THE INTERACTION OF STEROIDS WITH THE HYPOTHALAMIC-PITUITARY-TESTICULAR SYSTEM IN THE ADULT MALE RAT

PROEFSCHRIFT

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LIST OF ABBREVIATIONS

ABP	- androgen binding protein
ACTH	- adrenocorticotrophic hormone (corticotropin)
b.w.	- body weight
C.V.	- coefficient of variation
cyclic AMP	- cyclic adenosine-3':5'-monophosphoric acid
5α-dhTP	- 5α-dihydrotestosterone propionate
DNA	- deoxyribonucleic acid
EB	- oestradiol benzoate
FSH	- follicle-stimulating hormone (follitropin)
GnRF	- gonadotrophin releasing factor (gonadoliberin)
h(rs)	- hour(s)
HCG	- human chorionic gonadotrophin (human choriogonadotropin)
IU	- international unit
i.v.	- intravenous(ly)
kV	- kilovolt
LH	- luteinizing hormone (lutropin)
LHRF	- luteinizing hormone releasing factor (luliberin)
mA	- milliampère
min	- minute
n	- number
NIAMD	- National Institute of Arthritis and Metabolic Diseases
NIH	- National Institute of Health
P	- probability
R	- röntgen
RNA	- ribonucleic acid
RP	- reference preparation
rpm	- revolutions per minute
s.c.	- subcutaneous(ly)

S.D. - standard deviation

S.E.M. - standard error of the mean

TP - testosterone propionate

LIST OF TRIVIAL NAMES

Trivial names used in this study	Systematic names
acetylcholine	- β-acetoxyethyltrimethylammoniumhydroxide
5α-androstan-3α-ol-17-one	- 3α-hydroxy-5α-androstan-17-one
5α-androstan-3β-ol-17-one	- 3β-hydroxy-5α-androstan-17-one
5α-androstan-17β-ol-3-one	- 17β-hydroxy-5α-androstan-3-one
5β-androstan-3α-ol-17-one	- 3α-hydroxy-5β-androstan-17-one
5β-androstan-17β-ol-3-one	- 17β-hydroxy-5β-androstan-3-one
4-androstene-3β,6β-diol-17-one	- 3β,6β-dihydroxy-4-androsten-17-one
4-androstene-6β,17β-diol-3-one	- 6β,17β-dihydroxy-4-androsten-3-one
4-androstene-7α,17β-diol-3-one	- 7α,17β-dihydroxy-4-androsten-3-one
4-androstene-11β,17β-diol-3-one	- 11β,17β-dihydroxy-4-androsten-3-one
4-androstene-17β,19-diol-3-one	- 17β,19-dihydroxy-4-androsten-3-one
4-androsten-17α-ol-3-one	- 17α-hydroxy-4-androsten-3-one
4-androsten-17β-ol-3-one	- 17β-hydroxy-4-androsten- 3 -one
5-androsten-3β-ol-17-one	- 3β-hydroxy-5-androsten-17-one
corticosterone	- 11β,21-dihydroxy-4-pregnene-3,20-dione
corticotrophin	- adrenocorticotrophic hormone (corticotropin)
cortisol	- 11β,17α,21-trihydroxy-4-pregnene-3,20-dione
cortisone	- 17α,21-dihydroxy-4-pregnene-3,11,20-trione
dexamethasone	- 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione
5α-dihydrotestosterone	- 17β-hydroxy-5α-androstan-3-one
5α-dihydrotestosterone propionate	- 3-oxo-5α-androstan-17β-yl propionate

dopamine	- 3,4-dihydroxyphenylethylamine
epinephrine	- 1-(3,4-dihydroxyphenyl)-2-methylaminoethanol
norepinephrine	- 1-(3,4-dihydroxyphenyl)-2-aminoethanol
oestradiol-17α	- 1,3,5(10)-oestratriene-3,17α-diol
oestradiol-17β	- 1,3,5(10)-oestratriene-3,17β-diol
oestradiol benzoate	- 17β-hydroxy-1,3,5(10)-oestratriene-3-yl benzoate
1,3,5(10)-oestratrien-3-ol-17-one	- 3-hydroxy-1,3,5(10)-oestratrien-17-one
4-oestren-17β-ol-3-one	- 17β-hydroxy-4-oestren-3-one
5(10)-oestren-17β-ol-3-one	- 17β-hydroxy-5(10)-oestren-3-one
oestriol	- 1,3,5(10)-oestratriene-3,16α,17β-triol
oestrone	- 3-hydroxy-1,3,5(10)-oestratrien-17-one
5α-pregnane-17α,21-diol-3,20-dione	- 17α,21-dihydroxy-5α-pregnane-3,20-dione
4-pregnene-11β,21-diol-3,20-dione	- 11β,21-dihydroxy-4-pregnene-3,20-dione
progesterone	- 4-pregnene-3,20-dione
serotonin	- 5-hydroxytryptamine
testosterone	- 17β-hydroxy-4-androsten-3-one
testosterone-3-(O-carboxymethyl)-oxime	- 17β-hydroxy-4-androsten-3-(O-carboxymethyl)-oxime
testosterone propionate	- 3-oxo-4-androsten-17β-yl propionate

CHAPTER 1

INTRODUCTION AND SUBJECT OF THESIS

1.1 General introduction

Major functions of the mature male gonad are the production of gametes and steroid hormones. Extratesticular as well as intratesticular factors regulate these two male gonadal functions which are associated with two distinct cell compartments in the testis. It has been known for a long time that hypophysectomy is followed by gonadal atrophy and arrest of the spermatogenic process which only will proceed to a primary spermatocyte stage. The anterior pituitary gland appears to play an essential role in the regulation of the testis and systematic studies on the relationship between brain structures and the male gonad started in the late nineteen twenties and early thirties. The finding that testicular functions were controlled by the hypophysis stimulated almost simultaneously experiments on the reverse issue, i.e. whether testicular products can affect the hypophysis (Moore and Price, 1930). The connections between these systems are the anterior pituitary gonadotrophic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). It is now well established that the major control of pituitary function resides in the hypothalamus, although other brain structures also are involved in the regulation of the pituitary gland (Szentágothai et al. 1968; Mess and Martini, 1968; Halász, 1969). Hypothalamic gonadotrophin releasing factor (GnRF), reaching the anterior pituitary gland via a portal system, stimulates syn-

thesis (Redding et al. 1972) and release (Schally et al. 1968) of the hypophyseal gonadotrophins LH and FSH in special gonadotroph cells. The many mutually dependent and complicated interactions between hypothalamus, hypophysis and testis at different functional levels are intriguing and make their study very attractive.

