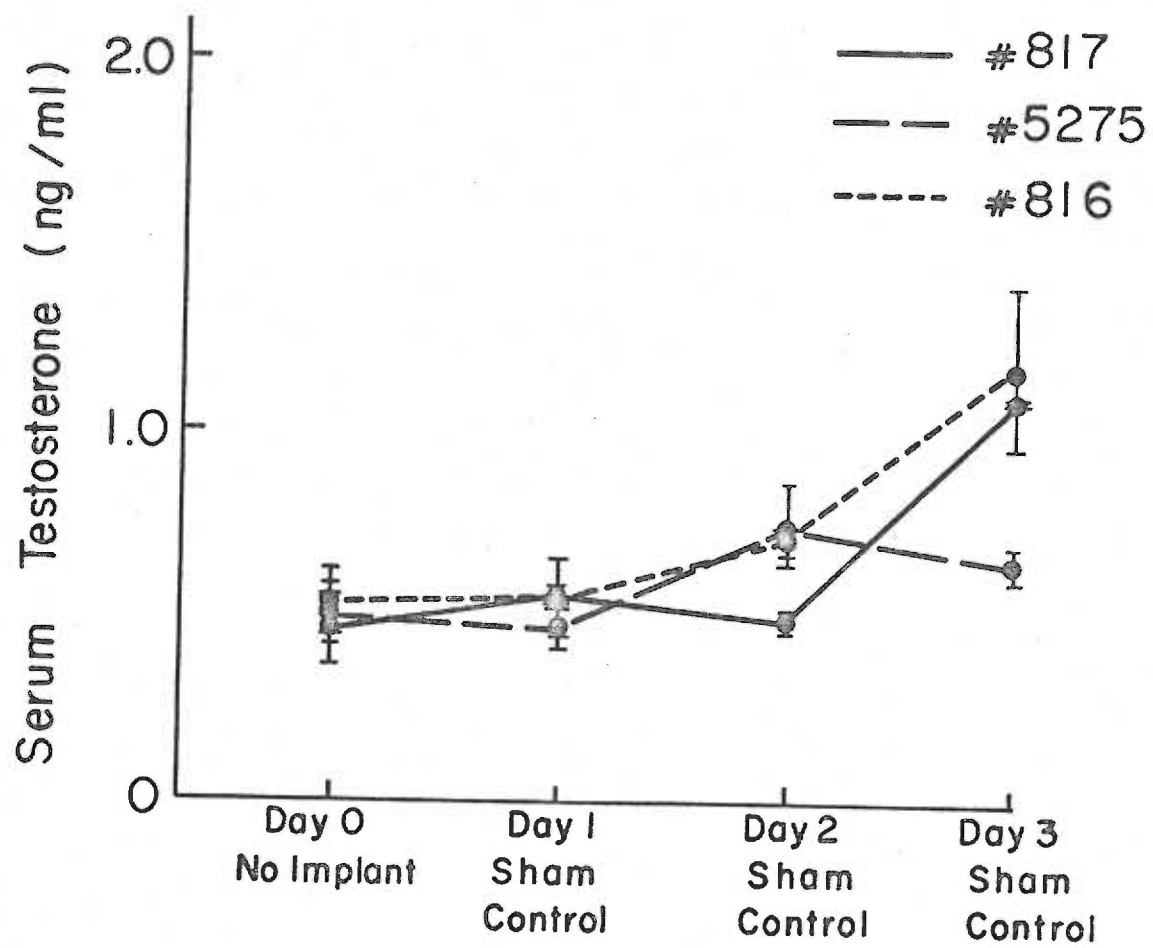
Appendix Figure 23

Appendix Figure 24. The effect of empty (sham) silastic capsules on serum luteinizing hormone (LH) levels in ovariectomized rhesus monkeys. The lines above the top of bars denote statistically significant differences by analysis of variance. The data are presented as means $\pm$ standard errors.



Appendix Figure 24

Appendix Figure 25. The concentration of serum testosterone in 3 ovariectomized rhesus monkeys influenced by empty (sham) silastic capsules. Each data point represents a mean $\pm$ standard error of 3 determinations taken about 20 minutes apart.



Appendix Figure 25

APPENDIX B

Acute stress influences various anterior pituitary hormones including adrenocorticotrophic hormone (Ganong, 1963), growth hormone (Meyer and Knobil, 1967), thyroid-stimulating hormone (Ducommun, Sakiz, and Guillemin, 1966), and prolactin (Neill, 1970). The influence of stress on LH secretion is not well documented for either rodent or primate species. Seyler and Reichlin (1973) showed that severe blood volume depletion induces an acute release of LH in the rat, and Proudftt and Schwartz (1974) reported that cardiac puncture reverses the pentobarbital blockade of ovulation in rats. Earlier, Brown-Grant, Davidson, and Greig (1973) had noted that certain nonsexual stimuli such as surgical trauma or handling in a novel environment induced ovulation in a small number of rats exposed to a constant-light regimen; and Davidson, Smith, and Bowers (1973) reported LH release in the female rat during mating. Furthermore, Ajika et al. (1972) demonstrated that ether anesthesia raised mean plasma levels of LH in ovariectomized rats. Finally, Dunn, Arimura, and Scheving (1972) showed that ether stress abolished the 24-hour rhythm in serum LH concentrations in adult male rats. These data suggest that under certain experimental conditions stress influences LH secretion in the rodent.

In primates, the influence of stress on LH secretion is less well defined. Charters, Odell, and Thompson (1969) were unable to demonstrate significant changes in plasma LH levels during a variety of surgical procedures in either menstruating or postmenopausal women, whereas Aona et al. (1972) showed that major surgical stress induced an acute release of LH in male patients. These data suggest that under certain conditions stress influences LH secretion in the primate.

Our experiments were designed primarily to determine whether sex differences play a role in the estrogen-mediated gonadotropin feedback mechanism in the rhesus monkey. The sex-specific differences discovered in both the negative and positive estrogen-gonadotropin feedback systems could very well be a function of a sexually dimorphic stress response, rather than, as we had supposed, a result of the estrogen treatment. This interpretation does not, however, militate against the basic conclusions that in the rhesus monkey there appears to be a sexually differentiated gonadotropin control system and that this control system is differentiated as a function of the fetal hormonal environment; this conclusion is buttressed by the fact that genetic female rhesus monkeys, androgenized in utero, behave more like their male counterparts than normal female controls.

On the basis of our own experience, this alternative interpretation remains only a remote possibility. In our work on the rhesus monkey, we have observed neither an acute suppression nor augmentation of LH release under the usual implantation-bleeding protocol described in the body of the thesis. Moreover, in Appendix A, Section IX, I have provided empirical support for my contention that acute stress did not interfere with the experimental design. In the experiment outlined in this section, the data show that the sham implant and subsequent bleeding procedures did not influence tonic serum LH levels in ovariectomized female rhesus monkeys, at least over the 30-hour time course of this experiment. The experiments outlined in the body of the thesis, however, were not specifically designed to delineate the potential role of stress in the gonadotropin-regulating system, and therefore, this possibility should be entertained.

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