INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again – beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106

76-8967

FELDMAN, Susan Carol, 1943-
TESTOSTERONE SYNTHESIS BY FETAL RAT TESTES
AND THEIR RESPONSE TO LH.

The City University of New York, Ph.D., 1976
Biology

Xerox University Microfilms, Ann Arbor, Michigan 48106

© COPYRIGHT BY

SUSAN CAROL FELDMAN

1975

TESTOSTERONE SYNTHESIS BY FETAL RAT TESTES
AND THEIR RESPONSE TO LH

by

Susan Carol Feldman

A dissertation submitted to the Graduate
Faculty in Biology in partial fulfillment
of the requirements for the degree of Doctor
of Philosophy, The City University of New York.

1975

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

June 27, 1975
Date

Max Hamburgh
Chairman of Examining Committee
Prof. M. Hamburgh, City College

July 7, 1975
Date

L.G. Moriber
Executive Officer
Prof. L.G. Moriber

Constance R. Martin
Prof. C. Martin
Hunter College
Institution

Robert A. Ortman
Prof. R. Ortman
City College
Institution

Eric Bloch
Prof. E. Bloch
Albert Einstein College of Medicine
Institution

Brij B. Saxena
Prof. B. Saxena
Cornell University
Institution

Michael Gordon
Prof. M. Gordon
Yale University
Institution

R.S. Rosenfeld
Prof. R. Rosenfeld
Montefiore Hospital
Institution

The City University of New York

Abstract

The ability of fetal rat testes (14.5, 15.5, 16.5, 18.5 and 20.5 days) to synthesize testosterone and respond to LH was studied as a function of testis age. Responsiveness to LH was measured as the change in the rate of testosterone synthesis at each age during short-term incubation. In addition, the concentrations of testosterone in the testes and genital tracts from fetuses aged 14.5 - 21.5 days was measured in order to correlate the *in vitro* results with the *in vivo* situation.

Testosterone secreted into the incubation medium and present in reproductive tissues was measured by radioimmunoassay. The assay used was that of Brenner, Gurerrero, Cekan, and Diczfalusy (1973). The antibody was used at a dilution of 1:30,000 and bound only testosterone and dihydrotestosterone. Testosterone standards were extracted from medium 199, and sample and standard extracts were not purified further prior to radioimmunoassay since testes did not metabolize ^{3}H-testosterone to any significant extent.

The concentration of testosterone secreted into the medium during incubation represented an accurate estimation of the total synthesized, within the limits of partitioning between medium and testes. Without LH the testes accounted for a constant fraction of the total synthesized (30%); with LH this decreased to 2% by 4 hours. LH did not affect the retention or permeability of the testes to testosterone. The concentration of testosterone synthesized without LH increased proportionately with time from 30 minutes onward, and with tissue weight both with and without LH at all ages studied.

Testosterone synthesis could not be demonstrated at 14.5 days.

From 15.5 to 20.5 days the response to LH increased with age, both in terms of the rate of synthesis with LH relative to the control level at each age, and in terms of the sensitivity to LH. At 15.5 days, 160 ng LH/ml increased the rate of synthesis 4-fold, to 1.3 ng/testis/hr. At 16.5 days, the same LH dose increased the rate 8-fold to 8.8 ng/testis/hr. At 18.5 days, 64 ng LH/ml increased testosterone synthesis 15-fold over base levels to a rate of 20 ng/testis/hr; and at 20.5 days, 16 ng/ml increased synthesis 20-fold to a rate of 36 ng/testis/hr. At 20.5 days testosterone synthesis was not affected by FSH or prolactin.

Testosterone was not detectable in testes and genital tracts from 14.5 day testes. From 15.5 to 17.5 days the concentration of testosterone in the testes increased approximately 10-fold and then remained constant until day 20.5. The concentration of testosterone in the genital tracts remained fairly constant with age.

The increase in *in vitro* testosterone synthesis with age from 15.5 to 18.5 days is consistent with a constant level of synthetic activity per Leydig cell; the increase from 18.5 to 20.5 days suggests increased capacity per cell. The change in sensitivity to LH with age is attributed to increased efficiency of LH utilization at a step preceding steroid synthesis. These findings are similar to those seen in the response of postnatal testes to injected LH (Odell et al, 1974) and imply similar regulatory mechanisms at both stages of testis function. The increase in the testicular testosterone concentration and its maintenance until birth is consistent with the idea that testis function is under gonadotropic regu-

lation at this time. The presence of testosterone in the fetus throughout sexual development implies a role for the fetal testis in the masculinization of the wolffian ducts and developing nervous system.

This thesis is dedicated to

Dr. Eric Bloch

whose friendship and scientific excellence made my years

in his laboratory an experience of learning and joy

and

Dr. Max Hamburgh

a great teacher and a good friend

ACKNOWLEDGEMENTS

There are many people whom I would like to thank for their help and support in making this thesis possible: the faculty at City College for their moral and financial support; the Department of Obstetrics and Gynecology at A.E.C.O.M. in whose department this study was carried out; Dr. Constance Martin, whose help with the thesis was invaluable; Dr. Robert Ortman, for his help in writing the thesis; Dr. Robert Rosenfeld, for his generous gift of the antibody used in this study; Dr. Robert Boyar, who taught me the radioimmunoassay for testosterone; Frieda Halpern; Dr. Fatma Aleem, for the use of her gas-liquid chromatograph; Olga Van Damme, for her technical assistance; David Starke, for drawing the figures; Brenda Zamboni, for preparing the manuscript.

I would like to thank the technical and secretarial staff on the first floor of the Ullmann building for their friendship over the years, particularly Barbara Kazmarek, Audrey Klein, and John Hong.

Finally, there are those special people to whom I am extremely grateful for allowing me to share their lives: Rudolf Wittenberg, Knut Larsson, Benjamin Thysen, Chhanda Gupta, Sion Mohaber, Michael Gatz, Fred Schatz, Nora Harlow, Lester Nafzger, Corinne Johnson, Lucy Halboth, Sam Feldman, Herb and Joanna Schaumberg, Eric and Dale Heyer, and my best friend, Robert Muller.

Table of Contents

Title Page 1

Copyright Page 2

Approval Page 3

Abstract 4

Dedication 7

Acknowledgements 8

Table of Contents 9

List of Tables 11

List of Figures 12

Introduction 13

Materials & Methods
- I. Materials
  - Animals 22
  - Incubation Medium 22
  - Solvents 22
  - Steroids 25
  - Pituitary Hormones 26
- II. Methods
  - Incubation of fetal testes 28
  - Determination of endogenous tissue testosterone 29
  - Extraction and purification of testosterone and other steroids
    - Extraction of steroids 29
    - Thin-layer chromatography 30
  - Radioactivity determination 31

Radioimmunoassay of Testosterone
- I. Introduction 33
- II. Materials
  - Phosphate buffer system 36
  - Charcoal 36
  - Antibody 37
  - Glassware 37
- III. Procedure 37

IV. Standard curve for the radioimmunoassay of testosterone
Antibody concentration 39
Standard curve 46
Blank value 54
Accuracy 55
Volume of extract bound 56
Specificity of the antiserum for testosterone 56

Results
I. The experimental design
Metabolism of testosterone 62
Measurement of testosterone by gas-liquid chromatography and radioimmunoassay 63
Partitioning of testosterone between medium and testes 65

II. Testosterone synthesis and LH responsiveness of fetal testes
Testosterone synthesis in the absence of LH 70
20.5 day testes 75
14.5, 15.5, 16.5 & 18.5 day testes 76
Response of fetal testes to LH
20.5 day testes 88
14.5, 15.5, 16.5 & 18.5 day testes 92
Sensitivity and responsivity to LH 104
Specificity of the response to LH 108
Endogenous testosterone concentrations in fetal reproductive tissues 108

Discussion 111

Summary & Conclusions 127

Bibliography 132

List of Tables

Table No.	Title	Page
I	Composition of Medium 199	23-24
II	Radioimmunoassay of Testosterone	48-49
III	Steroid Specificity of the Antibody	58
IV	Partitioning of Testosterone Between Testes & Incubation Medium with and without LH	67
V	Testosterone Synthesis by 20.5 Day Fetal Testes	72
VI	Testosterone Synthesis as a Function of Testis Age and Length of Incubation	77
VII	Testosterone Synthesis by Fetal Testes: Proportionality to Tissue Weight	81
VIII	Testosterone Synthesis by 20.5 Day Fetal Testes: The Effect of LH Stimulation	85
IX	Testosterone Synthesis by Fetal Testes as a Function of Age and LH Stimulation	94
X	Testosterone Synthesis by Fetal Testes: Proportionality to Tissue Weight	103
XI	Maximum Response to LH Reached as a Function of LH Dose and Fetal Testis Age	107
XII	Endogenous Testosterone Concentrations in Testes and Genital Ducts with Fetal Age	110

List of Figures

Figure No.	Title	Page
1.	Radioimmunoassay of testosterone: displacement of ^{3}H-testosterone bound to the antibody	41
2.	Log-logit transformation of Figure 1	43
3.	Relationship between antibody concentration and sensitivity and slope of the displacement curve	45
4.	Radioimmunoassay of testosterone: displacement of ^{3}H-testosterone by testosterone extracted from medium 199	51
5.	Log-logit transformation of Figure 4	53
6.	Radioimmunoassay of testosterone: specificity of the antibody	60
7.	Synthesis of testosterone by 20.5 day testes	74
8.	Synthesis of testosterone as a function of age	79
9.	Testosterone synthesis by 20.5 day fetal testes in response to increasing concentrations of LH	83
10.	Synthesis of testosterone by 20.5 day fetal testes: the effect of LH	87
11.	Responsiveness of 20.5 day testes to LH	91
12.	Synthesis of testosterone as function of age: the effect of LH	96
13.	Testosterone synthesis by 18.5 day fetal testes in response to increasing concentrations of LH	100
14.	Responsiveness of fetal testes aged 15.5, 16.5, 18.5, & 20.5 days to LH	102
15.	Maximum response to LH reached as a function of testis age	106

Introduction

Sexual development of the male mammal from fetus to adult is characterized by two periods of testicular activity. The first of these, depending upon the species, is fetal or perinatal. The second is pubertal. During this first phase, which always includes some portion *in utero*, the testes differentiate; the Leydig cells proliferate and then regress; there is masculinization of the uro-genital region, via the stimulus of testicular testosterone; and the hypothalamic-pituitary-testicular axis is established. The pubertal phase of testicular activity is characterized by an increase in the number of Leydig cells under the influence of an integrated negative feedback axis, an increase in the amount of testosterone secreted by the testes, and the maturation of the secondary sexual characteristics.

The question of the regulation of testis function during the peri-natal period raises the problem as to the development of the hypothalamic-pituitary-testicular axis. If fetal testis function is not under pituitary regulation then it becomes necessary to postulate control mechanisms unique to the fetus. In order to resolve this problem it is necessary to know to what extent the testis, pituitary, and hypothalamus are functional and responsive to each other and to characterize these responses in terms of their similarity to those found in the adult.

Two major characteristics of adult testes are the ability to synthesize testosterone from endogenous precursors and to respond to LH by increasing the rate of testosterone synthesis. A recent review of the pathways of testicular biosynthesis of testosterone by Eik-Nes (1970) states that testosterone is synthesized originally

from acetate. The predominant pathway appears to be: acetate to squalene to lanosterol to cholesterol. From cholesterol the first steroid formed is pregnenolone - the central precursor for most steroid hormones. The synthesis of testosterone from pregnenolone can occur via several pathways depending upon the order in which the side chain of pregnenolone is cleaved and ring A converted to the Δ^4-3-keto structure. Pregnenolone is converted to progesterone (via the action of the enzyme complex 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$-steroid isomerase) to 17α-hydroxyprogesterone, and then to testosterone via androstenedione. The alternate pathway involves pregnenolone conversion to 17α-hydroxypregnenolone to dehydroepiandrosterone and androstenediol to testosterone. These pathways are not completely separate as 17α-hydroxypregnenolone can be converted to 17α-hydroxyprogesterone and dehydroepiandrosterone is a precursor for androstenedione. According to studies by Hall (1970) the regulation of testosterone synthesis by LH occurs in the conversion of cholesterol to pregnenolone via increased hydroxylation of cholesterol at carbon-22, although this may not be the only locus of action of LH.

The endocrine status of the fetal testis has been largely inferred *in vivo* and *in vitro* from studies on the role of the fetal testis in the masculinization of the wolffian ducts and urogenital sinus. In the mammal the gonad differentiates from the germinal ridge into a structure which initially has both cortical and medullary elements - the "indifferent gonad". In the male, as the cortex regresses the seminiferous tubules and interstitial elements, including the Leydig cells, differentiate. In the rat the testis is first recognizable on day 14.5 and the cortex disappears by day 17.5

(Schlegel et al, 1966). In the mouse this process is advanced by approximately two days. In the rat the Leydig cells are recognizable with ordinary staining techniques, on day 15.5, and they rapidly increase in number by day 16.5. Thereafter, the number of Leydig cells increases more slowly until a maximum is reached on day 19 and then it declines (Roosen-Runge and Andersen, 1959). One of the key enzyme systems of testosterone biosynthesis and characteristic of the Leydig cells appears on day 13.5 (3β-hydroxysteroid dehydrogenase, $\Delta 4,5$-steroid isomerase), before the Leydig cells are histologically distinguishable (Niemi and Ikonen, 1961). In the mouse, (Scheib and Lombard, 1971) this enzyme is first detectable in cells either within or close to, the walls of the seminiferous tubules. As development proceeds, these cells are displaced into the inter-tubular spaces. The exact origin of the Leydig cells, either in the fetus or in the pre-pubertal male is not completely resolved.

The development of the mammalian genital tract also goes through a sexually indifferent stage, at which both sets of gonaducts, wolffian and mullerian, are present. The wolffian duct, which becomes the vas deferens in the male, extends into the urogenital sinus at the time of gonadal differentiation. The mullerian duct appears in the rat on day 14 and reaches the sinus by day 18, although in the male it begins to regress about day 16 (Price and Pannebecker, 1956). As the testis matures the wolffian duct increases in diameter and seminal vesicle and prostatic buds appear from the posterior end of the wolffian duct and the sinus, respectively (days 18 and 19).

In the rabbit, gonadectomy at the time of testis differentiation results in regression of the wolffian ducts and persistence

of the mullerian ducts. Unilateral implantation of a fetal testis results in retention of the wolffian duct on that side only (Jost, 1953). In the rat (Wells and Fralick, 1951) and mouse (Raynaud, 1950) in which castration _in utero_ at the time of testis differentiation is not possible, gonadectomy near the end of gestation results in a variable amount of wolffian duct degeneration. The extent of masculinization varies with the age at castration. _In vitro_ culture of genital tracts from male rat fetuses (Price and Pannabecker, 1956; Picon, 1969; Bloch, unpublished) supports the idea that the fetal testis is responsible for the masculinization of the internal reproductive structures. Similar studies and findings have been reported for the guinea pig (Price et al, 1967).

That the fetal testis acts via its secretion of testosterone comes from a number of studies. Organ culture of fetal rat genital tracts with C-19 steroids, primarily testosterone, results in masculinization of the wolffian ducts and urogenital sinus (Price and Pannabecker, 1956; Josso, 1970). Injection of a number of androgens, including dihydrotestosterone and methyltestosterone, into pregnant rats from days 14 through 21, results in a large number of female offspring with seminal vesicles and vas deferens (Schultz and Wilson, 1974). Injection of antibodies to testosterone (Goldman, 1972) and inhibitors of testosterone synthesis into pregnant rats and rabbits (Goldman, 1969; Bloch et al, 1971) result in male fetuses with hypospadias and reduced ano-genital distance. Wilson and Laznitiski (1971) have demonstrated that genital tracts can metabolize testosterone to dihydrotestosterone; and recently, Gupta and Bloch (in press) have shown that genital tracts from 14.5 day fetuses contain

a receptor specific for testosterone. This latter point is of particular interest since in some forms of testicular feminization, while circulating levels of testosterone are normal, the tissues either cannot bind testosterone or cannot metabolize it to dihydrotestosterone (Faiman and Winter, 1974).

Testosterone has been demonstrated in the plasma and testes of fetuses and neonates from a number of species, including: sheep (Attal, 1969); rhesus monkey (Resko, 1970); guinea pig (Resko, 1970) and human (Huhtaniemi et al, 1970). In one-day old neonatal rats testosterone has been demonstrated in testes and plasma (Resko et al, 1968).

At the time of sexual differentiation of the gonaducts, fetal testes have been shown to be capable of synthesizing testosterone *in vitro* from steroidal and nonsteroidal precursors. Weniger et al, (1967 a, b) have demonstrated testosterone and androstenedione synthesis from acetate by fetal mouse and calf testes. Lipsett and Tullner (1965) have shown increasing synthesis of these two androgens from pregnenolone, with increasing fetal age, by rabbit testes. Human fetal testes can synthesize conjugated and nonconjugated steroids from acetate (Lamont et al, 1970) and pregnenolone (Rice, 1966; Sierra et al, 1970) and from progesterone (Bloch, 1964). Noumora and co-workers (1966) have demonstrated testosterone and androstenedione synthesis from progesterone on day 13.5 and pregnenolone on day 15.5, in the rat. In all of these studies there is a marked correlation between the onset of testicular testosterone synthesis and gonaduct differentiation.

If fetal testes synthesize testosterone, is testosterone synthesis under fetal pituitary regulation? In order to demonstrate this, it is necessary to show that fetal testes can respond to LH with increased testosterone synthesis and that the pituitary not only contains, but secretes, biologically active LH. There is little evidence in the literature for responsiveness of fetal testes to LH. Recently, Weniger (1974) has shown that testosterone synthesis from acetate is increased four-fold with LH; however, this study used only mouse testes of one age. Scheib and Lombard (1971) have demonstrated that the fetal mouse testis can respond to LH secreted by adult pituitaries, as early as day 12.5; the measure used was increased 3β-hydroxysteroid dehydrogenase, Δ^4,5-steroid isomerase activity.

In many species in which sexual differentiation is completed before birth the pituitary has been shown to contain the full complement of trophic hormones, including LH. In the calf fetus LH and FSH are demonstrable, by immunofluorescence, at the time of the appearance of the interstitial cells, day 59 (Dubois, 1971). Foster and co-workers (1972) have determined circulating LH and FSH levels in fetal lambs from the last two-thirds of prenatal development through day 18 post-natal. Gonadotropin levels decreased precipitously at birth, as did the number of Leydig cells. In the human, utilizing several highly specific antisera for LH, the pituitary has been shown to synthesize LH, but its secretion remains in doubt (Pasteels, 1974).

Analysis of gonadotropin content of the rat fetal pituitary has not been reported. Light and electron microscopy studies indicate that the late fetal pituitary may contain LH and FSH. Fink and co-workers (1971) and Svalander (1974) have described cells with granules

characteristics of the adult gonadotrophs; however, Behrens and Martin (1972) were unable to demonstrate secretory gonoadotrophs before post-natal life. Using the Parlow assay, Kuznetsova (1970) measured LH activity in pituitaries of both sexes on the day before birth, and demonstrated that the pituitary is capable of modulating its production of LH in response to exogenous gonadotropin administration.

Hypothalamic involvement in the regulation of this first phase of testicular activity is unclear. Studies of hypothalamic differentiation in rats indicates that all of the nuclei are recognizable prenatally, however, the primary portal connection between the hypothalamus and pituitary is not completed until day 5 post-natal (Coggeshall, 1964; Daikoko, 1967; Fink, 1971). Decapitation of rat fetuses before day 17 results in decreased testicular weight and Leydig cell volume (Eguchi and Morikawa, 1968) and decreased conversion of pregnenolone to testosterone *in vitro* (Noumora et al, 1966). Bilateral castration of male fetuses on day 21 leads to a significant increase in leucine incorporation by cell bodies of the arcuate nucleus and basophils of the anterior pituitary when these structures are looked at 24 hours later (Nakai et al, 1972). In the guinea pig, LRF has been demonstrated in the fetal hypothalamus by immunofluorescence (Barry and Dubois, 1974), and in the lamb (Foster et al, 1972) prenatally. In the rat there is evidence that adrenal function is under pituitary, and possibly hypothalamic, control during the last two or three days of gestation (Jost, 1970).

The question posed originally, as to the mechanisms involved in the regulation and development of testis function in the fetal-perinatal period has not been answered. Although there has been a

great deal of work done on the changes taking place at puberty in testis function, the fetal period has been largely ignored. This has been due to the lack of techniques capable of accurately measuring small changes in functional status of the various tissues involved. The development in the last several years of highly sensitive and specific competitive-protein-binding assays for the measurement of protein and steroid hormones now makes such studies possible.

The experiments in the following sections make use of such a technique, a radioimmunoassay for testosterone, in order to answer two questions about the development of testicular function in the fetal rat: does the ability of fetal rat testes to synthesize testosterone from endogenous precursors change with development, and do fetal testes respond to gonadotropin stimulation with increased testosterone synthesis? These questions are basic to an understanding of the development of testis function and the mechanisms controlling it. Testosterone synthesis has been demonstrated in this species only with the use of steroid precursors (Noumora et al, 1966) and responsiveness to LH has not been investigated. These studies are viewed as the first in a proposed series of experiments on the mechanisms involved in the development of the hypothalamic-pituitary-testicular axis.

The design of the experiments is relatively straight-forward. Testicular function and responsivity to LH at each age in development will be measured in terms of the concentration of radio-immunoassayable testosterone secreted into the incubation medium. The experiments are divided into four groups: 1) standardization of the radioimmunoassay and its modification for use in this system; 2) demonstration that the concentration of testosterone in the

medium at any time is an accurate reflection of the total testosterone synthesized; 3) the synthesis of testosterone by fetal testes aged 14.5 to 20.5 days and their responsiveness to LH; and 4) measurement of endogenous testosterone concentrations in testes and genital tracts at the relevant ages.

Materials and Methods

I. Materials

A. Animals

Sprague-Dawley descended rats (CD) were bred by the supplier, Charles River Farms (Fall River, Mass.). Mating was ascertained by the presence of sperm in the vaginal smears on the morning after the animals were placed together. Fetal age was calculated as 0.5 days on the morning after mating, but may have varied by as much as 12 hours. One week before the fetuses were to be used, pregnant rats were shipped to our laboratory and housed at the Animal Institute. Animals were housed in air-conditioned quarters (22° C) artifically lighted (fluorescent light; 14 hours light; 10 hours dark). Food (Purina rat chow) and water were freely available at all times.

B. Incubation Medium

The medium used for the incubation of fetal testes was Medium 199 (Microbiological Associates, Baltimore, Md.). The composition is given in Table I. Medium was stored in 100 ml lots at 4°C. The medium was used immediately upon opening, and any remaining medium discarded.

C. Solvents

All solvents used, except diethyl ether and ethanol, were of spectrophotometric quality (suitable for use in spectrophotometry) and were not purified further. Diethyl ether, anhydrous analytical grade, was obtained from Fisher, but other brands were used as needed. The ethanol used was absolute (100 %), reagent grade.

Table I

Composition of Medium 199[a]

(Microbiological Associates)

Components	mg/liter
L-Alanine	25.0
L-Arginine HCl	70.0
L-Aspartic Acid	30.0
L-Cysteine HCl	0.1
L-Cystine	20.0
L-Glutamic Acid	67.0
L-Glutamine	100.0
Glycine	50.0
L-Histidine HCl·H_2O	22.0
Hydroxy-L-proline	10.0
L-Isoleucine	20.0
L-Leucine	60.0
L-Lysine HCl	70.0
L-Methionine	15.0
L-Phenylalanine	25.0
L-Proline	40.0
L-Serine	25.0
L-Threonine	30.0
L-Tryptophan	10.0
L-Tyrosine	40.0
L-Valine	25.0
Vitamins	
P-Aminobenzoic Acid	0.050
Ascorbic Acid	0.050
D-Biotin	0.010
Calciferol	0.100
D-Ca-Pantothenate	0.010
Cholesterol	0.200
Choline Chloride	0.500
Folic Acid	0.010
I-Inositol	0.050
Menadione	0.010
Nicotinamide	0.025
Nicotinic Acid	0.025
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.010
Thiamine HCl	0.010
DL-α-Tocopherolphosphate (Na_2)	0.010
Tween 80	5.000
Vitamin A	0.100

Table I

Components	mg/liter
Other Components	
Adenine $HCl \cdot 2H_2O$	12.10
Adenosine-5'-Monophosphoric acid, dihydrate (AMP) (Muscle Adenylic Acid)	0.20
Adenosine-5'-Triphosphate disodium, tetrahydrate (ATP)	1.08
Deoxyribose	0.50
Dextrose	1,000.00
Glutathione (Reduced)	0.05
Guanine $HCl \cdot H_2O$	0.33
Hypoxanthine	0.30
Phenol Red	20.00
Ribose	0.50
Sodium Acetate$\cdot 3H_2O$	83.00
Thymine	0.30
Uracil	0.30
Xanthine	0.34
Inorganic Salts	
$CaCl_2 \cdot 2H_2O$	186.0
$Fe(NO_3)_3 \cdot 9H_2O$	0.7
KCl	400.0
KH_2PO_4	60.0
$MgSO_4 \cdot 7H_2O$	200.0
NaCl	8,000.0
$NaHCO_3$	1,400.0
$Na_2HPO_4 \cdot 7H_2O$	90.0

a = composition supplied by the vendor: Microbiological Associates, Baltimore, Md.