1.2 Hypothalamic-pituitary unit

The hypothalamus is an area in the diencephalon lying at the base of the brain ventral to the thalamus and forming the floor and part of the lateral walls of the third ventricle. Anteriorly it is bounded by the optic chiasma and posteriorly by the mammillary bodies. The median eminence is adjacent to the pituitary stalk that links the two structures of this unit. The anatomical basis for the hypothalamic regulation of the anterior pituitary gland was established by Green and Harris (1947) by demonstrating a neurovascular link between the hypothalamus and the anterior pituitary gland. This fact was conclusively substantiated by the existence, the isolation and the synthesis of a specific hypothalamic decapeptide hormone GnRF by Schally and coworkers. Certain centres in the hypophysiotrophic area of the hypothalamus contain the GnRF producing neurons whose axons end at the capillary loops of the hypophyseal portal circulation. GnRF produced by these neurons under the influence of neurotransmitters (Schneider and Mc Cann, 1970; Wurtman, 1970; Kordon and Glowinski, 1972) is discharged into the primary capillary plexus of the portal system. From this capillary bed, originating in the median eminence, GnRF is transported to the anterior pituitary gland. As a consequence of such transport distinct gonadotroph cells in the anterior pituitary gland will secrete LH and FSH into the systemic circulation. Results suggesting

that stimulation of the adenylate-cyclase system and cyclic AMP may be involved in the action of GnRF on the anterior pituitary gland have recently been reviewed by Menon and Gunaga (1974). It is still disputed whether there exists only one releasing principle responsible for the production and release of both LH and FSH, but so far only one gonadotrophin releasing hormone has been identified and synthesized, which will promote synthesis (Redding et al. 1972) as well as release (Schally et al. 1968) of LH and FSH by the anterior pituitary gland in vivo and in vitro. In contrast to GnRF, the pituitary gonadotrophins appear to be species specific. The structure of these glycoproteins composed of two subunits designated the α- (or common) and the β- (or hormone specific subunits) (Pierce et al. 1971), has been elucidated for a number of species (Pierce et al. 1971; Papkoff et al. 1971; Shome and Parlow, 1974). It is generally believed that extrahypothalamic nervous structures such as the amygdala, cerebral cortex and hippocampus may influence the neurons that secrete GnRF (Szentágothai et al. 1968). Ablation of discrete areas of the central nervous system, hypothalamic and brain lesions and hypothalamic deafferentiation, which partially or totally interrupts the afferent pathways reaching the medial basal hypothalamus, provide useful methods for studying the role played by extrahypothalamic structures in regulating the action of the hypothalamic-pituitary-gonadal system. Central nervous system mediators such as epinephrine, norepinephrine, serotonin, dopamine and acetylcholine seem to be involved in transferring information from the extrahypothalamic centres to the hypothalamic nuclei (Ganong and Lorenzen, 1967; Kobayashi and Matsui, 1969; Fuxe and Hökfelt, 1969; Piva et al. 1969). Schneider and Mc Cann (1969) and Martini (1973) showed that dopamine and acetylcholine respectively stimulate LH release in vitro when added to incubation media containing hypothalamic fragments and anterior pituitary tissue. In addition, signals of diverse origin such as pineal gland secretions (Fraschini,

1969), testicular secretions (Moore and Price, 1930; Bogdanove, 1964), prostaglandins (Saksena et al. 1973), light, stress and emotions all have been shown to affect the hypothalamic-pituitary unit with respect to discharge of gonadotrophins. Finally, Martini and coworkers have suggested that complicated "short" loop feedback mechanisms regulate the secretion of LH (David et al. 1966) and FSH (Fraschini et al. 1968) and also the control of gonadotrophin releasing hormone (Motta, 1969).

1.3 Testis

Mature testicular tissue contains at least two different compartments: the interstitial compartment made up of connective tissue containing the Leydig cells, blood and lymph vessels and the non-vascularized tubular compartment containing as main components the Sertoli cells, forming a blood-testis barrier, and the germinal epithelium in different stages of spermatogenesis. It is now well recognized that the testis has binding sites with high affinity for labelled gonadotrophins (Castro et al. 1972; Means and Vaitukaitis, 1972; Catt et al. 1972; Leidenberger and Reichert, 1972), and is the main target organ for the gonadotrophic hormones LH and FSH.

It has been shown that LH can stimulate testicular steroidogenesis in vivo and in vitro after stimulating testicular adenyl cyclase activity (Kuehl et al. 1970; Eik-Nes, 1971; Dorrington et al. 1972) and after augmenting cyclic AMP production (Rommerts et al. 1972). The interstitial cell compartment is the predominant site for testosterone production in the male gonad (Christensen and Mason, 1965; Hall et al. 1969; Cooke et al. 1972). There is no agreement about the possible production of testosterone by the seminiferous tubules (Hall et al. 1969; Cooke et al.

1972; Bass et al. 1973). Steroids can, however, be transported from the Leydig cells to the seminiferous tubules (van Doorn et al. 1974). Production and secretion in vivo of several steroids by the normal testis of different species have been demonstrated by Eik-Nes (1970, 1971, 1972). Testosterone, however, appears to be quantitatively the main androgen steroid in systemic blood and is thought to be involved in the maintenance of the structural integrity of the accessory reproductive organs, in "long" loop feedback control of the gonadotrophins as well as in the spermatogenic process in the testis itself. It is, however, still not clear whether testosterone as such exerts these effects or following conversion to 5α-reduced androstane metabolites (or to oestrogens).

The effects of FSH in the male gonad are less clear, but it has been shown that tritiated FSH will specifically bind to seminiferous tubular membranes (Means and Vaitukaitis, 1972). Furthermore, FSH will specifically stimulate cyclic AMP production in seminiferous tubules (Dorrington et al. 1972; Cooke et al. 1972). Activation of adenyl cyclase induced by binding of FSH to isolated seminiferous tubular membranes was shown to result in an increment of intracellular levels of cyclic AMP and in activation of protein kinase activity (Means, 1974). In hypophysectomized rats, androgens (Walsh et al. 1934; Nelson et al. 1937; Ahmad et al. 1973) or LH (Steinberger, 1971) are capable of maintaining spermatogenesis when treatment is initiated immediately following hypophysectomy. But in long-term hypophysectomized animals showing testicular regression neither LH nor testosterone given alone are capable of initiating spermatogenesis and FSH has to be administered with LH or androgen in order to fully restore spermatogenic activity (Steinberger, 1971). Recently, evidence has been published that the Sertoli cells are affected by FSH (Hansson et al. 1973). It appears that under influence of FSH the Sertoli cell secretes an androgen binding protein having high affinity for the testicular secretion products

testosterone and 5α-dihydrotestosterone (Vernon et al. 1974). FSH administration in vivo to hypophysectomized rats results in increased levels of androgen binding protein in the testis (Hansson et al. 1973) and FSH also increased the androgen binding protein production by Sertoli cells in vitro (Fritz et al. 1974). Furthermore, it appears that the Sertoli cell is also an androgen target organ. It has been shown, that in the immature hypophysectomized rat androgen was capable of maintaining the secretion of androgen binding protein by the Sertoli cell, but after long-term regression of the seminiferous tubular epithelium in such animals FSH was again required in addition to androgen for complete restoration of the androgen binding protein production (Weddington et al. 1975). Finally, Odell et al. (1973) have postulated that FSH induces testicular sensitivity to LH, and it has been suggested that this might be an important cause of sexual maturation in the immature male.

1.4 Subject of thesis

The anterior pituitary gonadotrophic hormones LH and FSH play an essential role in testis physiology. On the other hand, factors from the testis (Moore and Price, 1930, 1932; Mc Cullagh, 1932) appear to control these hypophyseal gonadotrophins via a feedback regulation mechanism, since removal of the testes results in drastically increased circulating concentrations of these trophins (Gay and Bogdanove, 1969). It is not clear through which mechanism the male gonad regulates LH and FSH production and secretion. Detailed investigation of the feedback control between testicular products and the pituitary gonadotrophic hormones has been hampered by the lack of specific and sensitive analytical methods for quantification of testicular steroid hormones and pituitary gonadotrophins. The influence

of testicular and non-testicular steroid hormones on circulating LH and FSH in the mature male rat is, however, a matter of considerable physiological importance and has been the main subject of the current investigation. The introduction of sensitive assays for estimation of these gonadotrophins in blood and tissue made this study possible.

Production in vivo and secretion of testicular steroids by the isolated testis (Eik-Nes, 1970, 1971, 1972) and metabolism in vitro of radioactive androgens in testicular tissue (Folman et al. 1972; Rivarola and Podestá, 1972; Folman et al. 1973; Sowell et al. 1974; Pérez Lloret and Weisz, 1974) have been studied over the past years and many testicular steroids and radioactive androgen metabolites have been identified. It is not certain, whether all these metabolites occur also in the whole animal in vivo or bear on physiological functions at intragonadal or extragonadal androgen target levels. The androgenicity and the possible influence of these various androgen metabolites on androgen sensitive organs are still poorly documented. In this respect, the aim of the present study was to assess in adult male animals the effect of steroid hormones, and especially of testicular steroids, on circulating concentrations of the gonadotrophins LH and FSH which are essential for adequate function of the male gonad. Orchidectomized animals provide advantages to study the specificity of the effects of steroid hormones, because the influence of endogenous testicular products can be ruled out in this animal preparation. The androgenicity of the various compounds tested was evaluated by the classical bioassay test measuring weights of accessory reproductive organs in castrated animals (Young and Cornell, 1963). Furthermore, the influence of androgenic and oestrogenic steroids on the release of LH and FSH induced by GnRF was investigated. In addition to the testis, the adrenal cortex is another source of oestrogenic and androgenic steroids. It was therefore deemed necessary to investigate the possible involvement of adrenal steroid hormones in the regulation of the hypotha-

lamic-pituitary-testicular system. In order to elucidate which testicular tissue compartment produces the testicular factors involved in feedback control of LH and FSH, effects of testicular X-irradiation (destroying the germinal epithelium in the tubular compartment) in adult male animals were studied. Under the different experimental conditions in this study, the functions of the hypothalamic-pituitary unit and the testis were visualized by measuring blood serum concentrations of LH, FSH and testosterone.

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CHAPTER 2

ANALYTICAL METHODS FOR THE DETERMINATION OF TESTOSTERONE AND THE GONADOTROPHINS LH AND FSH

2.1 Introduction

Lack of reliable analytical methods for estimation of hormones influencing the hypothalamic-pituitary-testicular axis has been a crucial issue for many years. Specific, sensitive and reliable quantification of testosterone in systemic blood has only been possible since the nineteen sixties, and the available methods for estimation of this hormone in biological samples up to 1970 have been reviewed by van der Molen (1970). Quantitative determination of the gonadotrophins has for a long time only been possible with classical biological assays using the ovarian ascorbic acid depletion test (Parlow, 1961) for LH measurement and the human chorionic gonadotrophin augmentation test (Steelman and Pohley, 1953) for FSH measurement. Lack of sensitivity of the bioassay techniques prevented, however, estimation of these hypophyseal gonadotrophic hormones in blood samples of individual rats. The advent of radioactive labels of high specific activity and radioimmunoassay techniques in this walk of endocrinological research is a significant landmark and, nowadays new information and progress in this field mainly depend on data obtained with these newcomers to our technical armamentarium.

Principles, theoretical aspects, methodology and computer data processing of radioimmunoassays have been described extensively (see: Diczfalusy, 1969, 1970; Odell and

Daughaday, 1971; Zettner, 1973; Zettner and Duly, 1974; Skelley et al. 1973; Rodbard, 1974). Once the advantages of radioimmunoassay (high sensitivity and specificity of detection; simplicity and practicability) were recognized, these methods conquered many laboratories replacing many of the older analytical methods. The vast literature on this technology and its applications, which has appeared since the publication of Yalow and Berson (1959), gives a proper reflection of the impact of the technique. Radioimmunoassay methods have permitted sensitive quantification of a broad spectrum of hormonal and non-hormonal substances (for review see: Skelley et al. 1973) and have contributed to new and significant information that has promoted better knowledge of the general physiology. Application of radioimmunoassay techniques for the estimation of testosterone and pituitary gonadotrophins in order to study biological relationships in the hypothalamic-pituitary-testicular axis has been explored and evaluated in the present investigation.

2.2 Radioimmunoassay of testosterone

2.2.1. method

A rabbit antiserum was raised against testosterone-3-(O-carboxymethyl)-oxime bovine serum albumin. This latter complex was synthesized after conversion of testosterone to testosterone-3-(O-carboxymethyl)-oxime and coupling the latter to bovine serum albumin according to the method of Erlanger et al. (1957). Three months after the immunization procedure (Verjans et al. 1973) was initiated, an antiserum was obtained that was deemed suitable for measurement of testosterone. A flow sheet of the method in which this antiserum was used for testosterone quantification in blood

TABLE 2.2.1.