D. Steroids

The steroids used were testosterone (17β-hydroxyandrost-4-ene-3-one), progesterone (pregn-4-ene-3,20-dione), 17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3,20-dione), epitestosterone (17α-hydroxyandrost-4-ene-3-one), androstenedione (androst-4-ene-3,17-dione), dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), dehydroepiandrosterone (3β-hydroxyandrost-5-ene-17-one), androstanediol (5α-androstane-3α,17β-diol), androstanedione (5α-androstane-3,17-dione), androsterone (3α-hydroxy-5α-androstan-17-one) and androstenediol (androst-5-ene-3β, 17β,-diol).

Steroids were obtained from Steraloids, Inc. (Pawling, N.Y.) and recrystallized prior to use. The melting point of each steroid was taken as the index of purity. Purified steroids were stored in absolute ethanol.

Radioactive steroids, 1,2,6,7-^{3}H-testosterone (specific activity of 85 Ci/mM) and 4-^{14}C-dihydrotestosterone (specific activity of 50.6 mCi/mM) were obtained from the New England Nuclear Corporation (Boston, Mass.). An 0.25 mCi aliquot of each steroid was diluted in benzene:methanol (9:1) to final concentrations of 50 Ci/ml (^{3}H-testosterone) or 2 Ci/ml (^{14}C-dihydrotestosterone). Every two weeks, or as needed, an aliquot of the stock solution was purified according to the procedure described below.

1. Purification of radioactive steroids

Radioactive steroids were purified by column chromatography. The column bed was Sephadex-LH-20 (Pharmacia Chemicals, Piscataway, N.J.) and the solvent system was toluene:methanol (85:15).

The column was glass, 50 cm in height with an inside diameter of 1 cm. The base of the column was packed with a small amount of glass wool and covered with a scintered glass filter, to prevent leakage of the Sephadex particles.

Sephadex, 20 g, was swelled overnight in an excess of solvent system. The next day the Sephadex was carefully poured into the column and packed to a height of 30 cm. A 5 Ci aliquot of the radioactive steroid was dried under N_2 and dissolved in 1 ml of the solvent mixture. This was carefully applied to the column bed and allowed to penetrate to the top of the bed. The column was then eluted with solvent mixture. Fractions of 2 ml were collected in disposable glass tubes and an 0.01 ml aliquot counted. The fraction, or fractions, containing the highest number of counts was quantitatively transferred to a glass scintillation vial and stored at 4° C.

E. Pituitary hormones

Rat luteinizing hormone (LH, 97 μg), follicle stimulating hormone (FSH, 100 μg) and prolactin (100 μg) were supplied by the Rat Pituitary Hormone Distribution Program of the Institutes of Arthritis and Metabolic Disease, National Institutes of Health, in the amounts indicated. All hormones were received lyophilized and were immediately solubilized and stored according to instructions supplied by the NIAMD.

The biological potency of LH (NIAMD-rat $LH-I_3$) was equal to that of NIH-LH-SI (based on the ovarian ascorbic acid depletion assay; Parlow, 1961) with an FSH contamination of less than 0.04 x NIH-FSH-SI (HCG-augmentation assay; Steelman and Pohley, 1953). The

FSH (NIAMD-rat FSH-I$_3$) at a biological potency of 100 x NIH-FSH-SI (HCG-augmentation assay) had an LH contamination of less than 0.002 x NIH-LH-SI (ovarian ascorbic acid depletion assay).

Each hormone was dissolved in 1.0 ml buffer (0.01 M phosphate buffer, pH 7.6, containing 0.015 M NaCl and 1% merthiolate). Aliquots of .025 ml were placed in screw-capped glass tubes, frozen in a dry-ice-acetone bath and stored at -20° C.

Rat prolactin (NIAMD-rat prolactin-I-1) was solubilized in 1.0 ml $NaHCO_3$ (0.01 M). Aliquots of .025 ml were placed in 12 mm capped styrene tubes (Falcon Plastics, Ga.), frozen in a dry-ice-acetone bath and stored at -20° C.

Before use, one or more tubes of each hormone was diluted to 0.1 ml with medium 199.

II. Methods

A. Incubation of fetal testes

Rats were killed by ether inhalation, uteri removed and placed in glass Petri dishes containing a few drops of cold medium 199. Fetuses were decapitated, and the testes placed in ice-cold medium 199 as rapidly as was possible. At no time did it take more than 4 hours to dissect out the number of testes needed. Testes from older age fetuses were removed from the genital ducts; from 14.5 to 16.5 days gestation a small amount of adhering duct was retained in order to avoid damaging the testes. The combined testes were subdivided into groups of predetermined number. Each group of testes was weighed on a Roller-Smith Precision Balance and placed in a 10 ml ehrlenmeyer flask containing 3 ml of cold medium 199. The flasks were gassed with O_2:CO_2 (95:5) for 30 seconds, covered with a rubber stopper and placed in a Dubnoff metabolic shaking incubator at 37° C. In most of the experiments the testes were incubated for 30 minutes (preincubation) after which time the medium was discarded. Each flask then received 3 ml of cold medium 199 with or without added substrate (^{3}H-testosterone, pituitary hormones). An 0.2 ml aliquot of medium was removed and placed in a glass scintillation vial for radioimmunoassay ("zero" time). The flasks were then gassed, stoppered, and returned to the incubator. Testes were generally incubated for 4 to 8 hours. At intervals, generally 0.5, 1, 2 and 4 hours, 0.2 ml aliquots of medium were removed for determination of testosterone concentration by radioimmunoassay. After each aliquot was removed the samples were gassed before being returned

to the incubator. At the termination of the experiment the remaining medium was transferred to glass scintillation vials and stored at -20° C. In certain experiments the concentration of testosterone in the testes was determined at the end of the incubation. The testes were placed in 0.5 ml phosphate buffer (0.2 M; pH 7.8) and homogenized. An 0.2 ml aliquot of the homogenate was reserved for testosterone determination by radioimmunoassay, the remainder frozen.

The concentration of testosterone in the 0.2 ml aliquot was divided by the fraction of the total medium represented by the aliquot. To determine the concentration in 3 ml medium the concentrations of testosterone in all previous 0.2 ml aliquots was added to this value.

B. Determination of endogenous tissue testosterone

In these studies the relevant tissues (testes, genital tracts, lungs) were excised and placed directly into scintillation vials containing 0.5 ml phosphate buffer (0.2 M; pH 7.8). Tissues were homogenized as above and an 0.2 ml aliquot used for the determination of testosterone by radioimmunoassay.

C. Extraction and purification of testosterone and other steroids

1. Extraction of steroids

Testosterone and other steroids were extracted from the incubation medium and tissue homogenates with diethyl ether. When steroids were to be determined by radioimmunoassay the following procedure was utilized. Approximately 100 - 300 cpm of ^{3}H-testosterone (dissolved in phosphate buffer system, pp.36) were added to each 0.2 ml aliquot of medium or tissue homogenate to correct for pro-

cedural losses. Diethyl ether (10 ml) was added to each vial and the samples were mixed on a Vortex-Genie mixer for 45 seconds. Samples were then frozen in a dry-ice-acetone bath (-76° C), and when the aqueous phase appeared frozen (20 - 30 seconds) the ether fractions were decanted into conical glass centrifuge tubes. The ether extract was evaporated under N_2 at a temperature of 45° C. Sample residues were dissolved either in 1.0 ml of the phosphate buffer system for testosterone analysis by radioimmunoassay or in 1.0 ml benzene for separation on thin-layer chromatography. In both cases the extracts were stored at 4° C.

2. Thin-layer chromatography (TLC)

In several experiments TLC was used to separate ^{3}H-testosterone from other radioactive steroids. Two solvent systems were used. System I (chloroform:ether:ethanol, 9:1:0.3) separated testosterone, dihydrotestosterone, and androstenedione, (R_f 0.25, 0.32, 0.5, respectively). System II (hexane:ethyl acetate, 1:1) separated testosterone from dihydrotestosterone and androstenedione, which were counted as one fraction (R_f 0.14, 0.25, 0.22, respectively).

Pre-prepared plates of silica gel (0.25 mm thick; 20 x 20 cm) containing a fluorescent compound (F-254) were obtained from E. Merck (Darmstadt, Germany). The plates were activated by heating at 90° C for 1 hour, and stored in a glass dessicator. An 0.5 cm strip of gel was scraped from each side of the plate and the gel separated into lanes 1 cm wide for known standards and 2 cm wide for unknown samples. The unknown sample lanes were separated by 0.5 cm lanes. The origin was marked at 1 cm from the edge of the gel. Samples and standards were applied to the plates in benzene, ethanol, or ethyl acetate

using microliter pipettes. In general, standards were applied in 5 - 20 μl amounts and unknowns in 20 - 100 μl volumes. Solvents were evaporated under N_2.

Chromatography was performed in glass tanks (26 x 30 cm) equipped with removable covers. Tanks were rinsed in distilled water, and when dry were lined with filter paper (Whatman #2). The solvent mixture, prepared fresh, was poured into the tanks (150 ml per tank). After the tanks were equilibrated for a minimum of 2 hours the plates were put in. When the solvent front had reached a height of 15 cm the plates were removed and dried at room temperature.

Standards were visualized either by fluorescence under UV (254 nm) or by spraying with ethanol:sulfuric acid (1:1) and heating for 10 minutes at 100° C. Silica gel strips, corresponding to the Rf values of the standards, were scraped from the plates and placed in glass centrifuge tubes. Steroids were eluted from the gel with 2 washings in benzene:methanol (9:1). The eluates were dried under N_2, redissolved in 1 ml methanol, and an aliquot counted.

D. Radioactivity determination

Counting of samples containing ^{3}H and ^{14}C was done by liquid scintillation counting. The counter used was a Nuclear Chicago Mark I Liquid Scintillation Spectrometer. The scintillation fluid used was Hydromix (Yorktown Products, N.Y.). The machine was calibrated to give maximum efficiency for ^{3}H and ^{14}C (52% and 87%, respectively) when counted on two channels. Samples were counted until sufficient numbers of counts had accumulated to achieve a counting error of 1 - 2%.

The efficiency of the counting process is a function of the energy of the emitted electrons, capture of the energy by "inert" molecules, and quenching of the phosphorescence due to contaminating solutes or solvents in which the sample containing the isotope is dissolved. In order to determine the loss of efficiency ^{3}H and ^{14}C-toluene standards (New England Nuclear Corp., Boston, Mass.) were counted in the appropriate solvents. Sample results were expressed as cpm; in general sample efficiency ranged from 20 - 30% for ^{3}H and 40 - 50% for ^{14}C.

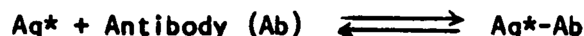
Radioimmunoassay of Testosterone

The concentration of testosterone in medium and tissues was determined by radioimmunoassay (RIA). The assay method of Brenner, Guerrero, Cekan and Diczfalusy (1973) for the measurement of plasma testosterone was modified for the detection of testosterone present in tissues and secreted into medium 199 during the incubation of fetal testes.

I. Introduction

Radioimmunoassay is a particular class of competitive protein-binding assays, in which the protein used is an antibody prepared against a non-antigenic hapten (e.g, testosterone), coupled to an antigenic molecule (e.g, bovine serum albumin). These assays take advantage of the fact that the binding of the antigen to the antibody is specific and reversible.

The binding of the labeled antigen (Ag*) to the antibody results in an antigen-antibody complex:



The addition of unlabeled antigen (Ag) to the complex results in competition for the available sites on the antibody molecule. This results in a decrease in the number of labeled antigen molecules associated (bound) to the antibody:

In order to determine the amount of labeled antigen displaced from the antibody by the addition of unlabeled antigen, the antigen molecules free in solution are separated from the antigen bound to the antibody. This is accomplished in most steroid assays by the use of non-specific adsorbants, such as charcoal or floricil.

Once the free and bound steroid molecules have been separated, the number of counts bound to the antibody is determined. Repetition of the above steps at several concentrations of competing (unlabeled) steroid enables one to construct a curve for the determination of the amount of steroid in unknown samples.

The slope of the displacement curve, the change in displacement over the range of competing concentrations, is dependent upon the weight of the labeled antigen (tracer) and the concentration of binding protein (antibody). For most assays the weight of the tracer is considered negligible due to the use of compounds with high specific activities. The antibody concentration which, in general, gives the most satisfactory displacement curve is one which binds approximately 50% of the labeled steroid in the absence of competing antigen (Odell et al, 1971).

For each antibody used the optimum concentration depends on the association constant (affinity) of the antibody for the antigen and on the conditions of the assay (pH; buffer; temperature) (Murphy, 1971). Thus, under the specific assay conditions employed it is necessary to determine the antibody concentration which: a) binds approximately 50% of the tracer in the absence of unlabeled antigen; b) gives maximum uniform displacement over the range of competing concentrations; c) gives a high degree of precision in the number of counts displaced at each concentration; both within and between assays ($\pm$ 10%; Murphy, 1971); and d) gives the smallest degree of non-specific displacement of counts in the absence of competing antigen (blank).

Although antibodies are relatively specific for the antigens against which they are prepared, the use of steroid haptens as antigens

results in a loss of specificity. This is due mainly to three factors: 1) coupling the steroid to another molecule obscures functional groups unique to that steroid; 2) the antibody is made against the antigenic molecule as well as the hapten; and 3) the coupling reaction may distort the shape of the hapten such that the antibody does not recognize the "native" form of the molecule. The first point is probably the most important in the preparation of steroid-specific antibodies. In general, the further the coupling is made from a unique or distinctive immunologically active site, the more specific is the resultant antibody (Niswender and Midgely, 1970). Antibody molecules which are reactive to the antigenic molecule (e.g., bovine serum albumin) can be removed by adsorption of the antiserum with the particular molecule used. Changes which affect the shape of the antigen and which might result in antibodies with low binding affinities can be minimized by successive immunizations to produce a relatively highly specific antibody (Zimmering, 1970).

Thus, in setting up a radioimmunoassay it is necessary to determine the extent to which other steroids, not necessarily having similar biological activity, can displace labeled antigen from the antibody. The ideal assay is one in which the antibody is sufficiently specific and has a sufficiently high binding affinity to obviate the need for procedures to purify and separate the antigen from any interfering and/or competing molecules. Since most antibodies do not meet these critera, each procedure used to extract, separate, and/ or purify the steroid must be analyzed for its effect on the binding of the antigen to the antibody. Unless there is no affect of these procedures on the displacement of labeled antigen bound to the anti-

body, the best standard curve is one in which the standards are prepared and processed in the same way and at the same time as experimental samples.

In summary, setting up the radioimmunoassay involves the following determinations: 1) the antibody concentration which gives maximum displacement of labeled antigen over the range of competing concentrations (i.e., sensitivity and responsivity); 2) estimation of the precision of label displacement at each competing concentration, both within (intra-assay) and between (inter-assay) assays; 3) effect of purification procedures on the slope of the curve and precision of the assay (recovery or accuracy of the assay); and 4) specificity of the antiserum for the antigen used.

II. Materials

A. Phosphate buffer system (PBS)

The buffer system used in the radioimmunoassay consisted of an 0.2 M phosphate buffer (pH 7.8) containing 0.1% gelatin, 0.9% NaCl, and 0.01% sodium azide. The buffer was prepared fresh every two weeks and stored in an all-glass stoppered bottle at 4° C.

B. Charcoal

Charcoal (Norit A, particle size 4 - 5μ) was obtained from Fisher Scientific Co. and prepared for use in the radioimmunoassay by washing with distilled water (10 g in 100 ml) to remove the fine particles. The residue was dried overnight at 100° C and stored in a stoppered jar or flask. A 1% (wt/vol) suspension in PBS was prepared every two days and stored at 4° C. To ensure an even suspension of charcoal particles the charcoal was mixed with a magnetic stirrer for one-half hour prior to use.

C. Antibody

The antibody used was generously supplied by Dr. R. Rosenfeld, Steroid Research Institute, Montefiore Hospital and Medical Center. The antibody had been prepared against 3-carboxymethoxy-testosterone-bovine serum albumin, dispersed in Freund's adjuvant 1:1. Rabbits were injected with the antigen for 3 - 4 months (1 mg/injection, subcutaneously). The antibody obtained was stored in a glass tube at -20° C. As needed, antibody was diluted 1:100 with PBS. Each day a fresh aliquot of 1:100 dilution was removed and diluted 1:10,000 with PBS (vol/vol). The addition of equal volumes of antibody, and labeled and unlabeled antigen to the reaction tubes resulted in a final antibody concentration of 1:30,000.

D. Glassware

All glassware used in the radioimmunoassay was first cleaned according to ordinary procedures (detergent, distilled water), rinsed with methanol, and dried at a temperature of 800° F in a G.E. self-cleaning oven.

III. Procedure

The procedure used for the radioimmunoassay of testosterone was taken from that of Brenner, Guerrero, Cekan, and Diczfalusy (1973), and involved the following steps:

1. Preparation of antibody-^{3}H-testosterone complex. ^{3}H-testosterone was bound to the antibody by adding equal volumes of both reactants, previously diluted to the concentrations indicated (pp 37), to a glass scintillation vial. After mixing, the solution was allowed to equilibrate for a mimimum of 2 hours at room temperature.

2. Testosterone standards. To 12 x 75 mm glass tubes (Kimble), 0.1 ml of a known concentration of testosterone (12.5, 25, 50, 75, 100, 200 pg) or of a sample extract was added. All samples were prepared in duplicate or triplicate. Two tubes received 0.1 ml of PBS ("zero" testosterone).

3. Reaction. An 0.2 ml aliquot of the antibody-^{3}H-testosterone mixture was added to each tube, and the tubes mixed for 15 seconds. Tubes were then placed in a 60° C water bath for 10 minutes, and then in a 30° C water bath for 30 minutes.

4. Separation of bound and free testosterone. Testosterone, free in solution, was separated from that bound to the antibody by adsorption of the free steroid onto charcoal. The tubes from Step 3 were placed in an ice-water bath, and an 0.5 ml aliquot of the charcoal suspension was added to each tube. Charcoal was added to all tubes within 4 minutes. The tubes remained in the ice-water bath for 20 minutes, during which time they were gently shaken to maintain an even charcoal suspension. Charcoal, with the adsorbed free steroid, was separated from the antibody-testosterone complex (bound steroid, B) by centrifugation for 10 minutes at 3,000 rpm at 4° C. The centrifuge used was a Sorvall RC-2B refrigerated centrifuge (Sorvall, Norwalk, Conn.) with a rotor designed to hold 96 12 x 75 tubes. The supernatant fraction from each tube was decanted into glass scintillation vials and counted according to the procedures previously described. The counting efficiency ranged from 20 - 25%.

5. Expression of results. The bound counts (B) obtained at each added testosterone concentration were expressed in

either of two ways: a) as a percentage of the total counts added to each tube (% counts bound: % B): or b) as the ratio of:

$$\frac{\text{\% counts bound at concentration}_a\ (B_a)}{\text{\% counts bound at "zero" testosterone}\ (B_o)}$$

For the standard curve the results were plotted as B/B_o vs testosterone added (in pg). For the range of competing concentrations used the results are shown in Figure 1. The same results plotted on a logit-log scale converts the curve in Figure 1 to a straight line (Fig. 2), and allows for the determination of the slope of the line.

IV. Standard curve for the radioimmunoassay of testosterone

As was discussed previously, the setting up of a radioimmunoassay involves the determination of the antibody concentration giving maximal sensitivity and uniformity of displacement over the range of competing concentrations; estimation of the precision of the assay; effect of purification procedures on the slope of the displacement curve; and determination of the specificity of the antiserum for testosterone.

A. Antibody concentration

In order to prepare a standard curve having both maximum sensitivity and steepness of slope, the relationship between the dilution of antibody used and the displacement of ^3H-testosterone bound to the antibody by increasing concentrations of competing testosterone was studied. The results are shown in Figure 3.

Figure 1. Radioimmunoassay of testosterone: displacement of ^{3}H-testosterone bound to the antibody (mean $B/B_o \pm$ S.D.) by testosterone standards prepared in buffer. The concentrations (and numbers of determinations per concentration) of competing testosterone were: 12.5 (40), 25 (39), 50 (39), 75 (39) 100 (59), 200 (28) pg. The assay was performed according to the procedures described in the text; the final antibody concentration used was 1:30,000.

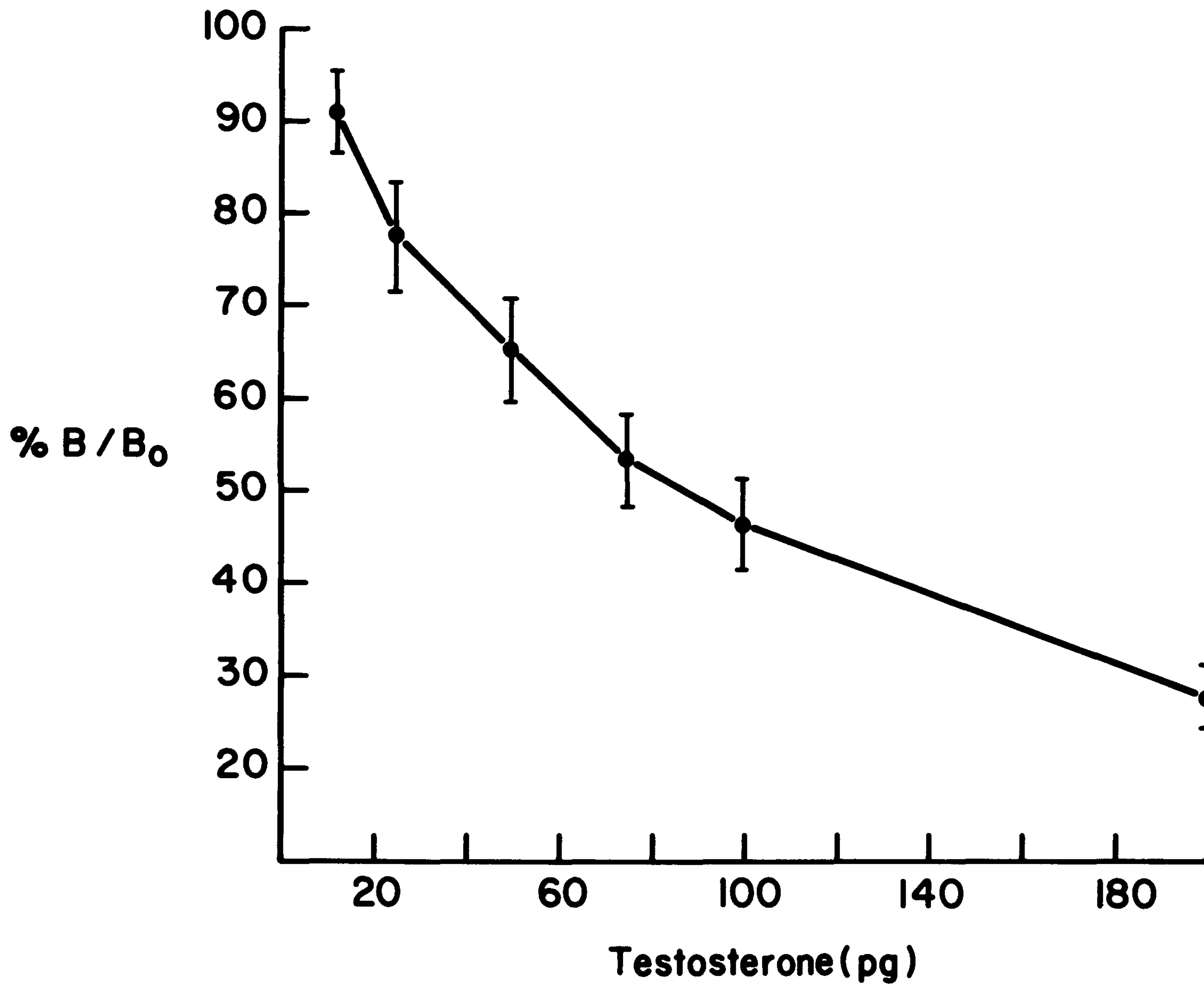
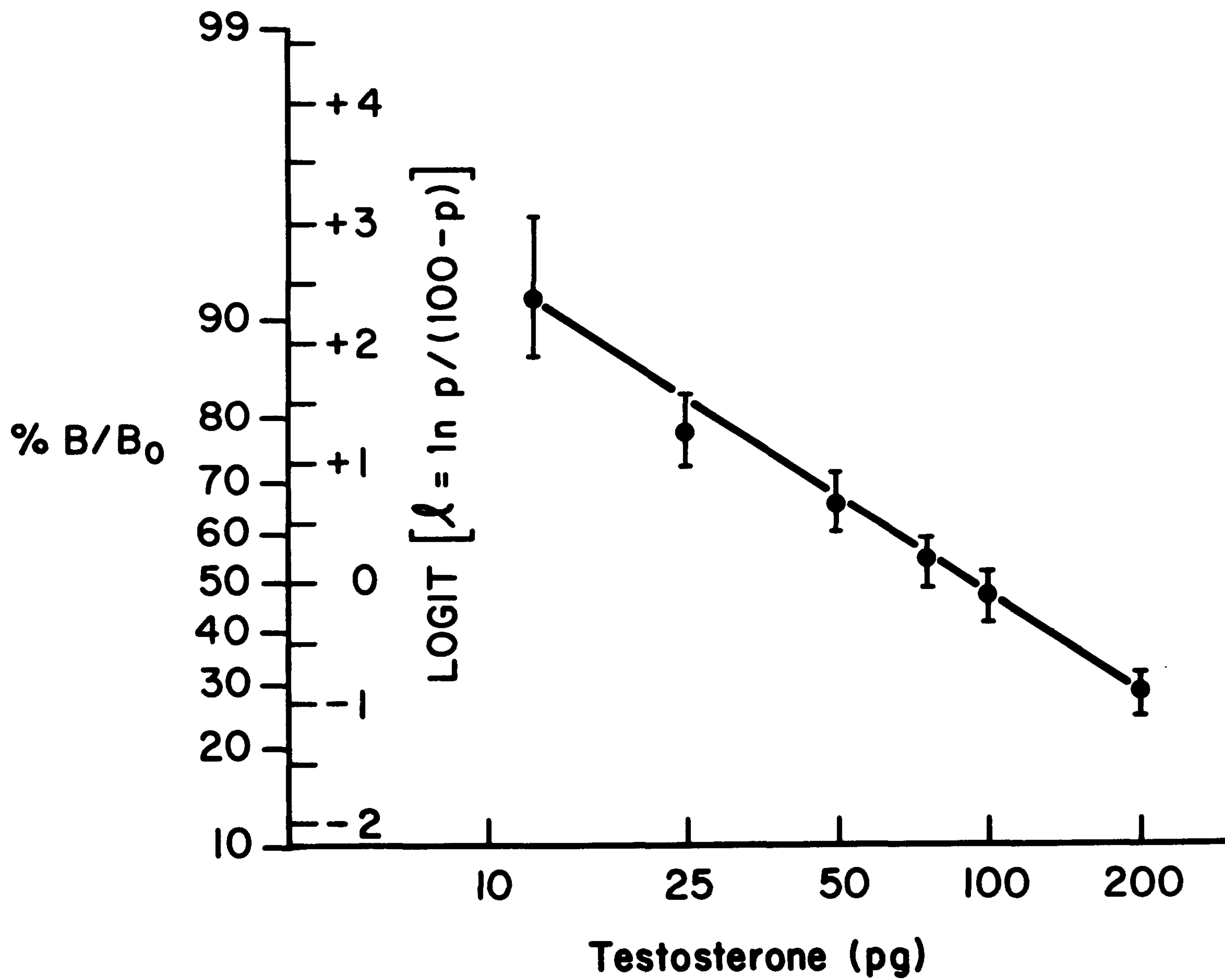