Procedure for the radioimmunoassay of testosterone in blood plasma or serum or testicular tissue

Blood plasma or serum	Testicular tissue
- Add 30,000 dpm ^{3}H-testosterone to plasma or serum (50-500 µl)	- Add 30,000 dpm ^{3}H-testosterone to tissue (10-100 mg)
- Extract with 1,5 ml hexane:ether - 8:2 (v/v)	- Sonify tissue in water
	- Precipitate proteins with acetone
	- Evaporate supernatant until only water remains
	- Extract water layer with 3 ml hexane:ether - 8:2 (v/v)
- Transfer organic layer to pre-eluted aluminium oxide column	
- Elute column with 1.6 ml hexane:ether - 8:2 (v/v)	
6 x 1.6 ml ethanol (0.45%) in hexane	
2 x 1.6 ml ethanol (0.95%) in hexane	
- Divide last eluates over two tubes	
- Evaporate solvents in each of the tubes	
- Add 250 µl of antibody solution and incubate at 4°C for 16 h	
- Take 50 µl for recovery estimation	
- Add 500 µl of dextran-coated charcoal suspension	
- Incubate for 5 min at 4°C	
- Centrifuge for 10 min with 3,000 rpm	
- Take 500 µl of supernatant for counting of bound radioactivity	

plasma, serum or testicular tissue is shown in Table 2.2.1. In order to obtain the standard curve known standard amounts of testosterone were treated in the same fashion as the unknown samples. Purification of radioactive testosterone, solvents for extraction and chromatography, preparation of aluminium oxide (Al_2O_3) micro-columns for chromatography, antibody buffer and dextran-coated charcoal suspension have been described by Verjans et al. (1973).

2.2.2. results

Several methods for separation of antibody bound and free testosterone were compared and the dextran-coated charcoal method was found to be the most practical one (Verjans et al. 1973) and employed throughout the rest of the studies.

The effects of the addition of increasing amounts of testosterone or of various other steroids to the binding of radioactive testosterone by the diluted antiserum are depicted in Fig. 2.2.1.

5α-Dihydrotestosterone exhibits considerable cross reaction with this testosterone-3-(O-carboxymethyl)-oxime bovine serum albumin antiserum. It has been reported that this cross reaction of 5α-dihydrotestosterone also occurs with several other antisera elicited against testosterone bovine serum albumin or testosterone thyroglobulin complexes, irrespective of the site of coupling of these proteins to the steroid via either the 1,7 (Kohen et al. 1975), 3,11 or 17 (Bosch et al. 1974) position in testosterone. The presence of 5α-dihydrotestosterone in human male and female blood plasma (Ito and Horton, 1970; Tremblay et al. 1970) and in rat serum (Coyotupa et al. 1973) and testicular tissue (Folman et al. 1972) has been demonstrated. Therefore, inclusion of a chromatographic procedure on

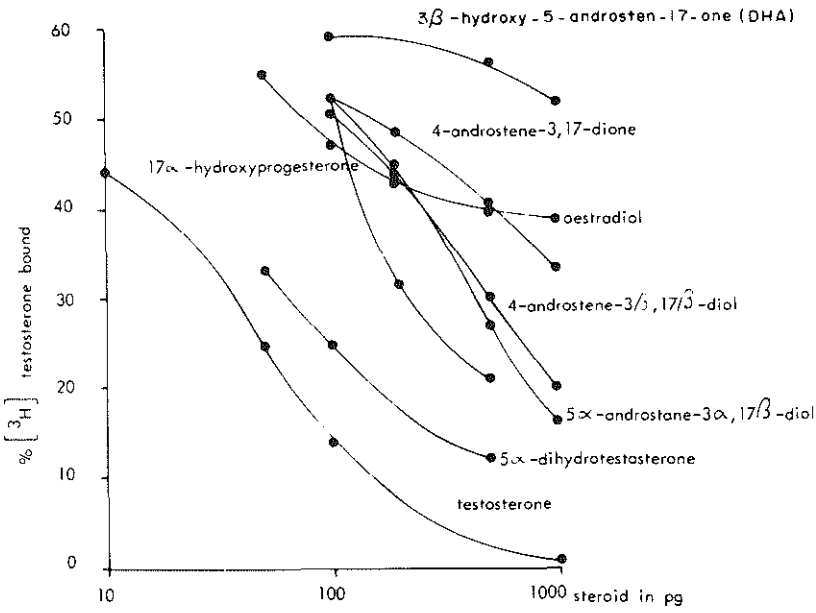

FIG. 2.2.1. Displacement of tritiated testosterone from testosterone antiserum (1:20,000 diluted) by various steroids.

Al_2O_3 micro-columns separating testosterone from 5α-dihydrotestosterone, should improve specificity of the described radioimmunoassay. We found, however, for rat testis tissue no differences between testosterone concentrations obtained with a gas-liquid chromatography procedure (Brownie et al. 1964) and this radioimmunoassay method omitting the chromatography step on the Al_2O_3 column (Verjans et al. 1973). This is in agreement with the observation that testicular tissue concentrations of 5α-dihydrotestosterone in mature rats are minute (Folman et al. 1972). Recovery of added tritiated testosterone after extraction from rat testicular

tissue (Table 2.2.1.) was 92 ± 2 (S.D.)%. After extraction and Al_2O_3 micro-column chromatography of blood plasma samples (Table 2.2.1.) a recovery of 67 ± 8 (S.D.)% was obtained. Recovery of added tritiated testosterone after incubation with diluted antiserum (Table 2.2.1.) was 90 ± 5 (S.D.)%. Results from accuracy testing of this method are given in Table 2.2.2. Mean blank values were

TABLE 2.2.2.

Accuracy of the estimation of testosterone by radioimmunoassay

pg testosterone added to water	n	pg testosterone found (mean)	S.D. (in pg)	Coefficient* of variation	% recovery
0	5	3	1	33.0	
50	5	47	4	8.5	94
100	5	96	8	8.3	96
150	5	152	8	5.3	101
200	5	206	35	17.0	103
300	5	260	35	13.5	87

*Coefficient of variation(%) = (S.D./mean x 100%)

low, varying from zero to four pg, and the sensitivity of the method was in the order of 25 pg. Data on the precision of the method as applied to human male blood plasma are summarized in Table 2.2.3. In addition, reliability of the described radioimmunoassay was evaluated by comparison of testosterone concentrations in the same samples of human blood plasma or rat testicular tissue assayed by a gas-liquid chromatography method described by Brownie et al. (1964). For rat testis tissue samples a correlation coefficient of 0.95 (n=54) was found when the same samples were

TABLE 2.2.3.

Precision of multiple estimations of testosterone in human male plasma performed in four different assays using radioimmunoassay. Mean values for each assay (intra-assay) and mean values calculated from the mean values obtained in the different assays (inter-assay) are given

			Testosterone (µg/100 ml)	
Plasma	Assay number	Number of replicates in each assay	Intra-assay means ± S.D. (C.V.*)	Inter-assay means ± S.D. (C.V.*)
M 1	1	6	0.50 ± 0.12 (24%)	0.49 ± 0.03 (6%)
	2	7	0.45 ± 0.02 (5%)	
	3	8	0.51 ± 0.06 (13%)	
	4	6	0.48 ± 0.07 (14%)	
M 2	1	7	0.75 ± 0.09 (12%)	0.73 ± 0.05 (7%)
	2	7	0.78 ± 0.10 (13%)	
	3	7	0.72 ± 0.09 (12%)	
	4	6	0.66 ± 0.05 (7%)	
M 3	1	7	0.72 ± 0.08 (10%)	0.69 ± 0.04 (6%)
	2	8	0.71 ± 0.09 (13%)	
	3	8	0.66 ± 0.07 (10%)	
	4	8	0.65 ± 0.05 (7%)	
M 4	1	6	0.44 ± 0.03 (7%)	0.44 ± 0.03 (7%)
	2	7	0.43 ± 0.06 (14%)	
	3	8	0.41 ± 0.05 (13%)	
	4	7	0.47 ± 0.04 (8%)	
M 5	1	7	0.58 ± 0.05 (9%)	0.68 ± 0.12 (17%)
	2	7	0.80 ± 0.10 (13%)	
	3	8	0.57 ± 0.06 (10%)	
	4	7	0.75 ± 0.03 (5%)	
M 6	1	3	0.49	0.41 ± 0.06 (15%)
	2	8	0.36 ± 0.10 (28%)	
	3	8	0.37 ± 0.07 (19%)	
	4	8	0.42 ± 0.06 (15%)	

*Coefficient of variation (%) = (S.D./mean x 100%)

assayed with radioimmunoassay and gas-liquid chromatography. For human female plasma this value was 0.80 (n=36) and for human male plasma a correlation coefficient of 0.87 (n=92) could be determined. In a few assays for the determination of rat serum testosterone levels, thin layer chromatography on silicagel using the solvent system toluene:methanol - 9:1 (v/v) was employed in order to separate 5α-dihydrotestosterone from testosterone. The results obtained for rat serum testosterone concentrations were essentially the same as those measured with the authentic method (Table 2.2.1.) not employing thin layer chromatography.

2.2.3. discussion

Accuracy and precision of the radioimmunological estimation of testosterone are acceptable. Specificity of detection of the procedure including Al_2O_3 micro-column chromatography appears to be satisfactory as reflected by the correlation coefficients found between testosterone concentrations in samples analysed with radioimmunological endpoint of detection and a technique based on gas-phase chromatography and electron capture as endpoint of detection. Data from other investigators show moreover that when testosterone is estimated in the same blood plasma samples by radioimmunoassay and gas-liquid chromatography (Collins et al. 1972), competitive protein binding (Furuyama et al. 1970; Dessypris and Adlercreutz, 1972; Dufau et al. 1972) or double isotope derivative assay (Dufau et al. 1972) the measured testosterone concentrations are in agreement. It appears therefore reasonable to conclude that satisfactory assay of testosterone in human blood plasma (van der Molen et al. 1971) can be done with radioimmunoassay technique. In addition, the correlation found between testosterone concentrations by radioimmunoassay and gas-liquid chromato-

graphy in the same rat testis tissue samples supports the validity of the radioimmunoassay method for testosterone estimations in tissues containing high concentrations of this hormone. Sensitivity of detection and practicability of the radioimmunoassay method offer, however, distinct advantages over other currently available techniques for testosterone determination and made the radioimmunoassay the method of choice in the present study.

2.3 Radioimmunoassay of rat LH and rat FSH

2.3.1. method and results

The radioimmunoassay systems used for the estimation of rat LH and rat FSH have been published by Uilenbroek (1974). These techniques are essentially a modification of the radioimmunoassay system introduced by Niswender et al. (1968b) and utilize purified ovine gonadotrophin preparations for the immunization procedures and purified rat gonadotrophin preparations for radioiodination. The techniques used in the present investigations are therefore referred to as "OR rat LH" radioimmunoassay and "OR rat FSH" radioimmunoassay according to the convention of Niswender et al. (1968a). Flow sheets of these methods are shown in Tables 2.3.1. and 2.3.2.

Production of antisera, antiserum dilutions, buffer solutions, procedures for labelling of rat LH and rat FSH with ^{125}I and specific activity of the labelled hormones have been described in detail by Uilenbroek (1974). The purified rat LH and rat FSH preparations used as reference standards and for the labelling were obtained from The National Institute of Arthritis and Metabolic Diseases (NIAMD), Bethesda, Maryland, U.S.A. and the biological activities of these preparations as measured by bioassay

TABLE 2.3.1.

Procedure for the radioimmunoassay of LH in blood serum of male rats ("OR rat LH" system)

- Standard series over the range 0-500 ng NIAMD rat LH RP-1
- Blood serum samples (100 µl) are adjusted to 500 µl with buffer
- Add 200 µl of diluted rabbit anti-ovine LH-serum
- Incubate for 24 h at 4°C
- Add 100 µl of solution of ^{125}I-labelled NIAMD rat LH I-1 (10,000 cpm)
- Incubate for 72 h at 4°C
- Add 200 µl of diluted donkey anti-rabbit γ globulin serum
- Incubate for 48 h at 4°C
- Centrifuge for 20 min at 3,000 rpm and aspirate supernatant
- Wash precipitate with buffer
- Centrifuge for 20 min at 3,000 rpm and aspirate supernatant
- Count precipitate for antibody bound radioactivity

techniques are given in appendix paper 1. Rat serum samples were analysed using a constant volume of 100 µl throughout all assays for both LH and FSH and LH and FSH concentrations were expressed on the basis of the widely used reference standard preparations NIAMD rat LH RP-1 and NIAMD rat FSH RP-1 respectively.

Standard curves, specificity of antiserum, accuracy, precision and sensitivity of the "OR rat LH" and the "OR rat FSH" radioimmunoassays have been published in detail

TABLE 2.3.3.

Precision of estimation of LH in male rat serum samples (100 µl) performed in different assays using the "OR rat LH" radioimmunoassay. Mean values for each assay (intra-assay) and mean values calculated from the mean values obtained in the different assays (inter-assay) are given

Serum	Number of assay	Number of replicates per assay	ng NIAMD rat LH RP-1/ml serum: Intra-assay means ± S.D. (C.V.*)	ng NIAMD rat LH RP-1/ml serum: Inter-assay means ± S.D. (C.V.*)
IV	1	5	906 ± 189 (21%)	921 ± 80 (9%)
	2	4	1,029 ± 171 (17%)	
	3	4	917 ± 41 (4%)	
	4	4	834 ± 203 (24%)	
V	1	5	285 ± 42 (15%)	242 ± 47 (19%)
	2	4	248 ± 17 (7%)	
	3	4	170 ± 5 (3%)	
	4	4	228 ± 16 (7%)	
	5	4	280 ± 18 (6%)	
VI	1	4	63 ± 28 (44%)	82 ± 17 (21%)
	2	4	89 ± 12 (13%)	
	3	4	73 ± 10 (14%)	
	4	4	101 ± 6 (6%)	

*Coefficient of variation (%) = (S.D./mean x 100%)

TABLE 2.3.2.