100
90
80
70
60
50
40
30
20
% B / B0
20
60
100
140
180
Testosterone (pg)

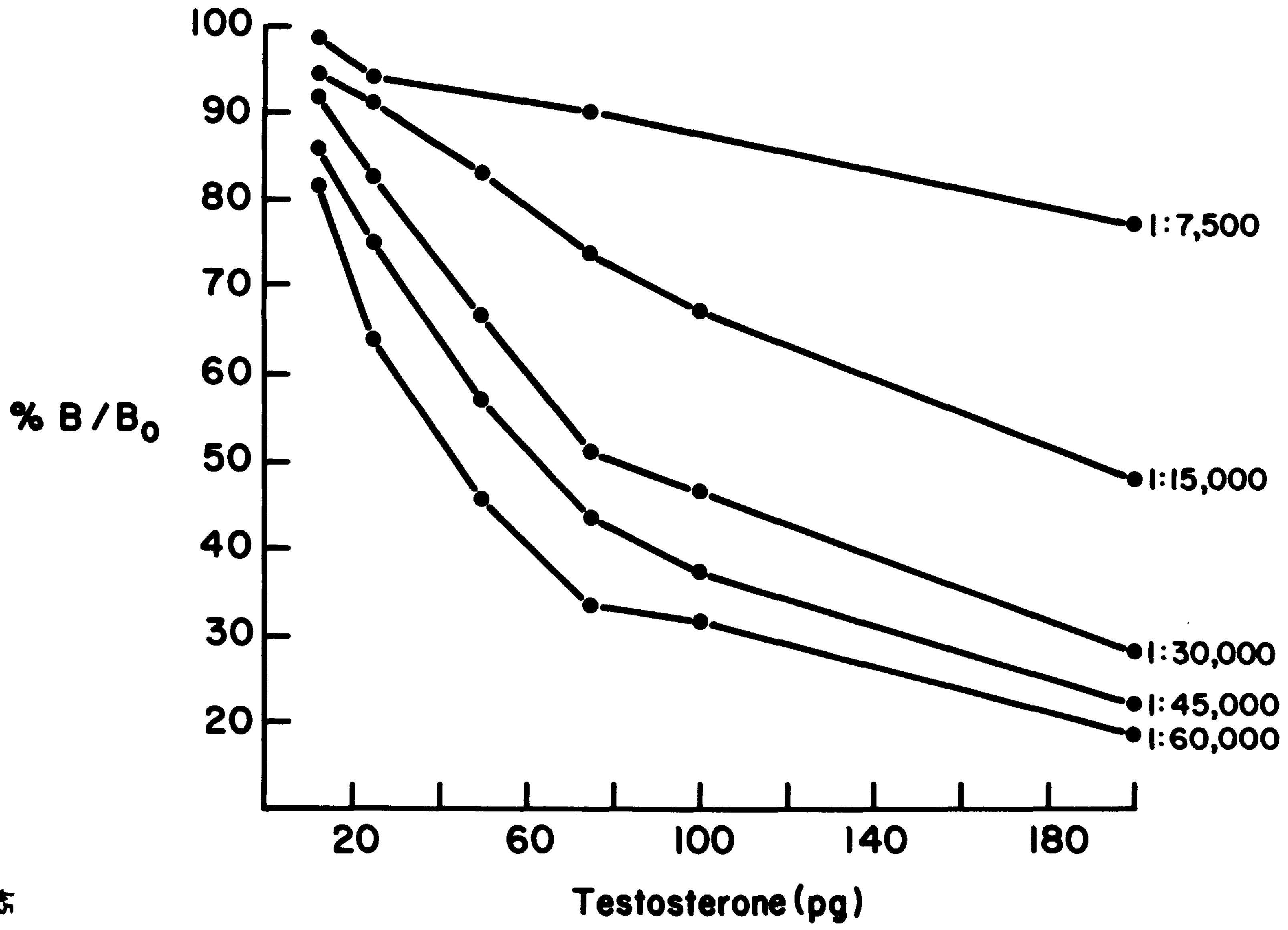
Figure 2. A log- logit transformation of the curve in Figure 1. The equation for the line is:

$$\log C = -0.37 \text{ logit} + 1.9542$$



99
90
80
70
60
50
40
30
20
10
% B/B0
+4
+3
+2
+1
0
-1
-2
LOGIT [ℓ = ln p/(100-p)]
10
25
50
100
200
Testosterone (pg)

Figure 3. Relationship between final antibody concentration and sensitivity and slope of the displacement curve over the range of competing testosterone concentrations 12.5 - 200 pg. Numbers in the figure represent the final antibody concentrations in the reaction tubes; each point represents the mean of 5 determinations. Testosterone standards were prepared in buffer and bound according to the procedures outlined in the text.



45

Increasing the antibody dilution increased the sensitivity of the assay. At a testosterone concentration of 12.5 pg, the B/B_o value decreased from 98.5, at a dilution of 1:7500, to 81.5, at a dilution of 1:60,000. The percentage of the total counts displaced over the entire range of 12.5 to 200 pg increased from 5.5% at a dilution of 1:7500, to 32% at a dilution of 1:30,000. Increasing the antibody dilution to 1:60,000 sacrificed the ability to read a wide range of testosterone concentrations for increased displacement in the range 12.5 to 50 pg. At a dilution of 1:60,000 the displacement of counts from 12.5 to 50 pg was 35%; this decreased to 14% from 75 to 200 pg. At a dilution of 1:30,000, displacement of counts from 12.5 to 50 and from 75 to 200 pg was uniform, 25.7%. A dilution of 1:30,000 was selected as the final working concentration of antibody to be used in all studies. The results shown in Figure 3 indicate that at this concentration small errors in antibody dilution would not result in major changes in slope or sensitivity of the displacement curve.

B. Standard curve for the radioimmunoassay of testosterone

The displacement curve shown in Figs. 1 - 2 describes the displacement of ^{3}H-testosterone by testosterone standards prepared in buffer. The experimental conditions, however, called for the measurement of testosterone extracted from the incubation medium or tissue. For the standard curve to most accurately reflect the experimental situation, testosterone standards were prepared in medium 199, extracted with diethyl ether (according to the procedure previously described), diluted in 1 ml PBS, and then bound according to the procedures outlined above.

Tables II A & B

Testosterone, in concentrations of 12.5 to 200 pg, prepared in buffer or extracted from medium 199, were assayed using the radioimmunoassay for testosterone described in the text; at an antibody concentration of 1:30,000.

a = Results are expressed as the ratio of percent of the counts bound at a specific concentration of unlabeled testosterone (B) to that bound in the absence of testosterone (B_o). The percent of counts bound (B_o) for buffer and extracted medium 199, was 45.7 $\pm$ 7.4 and 47.2 $\pm$ 5.4, respectively. Numbers of assays performed and interassay variation (coefficient of variation = S.D./$\bar{M}$) at each concentration are shown.

b = Deviation from the mean of replicate samples at each concentration are shown as the mean coefficient of variation $\pm$ S.D. The number of mean determinations at each concentration is shown.

c = Recovery of testosterone extracted from medium 199, determined by reading B/B_o values from buffer standard curve (Figure 1). Results are expressed as mean recovery $\pm$ S.D., numbers in parentheses indicate number of determinations at each concentration of testosterone.

d = Underlined pairs of values are significantly different ($P<.05$).

Table IIA

Radioimmunoassay of Testosterone

Testosterone concentration (pg)	Buffer Testosterone Standards				
	Inter-assay Precision[a]			Intra-assay Precision[b]	
	No. of assays	B/B_o (M ± S.D.)	C.V.	No. of assays	C.V. (M ± S.D.)
12.5	40	91 ± 4.5	.05	11	.033 ± .01
25	39	79.6 ± 5.7	.07	11	.026 ± .01
50	39	65.3 ± 5.7	.09	11	.025 ± .015
75	39	53.5 ± 5.4[d]	.10	11	.028 ± .024
100	39	46.4 ± 5.1	.11	11	.035 ± .025
200	28	27.8 ± 3.5	.13	6	.032 ± .021

Table IIB

Radioimmunoassay of Testosterone

Testosterone concentration (pg)	Extracted Testosterone Standards					
	Inter-assay Precision			Intra-assay Precision		Percent Recovery[c]
	No. of assays	B/B_o (M ± S.D.)	C.V.	No. of assays	C.V. (M ± S.D.)	(M ± S.D.)
12.5	17	88.2 ± 4.3	.05	10	.026 ± .023	139 ± 47.5 (11)
25	28	79.6 ± 6.5	.08	10	.028 ± .021	115 ± 41 (21)
50	28	66.7 ± 3.8	.06	10	.035 ± .022	100 ± 16.7 (21)
75	22	56.9 ± 4.6	.08	10	.03 ± .02	93.8 ± 17.5 (16)
100	25	50.7 ± 5.7	.11	10	.02 ± .02	89 ± 15.5 (21)
200	16	31.6 ± 2.7	.09	9	.025 ± .02	93 ± 5 (10)

Figure 4. Radioimmunoassay of testosterone: displacement of ^3H-testosterone bound to the antibody (mean $B/B_o \pm S.D.$) by testosterone standards extracted from medium 199. The concentrations (and number of determinations per concentration) were: 12.5 (17), 25 (28), 50 (28), 75 (22), 100 (25), 200 (16) pg testosterone. Testosterone standards were extracted from medium 199 and bound according to the procedures in the text; the final antibody concentration used was 1:30,000.

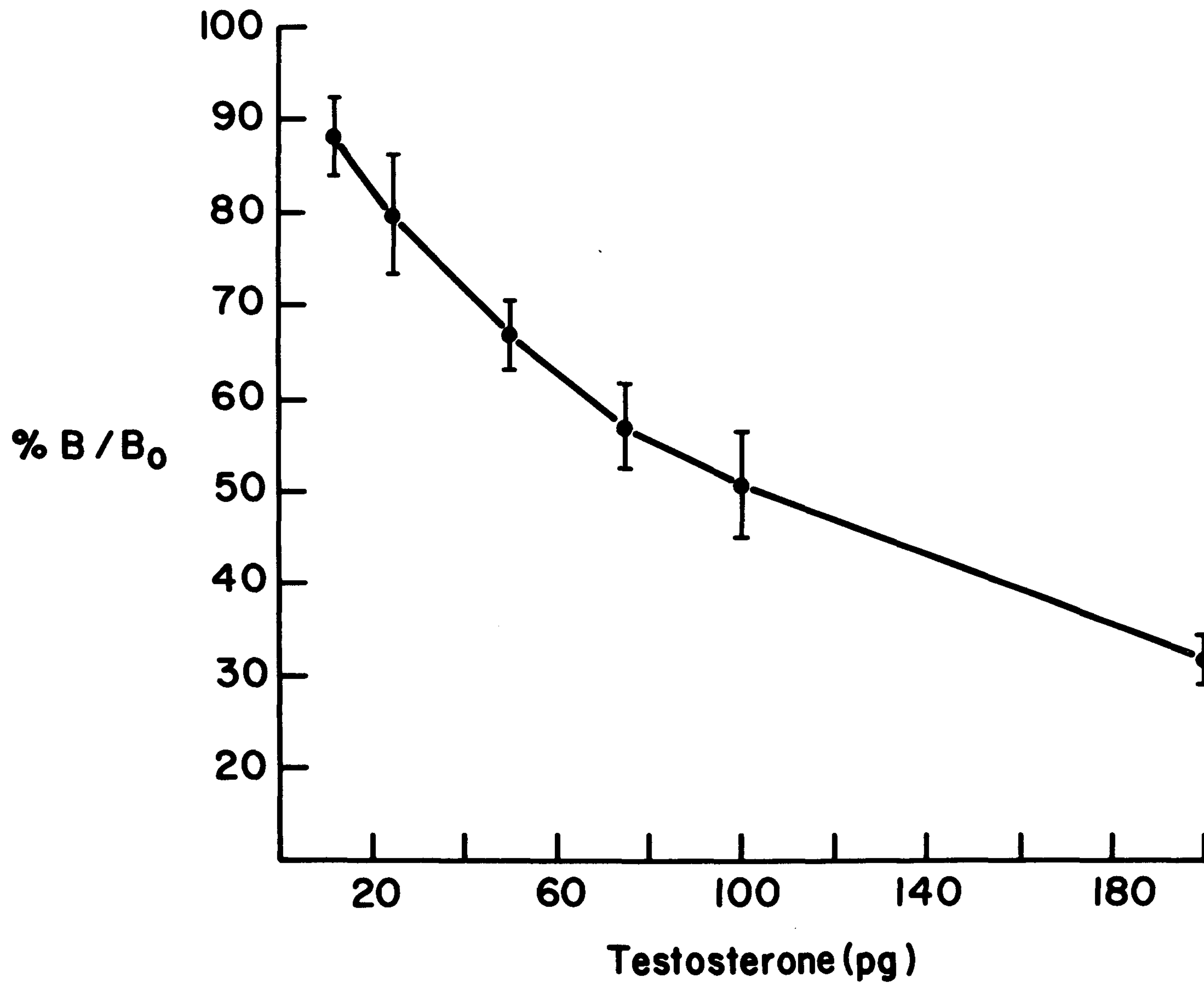
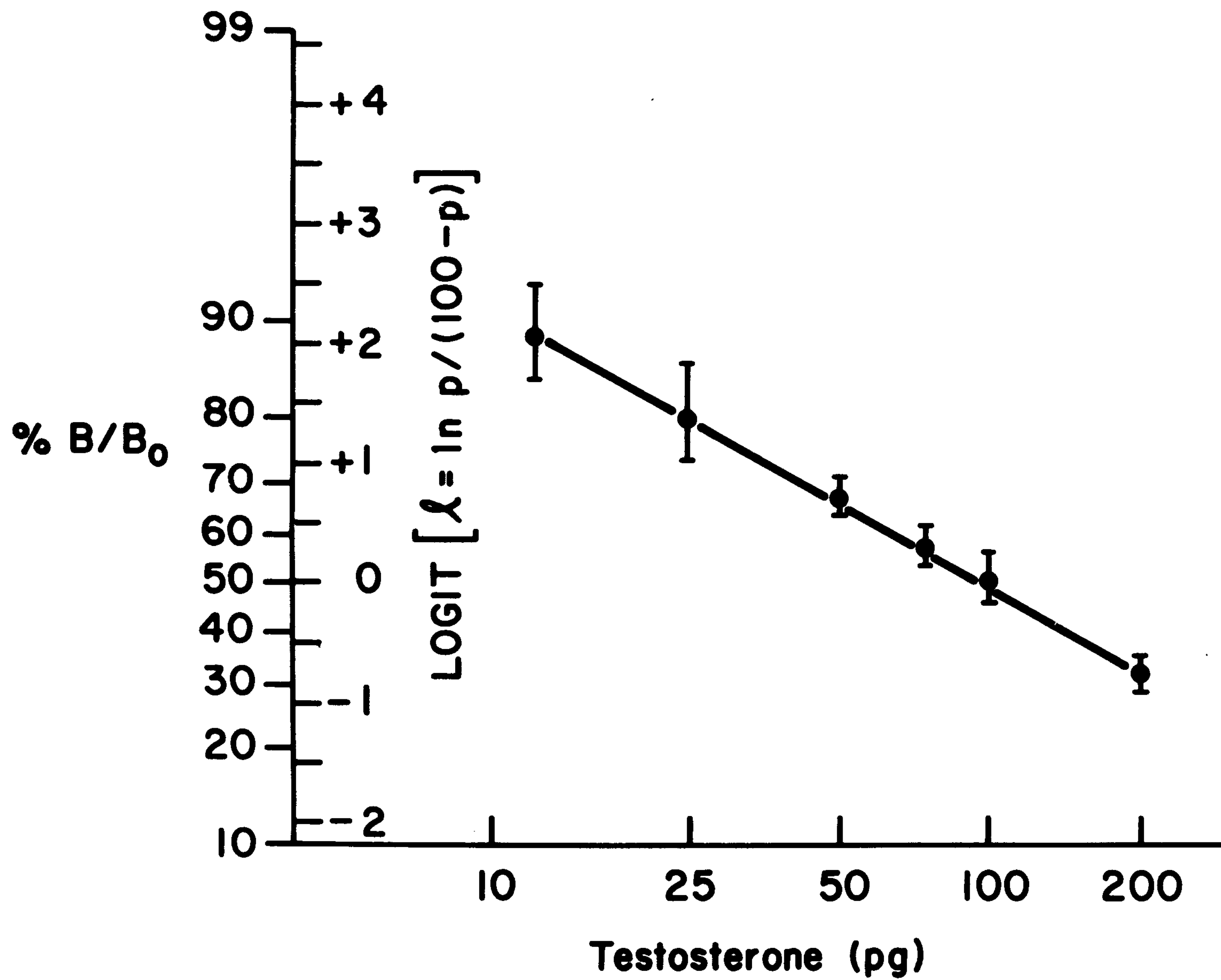

100
90
80
70
60
50
40
30
20
% B / B0
20
60
100
140
180
Testosterone (pg)

Figure 5. A log- logit transformation of the standard curve for the radioimmunoassay of testosterone given in Figure 4. The equation for the line is:

$$\log C = -0.425 \text{ logit} + 1.9731$$


99
90
80
70
60
50
40
30
20
10
% B/B$_0$
+4
+3
+2
+1
0
-1
-2
LOGIT [ℓ = ln p/(100-p)]
10
25
50
100
200
Testosterone (pg)

The displacement of ^{3}H-testosterone by testosterone standards (12.5 to 200 pg) either prepared in buffer or extracted from medium 199 is given in Table II A - B, along with inter and intra-assay precision. The standard curve for the radioimmunoassay of testosterone extracted from medium 199 is shown in Figures 4 - 5. Extraction of testosterone had no significant effect on concentrations below 75 pg. At 75, 100, and 200 pg extraction significantly decreased the displacement of ^{3}H-testosterone. This resulted in a curve with a somewhat shallower slope (Figure 5) than that seen without extraction (Figure 2), although the significance of this was not evaluated statistically.

The equation which defines the displacement of ^{3}H-testosterone by extracted testosterone standards does not apply at concentrations over 1 ng. At 1 and 10 ng the resultant B/B_o values are above those predicted by the equation (8.4 and 4.0, respectively).

Extraction of testosterone did not affect the precision of the assay. The intra-assay variation for buffer testosterone standards ranged from 0.025 (at 50 pg) to 0.035 (100 pg); and for extracted standards from 0.02 (at 100 pg) to 0.035 (at 50 pg). The inter-assay precision for buffer and extracted testosterone standards ranged from 0.05 to 0.13, and 0.05 to 0.11, respectively.

1. Blank value

The extraction of standards from medium 199 raised the problem of the displacement of a specific number of counts in the absence of added testosterone. This would most likely be due to materials present in the extract, derived either from the medium or ether. In 32 assays the resultant B/B_o values, when read off of the

curve in Figure 2 (non-extracted testosterone standards) corresponded to 5.8 $\pm$ 6.7 pg testosterone. For this reason, most samples were read above 25 pg, and any value below 12.5 pg was considered "zero". The blank value could not be subtracted from the final results since it is unclear if it changed as a function of the concentration of added testosterone.

2. Accuracy

The ability to correctly measure the amount of testosterone in a given sample, i.e, the accuracy, is dependent upon the loss of material during sample handling and on interference in the binding of testosterone to the antibody. Using the extraction procedure previously described, loss of testosterone during sample handling was negligible: 85 - 95% of ^{3}H-testosterone added to more than 100 samples was recovered after extraction. Since loss of material was small and testosterone standards were extracted at the same time as the experimental samples, the concentrations of testosterone in unknown samples were not corrected to reflect this loss.

In order to assess the interference on testosterone binding by materials present in the ether extract, testosterone standards extracted from medium 199 were bound and the resultant B/B_o values converted to pg testosterone using the curve in Figure 1. The results are shown in Table II B. At 12.5 pg the concentration of testosterone was overestimated whereas above 50 pg there was a tendency to underestimate the concentration of testosterone present. The consistent underestimation of testosterone concentrations above 75 pg is predicted by the significant decrease in the mean B/B_o values obtained

for extracted vs buffer standards. Because of the poor precision in recovery at testosterone concentrations below 50 pg and the under-estimation of testosterone at concentrations above 50 pg, testosterone standards were routinely extracted from medium 199.

3. Volume of extract bound

In order to further assess the contribution of the medium and extraction procedures to the radioimmunoassay of testosterone, standards (25, 50, 100, 200 pg) were prepared in medium 199 such that 0.1 and 0.01 ml aliquots of the resulting extract contained equal testosterone concentrations. Decreasing the amount of extract bound by ten-fold resulted in a small (0.7 - 5.4%) decrease in the mean B/B_o values at the range of concentrations teste. Since the results did not change consistently with the concentration of testosterone being assayed, it was concluded that decreasing the volume of extract bound was unlikely to significantly affect the binding of testosterone to the antibody.

4. Specificity of the antiserum for testosterone

The relative degree of specificity of the antiserum for testosterone was assessed in order to determine the need, if any, for chromatographic purification of the extracts. Ten steroids, known precursors or metabolites of testosterone were studied for their ability to displace ^3H-testosterone bound to the antibody (Figure 6; Table III). Only dihydrotestosterone significantly displaced ^3H-testosterone; at a B/B_o value of 0.5 (50% displacement) twice as much dihydrotestosterone as testosterone was required to displace the same amount of ^3H-testosterone (215 vs 100 pg, respectively); at 1 ng dihydrotestosterone was equivalent to 640 pg testosterone. The

metabolism of ^{3}H-testosterone by fetal testes was studied in order to determine the need to separate testosterone from dihydrotestosterone by chromatography prior to radioimmunoassay. Since dihydrotestosterone could not be identified as a metabolite of testosterone, no prior purification of the experimental samples was required (*See*: Results).