Procedure for the radioimmunoassay of FSH in blood serum of male rats ("OR rat FSH" system)

- Standard series over the range 0-200 ng NIAMD rat FSH RP-1
- Blood serum samples (100 µl) are adjusted to 500 µl with buffer
- Add 200 µl of diluted rabbit anti-ovine FSH-serum
- Incubate for 24 h at $4^{o}C$
- Add 100 µl of solution of ^{125}I-labelled NIAMD rat FSH I-1 (10,000 cpm)
- Incubate for 72 h at $4^{o}C$
- Add 200 µl of diluted donkey anti-rabbit γ globulin serum
- Incubate for 48 h at $4^{o}C$
- Centrifuge for 20 min at 3,000 rpm and aspirate supernatant
- Wash precipitate with buffer
- Centrifuge for 20 min at 3,000 rpm and aspirate supernatant
- Count precipitate for antibody bound radioactivity

by Uilenbroek (1974) and Welschen et al. (1975). Data on the precision of these radioimmunoassay systems as applied to multiple 100 µl samples of pooled serum obtained from adult male rats are summarized in Tables 2.3.3. and 2.3.4. Samples with various concentrations of gonadotrophins were analysed in triplicate, quadruplicate or quintuplicate in different assays performed on different days. The coefficients of variation of the mean estimate in each individual assay, reflecting the intra-assay precision, varied from 3 to 44% for LH (Table 2.3.3.) and from 2 to 17% for FSH (Table 2.3.4.). Mean values and standard deviations calculated from average values obtained in different assays, may give an impression about possible inter-assay variations.

TABLE 2.3.4.

Precision of estimation of FSH in male rat serum samples (100 µl) performed in different assays using the "OR rat FSH" radioimmunoassay. Mean values for each assay (intra-assay) and mean values calculated from the mean values obtained in the different assays (inter-assay) are given

Serum	Number of assay	Number of replicates per assay	ng NIAMD rat FSH RP-1/ml serum: Intra-assay means ± S.D. (C.V.*)	Inter-assay means ± S.D. (C.V.*)
I	1	4	1,243 ± 171 (14%)	1,209 ± 152 (13%)
	2	4	1,198 ± 140 (12%)	
	3	3	1,432 ± 33 (2%)	
	4	5	1,164 ± 55 (5%)	
	5	5	1,008 ± 77 (8%)	
II	1	5	396 ± 65 (16%)	455 ± 40 (9%)
	2	5	478 ± 44 (9%)	
	3	5	480 ± 36 (8%)	
	4	3	467 ± 78 (17%)	
III	1	4	323 ± 25 (8%)	308 ± 18 (6%)
	2	5	288 ± 41 (14%)	
	3	5	324 ± 15 (5%)	
	4	5	296 ± 15 (5%)	

*Coefficient of variation (%) = (S.D./mean x 100%)

For LH determination these latter coefficients of variation ranged from 9 to 21% and for FSH determination from 6 to 13%. From the comparison of these intra-assay and inter-assay coefficients of variation it appears that, in general, precision of the radioimmunoassay for rat FSH was slightly better than for rat LH.

2.3.2. discussion

The development of radioimmunoassay techniques for rat LH and rat FSH has made it possible to monitor gonadotrophin concentrations in serum of individual rats (Monroe et al. 1968; Niswender et al. 1968b; Daane and Parlow, 1971). For the present study the heterologous "OR rat LH" and "OR rat FSH" radioimmunoassay systems have been used. Accuracy and precision of these systems appear to be in the same range as those of the widely used homologous "RR rat LH" and "RR rat FSH" radioimmunoassay systems supplied by the NIAMD (Seki et al. 1971), while the sensitivity of both assay systems employed is higher than that of the NIAMD kits (Uilenbroek, 1974; Welschen et al. 1975). The specificity of the "OR rat LH and FSH" radioimmunoassays is similar to that of the highly specific NIAMD radioimmunoassays (Welschen et al. 1975). Slight interferences of serum components in the rat FSH radioimmunoassay have been reported (Swerdloff et al. 1971; Seki et al. 1971; Uilenbroek, 1974). Therefore, it was deemed necessary to measure all serum samples at the same volume level of 100 µl in this radioimmunoassay system. Furthermore, in order to avoid inter-assay variations, only sample values analysed in the same assay have been compared in this study.

All radioimmunoassay systems so far used for quantification of gonadotrophin hormones may not necessarily measure

biologically active but rather immunologically active hormones. Monroe et al. (1968) and Niswender et al. (1968b) have reported a good correlation between results of classical bioassay and radioimmunoassay for rat LH concentrations in pituitary extracts. Rat serum LH values estimated by radioimmunoassay, however, were found to be lower than those measured by bioassay (Monroe et al. 1968; Bogdanove et al. 1971). A satisfactory correlation between results of bioassay and radioimmunoassay has been found for FSH estimation in rat pituitary extracts and rat serum samples (Daane and Parlow, 1971; Shahmanesh et al. 1975a). Diebel et al. (1973), however, reported marked discrepancies between rat pituitary FSH levels estimated by bioassay and radioimmunoassay.

Recently, more sensitive bioassay techniques for measurement of gonadotrophins have been introduced. These new techniques are based on the interaction of gonadotrophins with specific gonadotrophin receptors in testicular tissue. Reichert and Bhalla (1974) developed a radioligand receptor-binding assay for measurement of human FSH employing homogenate of tubular elements of rat testes. Using testis homogenate of adult rats, Catt et al. (1972) introduced the radioligand receptor-binding assay for measurement of LH. Attempts to measure LH concentrations in rat serum with this latter method were, however, unsuccessful (Shahmanesh et al. 1975b). Dufau et al. (1974) described a sensitive bioassay in vitro for measurement of LH activity using collagenase-dispersed rat Leydig cells. Thus far, these newer receptor-binding techniques have not been applied for measurement of rat serum gonadotrophins. When LH concentrations were measured in rat pituitary tissue (Shahmanesh et al. 1975b) or human blood plasma (Dufau et al. 1974) by these receptor-binding methods and by radioimmunoassay, the latter technique tended to give lower LH results. The discrepancies observed were of quantitative rather than of qualitative nature.

Over the last years increasing evidence has accumulated

that several hormone preparations having similar immunological properties may differ widely in their biological potency (Robyn et al. 1971). Furthermore, it has been established that many protein and polypeptide hormones exist in more than one form, both in blood plasma and in tissues (Berson and Yalow, 1968; Rabinowitz et al. 1974). The complexity of immunoreactive conformations of several peptide hormones in plasma and in tissue has recently been reviewed by Yalow (1974). It has been demonstrated that different molecular forms of FSH in rat (Diebel et al. 1973) and monkey (Peckham et al. 1973) pituitary tissues and in rat serum (Bogdanove et al. 1974a) and of LH in human blood plasma (Robertson et al. 1975; Loeber and Lequin, 1975) may exist. Heterogeneity of polypeptide hormones may therefore explain some of the discrepancies found between bioassay and radioimmunoassay potency estimates. After certain treatments, the structure of the polypeptide hormone molecule could have been altered in such a way that either the immunological or the biological activity might have changed. In the male rat, it has been shown that long-term orchidectomy alters the physicochemical characteristics of pituitary (Diebel et al. 1973) and serum (Bogdanove et al. 1974a) FSH. Exogenous androgen treatment of such rats could reverse this effect (Diebel et al. 1973; Bogdanove et al. 1974a,b). Such effects and changes have not been observed for rat pituitary LH (Bogdanove et al. 1974b). These observations might indicate a steroid regulated pleomorphism of rat FSH in long-term castrates (Diebel et al. 1973). There is, however, no reason to believe that the hypothalamic-pituitary axis still responds in a normal way after long-term castration (Schwartz and Mc Cormack, 1972), and in the present study only short-term gonadectomized, adult male rats have been employed.

Although these data indicate, that certain limitations may be associated with the use of radioimmunoassay of rat serum gonadotrophins, this should not detract from the fact that radioimmunoassay techniques actually offer the only

possibility to estimate gonadotrophin activity in blood serum of individual rats. Recognizing the limitations of measuring gonadotrophins by radioimmunoassays, it may be concluded, however, that these assays offer a useful approach to the solution of physiological problems, which previously were not accessible for direct investigations.

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CHAPTER 3

EXPERIMENTS WITH GONADECTOMIZED,ADULT MALE RATS

3.1 Introduction

The results of the effects of injecting a standard dose of 100 µg/100 g b.w./day of various steroids during 7 days on serum gonadotrophin levels and ventral prostate weights in orchidectomized, adult male rats (appendix paper 6) prompted us to investigate and compare the influence of lower doses of some potent androgens on serum gonadotrophins and weights of accessory reproductive organs. Furthermore, we have examined whether the observed positive feedback effects of androgens in castrates (appendix paper 6) are associated with the presence of a 3β-hydroxy group in such steroids.

The present chapter provides results on the effects of administering 6.25, 12.5, 25, 50 or 100 µg/100 g b.w./day during 7 days of various androgens on weights of the ventral prostate and seminal vesicles and on serum gonadotrophin concentrations in gonadectomized, adult male rats. Information on the effects of injection during 7 days of 100 µg/100 g b.w./day of the various steroids, tested in the study of appendix paper 6, on weights of seminal vesicles in castrates has also been included here. Furthermore, results are reported on the influence of administering 50 µg/100 g b.w./day during 7 days of various 3β-hydroxy androstenes or androstanes on circulating gonadotrophin levels in castrates. Finally, we have studied the effect of exposure to ether anaesthesia during 2 min on serum levels

of LH, FSH and testosterone in gonadectomized male rats.

In the Discussion section of this chapter, we have tried to evaluate our data on effects of steroids on circulating gonadotrophins and weights of accessory reproductive organs in our experimental orchidectomized animal model (see also appendix papers 2, 3, 4, 6 and 7) in light of published information on: a) testicular steroid secretion in adult males, b) effects of castration on gonadotrophins, GnRF and circulating steroids and c) uptake and metabolism of steroids by the hypothalamic-pituitary axis.

3.2 Animals

Adult, male, Wistar rats (3 to 4 months old, body weights ranging from 250 to 330 g) were used. The animals were kept under constant light (14 h light and 10 h darkness), temperature (19-21°C) and humidity conditions. Laboratory rat chow and tap water were provided ad libitum. The animals were employed in experiments at least one week after arrival in the animal quarters.

3.3 Experimental animal model and hormone treatments

Rats were orchidectomized via the abdominal route under ether anaesthesia. Subcutaneous injections with various doses of different steroids or other compounds in sesame oil (0.04 ml oil/100 g body weight) were given immediately after surgery and continued daily during the next six days. Control castrated animals received sesame oil only (0.04 ml/100 g b.w.). Control normal animals were exposed to ether vapour for 10 min prior to the first injection with vehicle

only. The rats were injected each day between 11 a.m. and 1 p.m. Twenty hours after the last injection, the animals were weighed, exposed to ether vapour in a jar for approximately 2 min and then immediately decapitated. Blood was collected from the trunk and allowed to clot overnight at 4°C. Resulting blood serum was stored at -20°C until analysed for gonadotrophins and testosterone. Accessory reproductive organs were removed immediately following sacrifice. After dissection the seminal vesicles (emptied) and ventral prostates were weighed. All steroids employed were purchased from Steraloids (Pawling, New York, U.S.A.), unless otherwise stated in the appendix papers, and used without purification. Treatment with ACTH or dexamethasone was as indicated in appendix paper 4. Administration of gonadotrophin releasing factor (GnRF) to animals pretreated with steroids, was as stated in appendix paper 7. Three or more rats were used for each treatment group.

Each set of hormone treatments had its own controls both of castrated and normal rats. Owing to slight differences in age and body weights of the various animal groups used during the entire period of all experiments (2½ years) and to inter-assay variations in the hormone determinations, we have expressed some data relative to castrate control or normal control values. These latter informations were obtained from animals of the same age and runned in the same experiment.

Significance of differences between the absolute values of the relative weights of accessory reproductive organs (mg/100 g b.w.) and of serum testosterone and gonadotrophin levels (ng/ml) from the different treatment groups was determined using Student's t-test unless otherwise stated.

3.4 Results

Serum gonadotrophins and testosterone

Effect of exposure to ether vapour during 2 min on serum levels of LH, FSH and testosterone in gonadectomized male rats is shown in Table 3.4.1. One group of rats was

TABLE 3.4.1.

Effect of exposure to ether vapour during 2 min on mean (± S.D.) serum levels of LH, FSH and testosterone in gonadectomized, mature male rats (n=5) injected each day during 7 days with 0.04 ml sesame oil/100 g b.w.