Table III

Steroid Specificity of the Antibody

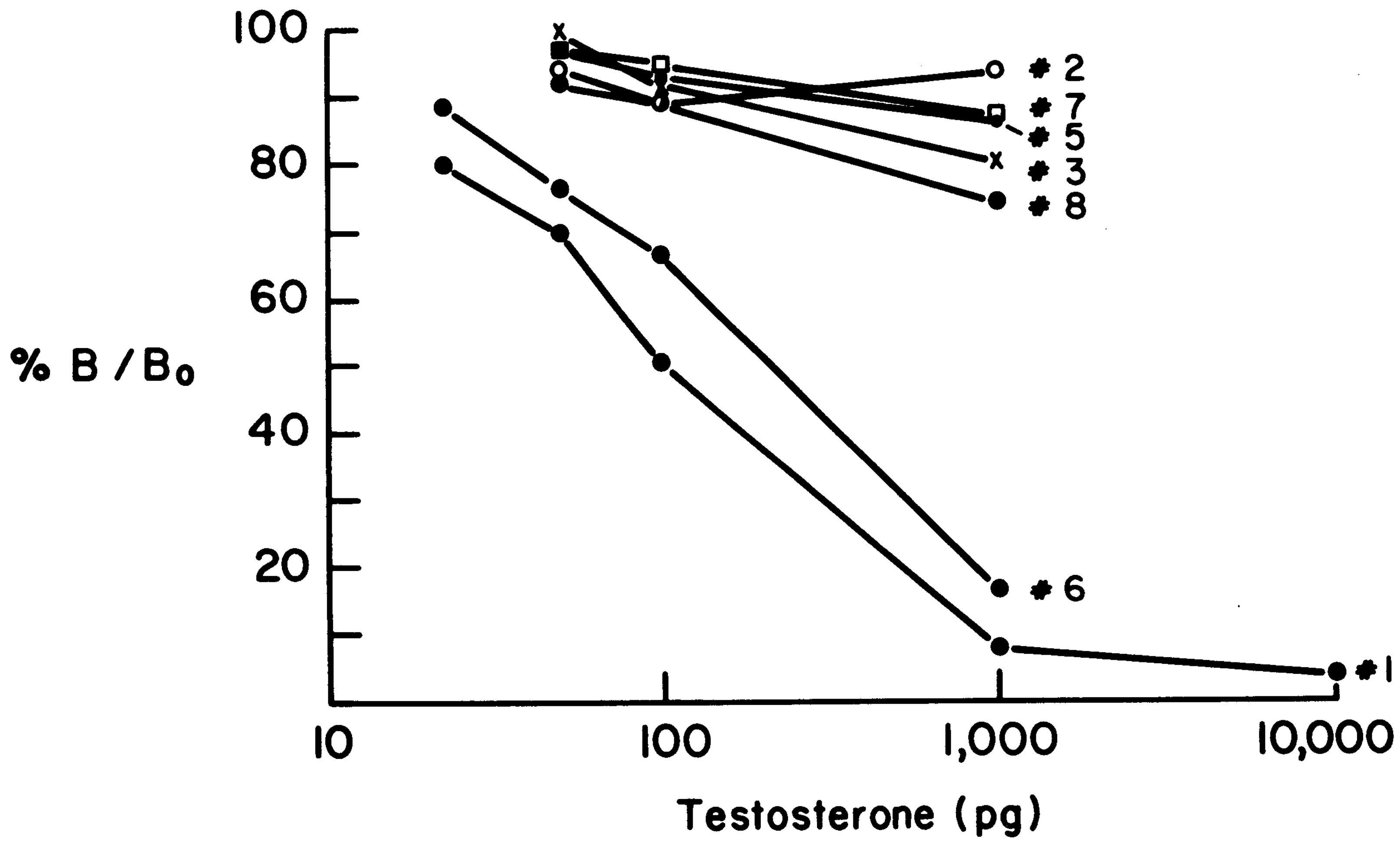
	Steroid[a]	B/B_o [b]	Testosterone equivalents in pg	Percent Displacement
#1	Testosterone	8.0	1,000	100
#2	Progesterone	93.8	2.8	<1
#3	17α-Hydroxy-progesterone	80.9	26	2.6
#4	Epi-testosterone	100	0	<1
#5	Androstenedione	86.1	11.0	1.1
#6	Dihydrotesto-sterone	16.6	640.0	64
#7	Dehydroepian-drosterone	87.3	9.0	<1
#8	5α-Androstane-3α, 17β-diol	74.7	36.0	3.6
#9	5α-Androstane-3, 17-dione	86.7	10.0	1
#10	5α-Androstane-3α-ol-17-one	96.8	1.8	<1
#11	Δ5-Androstene-3β, 17β-diol	79.5	29	2.9

Steroids, crystallized as described (see: Materials) were studied for their ability to displace ^3H-testosterone bound to the antibody utilizing the radioimmunoassay procedure described in the text. The resultant B/B_o values were converted to testosterone equivalents by reading the B/B_o values from the standard curve for testosterone in Fig. 6.

a = for systematic names see: Materials, Steroids.

b = concentration of competing steroids was 1,000 pg; B/B_o = 50% shown only for dihydrotestosterone (#6).

Figure 6. Radioimmunoassay of testosterone: specificity of the antibody for testosterone. The displacement of ^{3}H-testosterone bound to the antibody (B/B_o) by increasing concentrations of: testosterone (#1), progesterone (#2), 17α-hydroxyprogesterone (#3), androstenedione (#5), dihydrotestosterone (#6), dehydroepiandrosterone (#7), androstanediol (#8) was measured by the radioimmunoassay described in the text; the final antibody concentration was 1:30,000. Each point represents the mean of 4-6 determinations.


100
80
60
40
20
% B / B₀
10
100
1,000
10,000
Testosterone (pg)
2
7
5
3
8
6
1

Results

1. The experimental design

The majority of experiments carried out in this study depended upon the measurement of testosterone secreted into medium by fetal testes during short-term incubation, using the radioimmunoassay previously described. The feasibility of this approach requires the concentration of testosterone in the medium at any particular time to be an accurate reflection of the total testosterone synthesized.

The possibility that the concentration of testosterone in the medium does not represent an accurate estimate of the total could be due to any one of three factors. First, testosterone could be metabolized to other steroids. If such metabolites were capable of displacing testosterone from the antibody the amount of steroid produced would be incorrectly estimated to an even greater extent. Second, the fetal testes could produce other ether-soluble compounds which might, in some non-specific fashion, interfere with the radioimmunoassay. Third, testosterone could partition unequally between the tissue and medium compartments, again resulting in an inaccurate determination of the total amount synthesized.

The contributions of these three factors to the determination of testosterone synthesized during the incubation of 20.5 day testes were studied in the following experiments. In all cases, testes were prepared and incubated according to the procedures described previously. Analysis of testosterone concentrations in medium and tissues was performed by the radioimmunoassay previously described, unless otherwise stated.

A. Metabolism of testosterone

The measurement of testosterone as an accurate reflection of the total amount synthesized depends upon the extent to which testosterone is metabolized to other steroids. Since the previous studies on antibody specificity demonstrated that dihydrotestosterone can bind to the antibody used in the radioimmunoassay, particular attention was paid to the amount of testosterone reduced to that steroid.

Testes, 20.5 days, were incubated in triplicate, each flask containing 4 testes in 3 ml medium 199 and 1.4 Ci of ^{3}H-testosterone. At 0,1, and 4 hours of incubation an 0.2 ml aliquot of medium was removed and extracted with diethyl ether; 85 - 95% of the added radioactivity was recovered in the extract. Testosterone and its metabolites in medium 199 were analyzed by thin-layer chromatography using the solvent systems and procedures described previously (See: Methods, 2). After chromatography the zones were eluted and counted; none of the zones were rechromatographed.

Recovery of the label from TLC in eight out of the nine aliquots as testosterone, androstenedione, and dihydrotestosterone ranged from 86 - 100% of the counts put on the plates. In one instance, recovery was 58% ("zero" time). At "zero" time, testosterone accounted for 84.5 - 94.5% of the counts, the remainder moved as androstendione and dihydrotestosterone. At 4 hours the distribution of counts in the three zones had not changed; recovery of the label as testosterone was 86.4 - 96%. These results demonstrated that testosterone is not metabolized by 20.5 day fetal testes.

As will be seen later, LH greatly increased the synthesis

of testosterone at this age. With this in mind the above experiment was repeated in order to see if the effect of LH on testosterone synthesis also increased testosterone metabolism. Testes (4/flask) were again incubated in triplicate as above in medium containing LH (16 ng/ml). Recovery of the label from TLC in nine aliquots as testosterone, dihydrotestosterone, and androstenedione again ranged from 86 - 100%. At "zero" time, recovery of non-metabolized testosterone ranged from 88.7 - 90.4% and at 4 hours from 83.4 - 90.5%. Thus, LH had no effect on the metabolism of testosterone by 20.5 day testes.

Because of the developmental nature of the questions being asked, the above studies were repeated using 16.5 day fetal testes. Testes were incubated in the same number and manner as above, both with and without LH (160 ng/ml). Aliquots of medium (0.2 ml) were removed at 0, 1, and 4 hours and extracted in diethyl ether; 85 - 95% of the counts were recovered after extraction. Testosterone and its metabolites were analyzed by TLC using the same solvent systems as above. Recovery of the label in the three fractions of interest accounted for 80 - 85% of the total. In the absence of LH testosterone accounted for 88 - 92% of the total counts at "zero" time, and 89.3 - 96.7% at 4 hours. With LH, testosterone again accounted for 84 - 95% of the counts at "zero" time and 88 - 95% at 4 hours.

In conclusion, underestimation of the total amount of testosterone synthesized due to metabolism is unlikely to be a significant source of error.

B. Measurement of testosterone secreted into medium using gas-liquid chromatography (GLC) and radioimmunoassay

Although 20.5 day testes did not metabolize ^{3}H-testosterone,

the possibility remained that this did not give an accurate estimation of metabolic activity and that the testes produced other ether-soluble compounds which, by interfering with the measurement of testosterone by radioimmunoassay could result in erroneous testosterone values. Gas-liquid chromatography was used to provide an independent measure of the amount of testosterone present in the medium of one sample. This procedure was kindly performed by Dr. Chhanda Gupta of our laboratory.

Testes, 20.5 days, were incubated in 3 ml medium containing LH (16 ng/ml) for 4 hours. Aliquots of medium (0.2 ml) were removed at 0, 1, and 4 hours and analyzed for testosterone by radioimmunoassay. At 4 hours the concentration of testosterone in the medium was 457 ng.

The remaining 2.4 ml of medium to which had been added ^{3}H-testosterone (1,000 cpm; 40 pg) and ^{14}C-dihydrotestosterone (1,000 cpm; 1.5 ng) was extracted with diethyl ether according to the procedure described above (10 ml ether per 0.2 ml medium). The ether extract was run through a Sephadex LH-20 column to remove impurities (See: Materials, D., Purification of Radioactive Steroids). Recovery of both labels was 80%. The sample was dried and redissolved in 0.05 ml ethyl acetate for analysis by GLC. Chromatography was performed on a Hewlett-Packard Chromatograph (Series 402B) using XE60 (2% on 80/100 mesh Chromasorb G) as the solvent phase. The carrying gas was helium (20 p.s.i.), the flame gas hydrogen (40 p.s.i.), and the remainder air (20 p.s.i.). The temperature was 225° C.

Chromatography of half the sample resulted in 4 peaks with the following retention times: peak 1, 1.0 min; peak 2, 2.7 min;

peak 3, 3.5 min; peak 4, 3.9 min. Peak 2 corresponded to underivatized testosterone; none of the remaining peaks corresponded to dihydrotestosterone (retention time 4.3 min) and were not identified. The concentration of testosterone was determined by integrating the area under the curve, and gave a value of 168 ng. Correction of this value for procedural losses and aliquot taken resulted in a final value of 420 ng.

Comparison of the values for testosterone obtained by the two procedures, 457 vs 420 ng, indicated that radioimmunoassay of testosterone secreted into the medium gave an accurate reflection of the total present. No attempt was made to identify the other peaks since these compounds did not interfere with testosterone measurement by radioimmunoassay.

C. Partition of testosterone between medium and testes

The experiments in the preceding sections demonstrated that testosterone was not metabolized to any significant extent. The possibility remained that the concentration of testosterone in the medium represented an underestimation of the total testosterone synthesized. This would occur if the testes bound a significant fraction of the testosterone synthesized. The following experiments demonstrated that the testes do bind or retain a significant fraction of the testosterone synthesized. Nevertheless, as will be shown, testosterone in the medium is an accurate reflection of the totally synthesized, within certain limitations.

The first experiment was designed to measure the movement of ^{3}H-testosterone from medium to testes. To each of seven flasks, 2 testes/flask weighing 3.6 mg, was added 0.3 Ci of ^{3}H-testosterone (2×10^5 cpm); three of the flasks also contained LH (16 ng/ml).

Table IV

Fetal testes, aged 20.5 days, were incubated in 3 ml medium 199 either with or without LH (16.0 ng/ml). Testes were incubated at 37°C under $O_2:CO_2$ (95:5). At specified time intervals testes were removed from the incubation flask and homogenized in 0.5 ml phosphate buffer (pH 7.8; 0.2M). An 0.2 ml aliquot of incubation medium was removed for testosterone analysis.

A) Testes, 2 per flask, were incubated as above in medium containing 0.3μCi ^{3}H-testosterone (1,2,6,7-^{3}H-testosterone). At 0, 0.5, and 1 hour the testes were removed and treated as above and 0.2 ml of the homogenate counted according to the procedures described. (See Methods). An 0.2 ml aliquot of medium was also counted.

B) Testes, 4 per flask, were incubated as above in medium 199. At 0, 0.5, 1, and 4 hours testes were removed and treated as above. Aliquots (0.2 ml) of the tissue homogenate and of the incubation medium were removed, extracted with diethyl ether, and testosterone concentration determined by the radioimmunoassay previously described.

a = Testosterone concentration in 3 ml medium or testes (3.6 mg/testis) in cpm (A) or ng (B).

b = Ratio of testosterone concentration in testes:medium, expressed as absolute concentrations in total weight of testes to medium (total) or per unit weight testes:unit volume medium (w/v).

c = 1 flask per time point except "0" hours, no LH (2 flasks).

Table IV

Partitioning of Testosterone Between Testes and Incubation Medium with and without LH

Length of Incubation[c] (hrs)	Medium				Medium + LH			
	Testosterone conc.[a]		T/M[b]		Testosterone conc.		T/M	
	Testes	Medium	Total	W/V	Testes	Medium	Total	W/V
A. Partitioning of ^3H-Testosterone with Time of Incubation								
0	2×10^3	1×10^5	2	13	2×10^3	1.5×10^5	1.3	10
0.5	6×10^3	1×10^5	6	39	8×10^3	2.5×10^5	3	27
1	8×10^3	2×10^5	4	29	8×10^3	1.5×10^5	5.3	43
B. Partitioning of Testosterone Synthesized by Testes during Incubation								
0	4	$<.1$	-	-	5	$<.1$	-	-
0.5	2	6.5	30.8	126	13	35	37	155
1	3.5	11.5	30.4	127	7.5	61	12	88
4	7.5	28	26.8	65	10	521	2	7.8

At 0, 0.5, and 1 hour two appropriate flasks, one with and one without LH, were removed from the incubator. The testes were quickly removed from the medium, homogenized in 0.5 ml phosphate buffer (0.2 M; pH 7.8), and an aliquot (0.2 ml) counted. At the same time an 0.2 ml aliquot of medium was counted. The results are shown in Table IV. The equilibration of the labeled testosterone between the two compartments was rapid, occurring within the first 30 minutes. At "zero" time 2% of the total counts were found in the testes; this fraction increased to 6% of the total at 30 minutes, and then remained constant. LH had no effect on the partitioning of testosterone.

If the results are recalculated to take into account the differences in volume between the two compartments it becomes clear that the testes bind or retain a significant portion of the added testosterone. If the volumes are equalized the testes account for 0.12% of the incubation system. At the beginning of the incubation the testes contained about 13 times the number of counts present in the medium and at 30 and 60 minutes thirty to fourty times. LH did not affect the retention of testosterone by the testes.

The second experiment studied the movement of endogenously synthesized testosterone from tissue to medium as a function of the length of the incubation. Two flasks (4 testes/flask), one containing LH (16 ng/ml), were each incubated as above for 0, 0.5, 1, and 4 hours. Testosterone concentrations in medium and testes were measured by radioimmunoassay; their sum gave the total testosterone synthesized at each time. The results of the experiment are presented in Table IV.

In the absence of LH, the concentration of testosterone

in the testes increased two-fold, and the concentration in the medium three-fold. From 30 minutes to 4 hours the testes accounted for a constant (30%) fraction of the total testosterone synthesized.

The addition of LH to the incubation medium doubled the concentration of testosterone in the testes within the first 30 minutes. With continuing incubation this concentration remained relatively constant (within the limits of variation of testosterone synthesis by individual groups of testes). The effect of LH on the rate of testosterone synthesis was reflected in the increase in the concentration of testosterone in the medium. In the absence of LH testosterone synthesis increased three-fold during the four hour incubation, with LH there was a fifteen-fold increase in the amount of testosterone synthesized during the same time period. Thus, with LH, the rate of secretion was sufficiently great such that the testes accounted for a progressively smaller fraction of the total testosterone synthesized with time, i.e., from about 30% at 0.5 hours to 2% at 4 hours.

Recalculation of the results to account for differences in volume between the two compartments again indicated a tendency for the testes to concentrate testosterone.

In conclusion, measurement of testosterone in the medium provides an accurate reflection of total testosterone synthesis within the limits of testosterone partitioning between the two compartments. Metabolite formation is negligible and the presence of other compounds in the extract does not affect the radioimmunoassay.

II. Testosterone synthesis and LH responsiveness of fetal testes

In the previous section it was demonstrated that the

measurement of testosterone in the medium during the incubation of fetal testes provides a reliable estimation of the total amount of testosterone synthesized. The incubation system was then applied to the determination of testosterone synthesis and response to LH at different gestational ages.

The gestational ages studied were 14.5, 15.5, 16.5, 18.5 and 20.5 days. The first series of experiments was concerned with the demonstration of testosterone synthesis at each age in the absence of LH. The second study dealt with the ability of the testes at each age to respond to LH with increased testosterone synthesis. In the last section, the endogenous testosterone concentrations of testes and genital tracts were determined at ages 14.5 to 21.5 days, in order to correlate the *in vitro* findings with the *in vivo* situation.

A. Testosterone synthesis in the absence of LH

The experiments in this section were designed to answer two questions: do fetal testes of different ages synthesize testosterone, and if so, does the rate of synthesis change with age?

Synthesis of testosterone at each age was studied as a function of the length of incubation (i.e.,rate) rather than for a predetermined time. The rate of a sequence of enzyme catalyzed reactions in which one end-product accumulates must be proportional to the time of incubation, although not necessarily linear. This proportionality may cease at some point because of feedback inhibition, change in substrate concentration, depletion of required cofactors, enzyme inactivation, or other variables, frequently dependent upon

Table V

Testes were incubated under standard conditions. At selected time intervals 0.2 ml aliquots of medium were removed, extracted, and the concentration of testosterone determined by radioimmunoassay.

a = Testosterone in ng/testis shown as $\bar{M} \pm$ S.D. and C.V. (coefficient of variation S.D./$\bar{M}$). The number of testes/flask varied with experiment: 2, 4, 8, 16 testes/flask in experiments I and II; 2, 4 testes/flask in experiment III; 4 testes/flask in experiments IV, V, VII. Experiment VI had 2 flasks at 0.5 hours, 1 flask/time at 1 and 4 hours.

b = Mean rate $\pm$ S.D. for each experiment from 30 minutes to the end of the incubation.

c = Mean rate $\pm$ S.D. of all flasks as Δrate/Δtime. The rate of synthesis from 0 - 0.5 hours is significantly greater ($P < .01$) than the mean rate for the remainder of the incubation (1.26 ± 0.78).

Table V

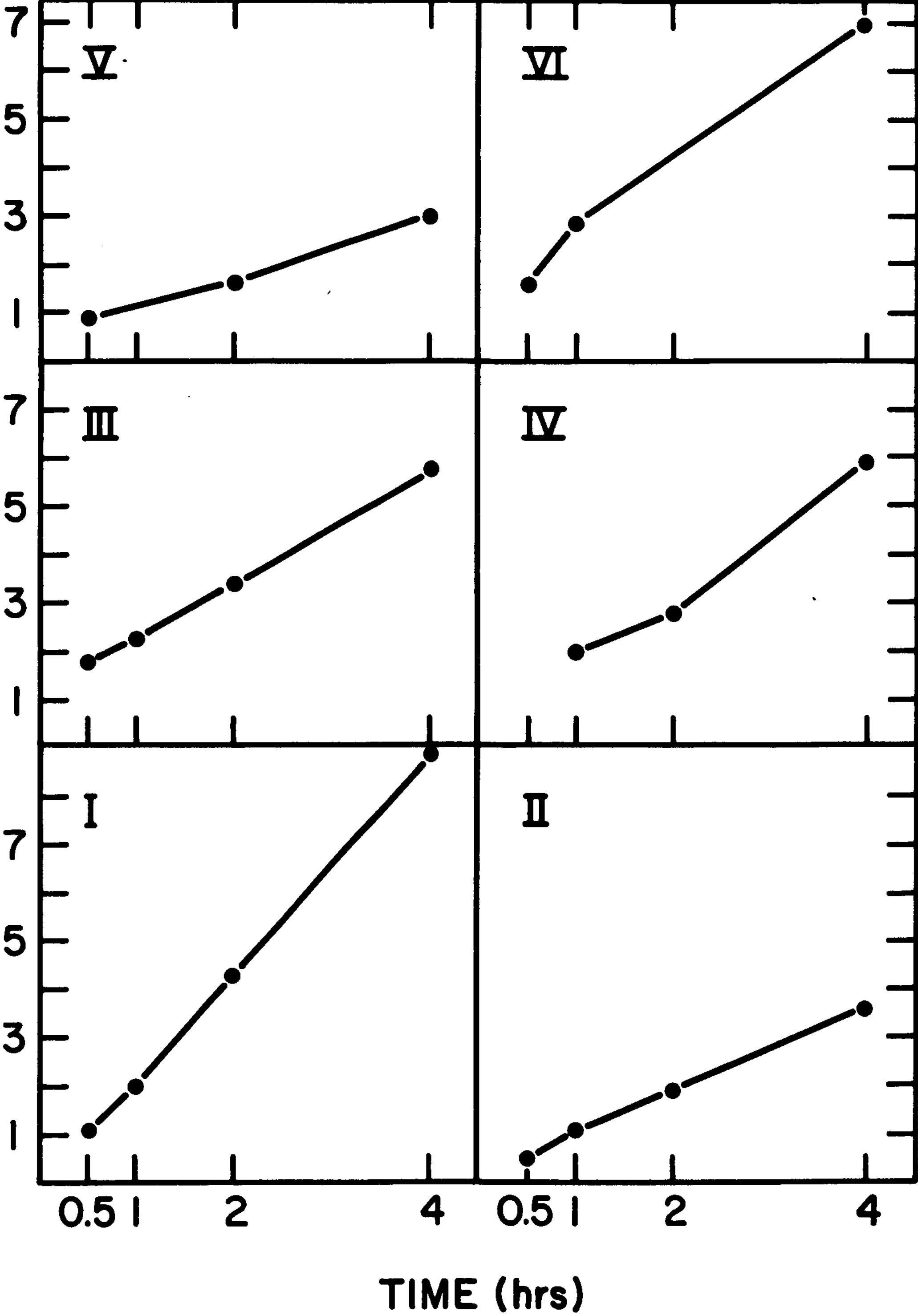
Testosterone Synthesis by 20.5 Day Fetal Testes

Experiment	Number of Flasks	Length of Incubation (hours)[a] 0.5	1	2	4	8	Rate[b] (ng/testis/hr)
I	4	1.16 ± 0.5	2 ± 0.5	4.3 ± 0.7	8.9 ± 0.4	16 ± 4.5	2.28 ± 1.1
II	4	0.5 ± 0.13	1.1 ± 0.25	1.9 ± 0.6	3.6 ± 0.6	-	1 ± 0.5
III	2	1.8 ± 0.16	2.3 ± 0.6	3.4 ± 0.1	5.8 ± 1	8.4 ± 2	1.44 ± 1.3
IV	2	-	2 ± 0.05	2.8 ± 0.04	5.9 ± 1	7.5 ± 0.7	1.2 ± 0.7
V	1	0.87	-	1.6	3	-	0.96 ± 0.66
VI	4	1.6 ± 0.6	2.8	-	7	-	1.9 ± 1.08
Average synthesis		1.1 ± 0.7	1.8 ± 0.6	3.5 ± 1.2	6.1 ± 2.4	11 ± 5	1.6 ± .78
C.V.		.66	.33	.34	.39	.45	

Rate of Testosterone Synthesis with Time[c]

Time Interval (hrs)	0 - 0.5	0.5 - 1	1 - 2	2 - 4	4 - 8
Rate (ng/testis/hr)	2.8 ± 1.2	1.3 ± 0.66	1.3 ± 0.7	1.4 ± 0.7	1.15 ± 1.1
C.V.	0.43	0.45	0.55	0.5	0.95

Figure 7. Synthesis of testosterone by 20.5 day fetal testes as a function of time of incubation in 6 individual experiments. Testes were incubated under standard conditions and at specified time intervals 0.2 ml aliquots of medium were removed, extracted, and the concentration of testosterone determined by radioimmunoassay. Mean testosterone synthesized, in ng/testis, shown for each experiment. The number of flasks/experiment and testes/flask is: I and II, 4 flasks each (2, 4, 8, 16 testes/flask); III, 2 flasks (2, 4 testes/flask); IV, 2 flasks (4 testes/flask); V, 1 flask (4 testes/flask); VI, 2 flasks at 0.5 hours, 1 flask each at 1 and 4 hours (4 testes/flask).


Testosterone (ng / testis)
TIME (hrs)
V
VI
III
IV
I
II
1
3
5
7
0.5
1
2
4

the conditions of a particular *in vitro* sytem. Further, enzyme-catalyzed reactions are directly proportional to enzyme concentration, or, in a crude tissue system, to tissue concentration. In order to obtain valid data on reaction velocities, which will in turn permit comparisons with each other, such proportionalities must be demonstrated. For these studies, testosterone synthesis by 20.5 day testes was used as a model system with which to demonstrate proportionality of synthesis to time and tissue weight.

1. Testosterone synthesis by 20.5 day testes

The synthesis of testosterone by 20.5 day testes was studied in six experiments comprising 17 flasks and four tissue concentrations. The results of these studies are presented in Figure 7 and Table V. The mean rate of testosterone synthesis was 1.6 $\pm$ 0.78 ng/testis/hr. At this age testosterone synthesis was usually linear with time from 30 minutes onward, although there was a slight tendency for the rate to decrease after 4 hours. The initial rate of synthesis (0 - 30 minutes) was significantly greater than the mean rate for the remainder of the incubation, 2.8 vs 1.6 ng/testis/hr. This result was not unexpected since the studies on the partitioning of endogenously synthesized testosterone demonstrated that the concentration of testosterone in the testes decreased from 0 to 30 minutes, while the concentration of testosterone in the medium increased from "non-detectable" to 6.5 ng at 30 minutes. Thus, from 0 to 30 minutes the concentration of testosterone in the medium represents not only synthesis but also release of preformed testosterone.

The synthesis of testosterone up to 4 hours was not affected by the concentration of testosterone in the medium nor by the incu-

bation conditions. This is shown not only by the relative constancy in the production rate, but also by the proportionality of synthesis with tissue concentration (Table VII). At 4 hours the concentration of testosterone in the medium increased proportionately with the concentration of tissue in the incubation flask, whether the results are based on the number or weight of the testes.

2. Testosterone synthesis by 14.5, 15.5, 16.5, and 18.5 day fetal testes

The incubation system was used to study the rate of testosterone synthesis at younger gestational ages. The number of experiments at each age was reduced from that at 20.5 days, since the variation in rate over 6 experiments and 17 flasks (61.9%) was not too different from that of within experiment flasks (range: 42 - 69.7%).

In the rat the testis is first recognizable on day 14.5 of gestation. At this age testosterone was not measureable by radioimmunoassay at any time up to 18 hours of incubation.

The ability of fetal testes to synthesize testoserone appeared on day 15.5. At this age the mean rate of testosterone synthesis was 0.32 $\pm$ 0.22 ng/testis/hr. From 15.5 to 16.5 days the rate increased significantly to 0.68 $\pm$ 0.35 ng/testis/hr. From 16.5 to 20.5 days the rate of synthesis increased with age, although the increase was not statistically significant ($P < .05$). At 18.5 days the mean synthetic rate was 1.02 $\pm$ 0.06 ng/testis/hr, and at 20.5 days, 1.6 $\pm$ 0.78 ng/testis/hr (Figure 8; Table VI).

As was described above, the rate of synthesis at 20.5 days was constant from 0.5 to 4 hours, and then declined non-significantly

Table VI

Testosterone Synthesis as a function of Testis Age and Length of Incubation

Age	Number of Flasks	Length of Incubation (hours)[a]					Rate[b] (ng/testis/hr)
		0.5	1	2	4	8	
15.5	2	0.32 ± 0.13	0.48 ± 0.2	0.67 ± 0.2	1.2 ± 0.5	1.8 ± 0.7	0.32 ± 0.22
16.5	3	0.67 ± 0.13	0.95 ± 0.2	1.7 ± 0.3	2.8 ± 0.2	4.8 ± 0.8	0.68 ± 0.35
18.5	5	0.5 ± 0.05	0.91 ± 0.2	2 ± 0.5	4.2 ± 1.4	-	1.02 ± 0.06
20.5[c]	17	1.1 ± 0.7	1.8 ± 0.6	3.5 ± 1.2	6.1 ± 2.4	11 ± 5	1.6 ± 0.78

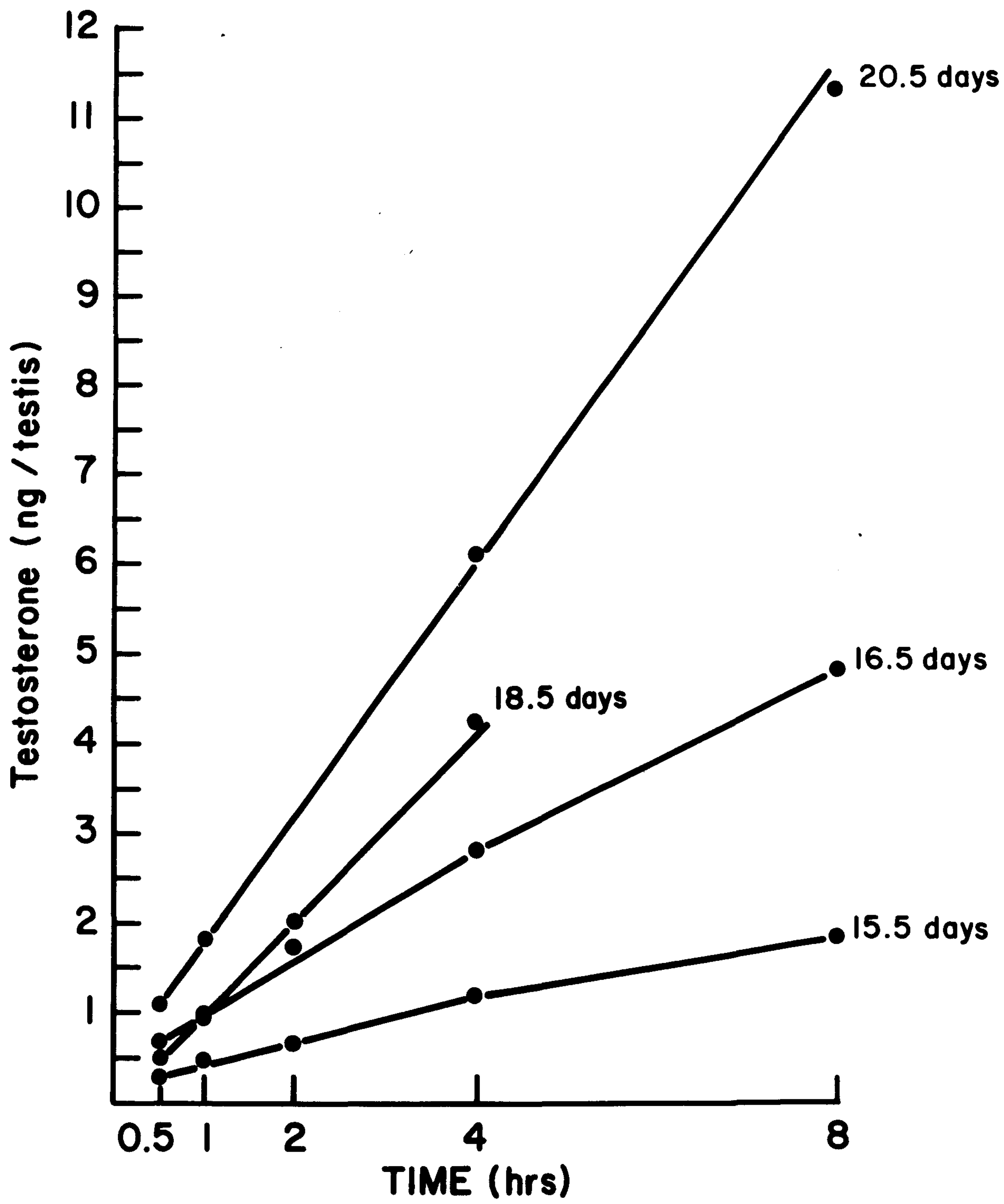
Testes were incubated under standard conditions. At selected time intervals 0.2 ml aliquots of medium were removed, extracted, and the concentration of testosterone determined by radioimmunoassay.

a = Testosterone in ng/testis shown as $\bar{M} \pm$ S.D. The number of testes/flask varied with age: 8 and 16 at 15.5 days; 4 and 8 at 16.5 days; 4, 8, 16 at 18.5 days.

b = Mean rate ± S.D. for each age from 30 minutes to the end of the incubation.

c = From Table V.

Figure 8. Synthesis of testosterone as a function of age and length of incubation by fetal testes aged 15.5, 16.5, 18.5, and 20.5 days. Each point represents mean synthesis (in ng/testis): at 15.5 days, 2 flasks (8, 16 testes/flask); at 16.5 days, 3 flasks (4, 8 testes/flask); at 18.5 days, 5 flasks (4, 8, 16 testes/flask); at 20.5 days (2, 4, 8, 16 testes/flask). Testes were incubated under standard conditions. At selected time intervals 0.2 ml aliquots of medium were removed, extracted, and the concentration of testosterone determined by radioimmunoassay.


Testosterone (ng / testis)
12
11
10
9
8
7
6
5
4
3
2
1
0.5
1
2
4
8
TIME (hrs)
20.5 days
18.5 days
16.5 days
15.5 days

from 4 to 8 hours. This pattern was repeated at 15.5 and 16.5 days. At 18.5 days there was a tendency for the rate to decrease from 0.5 to 1 hour, followed by an increase in rate from 1 to 2 hours. The possible significance of this short-term decline in rate is unclear; more experiments are needed to resolve this point. As at 20.5 days, the initial synthetic rate (0 to 30 minutes) was somewhat greater than the rate for the remainder of the incubation, although not significantly so.

Testosterone synthesis was proportional to tissue weight at 16.5 days and at 18.5 days for 4 and 16, but not 8 testes. At 15.5 days the results are based on one flask per tissue concentration, and so no conclusions regarding proportionality of testosterone synthesis with tissue weight can be drawn at this age (Table VII).

The finding that the synthesis of testosterone at each age was proportional to time from 0.5 to 4 hours meant that any change in rate with LH would most likely be a reflection of the synthetic capabilities of the testes and not an artifact of the incubation system. With this in mind, the incubation system was used to determine the responsiveness of fetal testes to LH.

B. The response of fetal testes to LH

The ability of fetal testes to synthesize testosterone did not necessarily indicate that the testes would respond to LH with increased testosterone synthesis. However, it did seem likely that once LH responsiveness had been demonstrated at a particular age, older age testes would also respond to LH, and that the responsiveness would increase, although not necessarily significantly, with age.

Table VII

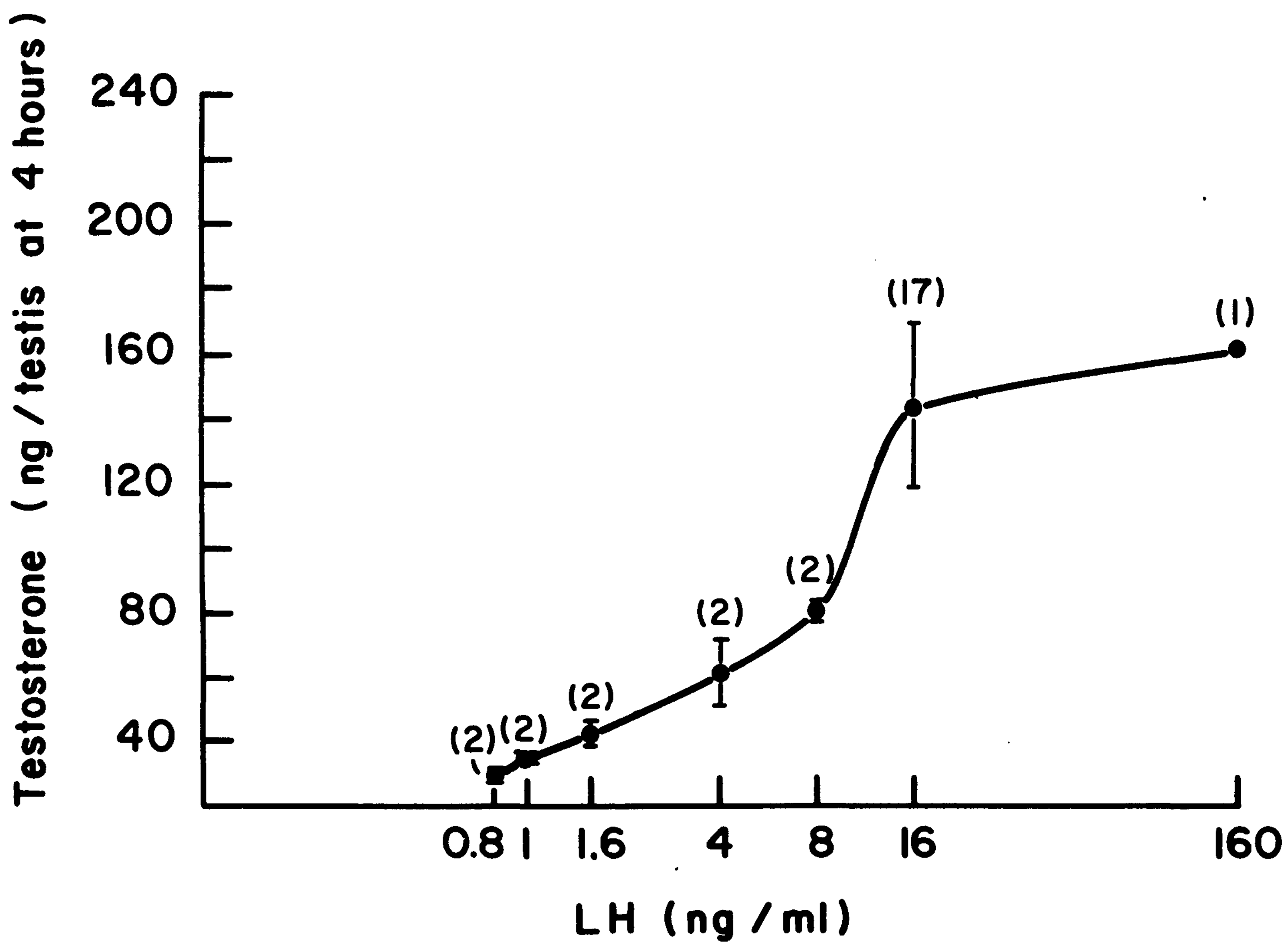
Testosterone Synthesis by Fetal Testes: Proportionality to Tissue Weight

	Number of Testes per Flask[a]			
Age	2	4	8	16
15.5	-	-	13 (29)	13 (21)
16.5	-	12 ± 0.4	21 (18)	-
18.5	-	19 ± 6 (18 ± 7)	22 (22)	71 (72)
20.5	11 ± 6.6 (6 ± 5)	26 ± 11 (16 ± 12)	50 ± 22 (26 ± 19)	102 ± 45 (50 ± 54)

Testes were incubated under standard conditions; the concentration of testosterone in the medium at 4 hours was determined by radioimmunoassay. The number of flasks per tissue concentration at each age was: at 15.5 days 1 flask/tissue concentration; at 16.5 days 2 flasks with 4 testes, 1 flask with 8 testes; at 18.5 days 3 flasks of 4 testes, 1 flask at 8 testes, 1 flask at 16 testes; at 20.5 days 3 flasks at 2 testes/flask, 3 flasks at 4 testes/flask, 2 flasks at 8 testes/flask, 2 flasks at 16 testes/flask.

a = Concentration of testosterone at 4 hours; results shown as testosterone in ng/3 ml medium 199. Value in parentheses: testosterone in ng/mg wet weight x number of testes.

Figure 9. Testosterone synthesis by 20.5 day fetal testes in response to increasing concentrations of LH added to the incubation medium. Testes, 4/flask (for 16 ngLH/ml 2, 4, 8, 16 testes/flask) were incubated under standard conditions in medium 199 to which had been added LH (0.8 to 160 ng/ml). The concentration of testosterone in the medium at 4 hours (mean $\pm$ S.D.) was determined by radioimmunoassay; the number of flasks/LH dose is given in parentheses. The increase in testosterone synthesized with LH dose is significant ($P<.05$) from 0.8 to 16.0 ng/ml.



Testosterone (ng / testis at 4 hours)
240
200
160
120
80
40
(2)
(2)
(2)
(2)
(2)
(17)
(1)
0.8
1
1.6
4
8
16
160
LH (ng / ml)

Table VIII

Testes were incubated under standard conditions in medium 199 containing LH (16.0 ng/ml). At selected time intervals 0.2 ml aliquots of medium were removed, extracted, and the concentration of testosterone in the medium determined by radioimmunoassay.

a = Testosterone in ng/testis shown as mean ± S.D. and C.V. (coefficient of variation = S.D./$\bar{M}$). The number of testes/flask varied with the experiment: 2, 4, 8, 16 testes/flask in experiments I and II; 4, 8 testes/flask in experiment III; 4 testes/flask in experiemnts IV, V, VI. Experiment VI had 2 flasks at 0.5 hours; 1 flask each at 1 and 4 hours.

b = Mean rate ± S.D. of all flasks as Δrate/Δtime. The rate of synthesis from 1 - 4 hours is significantly greater (P<.02) than the rate for 0 - 0.5 and 4 - 8 hours.

Table VIII

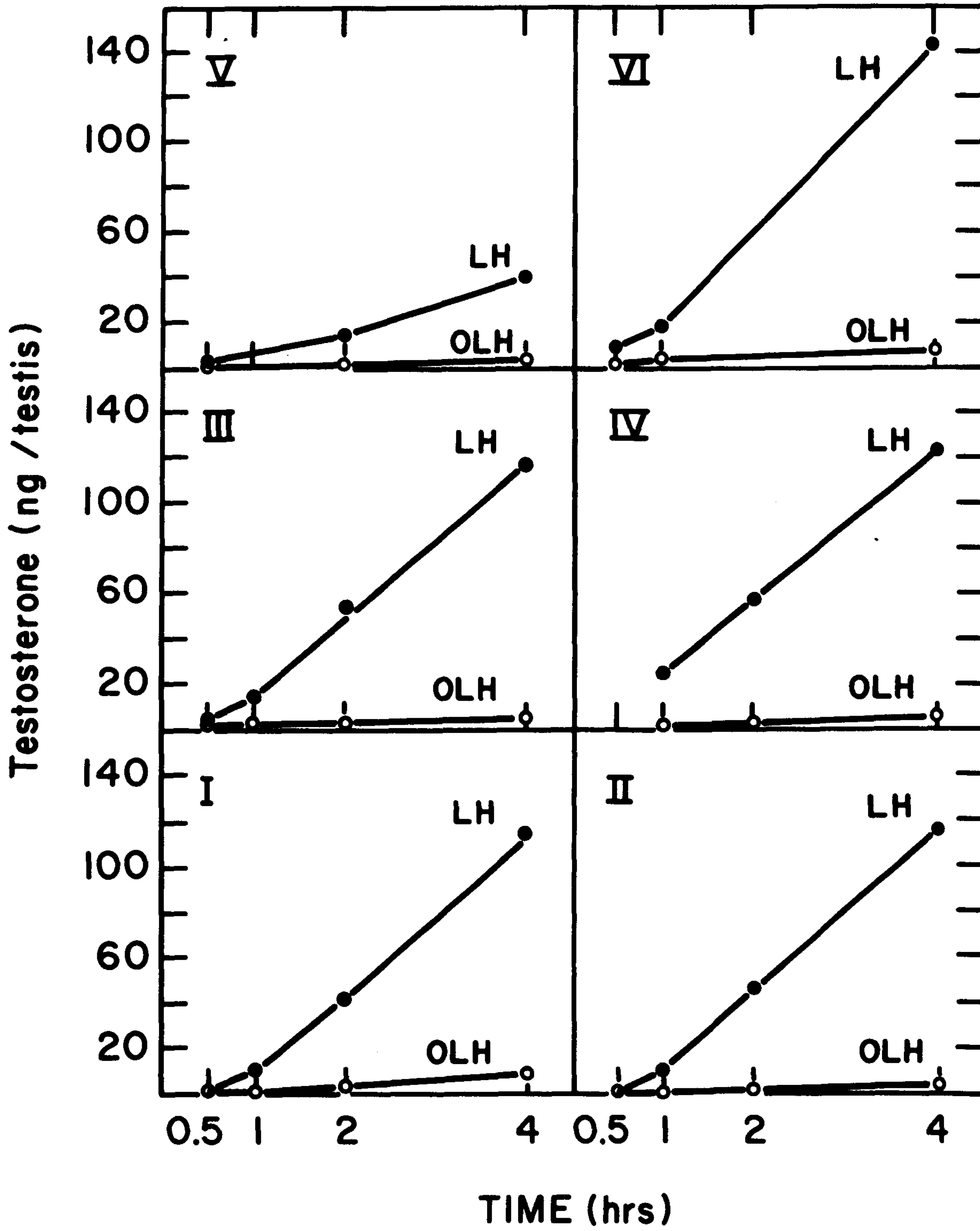
Testosterone Synthesis by 20.5 Day Fetal Testes: The Effect of LH Stimulation (16.0 ng/ml)

Experiment	Number of Flasks	Length of Incubation[a] 0.5	1	2	4	8
I	4	2 ± 0.9	11 ± 5	42 ± 20	115 ± 47	225 ± 119
II	8	1.6 ± .6	10 ± 3	47 ± 5	116 ± 17	-
III	2	4.7 ± 1.7	15 ± 4	53 ± 8	116 ± 38	198 ± 118
IV	1	-	25	57	123	204
V	1	2	-	15	40	-
VI	4	8.8 ± 2	18	-	143	-
Average synthesis		2 ± 0.9	12 ± 5	47 ± 13	121 ± 26	210 ± 10
C.V.		0.45	0.42	0.28	0.21	.05

Rate of Testosterone Synthesis with Time[b]

Time Interval (hrs)	0 - 0.5	0.5 - 1	1 - 2	2 - 4	4 - 8
Rate (ng/testis/hr)	6.9 ± 4.7	15 ± 5.8	36 ± 9	39 ± 10	27 ± 16
C.V.	0.68	0.39	0.25	0.26	0.59

Figure 10. Synthesis of testosterone by 20.5 day fetal testes as a function of time of incubation: the effect of LH (16.0 ng/ml). Mean testosterone synthesis with LH (●—●) and without LH (OLH, o—o) for the 6 experiments shown in Figure 7. Testes were incubated under standard conditions in medium 199 containing LH (16.0 ng/ml). At specified time intervals 0.2 ml aliquots of medium were removed and the concentration of testosterone determined by radioimmunoassay. Mean testosterone synthesized, in ng/testis, shown for each experiment. The number of flasks/experiment and testes/flask is: I, 4 flasks (2, 4, 8, 16 testes/flask); II, 8 flasks (2, 4 8, 16 testes/flask); III, 2 flasks (4, 8 testes/flask); IV and V 1 flask, each (4 testes/flask); VI, 2 flasks at 0.5 hours; 1 flask each 1 and 2 hours (4 testes/flask).



Testosterone (ng /testis)
140
100
60
20
V
LH
OLH
VI
LH
OLH
III
LH
OLH
IV
LH
OLH
I
LH
OLH
II
LH
OLH
0.5
1
2
4
TIME (hrs)

The increase in testosterone synthesis was studied not only in terms of the maximum increase in the rate of testosterone synthesis for each age, but also in terms of the capacity of the testes to sustain this rate for the duration of the experiment. Testosterone synthesis at 18.5 and 20.5 days was studied at increasing concentrations of LH in order to demonstrate a dose-response relationship between the concentration of LH in the incubation medium and the rate of testosterone synthesis. In addition, these studies also served to substantiate the finding of increased response to LH with increased age. This last point is of particular interest, since any hypothesis regarding the role of the fetal pituitary in the regulation of testis function must take into account the presumed low levels of circulating LH in the fetus.

1. Response of 20.5 day testes to LH

The rate of testosterone synthesis by 20.5 day testes was studied first, since the results at this age would determine if modifications in the incubation system would be necessary.

At 20.5 days the response of the testes to increasing concentrations of LH added to the incubation medium was studied first, in order to ascertain at what dose testosterone synthesis was stimulated maximally (Figure 9). The concentration to testosterone in the medium after 4 hours of incubation increased with the dose of LH, from 0.8 to 160 ng/ml. At a dose of 16 ng/ml testosterone synthesis was at or near maximal rate and this concentration of LH was used in all further experiments at this age.

At an LH concentration of 16 ng/ml testosterone synthesis was increased markedly over that seen without LH (Figure 10). Exami-

nation of testosterone synthesis with LH for the six experiments shown indicates that the rate of testosterone synthesis is constant from 1 to 4 hours. The maximum rate of synthesis was 39 $\pm$ 10 ng/testis/hr (Table VIII); this represented a twenty-fold increase in the rate of synthesis over that seen without LH (1.6 $\pm$ 0.78 ng/testis/hr). From an initial level of 6.9 ng/testis/hr (0 to 30 minutes), the rate doubled by 1 hour and between 1 and 2 hours doubled again to its maximum. Although the rate of synthesis declined significantly after 4 hours it remained above the unstimulated level (even after 18 hours in one experiment).

The marked increase in testosterone synthesis in response to LH is clearly a measure of the capacity of 20.5 day testes to synthesize testoserone. Calculation of this capacity, in terms of the concentrations of testosterone in the medium at any time is given by the response ratio:

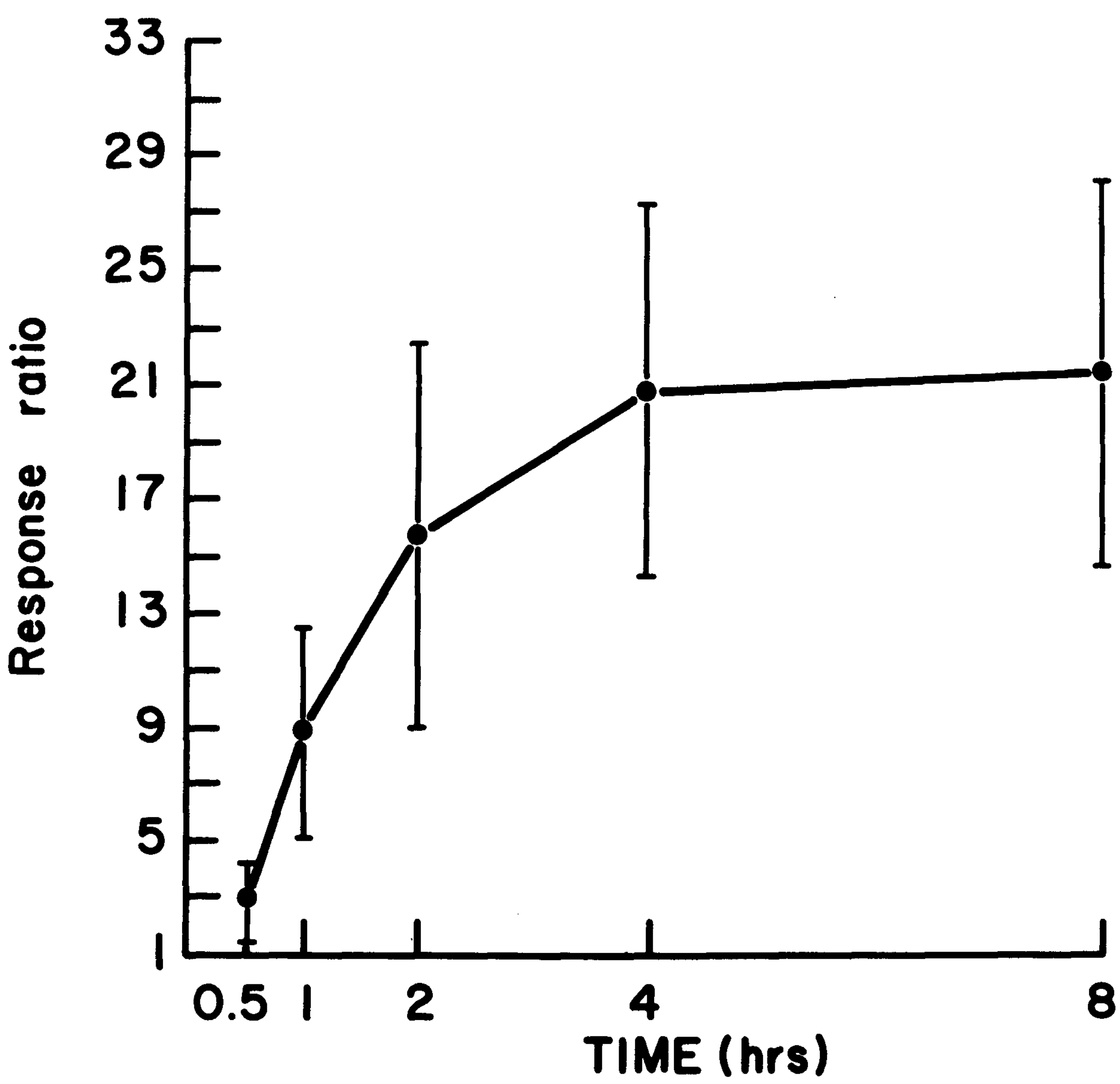
$$= \frac{\text{Testosterone (ng/testis) with LH at time "t"}}{\text{Testosterone (ng/testis) without LH at time "t"}}$$

The change in response to LH with time of incubation is shown in Figure 11 for the six experiments detailed in Table VIII. The increase in response paralleled the increase in the rate of synthesis with time, reaching a maximum of 20.5. The response to LH was highly reproducible among experiments; examination of experiment V in Figure 10 indicates that even when the rates of synthesis, both with and without LH were low, the difference (i.e., response ratio) was within expected values.

Figure 11. Responsiveness of 20.5 day testes to LH (16.0 ng/ml) with time of incubation. Response to LH was measured as the response ratio =

$$\frac{\text{testosterone (ng/testis with LH at time "t")}}{\text{testosterone (ng/testis) without LH at time "t"}}$$

Mean response ratio $\pm$ S.D. for the 6 experiments shown in Figures 7 and 10. Response increased significantly ($P < .05$) from 0.5 to 2 hours.


Response ratio
33
29
25
21
17
13
9
5
1
0.5
1
2
4
8
TIME (hrs)

The maximum response ratio was reached at 2 hours and did not decline for the remainder of the incubation. Recalculation of the response ratios in terms of the rates of testosterone both with and without LH, indicates that the change in rate under both conditions is similar, and a decline in rate with LH is matched by a corresponding decrease in the basal rate, the ratio of which is constant.

The incubation system was then applied to the determination of the responsiveness to LH by testes from younger aged fetuses.

2. Response of testes aged 14.5, 15.5, 16.5, and 18.5 days to LH

Except at 18.5 days, no attempt was made to study the synthesis of testosterone in response to increasing levels of LH. There were two major purposes to this study. The first was to determine if the ability to respond to LH occurred at a later stage in development than the ability to synthesize testosterone. The second purpose was to determine if testosterone synthesis with LH followed the same developmental pattern as testosterone synthesis without LH. The concentration of LH used at 15.5 and 16.5 days (160 ng/ml) was selected since at 18.5 days synthesis was maximum at 64 ng/ml.

Incubation of 14.5 day fetal testes with 360 ng LH/ml for up to 18 hours failed to reveal any evidence of responsiveness to LH. Since testes from this age did not synthesize measureable quantities of testosterone without LH, it is unclear if the lack of response to LH is due to an inability to synthesize testosterone, respond to LH, or both.

Table IX

Testes were incubated under standard conditions in Medium 199 containing LH (at 15.5 days, 160 ng/ml; at 16.5 days, 160 ng/ml; at 18.5 days, 64 ng/ml; at 20.5 days, 16 ng/ml). At selected time intervals 0.2 ml aliquots of medium were removed, extracted, and the concentration of testosterone in the medium determined by radioimmunoassay.

a = Testosterone in ng/testis shown as mean ± S.D. The number of testes/flask varied with age and flask: 8, 16 testes/flask at 15.5 days, 4, 8 testes/flask at 16.5 days; 4, 8, 16 testes/flask at 18.5 days, 2, 4, 8, 16 testes/flask at 20.5 days.

b = Mean rate ± S.D. in ng/testis/hr with time of incubation for each age. Underlined values indicate mean rate for that time interval is significantly different ($P < .05$) from rate for remainder of incubation.

Table IX

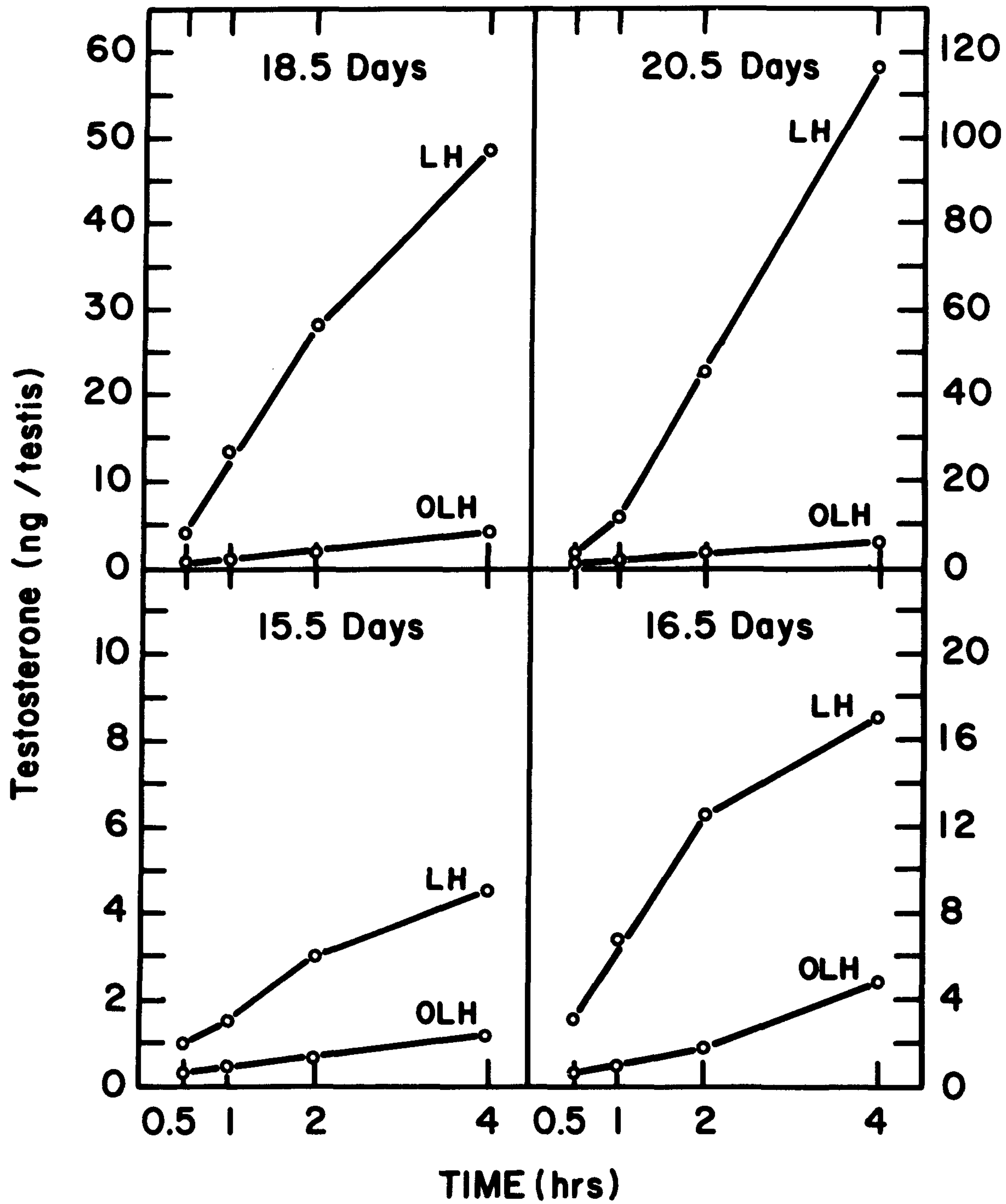
Testosterone Synthesis by Fetal Testes as a function of Age and LH Stimulation

Age	Number of Flasks	Length of Incubation[a] 0.5	1	2	4	8
15.5	3	1 ± 0.17	1.6 ± 0.5	2.9 ± 0.9	4.6 ± 0.9	6.9 ± 2
16.5	6	2.7 ± 0.9	6.6 ± 1.3	12 ± 3	17 ± 4	29 ± 6
18.5	6	6.6 ± 3.6	14 ± 6	28 ± 6	49 ± 17	
20.5	20	2 ± 0.9	12 ± 5	47 ± 13	121 ± 26	210 ± 10

Rate of Testosterone Synthesis with Time[b]

Age	Time Interval 0 - 0.5	0.5 - 1	1 - 2	2 - 4	4 - 8
15.5	7.2 ± 0.3	1.1 ± 0.8	1.3 ± 0.7	0.9 ± .06	0.6 ± 0.3
16.5	5.4 ± 1.7	8.8 ± 1.1	5.3 ± 1.1	3.1 ± 0.5	2.6 ± 1.1
18.5	6.3 ± 2.2	20 ± 13	15 ± 10	11 ± 0.6	
20.5	6.9 ± 4.7	15 + 5.8	36 ± 9	39 ± 10	27 ± 16

Figure 12. Synthesis of testosterone as a function of age and length of incubation: the effect of LH. Testes aged 15.5, 16.5, 18.5, and 20.5 days were incubated under standard conditions in medium 199 containing LH: at 15.5 and 16.5 days, 160 ng/ml; at 18.5 days, 64 ng/ml; at 20.5 days, 16.0 ng/ml. At selected time intervals 0.2 ml aliquots of medium were removed and the concentration of testosterone determined by radioimmunoassay. Each point represents mean testosterone synthesized (in ng/testis): at 15.5 days, 3 flasks (8, 16 testes/flask); at 16.5 days, 6 flasks (4, 8 testes/flask); at 18.5 days, 6 flasks (4, 8, 16 testes/flask; at 20.5 days, 20 flasks (2, 4, 8, 16 testes/flask). Testosterone synthesis in the absence of LH (OLH) is included at each age (Figure 8).


18.5 Days
20.5 Days
15.5 Days
16.5 Days
LH
OLH
Testosterone (ng / testis)
TIME (hrs)
0.5
1
2
4
0
10
20
30
40
50
60
80
100
120
2
4
6
8
12
16

One day later, at 15.5 days, the testes not only synthesize testosterone but respond to LH with increased testosterone synthesis. At this age, LH (160 ng/ml) increased the rate of synthesis from a mean of 0.32 ng/testis/hr to a maximum of 1.3 ng/testis/hr. The rate of synthesis with LH did not change significantly with time of incubation (Figure 12; Table IX). The capacity of 15.5 day fetal testes to respond to LH, as measured by the response ratios, was maximal at 2 hours; however, this value did not change significantly with time (Figure 14).

As expected, the ability to respond to LH increased with age (Figures 12, 14; Table IX). At 16.5 days, LH (160 ng/ml) increased the rate of synthesis ten-fold over the basal rate (0.68 vs 8.8 ng/testis/hr, respectively) and altered the linearity of synthesis with time. The maximum synthetic rate was reached between 30 minutes and 1 hour (Figure 12); by two hours the rate had declined to that seen with LH initially. This transitory increase in rate was reflected in the response level (i.e., response ratio) reached with time of incubation (Figure 14): the capacity for testosterone synthesis increased significantly from 30 minutes (4.0) to 2 hours (7.3) and then decreased slightly.

At 18.5 days testosterone synthesis was studied at four concentrations of LH, ranging from 1.6 to 160 ng/ml (Figure 13). An LH concentration of 64 ng/ml resulted in maximal testosterone synthesis, although the dose response curve could have reached a plateau between 16 and 64 ng/ml. At this age 64 ng LH/ml increased the synthesis of testosterone from the control rate of 1.02 ng/testis/hr to a maximum of 20 ng/testis/hr (Figure 12; Table IX). This increase represented

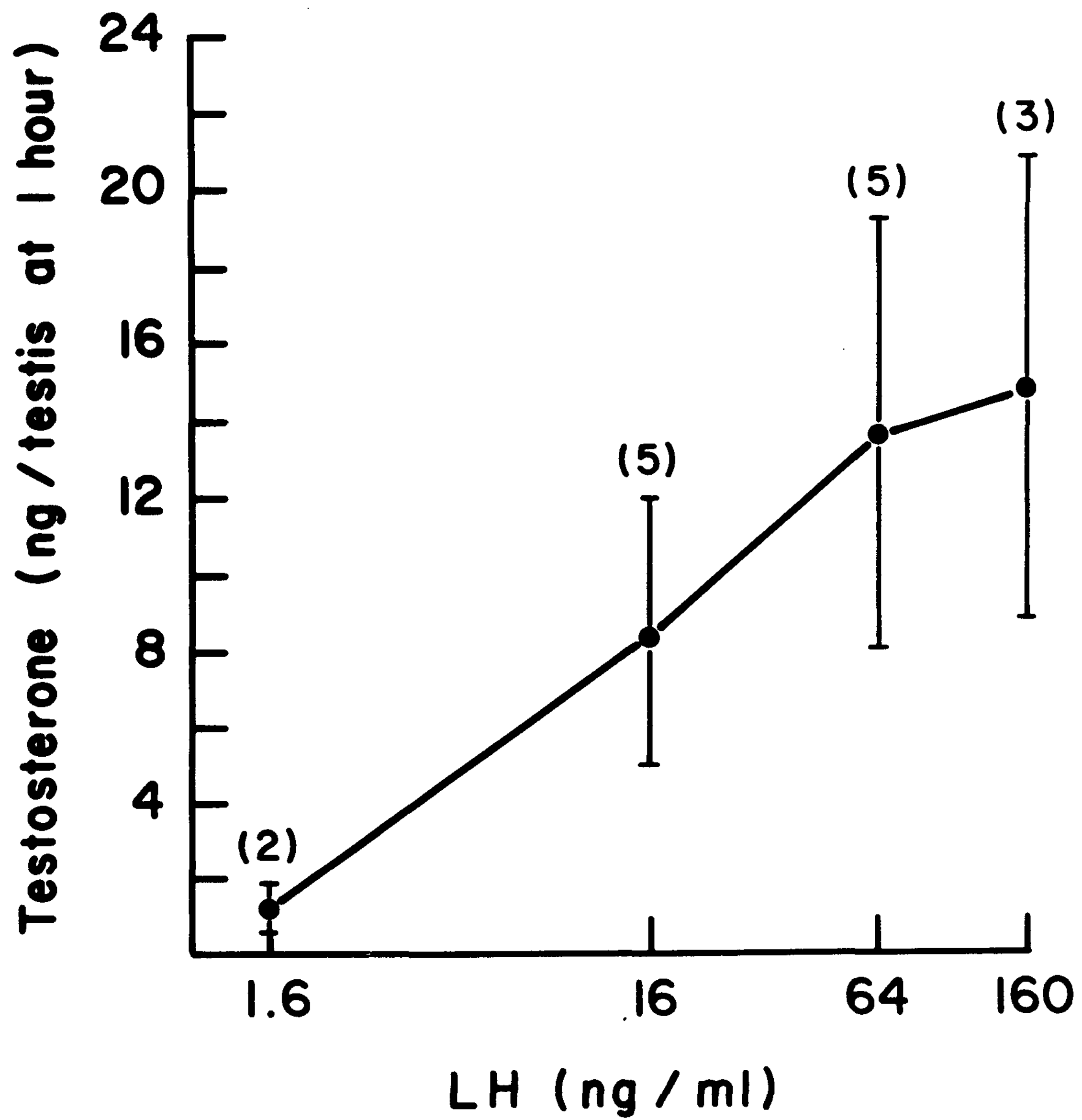
a maximum response level reached of 15.4 (response ratio) and occurred at 1 hour. Although both the rate and response ratios tended to change with time, the change was not significant. Since all experiments at this age were terminated at 4 hours of incubation, the possible significance of this trend could not be evaluated.

The synthesis of testosterone with LH, although proportional to time for only a portion of the incubation at most ages, was proportional to tissue concentration. Except at 15.5 days the concentration of testosterone in the medium at 4 hours was directly proportional to the concentration of tissue in the incubation flasks (Table X).

Comparison of the effects of LH on testosterone synthesis at each age indicated a developmental pattern in terms of both the rate of synthesis and the ability to increase synthesis over basal levels (i.e., response ratios). The maximum synthetic rate increased significantly with age from 15.5 to 20.5 days, while the unstimulated rate increased significantly only from 15.5 to 16.5 days. At 15.5 days, LH increased the rate of synthesis to that seen at 20.5 days without LH, but at the other ages the stimulated rate was greater than the basal rate at 20.5 days.

The capacity to synthesize testosterone in response to LH, as measured by the response ratios, also increased significantly from 15.5 to 18.5 days, but not from 18.5 to 20.5 days. Comparison of the change in maximum response level reached at each age with that of the change in the basal rate of testosterone synthesis with age, indicates that the pattern of increase is similar in both instances, (compare Figure 15 with Table VI). From 15.5 to 16.5 days the basal synthetic

Figure 13. Testosterone synthesis by 18.5 day fetal testis in response to increasing concentrations of LH added to the incubation medium. Testes (4, 8, 16/flask) were incubated under standard conditions in medium 199 to which had been added LH (1.6 to 160 ng/ml). The concentration of testosterone in the medium at 1 hour (mean ± S.D.) was determined by radioimmunoassay; the number of flasks/LH dose is given in parentheses. The increase in testosterone synthesized with LH dose is significant ($P < .05$) from 1.6 to 64 ng/ml.



Testosterone (ng / testis at 1 hour)
24
20
16
12
8
4
(2)
(5)
(5)
(3)
1.6
16
64
160
LH (ng / ml)

Figure 14. Responsiveness of fetal testes aged 15.5, 16.5, 18.5 and 20.5 days to LH with time of incubation. Response to LH (M $\pm$ S.D.) was measured as the response ratio =

$$\frac{\text{testosterone (ng/testis) with LH at time "t"}}{\text{testosterone (ng/testis) without LH at time "t"}}$$

Mean response ratio $\pm$ S.D. for 15.5 days (160 ngLH/ml); 16.5 days (160 ngLH/ml); 18.5 days (64 ngLH/ml); for the experiments shown in Figures 8 and 12. Mean response $\pm$ S.D. at 30 minutes for 15.5 days was 3.2 $\pm$ 0.3; 16.5 days 4 $\pm$ 0.9; 18.5 days 10 $\pm$ 6. At 1 hour mean response $\pm$ S.D. for 15.5 days was 3.2 $\pm$ 1; 16.5 days, 6.7 $\pm$ 1.6; 18.5 days, 15.6 $\pm$ 2. Mean response at 30 minutes for 18.5 day testes was significantly greater ($P < .05$) than at other ages. For 20.5 days see Figure 11.

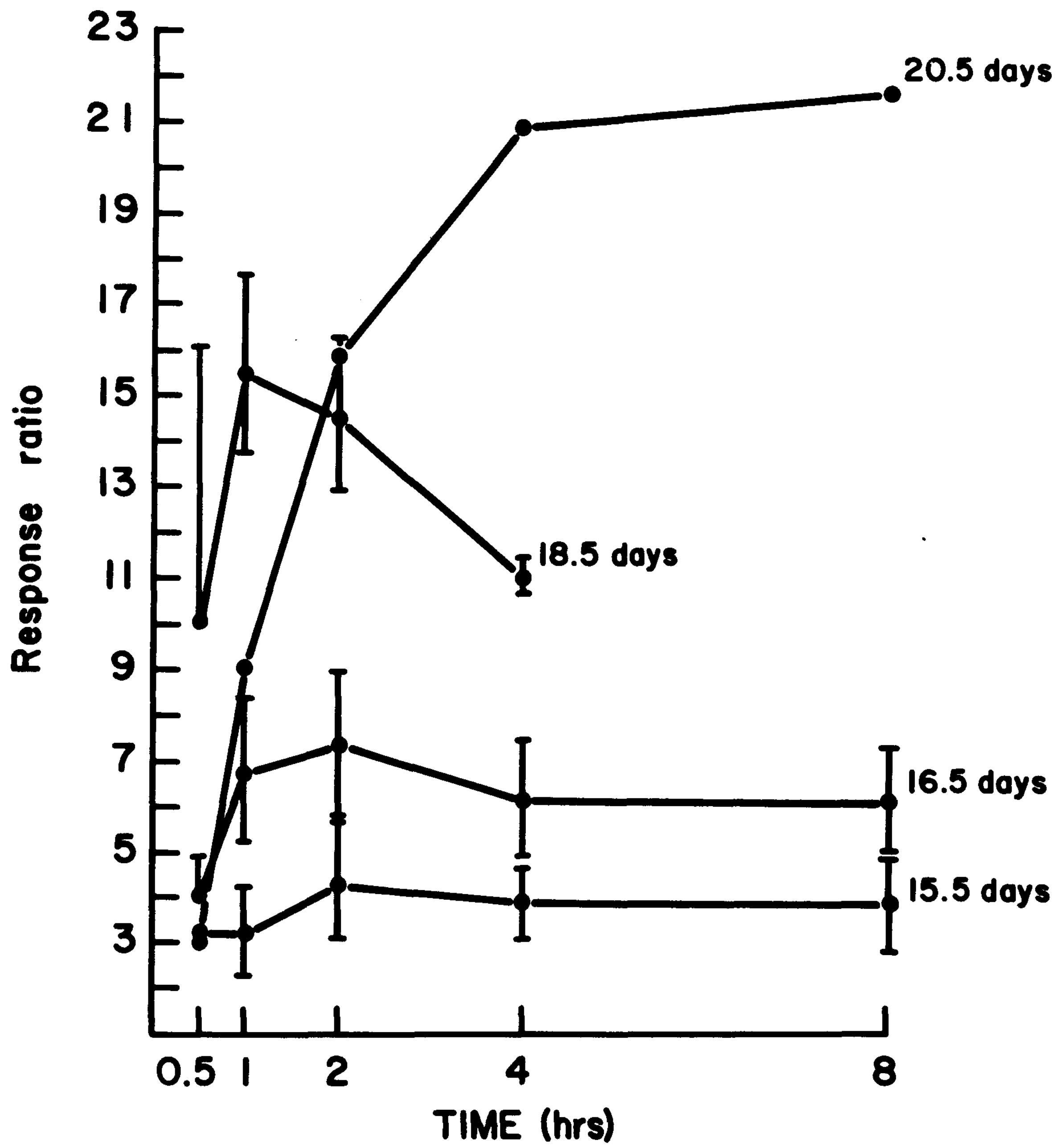

Response ratio
23
21
19
17
15
13
11
9
7
5
3
20.5 days
18.5 days
16.5 days
15.5 days
0.5
1
2
4
8
TIME (hrs)

Table X

Testosterone Synthesis by Fetal Testes: Proportionality to Tissue Weight

Age	Number of Testes per Flask[a] 2	4	8	16
15.5	-	-	40 ± 4 (66 ± 28)	58 (92)
16.5	-	66 ± 18 (57 ± 15)	142 ± 31 (101 ± 26)	-
18.5	-	146 ± 41 (146 ± 53)	266 ± 26 (276 ± 12)	523 (615)
205	217 ± 63 (100 ± 14)	381 ± 115 (231 ± 92)	1053 ± 202 (518 ± 28)	2061 ± 385 (1255 ± 195)

Testes were incubated under standard conditions in 3 ml medium 199 containing LH: at 15.5 and 16.5 days, 160 ng/ml; at 18.5 days, 16 and 64 ng/ml; at 20.5 days, 16 ng/ml. The concentration of testosterone in the medium at 4 hours was determined by radioimmunoassay. At each age the number of flasks per tissue concentration was: at 15.5 days 2 flasks at 8 testes/flask, 1 flask at 16 testes; at 16.5 days 3 flasks at 4 testes/flask, 2 flasks at 8 testes/flask; at 18.5 days 5 flasks at 4 testes/flask, 2 flasks at 8 testes/flask, 1 flask at 16 testes/flask; at 20.5 days 3 flasks at 2 testes/flask, 4 flasks at 4 testes/flask, 4 flasks at 8 testes/flask, 3 flasks at 16 testes/flask.

a = concentration of testosterone at 4 hours in: ng/3 ml medium 199.

Values in parentheses: testosterone in ng/mg wet weight x number of testes.

rate doubled, as did the maximum response level reached. From 16.5 to 18.5 days the rate of synthesis without LH increased about 1.5 times, and from 18.5 to 20.5 days increased slightly. The maximum response level reached from 16.5 to 18.5 days increased two-fold and by 20.5 days, about 30%. The possible implications of this developmental pattern will be discussed later.

At 20.5 days the response level at 30 minutes was significantly lower than that at 18.5 days. However, with increasing time of incubation, the response increased to a maximum level at 2 to 4 hours. The implications of the delay in reaching maximum response to LH for the development of fetal testis function with age will be discussed later.

3. Sensitivity and responsivity of fetal testes to LH

From the results presented in the previous sections it is clear that not only does the rate of synthesis increase with age, but that for any given dose of LH the magnitude of the response increases with age.

Examination of the data in Table XI demonstrates that testosterone synthesis at 20.5 days is more sensitive and responsive to LH stimulation than at any other age. At 15.5 days, 160 ng LH/ml increased testosterone synthesis four-fold; at 20.5 days a four-fold increase in synthesis (i.e. response ratio) was obtained using one-hundreth the dose, 1.6 ng/ml. At 16.5 days, 160 ng/ml increased synthesis eight-fold; at 20.5 days a comparable increase was obtained with 4 ng LH/ml. At 18.5 days, the increase in testosterone synthesis with 1.6 ng LH/ml is not significantly different from the control value, whereas at 20.5 days, an LH dose of 0.8 ng/ml increased testosterone

Figure 15. Maximum response to LH reached as a function of testis age. Response to LH measured as the response ratio =

$$\frac{\text{testosterone (ng/testis) with LH at time "t"}}{\text{testosterone (ng/testis) without LH at time "t"}}$$

Time, "t", of maximum response and LH dose (ng/ml) varied with age : at 15.5 days, 2 hours at 160 ngLH/ml; at 16.5 days, 2 hours at 160 ngLH/ml; at 18.5 days, 1 hour at 64 ngLH/ml, at 20.5 days, 4 hours at 16 ngLH/ml. Response increased significantly ($P < .05$) with age except from 18.5 to 20.5 days. Data taken from Figures 11 and 14.

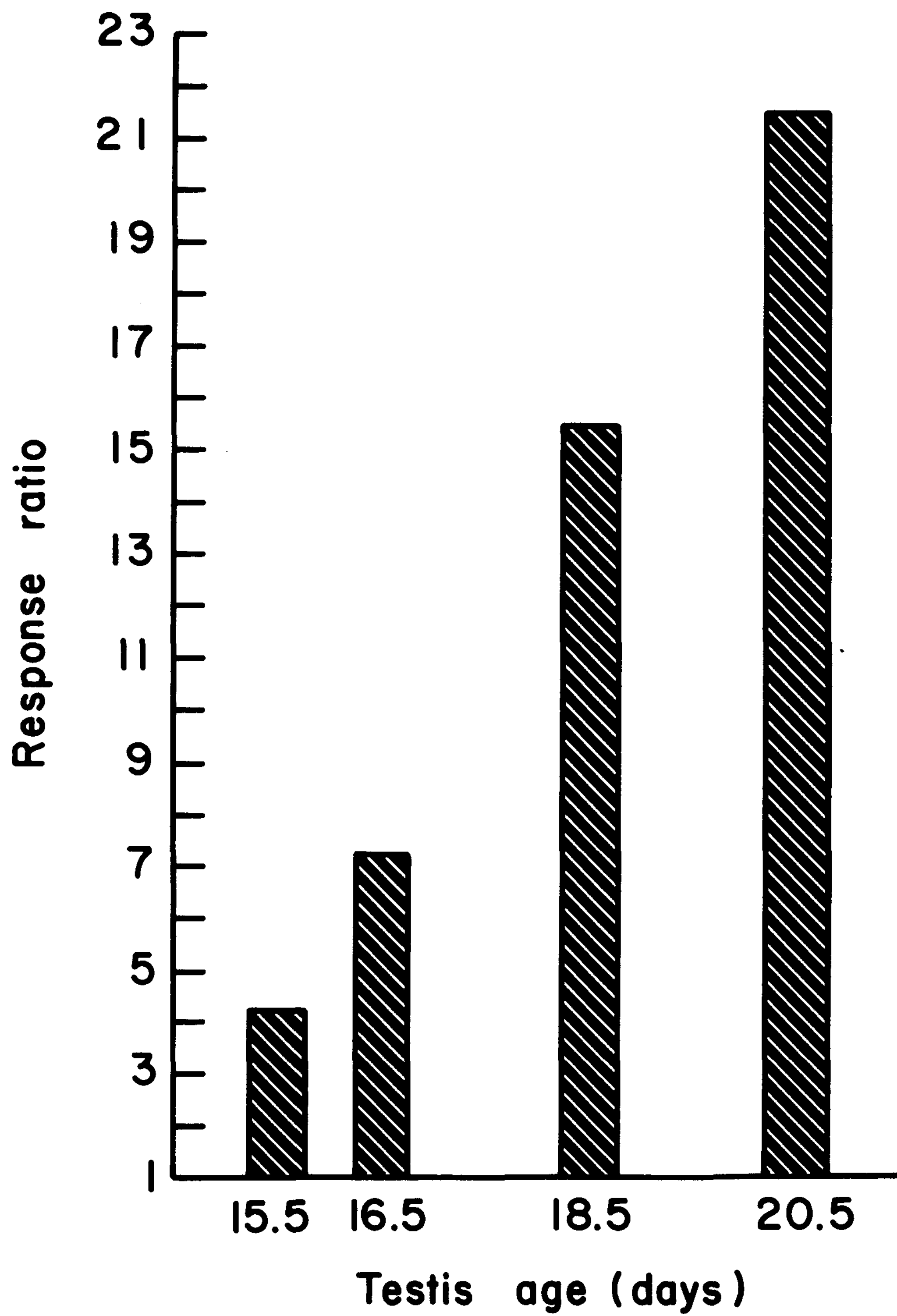

23
21
19
17
15
13
11
9
7
5
3
1
Response ratio
15.5
16.5
18.5
20.5
Testis age (days)

Table XI

Maximum Response to LH Reached as a function of LH Dose

and Fetal Testis Age

Testis Age (days)	LH (ng/ml)					
	0.8	1.6	4.0	16	64	160
15.5						4.3(3)
16.5				5.6(1)		8.2(5)
18.5		1.4(2)		9.4(5)	15.4(5)	16.4(3)
20.5	2.7(2)	3.9(2)	7.3(2)	20.5(17)		24.4(1)

Testes were incubated under standard conditions in medium 199 to which had been different concentrations of LH. The response to LH was measured as the response ratio:

$$= \frac{\text{testosterone (ng/testis) with LH at time "t"}}{\text{testosterone (ng/testis without LH at time "t"}}$$

At 15.5 days and 16.5 the mean maximum response to LH was reached at 2 hours; at 18.5 days at 1 hour, at 20.5 days at 4 hours. The numbers in parentheses indicate the number of determinations at each LH concentration for each age testis.

synthesis significantly. At 18.5 days maximal stimulation was reached at an LH concentration of 64 ng/ml; this contrasted with a maximal dose of 16 ng/ml at 20.5 days.

4. Specificity of the response to LH

The increase in testosterone synthesis by 20.5 day testes, in response to gonadotropic stimulation, appears to be specific for LH. The addition of FSH and Prolactin to the incubation medium, at concentrations (16 and 160 ng/ml) which resulted in maximal LH stimulation of testosterone synthesis, did not increase the rate of testosterone synthesis over that seen in the absence of LH. Prolactin (200 or 400 ng/ml) plus LH (16 ng/ml) did not increase synthesis over that of LH alone, after 4 hours of incubation. The effect of FSH and Prolactin with submaximal doses of LH was not studied; neither was the specificity of the response at other ages. Since no attempt was made to check either the potency of these particular lots of FSH and Prolactin or their stability under the incubation conditions, additional studies are needed to confirm the above findings.

5. Endogenous concentrations of testosterone in fetal reproductive tissues

The finding of both testosterone synthesis and responsivity to LH at all stages of testis development except 14.5 days, raised the question of the role of testosterone in the differentiation of the internal and external genitalia. The demonstration of testosterone in fetal reproductive tissues at the relevant gestational ages would provide strong support for a role for testosterone in the development of the secondary sexual characteristics. If the concentration of testosterone in the testes increased with age, this

would also tend to support the *in vitro* findings of a developmental pattern of testosterone synthesis.

Testosterone was measured in the testes, ducts, and lungs of fetuses aged 14.5 - 21.5 days. The results are presented in Table XII. Testosterone was not found in lung tissue at any age. Testosterone was first measureable in testes and ducts on day 15.5. The concentration of testosterone in the testes increased with age from 15.5 to 17.5 days (most markedly between 16.5 and 17.5 days), remained constant until day 20.5, and then appeared to decline, although this is unclear. The concentration of testosterone in the genital tracts showed no consistent pattern with age but tended to remain fairly constant. Thus, with age, synthesis and/or retention of testosterone was favored over release into the ducts. These results are tentative, and must be repeated due to the high amount of variation seen in replicate measurements at each age. However, these results are consistent with a role for testosterone in the differentiation and masculinization of the genital tracts.

Table XII

Endogenous Testosterone Concentrations in Testes and Genital Ducts with Fetal Age

Age	Number of Determinations	Testes	Genital Ducts	Testes/Duct[a]
14.5	2	<.1	<.1	-
15.5	1	0.25	0.1	2.5
16.5	3	0.56 ± 0.28	0.024 ± .018	23
17.5	1	2.6	0.46	5.6
18.5	2	2.6 ± 2.4	0.05 ± .05	52
19.5	3	2 ± 1.1	0.04 ± .04	50
20.5	2	3.5 ± 2.1	0.15 ± .06	23
21.5	2	0.85± .09	.026 ± .008	33

Testes and genital ducts were removed from fetuses and homogenized in 0.5 ml phosphate buffer (0.2M; pH 7.8). An 0.2 ml aliquot of the homogenate was extracted and the concentration of testosterone determined by radioimmunoassay. Number of determinations and mean testosterone ± S.D. (in ng/unit tissue) are shown. The number of testes and ducts for each determination varied: at 14.5 days 60, 37, testes and ducts; at 15.5 days 27 testes, 24 ducts; at 16.5 days 22, 34, 34 testes/determination, 20,36 ducts/determination; at 17.5 days 22 testes, 19 ducts; at 18.5 days 18, 24 testes/determination, 18, 24 ducts/determination; at 19.5 days 16, 32, 22 testes/determination, 16, 54, 20 ducts/determination; at 20.5 days 10, 20 testes/determination, 10, 20 ducts/determination; at 21.5 days, 34, 52 testes/determination, 33, 38 ducts/determination.

a = ratio of mean testosterone in testes to mean testosterone in ducts.

Discussion

The experiments presented demonstrate two important aspects of fetal testis function in the rat: an increasing capacity to synthesize testosterone *de novo* with increasing age, and the ability to respond to LH with increased testosterone synthesis. The *in vitro* demonstration of these two facets of testicular activity in the fetal period extends the time course for the differentiation and maturation of function of one part of the hypothalamic-pituitary-testicular axis into the fetal period. The endogenous levels of testosterone in the testes and genital tracts of male fetuses throughout the period of sexual differentiation of the fetus supports a role for testosterone in the expression of the male phenotype and confirms the *in vitro* findings.

Since the majority of the experiments were concerned with the *in vitro* synthesis of testosterone and responsiveness to LH, a great deal of emphasis was placed on determining the limitations of the incubation system. It was hoped that testosterone synthesis by 20.5 day testes *in vitro* could be used as a bioassay for pituitary function during fetal life. Although there is insufficient data at this time to decide whether or not this is feasible, as will be discussed later, it was determined that the concentration of radioimmunoassayable testosterone secreted into the incubation medium is a reliable index of testosterone synthesis with age and LH stimulation.

The radioimmunoassay for plasma testosterone developed by Brenner, Guerrero, Cekan, and Diczfalusy (1973) was modified to reflect the experimental conditions, i.e. the measurement of testosterone extracted from incubation medium and tissue. The more tedious, but

more accurate, practice of extracting a fresh set of testosterone standards for each group of experimental samples was followed. Each time an assay was performed, two standard curves - one for testosterone standards prepared in buffer, the other for standards extracted from the incubation medium - were performed. This enabled an accurate determination of the incubation conditions each time the assay was performed. The experimental results were thus accepted or rejected on the basis of the agreement of the B/B_0 values at each testosterone concentration for both sets of standards with those previously obtained. Replicate experimental samples were assayed on at least two successive days in order to account for any inter-assay variation. The concentration of antibody used in the assay and the specificity of the antibody for testosterone were carefully determined.

The studies on the specificity of the antibody for testosterone demonstrated that dihydrotestosterone significantly displaced ^3H-testosterone bound to the antibody. The need for purification of sample extracts prior to radioimmunoassay in order to separate these two steroids was assessed by determining the extent of testicular metabolism of testosterone. Testes from both early (16.5 days) and late (20.5 days) stages of development did not metabolize testosterone to any significant extent. LH, which markedly increased testosterone synthesis at both ages, did not affect the metabolism of testosterone. The results at 20.5 days were confirmed by gas-liquid chromatography, which, in addition, showed that other compounds present in the ether extract did not interfere with the radioimmunoassay of testosterone.

These results are in agreement with the studies of Noumora and co-workers (1966) who demonstrated that fetal rat testes at all

stages of development converted progesterone to testosterone and a small amount of androstenedione. In this respect fetal testes more closely resemble adult than pubertal testes. During the early stages of puberty testosterone is extensively metabolized to 5 - reduced steroids (Nayfeh et al, 1966) whereas in adulthood, testes synthesize predominantly testosterone during *in vitro* incubation (Dufau et al, 1972). The circulating levels of steroids in the male rat from immature through adult stages reflect this change in *in vitro* synthetic pathways: as the rat goes from puberty to adulthood, the relative concentrations of androstenedione and testosterone shift towards the latter (Resko et al, 1968).

The measurement of responsiveness to LH as a change in the *in vitro* synthesis of testosterone required that the concentration of testosterone in the medium accurately reflect the total amount synthesized. The synthesis of testosterone and its retention by the testes was studied using 20.5 day fetal testes. LH could conceivably have affected the concentration of testosterone in the medium by at least two separate mechanisms: an effect on synthesis or an effect on testis retention and/or permeability to testosterone. The studies on the partitioning of both radioactive and endogenously synthesized testosterone demonstrated that both with and without LH the testes retained a constant amount of testosterone after the first 30 minutes of the incubation. The major effect of LH was to rapidly increase the concentration of testosterone in the medium above that seen without LH, six-fold in the first hour, thus decreasing the relative importance of the testes' contribution to the total synthesized. Studies on the retention of added ^{3}H-testosterone by 20.5 day testes

indicated that LH had no effect on the permeability of the testes to testoserone. The conclusion was reached that the increase in the concentration of testosterone in the incubation medium is due to an effect of LH on the rate of testosterone synthesis, although this has not been conclusively demonstrated.

It was expected that since the synthesis of testosterone is controlled by a series of enzymatic reactions (See: Introduction) the synthesis of testoterone under the *in vitro* conditions employed in the present study should show proportionality with time and tissue concentration. At 20.5 days, as at all other ages, the unstimulated rate of testosterone synthesis was relatively constant from thirty minutes to 4 hours, allowing the calculation of a mean synthetic rate for each age testis. As was predicted from the studies on the partitioning of testosterone at 20.5 days, the initial rate of synthesis, 0 to 30 minutes, was greater than the mean rate for the remainder of the incubation. The high initial synthetic rate was explained on the basis of synthesis coupled with release of pre-formed testosterone. This has been tentatively demonstrated only for 20.5 day testes, but a similar mechanism is consistent with the results with younger aged testes. Testosterone synthesis was proportional to tissue weight at all ages except for 15.5 days. The implications of this finding at 15.5 days, if reproducible, are unclear, implying as they do that testosterone synthesis at this age is not enzymatically regulated.

LH increased the rate of testosterone synthesis at all ages, such that the increase in medium testosterone was linear with time for at least some portion of the total incubation. The tendency

for the rate of synthesis to decline after 4 hours without LH was more pronounced with LH, particularly at 16.5 and 20.5 days. Although it is possible that this decline was solely the result of the incubation conditions, it is more likely that it is a reflection of the ability of the testes to maintain a high level of response to LH. The same phenomenon is observed in the incubation of adult testes with and without LH (Dufau et al, 1972), and in the response of pubertal and adult, but not prepubertal, testes to the injection of LH (Odell et al, 1974).

Since no measurements of the concentrations of LH in the incubation medium were made during or after the incubation, it was not possible to determine if the decrease in rate seen with LH, relative to that seen without LH, is the result of LH degradation or a manifestation of testicular "exhaustion" or "insensitivity." If the decrease in rate were due to the degradation of LH or to its adsorbance onto the glass walls of the incubation flasks, the repeated addition of LH to the incubation medium should result in maintenance of the maximum stimulated rate. If the defect is testicular, or results from lack of substrate, repeated transfers of the testes to fresh medium with and without LH should result in a return to the high levels of synthesis seen originally.

The experiments demonstrating endogenous testosterone synthesis and responsiveness to LH with developmental age represent the first systematic study of these parameters of testis function in the rat fetus. The results on the synthesis of testosterone in the absence of LH were not entirely unexpected. The studies of Noumora and coworkers (1966) had demonstrated increased testosterone synthesis

from progesterone with increasing fetal age from 14.5 days on. In addition, Schlegel and co-workers (1966) had demonstrated that certain enzymes necessary for the conversion of pregnenolone to progesterone were present in the testes from day 15.5 and increased in activity to day 18.5.

The in vitro studies presented here demonstrated that testes aged 14.5 days do not synthesize or contain testosterone. These results are in agreement with those of Warren et al, (1972), who demonstrated only cholesterol in 14.5 day testes. Since testosterone synthesis at this age could not be demonstrated in the absence of LH, the failure to measure a response to LH was not unexpected. However, it is not clear if the lack of response is due only to an inability to synthesize testosterone. Other measures of responsiveness to LH, e.g., the presence of LH receptor molecules, increase in cyclic AMP and/or enzyme activity, and possible formation of testosterone precursors, are necessary to resolve this question. It should be recalled that Scheib and Lombard (1971) demonstrated increased 3β-hydroxysteroid dehydrogenase activity in 12.5 day mouse testes (equivalent to 14.5 day rat testes) when cultured with adult pituitary.

The increase in the rate of testosterone synthesis from 15.5 to 18.5 days seems to parallel the increase in the number of Leydig cells. According to Roosen-Runge and Anderson (1959) the number of Leydig cells increases from day 15 through day 19 and then declines; the greatest increase in the number of cells is seen between 15 and 16 days. The conversion of progesterone to testosterone follows this pattern of Leydig cell increase but declines only after birth (Noumora

et al, 1966). In the present study the rate of testosterone synthesis increased significantly from 15.5 to 16.5 days (0.32 vs 0.68 ng/testis/hr, respectively). From 16.5 days to 18.5 days the rate increased to 1.02 ng/testis/hr, and by 20.5 days to 1.6 ng/testis/hr. The increase in synthetic rate with age from 15.5 to 18.5 days would seem to parallel the increase in Leydig cell number. The continued increase in the rate of 20.5 days, in the face of declining numbers of Leydig cells is consistent with the idea that the capacity of the individual cells to synthesize testosterone increases at this time. In this respect it is interesting to recall that 3β-hydroxysteroid dehydrogenase, Δ^4,5-steroid isomerase activity increased during development to a maximum at day 17 and then was maintained at a relatively constant level until after birth (Schlegal et al, 1966).

The ability to respond to LH is based on a series of mechanisms and reactions only partially related to steroid synthesis. Thus, there was no a priori reason to expect that fetal testes would respond to LH. Although the mechanisms responsible for increased testosterone synthesis in response to LH have not been fully delimited (Hall, 1970) it is probable that cyclic AMP is involved (Moyle and Ramachandran, 1973)., Whatever the steps are that lead to increased hydroxylation of cholesterol at carbon-22, it would appear that a long series of intermediate reactions are necessary. Thus, it appeared likely that in the first few days after testis differentiation, one or more enzymes necessary for the full series of reactions to occur would be lacking. However, there were two lines of evidence that tended to indicate that testes from late-gestation fetuses (days 19-21) would respond to LH. The work of Goldman and co-workers (1971) had shown that unilateral castration of male rats on day 3 post-natal, resulted

in increased circulating levels of LH and weight of the remaining testis. The second line of evidence concerned demonstrations of LH (Kuznetzova, 1970) and gonadotrophs (Svalander, 1974) in the pituitaries of late-gestation rats.

The finding in the present study that responsiveness to LH could be demonstrated on day 15.5, the age at which the testes first demonstrated the ability to synthesize testosterone, rather than at a later stage, indicates that the Leydig cells possess both the receptors and enzymes necessary to respond to LH. These results suggest that the lack of response to LH at 14.5 days is due to an inability to synthesize testosterone.

The results on the increased rate of testoserone synthesis in response to LH stimulation demonstrated that both responsivity and sensitivity to LH increase with age. As testes age increased the concentration of LH required to increase testosterone synthesis maximally over basal levels decreased. Thus, 20.5 day testes were one-hundred-fold more sensitive to LH than 15.5 day testes, forty-fold more sensitive than 16.5 day testes, and 4-fold more sensitive than 18.5 day testes. The results would seem to suggest that the increase in sensitivity to LH can be dissociated from an increase in the ability of the individual Leydig cells to synthesize testosterone, except from 18.5 to 20.5 days. First, the basal rate of testosterone synthesis seemed to increase as a function of the number of Leydig cells, without assuming a change in capacity per cell. Second, comparison of the maximum response ratios reached at 15.5, 16.5, and 18.5 days, irrespective of the dose of LH needed to get that response, indicates that this measure of testis function also increases proportionately with the

number of Leydig cells (compare Figure 15 with Table VI). The increase in sensitivity, however, implies that the ability to utilize LH becomes more efficient with age, and that this change in LH utilization occurs at some point prior to the steps involving the direct synthesis of testosterone.

From 18.5 to 20.5 days the increase in the rate of testosterone synthesis with LH must in part reflect the increased capacity of the Leydig cells to synthesize testosterone. However, that this is not the only contributing factor at this age is implied by the time delay at 20.5 days in reaching the maximum response level. At 20.5 days the response level reached at 30 minutes was significantly less than that seen at 18.5 days. The fact that the response to LH at 20.5 days continued to increase with time could be interpreted as "activation" of the mechanisms needed to respond to LH, but not to synthesize testosterone. The rate of testosterone synthesis without LH decreased significantly during the first 30 minutes of the incubation and then remained relatively constant, and was twice that seen at 18.5 days.

From these results it is possible to draw some inferences regarding the development and regulation of testis function in the fetal rat. Testosterone synthesis and responsiveness to LH are the result of two partially independent processes both of which change with age. The first process concerns the capacity of the Leydig cells to synthesize testosterone. The second concerns the development of the systems necessary to respond to LH. Testosterone synthesis involves the ability to synthesize cholesterol and its precursors from acetate, and the enzyme systems necessary to transform cholesterol into C-19 steroids. Since the rate of testosterone synthesis is low at 15.5

days and seems to increase proportionately with the number of Leydig cells, this would imply that from 15.5 to 18.5 days the synthetic capacity per Leydig cells is constant. From 18.5 to 20.5 days the increase in synthetic rate with decreasing numbers of Leydig cells can be explained by assuming increased synthetic acti\'ty per cell. The factors which control the activity of steroidogenic enzymes in the Leydig cell (fetal or adult) are not clear. These could include increased metabolism, increased cytochrome P-450, or a change in the hormonal milieu. The possibility also exists that the increase in the rate of testosterone synthesis with age is a reflection not of increased steroid synthesis but of the predominance of alternate synthetic pathways at younger ages. This latter point is unlikely based on the work of Noumora et al, (1966), although no attempt was made in the present study to measure total steroid synthesis.

With respect to the second process, the response of fetal testes to LH increased with age in a manner suggesting that the change in sensitivity to LH reflects a series of events prior to steroid synthesis. It would be interesting in this regard to demonstrate either a change in receptor affinity for LH, a decrease in the amount of cyclic AMP needed to increase testosterone synthesis with increasing age, or an increase in the concentration of cyclic AMP in the Leydig cells or reduced levels of phosphodiesterase. If the rate-limiting step is beyond cyclic AMP then it may be necessary to look for changes in protein synthesis or activity of non-steroid related enzymes. The possibility remains that LH selectively alters the pathways involved in steroid synthesis to reactions favoring testosterone production, although this is a moot point at present (Eik-Nes, 1970).

The same pattern of responsiveness to LH has been demonstrated at the only other time the Leydig cells increase in number - the transition from immature to adult testes. In a recent study, Odell and co-workers (1974) demonstrated that the level of circulating testosterone increased in response to injected LH from day 10, when the Leydig cells are not easily demonstrable, to day 60, adulthood. With increasing age the testes became more responsive and sensitive to LH stimulation. The response levels reached at puberty and adulthood, as measured by the increase in testosterone synthesis over base level, are almost identical (16 and 18, respectively) to that seen in the present study with 18.5 and 20.5 day fetal testes _in vitro_ (15.4 and 20.5, respectively). Thus, the synthesis of testosterone in response to LH in puberty and adulthood, which is under the regulation of the negative feedback axis, is similar to the response of fetal testes. Odell and co-workers attribute the increase in responsivity to LH to an effect of FSH on the testis. Whether this mechanism could be responsible for the increase in sensitivity and response seen in the fetus is unclear, since there is no evidence that FSH or LH are present in the fetal circulatory system. The important point from the viewpoint of fetal testis function is the implication that hormones other than LH can play a role in the development of testis function.

If the response of immature and pubertal testes to injected LH can be used as a model for the prediction of the _in vivo_ fetal situation, then it is not necessary to assume that the large concentrations of LH used in these _in vitro_ studies are present _in vivo_. Negro-Vilar and co-workers (1973) demonstrated that from 15 days to 30 days the concentration of LH in the plasma reaches a maximum; this

increase in LH is less than 2-fold, 0.04 to 0.06 ng/ml plasma. At the same time the level of testosterone in the plasma increases markedly (Resko et al, 1968) and the response of the testes, in terms of circulating testosterone, to LH triples (Odell et al, 1974). Thus, it is the ability of the fetal testes to respond to LH, rather than the absolute concentration of LH, that is the key to the rising levels of testosterone at puberty.

By analogy, one could expect that in the fetus the concentration of circulating LH could be maintained at a minimum while the concentration of testosterone in plasma could increase due to the increased responsiveness of the testes to LH. Unfortunately, there is no data available on the levels of LH or testosterone in the fetal rat circulation. Decapitation of rat fetuses on day 17 leads to decreased conversion of pregnenolone to testosterone at later ages (Noumora et al, 1966) and decreased testicular weight and Leydig cell volume (Eguchi and Morikawa, 1968). Gonadotrophs have been identified in the fetal rat pituitary from day 19 onwards (Fink et al, 1971; Svalander, 1974) although Behrens and Martin (1972) claim that these cells do not have the appearance of actively secreting cells until after birth. LH has been assayed in pituitaries from male and female fetuses on the day before birth (Kuznetsova, 1970) and castration at this time results in increased leucine incorporation by the basophils of the anterior pituitary (Nakai et al, 1972).

If fetal testes are not under gonadotrpic regulation from the fetal pituitary then the increase in Leydig cell number with development has to be explained by some other mechanism. In the human it is believed that HCG is responsible for the increase in Leydig cell

number at the early stages of testis maturation and, only later, during the stage of wolffian duct virilization, does the fetal pituitary regulate testis function (Grumbach, 1973). In the rat there is evidence that the placenta secretes a luteotrophic factor (LTH) which is capable of maintaining pregnancy during the second half of gestation. Whether or not this placental prolactin complex also has LH-like activity is still unclear (Heap et al, 1973). It is possible that other pituitary secretions, e.g., FSH and Prolactin play a role in the initiation of testosterone synthesis by the fetal testis early in development and/or are responsible for the increased sensitivity to LH seen with age. Testes aged 20.5 days were unresponsive to FSH and Prolactin *in vitro*, however, long-term exposure *in vivo* may affect testosterone synthesis or Leydig cell number.

The measurement of testosterone in testes and genital ducts not only supports a role for testosterone in the virilization of the wolffian ducts (*See:* Introduction) but may provide a clue as to the time of appearance of testicular regulation. The present study indicates that there is an abrupt increase in testicular testosterone on day 17.5, and then a maintenance of this level through day 20.5. These findings are in essential agreement with those of Warren and co-workers (1973) who demonstrated, by electron-capture following gas-phase chromatography, a marked increase in testicular testosterone on day 18.5 and a maintenance of this level until day 20.5.

The level of testosterone in the testes *in vivo* is a reflection of synthesis, catabolism, retention, and rate of secretion. If one assumes that *in vivo* the rate of synthesis increases proportionately to the Leydig cell number, then the increase in testosterone at

17.5 days seen in this study, and at 18.5 days in Warren's study, is greater than expected. If the *in vitro* results on the retention of testosterone by the testes and on the increase in the rate of testosterone synthesis with age are valid *in vivo* then the increased concentration of testosterone in the testes must reflect increased synthesis. This increased synthesis could be the result of gonadotropic stimulation.

The finding of testosterone in the wolffian ducts throughout the period of sexual differentiation does not mean that testosterone is responsible for wolffian duct virilization. The findings by Gupta and Bloch (in press) of a specific binding protein in the cytosol of the male wolffian duct from 14.5 days onward which binds mainly testosterone, and the demonstration that testosterone is metabolized to dihydrotestosterone by the cells of the urogenital sinus (Wilson and Laznitski, 1971) are consistent with the idea that testosterone or its metabolites are necessary for masculinization *in vivo*.

Whether or not testosterone influences the development of the hypothalamus and pituitary in the fetal period is a moot point. The finding of testosterone in the genital tracts does not mean that it is present in the fetal circulation, although this is likely. The inability to measure testosterone in lung tissue is not indicative of a lack of testosterone in the general circulation. In this study only enough lung tissue was taken to serve as a tissue "blank" in the radioimmunoassay. Clearly, the measurement of testosterone levels in fetal plasma during the relevant ages is necessary.

One of the aims of the present study was to develop an assay

for fetal pituitary function based on the synthesis of testosterone by 20.5 day testes. While the data presented are incomplete as to the number of concentrations of LH used, and linearity of response over a segment of the dose-response curve has not been fully documented, the data presented on dose-response and specificity of response suggest that further work may make such an assay feasible. A similar bioassay for LH, based on the responsiveness of intact adult testes and Leydig cell cultures from adult testes (Dufau et al, 1974) has been proposed. The use of fetal testes as an assay for fetal pituitary function seems desireable in light of the fact that the kind of FSH secreted by the male pituitary seems to change with the level of circulating androgens (Bogdanove et al, 1974). If fetal LH also changes with the level of circulating androgens then it is likely that it may not have the same degree of biological activity in an assay using the response of an adult tissue.

The demonstration of a developmental pattern for testosterone synthesis and responsiveness to LH _in vitro_, as well as endogenous testicular testosterone, extends the time course for maturation of one part of the hypothalamic-pituitary-testicular axis well into the fetal period. These studies suggest that the developing nervous system and pituitary are exposed to a very potent androgen, testosterone, throughout most of their developmental histories. It is reasonable to expect, therefore, that fetal testosterone, as well as testosterone synthesized during the first three days of post-natal life, plays a role in the masculinization of the hypothalamus as well as the

internal and external genitalia. Finally, the similarity in the development of responsivity to LH in both the fetal and peripubertal periods implies that similar mechanism control the development and expression of testis function at both stages of sexual activity.

Summary and Conclusions

Two aspects of the development and maturation of fetal testis function in the rat were studied: the capacity to synthesize testosterone from endogenous precursors and respond to LH with increased testosterone synthesis. The ability to synthesize testosterone and respond to LH were studied in fetal testes aged 14.5, 15.5, 16.5, 18.5 and 20.5 days. Responsivity to LH was measured as a change in the concentration of radioimmunoassayable testosterone secreted into medium 199 during short term incubation. A second series of experiments concerned the measurement of the endogenous testosterone concentrations in fetal testes and genital tracts from 14.5 to 21.5 day fetuses; these results were used to provide *in vivo* confirmation of the *in vitro* findings.

Testes were incubated in 3 ml medium 199 at a temperature of 37° C in an atmosphere of O_2:CO_2 (95:5). At selected time intervals aliquots of medium were removed and testosterone extracted with diethyl ether (10 ml ether/0.2 ml medium) by freezing the aqueous phase in a dry-ice-acetone bath. The residue of the extract was dissolved in 1 ml of a protein-containing buffer (.02 M phosphate buffer, pH 7.8 plus 1% gelatin) for analysis by radioimmunoassay.

The radioimmunoassay technique utilized was that of Brenner, Guerrero, Cekan, and Diczfalusy (1973) for the measurement of plasma testosterone. The final antibody dilution used was 1:30,000. The antibody bound only testosterone and dihydrotestosterone to any significant extent; at 50% displacement (B/B_0) twice as much of the latter steroid was required to displace an equivalent amount of testo-

sterone. The assay of Brenner et al, was modified for use in the present experiments in two ways: First, testosterone standards were extracted from medium 199, since there was a definite effect of the medium and extraction process on the displacement of ^{3}H-testosterone bound to the antibody. Second, sample extracts were not purified prior to being assayed, as studies on the metabolism of ^{3}H-testosterone by testes from 16.5 and 20.5 day fetuses, both with and without LH, showed no evidence of dihydrotestosterone formation. These results at 20.5 days were confirmed by gas-liquid chromatography.

The use of the concentration of testosterone secreted into the incubation medium as the measure of testis response to LH required the demonstration that the concentration of testosterone in the medium at any time was an accurate reflection of total testosterone synthesis. Studies on the partitioning of both ^{3}H-testosterone added to the medium and endogenously synthesized testosterone demonstrated that the testes bound or retained a significant fraction of the total. In the absence of LH, this fraction accounted for 30% of the total and did not change with time of incubation. With LH, the increase in medium testosterone was such that the testes accounted for 2% of the total after 4 hours of incubation. LH did not affect the retention or permeability of the testes to testoserone; therefore, the increase in medium testosterone with LH was due to the effect of LH on the rate of testosterone synthesis.

In the absence of LH, testosterone synthesis was not demonstrable by testes from 14.5 day fetuses. Testosterone synthesis was first demonstrable at 15.5 days; the mean synthetic rate at this age

was 0.32 ± 0.22 ng/testis/hr. This increased significantly at 16.5 days to a mean rate of 0.68 ± 0.35 ng/testis/hr. At 18.5 days the mean rate was 1.02 ± 0.06 ng/testis/hr; and by 20.5 days had increased to 1.6 ± 0.78 ng/testis/hr.

The ability of fetal testes to respond to LH added to the incubation medium was measured as change in the rate of testosterone synthesis relative to that seen in the absence of LH. At 14.5 days, testosterone synthesis was not demonstrable with LH (360 ng/ml). At 15.5 days, LH, 160 ng/ml, increased testosterone synthesis four-fold, to a maximum rate of 1.3 ng/testis/hr. At 16.5 days, the same dose of LH increased testosterone synthesis eight-fold, to a maximum rate of 8.8 ng/testis/hr from thirty minutes to one hour. At 18.5 days, a dose of 64 ng/ml increased synthesis fifteen-fold to a maximum rate of 20 ng/testis/hr. The response of 20.5 day testes to LH showed both increased sensitivity and responsivity to that seen at younger ages: at 1 to 4 hours of incubation the maximum rate of testosterone was 36 ng/testis/hr, at 20-fold increase in the control rate. The concentation of LH needed to get this response was 16 ng/ml, one-fourth the dose used at 18.5 days. Testes of this age were 100-times as sensitive to LH as 15.5 day testes, forty-fold as sensitive as 16.5 day testes, and four-fold as sensitive as 18.5 day testes. At 20.5 days the response to gonadotropic stimulation was specific for LH; neither FSH nor prolactin increased testosterone synthesis above control values. As in the absence of LH, testosterone synthesis with LH was proportional to tissue weight.

Testosterone was not measureable in testes and genital tracts

from 14.5 day fetuses. From 15.5 to 17.5 days the endogenous concentration of testosterone in the testes increased ten-fold, and then remained relatively constant until day 20.5. The concentration of testosterone in the ducts remained fairly constant from 15.5 to 21.5 days.

Based on the *in vitro* findings presented here and the work of others, the following conclusions as to the maturation and regulation of testis function were reached:

1. The increase in the basal synthetic rate with age is consistent with the idea that from 15.5 to 18.5 days the capacity of the individual Leydig cells to synthesize testosterone is constant; the increase in rate appears to parallel the reported increase in Leydig cell number. The increase in basal rate from 18.5 to 20.5 days, when the Leydig cells are regressing, indicates that at this time capacity per cell increases.

2. The increase in sensitivity to LH with age to a maximum at 20.5 days implies that the efficiency of LH utilization increases with age. The change in LH utilization occurs at some step(s) preceding steroid synthesis; this is shown from a comparison of the results at 18.5 and 20.5 days. At 20.5 days, although the initial rate of synthesis (0 to 30 minutes) is greater than at 18.5 days, the initial response to LH is significantly less than that at 18.5 days and increases to a maximum gradually with time of incubation.

3. The increase in endogenous testicular testosterone and its maintenance until shortly before birth, indicates that at this time testis function may be under gonadotropic regulation.

4. The presence of testosterone in the testes and genital tracts supports the idea that testicular testosterone is responsible for the virilization of the wolffian ducts, and, by inference, the developing nervous system.

5. The change in responsiveness to LH with age seen in the fetal period is similar to that seen during the maturation of testis function postnatally (Odell et al, 1974) and imply that the mechanisms regulating testis function may be similar at both stages of development.

Bibliography

Attal, J. (1969) Levels of testosterone, androstenedione, estrone, and estradiol-17β in the testes of fetal sheep. Endocrinology, 85, 280-289.

Behrens, U. & Martin, C. (1972) Ultrastructural changes in the pars distalis of the maturing male rat. J. Morph., 136, 53-78.

Bloch, E., Lew, M. & Klein, M. (1971) Studies on the inhibition of fetal androgen formation: inhibition of testosterone synthesis in rat and rabbit fetal testes with observations on reproductive tract development. Endocrinology, 89, 16-31.

Bloch, E. (1967) The conversion of 7-^{3}H-pregnenolone and 4-^{14}C-progesterone to testosterone and androstenedione by mammalian fetal testes _in vitro_. Steroids, 9, 415-430.

Bloch, E. (1964) Metabolism of 4-^{14}C-progesterone by human fetal testes and ovaries. Endocrinology, 74, 833-845.

Bogdanove, E.M., Campbell, G.T., Blair, E.D., Mula, M.E., Miller, A.E. & Grossman, G.H. (1974) Gonad-pituitary feedback involves qualitative change: androgens alter the type of FSH secreted by the rat pituitary. Endocrinology, 95, 219-228.

Brenner, P.F., Guerrero, R., Cekan, Z., & Diczfalusy, E. (1973) Radioimmunoassay method for six steroids in human plasma. Steroids, 22, 775-794.

Coggeshall, R.E. (1964) A study of the diencephalic development in the albino rat. J. Comp. Neur., 122, 241-269.

Daikoko, S., Morishita, H., Hashimoto, T. & Takahashi, A. (1967) Light-microscopic studies on the development of the interrelationship between the neurosecretory pathway and the portal system in rats. Endocr. Jap.,14, 209-224.

Dubois, A.P. (1971) Sur l'apparātion des secrétions hormonales dans l'hypophyse foetale des bovins : mise en evidence par immunofluorescence des cellules gonadotropes et des cellules thyreotropes. C.R. Acad Sci Paris, 272, 1793-1795.

Dufau, M.L., Mendelsohn, C.R. & Catt, K.J. (1974) A highly sensitive in vitro bioassay for luteinizing hormone and chorionic gonadotropin: testosterone production by dispersed leydig cells. J. Clin. Endocr. Metab., 39, 610-613.

Dufau, M.L., Catt, K.J. & Tsuruhara, T. (1972) A sensitive gonadotropin responsive system: radioimmunoassay of testosterone production by the rat testis in vitro. Endocrinology, 90, 1032-1040.

Eguchi, Y. & Morikawa, Y. (1968) Changes in pituitary-gonadal interrelations during perinatal days in the rat. Anat. Rec., 161, 163-170.

Eik-Nes, K.B. (1970). Synthesis and secretion of androstenedione and testosterone. In The Androgens of the Testis, p. 10-23. Ed. K.B. Eik-Nes. New York: M. Dekker.

Faiman, C. & Winter, J.S.D. (1974) The control of gonadotropin secretion in complete testicular feminization. J. Clin. Endocr. Metab., 39, 631-638.

Fink, G. & Smith, G.C. (1971) Ultrastructural features of the developing hypothalamo-hypophysial axis in the rat. Z. Zellforsch. Mikro. Anat., 119, 208-226.

Foster, D.L., Jackson, G.L., Cook, B. & Nalbandov, A.V. (1972) Regulation of luteinizing hormone (LH) in the fetal and neonatal lamb. IV. Levels of LH releasing activity in the hypothalamus. Endocrinology, 90, 684-689.

Goldman, A.S., Baker, M.K., Chen, J.C. & Wieland, R.G. (1972) Blockade of masculine differentiation in male rat fetuses by maternal injection of antibodies to testosterone-3-bovine serum albumin. Endocrinology, 90, 715-716.

Goldman, A.S. (1969) Congenital effectiveness of an inhibitor of 3β-hydroxysteroid dehydrogenase administered before implantation of the rat blastula. Endocrinology, 84, 1206-1212.

Goldman, B.D., Garcia, Y.R., Camberi, I.A., & Porter, J.C., (1971) Serum gonadotropin concentrations in intact and castrated neonatal rats. Endocrinology, 88, 771-776.

Grumbach, M., and Kaplan, S.L. (1973) Ontogenesis of growth hormone, insulin, prolactin and gonadotropin secretion in the human fetus. In Foetal and Neonatal Physiology, p. 462-487. Ed. Comline, K.S., Cross, K.W., Dawes, G.S. & Nathanielsz, P.W. London: Cambridge University Press.

Gupta, C. & Bloch, E. (1975) Testoserone binding protein in reproductive tracts of fetal rats. J. Biol. Chem. (in press).

Hall, P.F. (1970) Gonadotrophic regulation of testicular function. In The Androgens of the Testis, p. 73-116. Ed. K.B. Eik-Nes. New York: M. Dekker.

Heap, R.B., Perry, J.S. & Challis, J.R.G. (1973) The hormonal maintenance of pregnancy. In Handbook of Physiology, Section 7: Endocrinology, 11. p. 218-230. Ed. Greep, R.O. & Astwood, E.B. Maryland: Williams & Wilkins Co.

Huhtaniemi, I., Ikonen, M. and Vihko, R. (1970) Presence of testosterone and other neutral steroids in human fetal testes. Biochem. Biophys. Res. Comm.,38 , 715-720.

Josso, N. (1970) Action de l'androstenediol, de la dehydroepiandrosterone et de son sulfate sur le maintien du canal de wolff du foetus de rat, en culture organotypique. C.R. Acad Sci Paris, 270, 1925-1927.

Jost, A., Dupouy, J.P. & Geloso-Meyer, A. (1970) Hypothalamo-hypophyseal relationships in the fetus. In The Hypothalamus, p. 1-11. Ed. Martini, L., Motta, M., Fraschini, F. New York: Academic Press.

Jost, A., (1953) Problems of fetal endocrinology: The gonadal and hypophyseal hormones. Rec. Prog. Horm. Res. 8, 379-384.

Kuznetsova, L.V. (1970) Influence of testosterone proprionate on the luteinizing-hormone content of the rat-fetus hypophysis. Ontogenez, 2, 1, 182-185.

Lamont, K.G., Perez-Palacios, G., Perez, H.E. and Jaffe, R.B. (1970) Pregnenolone and pregnenolone sulfate metabolism by human fetal testes in vitro. Steroids, 16, 127-140.

Lipsett, M.B., & Tullner, W.W. (1965) Testosterone synthesis by the fetal rabbit gonad. Endocrinology, 77, 273-277.

Moyle, W.R. & Ramachandran, J. (1973) Effect of LH on steroidogenesis and cyclic AMP accumulation in rat leydig cell preparations and mouse tumor leydig cells. Endocrinology, 93, 127-134.

Murphy, B.E.P. (1971) Hormone assay using binding proteins in blood. In Principles of Competitive-Protein Binding Assays. p. 108-125. Ed. Odell, W.D. & Daughaday, W.H. Philadelphia: J.B. Lippincott.

Nakai, T., Sakanoto, S. & Kigawa, T. (1972) Hypothalamic-pituitary-testicular interrelations in fetal rats: effects of castration on ^{3}H-leucine uptake in pituitary basophilic cells. Endocr. Jap., 19, 133-137.

Nayfeh, S.N., Barefoot, S.W. & Baggett, B. (1966) Metabolism of progesterone by rat testicular homogenates. II. Changes with age. Endocrinology, 78, 1041-1048.

Negro-Vilar, A., Krulich, L. & McCann, S.M. (1973) Changes in serum prolactin and gonadotropins during sexual development of the male rat. Endocrinology, 93, 660-664.

Niemi, M. & Ikonen, M. (1963) Histochemistry of the leydig cells in the postnatal prepubertal testis of the rat. Endocrinology, 72, 443-448.

Niswender, G.D. & Midgely, A.R. Jr. (1970) Hapten-radioimmunoassay of steroids. In Immunologic Methods in Steroid Determination. p. 149-166. Ed. Peron, F.G. & Caldwell, B.V. New York: Appleton-Century Crofts.

Noumora, T., Weisz, J., & Lloyd, C., (1966) In vitro conversion of 7-^{3}H-progesterone to androgens by the rat testis during the second half of fetal life. Endocrinology 78, 245-253.

Odell, W.D., Swerdioff, R.S., Bain, J., Wollesen, F., & Grover, P.K. (1974) The effect of sexual maturation on testicular response to LH stimulation of testosterone secretion in the intact rat. Endocrinology, 95, 1380-1384.

Odell, W.D., Abraham, G.A., Skowsky, W.R., Hescox, M.A. & Fisher, D.A. (1971) Production of antisera for radioimmunoassays. In Principles of Competitive-Protein Binding Assays, p. 58. Ed. Odell, W.D. & Daughaday, W.H. Philadelphia: J.B. Lippincott.

Pasteels, J.L., Gausset, P., Danguy, A. & Ectors, F. (1974) Gonadotropin secretion by human fetal and intact pituitaries. In International Symposium on Sexual Endocrinology of the Perinatal Period, p. 13-35. Ed. Forest, M.G. & Bertrand, J. Paris: I.N.S.E.R.M.

Picon, R. (1969) Modifications, chez le rat, au course du developpement du testicle, de son action inhibitaire sur les canaux de muller in vitro. C.R. Acad. Science, 271, 2370-2372.

Price, D., Ortiz, E., & Zaaijer, J.J.P. (1967) Organ culture studies of hormone secretion in endocrine glands of fetal guinea pigs. II. Secretion of androgenic hormone in adrenals and testes during early stages of development. Anat. Rec., 157, 27-41.

Price, D., & Pannabecker, R. (1956) Organ culture studies of fetal reproductive tracts. Ciba Foundation Colloquia Aging, 2, 3-13.

Raynaud, A. (1950) Developpement des canaux de muller chez des foetus males de souris dont les testicules ont ete irradies, an moyens des rayons X, a l'age de 14 jours. Ann. Endocr., 11, 32-48.

Resko, J.A. (1970) Androgens in systemic plasma of male guinea pigs during development and after castration in adulthood. Endocrinology, 86, 1444-1447.

Resko, J.A. (1970) Androgen secretion by the fetal and neonatal rhesus monkey. Endocrinology, 87, 680-687.

Resko, J.A., Feder, H.H., & Goy, R.W. (1968) Androgen concentrations in plasma and testis of developing rats. J. Endocr., 40, 485-491.

Rice, B.F., Johanson, C.A. & Sternberg, W.H. (1966) Formation of steroid hormones from acetate-1-^{14}C by a human fetal testis preparation grown in organ culture. Steroids, 7, 79-90.

Roosen-Runge, E.C. & Anderson, D. (1959) The development of the interstitial cells in the testis of the albino rat. Acta Anatomica, 37, 125-137.

Scheib, D. & Lombard, M-N (1971) Etude histo-enzymologique de l'activité de la Δ_5-3β-hydroxysteroide deshydrogenase du testicule de souris blanche. Arch. Anat. Micros. Morphol. Expt., 60, 205-218.

Schlegel, R.J., Farias, E., Russo, N.C., Moore, J.R. & Gardner, L.I. (1967) Structural changes in the fetal gonads and gonaducts during maturation of an enzyme, steroid 3β-ol-dehydrogenase, in the gonads, adrenal cortex and placenta of fetal rats. Endocrinology, 811, 565-572.

Schultz, F.M. & Wilson, J.D. (1974) Virilization of the wolffian duct in the rat fetus by various androgens. Endocrinology, 94, 979-986.

Sierra, G.B., Perez-Palacios, G. & Jaffe, R.B. (1970) De Novo testosterone biosynthesis in the human fetal testis. J. Clin. Endocr., 30, 128-130.

Svalander, C. (1974) Ultrastructure of the fetal rat adenohypophysis. Acta Endocr. Suppl. 188, 76, 5-113.

Warren, D.W., Haltmeyer, G.C. & Eik-Nes, K.B. (1973) Testosterone in the fetal rat testis. Biol. Repro., 8, 560-565.

Wells, L.J. & Fralick, R. (1951) Production of androgen by the testes of fetal rats. Am. J. Anat., 89, 63-107.

Weniger, J-P., & Zeis, A. (1974) Sur la sensibilite du testicule embryonnaire de souris a l'hormone luteinisante. C.R. Acad Science Paris, 279, 1629-1631.

Weniger, J-P., Ehrhardt, J-D. & Fritig, B. (1967a) Secretion de testosterone et d'androstenedione par les testicules foetaux de veau cultives in vitro. C.R. Acad Sci Paris, 264, 1911-1912.

Weniger, J-P., Ehrhardt, J-D. & Fritig, B. (1967b) Secretion de testosterone et d'androstenedione par les testicules embryonnaires de souris cultives in vitro. C.R. Acad Sci Paris, 264, 1069-1071.

Wilson, J.D. & Lasnitzki, I. (1971) Dihydrotestosterone formation in fetal tissues of the rabbit and rat. Endocrinology, 89, 659-668.

Zimmering, P. (1970) In Immunologic Methods in Steroid Determination p 167. Ed. Peron, F.G. & Caldwell, B.V. New York: Appleton-Century Crofts.