Treatment	LH (ng/ml)	FSH (ng/ml)	testosterone (ng/ml)
ether	373 ± 79	1,287 ± 184	0.04 ± 0.03
no ether	428 ± 48	1,224 ± 74	0.03 ± 0.03

weighed, exposed to ether and then decapitated immediately after removal from the ether jar, the other group was first weighed and then immediately decapitated. In these castrated rats ether anaesthesia did not significantly affect the blood serum parameters investigated (Table 3.4.1.). Effects of treating of castrates with ACTH or dexamethasone on circulating hormone levels are summarized in appendix paper 4.

Influence of increasing doses of 4-androstene-3,17-dione, testosterone (appendix paper 3), 4-androstene-3β, 17β-diol, 5α-dihydrotestosterone (Verjans and Eik-Nes,

1976), 5α-androstane-3α,17β-diol (Verjans and Eik-Nes, 1976) or 5α-androstane-3β,17β-diol on serum concentrations of LH and FSH in gonadectomized rats are summarized in Tables 3.4.2. and 3.4.3. respectively. Injection of 25 µg/100 g b.w./day of 5α-dihydrotestosterone or 5α-androstane-3α,17β-diol during 7 days was sufficient to keep circulating LH in castrated rats at values encountered in normal rats ($P < 0.05$). These 5α-reduced androstanes exhibited a higher potency than the other steroids used (Table 3.4.2.) in curbing augmentation of serum LH postorchiectomy. Administration of relatively low doses of androgens to castrates resulted in circulating LH levels higher than those found in castrate controls. This rise was significant ($P < 0.05$) following administration of 25 µg testosterone, 50 µg testosterone, 4-androstene-3β,17β-diol or 5α-androstane-3β,17β-diol and 100 µg 4-androstene-3β,17β-diol. In order to prevent the increase in circulating FSH levels postgonadectomy, higher doses of 5α-reduced testosterone metabolites were required than for curbing the LH rise (Tables 3.4.1. and 3.4.2.). At the doses tried testosterone, 4-androstene-3,17-dione, 4-androstene-3β,17β-diol or 5α-androstane-3β,17β-diol were less effective in suppressing serum FSH than were 5α-dihydrotestosterone or 5α-androstane-3β,17β-diol. Injection of low doses of the six androgens tested tended to elevate circulating FSH levels over castrate control levels. This effect was significant ($P < 0.05$) after injection of 25 µg 5α-androstane-3β,17β-diol and 50 µg of 4-androstene-3β,17β-diol or 5α-androstane-3β,17β-diol (Table 3.4.3.). In addition, we observed that administration of 50 µg 4-androstene-3β,17α-diol or 5-androstene-3β,17β-diol resulted in significantly ($P < 0.05$) higher mean values of circulating LH and FSH levels than values in castrate control animals (Table 3.4.4.). Various steroids from the 4-androstene, 5-androstene, 5α-androstane and 5β-androstane series were administered only in a dose of 100 µg/100 g b.w./day to gonadectomized rats and effects of these steroids on serum LH and FSH are given in appendix

TABLE 3.4.2.

Effects of various doses (µg/100 g b.w./day for 7 days s.c.) of different steroids on serum LH levels (ng/ml) in gonadectomized, mature male rats. Mean levels ± S.D. ($n \geq 3$) are expressed as % of castrate control values (=100%)

steroid / dose	4-androstene-3,17-dione	testosterone	4-androstene-3β,17β-diol	5α-dihydro-testosterone	5α-androstane-3α,17β-diol	5α-androstane-3β,17β-diol
oil	100 ± 20	100 ± 17	100 ± 16	100 ± 19	100 ± 19	100 ± 20
6.25	110 ± 35	101 ± 13	140 ± 21	118 ± 28	74 ± 19	82 ± 13
12.5	132 ± 43	98 ± 11	143 ± 26	120 ± 30	54 ± 14	97 ± 18
25.0	136 ± 26	155 ± 20	160 ± 45	33 ± 11	35 ± 8	143 ± 18
50.0	94 ± 27	139 ± 14	139 ± 8	6 ± 2	31 ± 10	169 ± 19
100.0	110 ± 24	71 ± 10	153 ± 27	< 5	< 5	107 ± 28
normal, oil	18 ± 6	23 ± 5	20 ± 6	22 ± 6	22 ± 6	18 ± 5

TABLE 3.4.3.

Effects of various doses (µg/100 g b.w./day for 7 days s.c.) of different steroids on serum FSH levels (ng/ml) in gonadectomized, mature male rats. Mean levels ± S.D. ($n \geq 3$) are expressed as % of castrate control values (=100%)

steroid / dose	4-androstene-3,17-dione	testosterone	4-androstene-3β,17β-diol	5α-dihydro-testosterone	5α-androstane-3α,17β-diol	5α-androstane-3β,17β-diol
oil	100 ± 12	100 ± 20	100 ± 16	100 ± 18	100 ± 18	100 ± 12
6.25	106 ± 14	115 ± 9	104 ± 12	112 ± 7	114 ± 9	110 ± 49
12.5	123 ± 12	127 ± 13	124 ± 21	121 ± 17	117 ± 8	108 ± 11
25.0	123 ± 45	98 ± 13	131 ± 10	108 ± 5	95 ± 2	138 ± 9
50.0	120 ± 21	79 ± 16	145 ± 17	76 ± 11	89 ± 10	147 ± 14
100.0	106 ± 16	63 ± 14	107 ± 20	36 ± 10	23 ± 3	96 ± 8
normal, oil	29 ± 8	28 ± 7	30 ± 7	31 ± 5	31 ± 5	29 ± 8

TABLE 3.4.4.

Effects of various 3β-hydroxy androstenes or androstanes (50 μg/100 g b.w./day for 7 days s.c.) on mean (± S.D.) serum gonadotrophin levels (ng/ml) in gonadectomized, mature male rats (n=4)

Treatment	Serum LH (ng/ml)	Serum FSH (ng/ml)
sesame oil	293 ± 81	1,173 ± 65
50 μg 4-androstene-3β,17α-diol	557 ± 23*	1,338 ± 80*
50 μg 5-androstene-3β,17β-diol	537 ± 55*	1,312 ± 20*
50 μg 5α-androstan-3β-ol	378 ± 135	1,057 ± 90
50 μg 5α-androstan-3β-ol-17-one	430 ± 74	1,322 ± 170
50 μg 5β-androstan-3β-ol-17-one	366 ± 211	1,193 ± 214
normal, sesame oil	57 ± 16	390 ± 10

*$P < 0.05$, compared with castrate controls receiving vehicle only

paper 6. Effects of oestrogens and oestrenes on these parameters are recorded in appendix papers 2 and 6.

Weights of accessory reproductive organs

Results of daily injections during 7 days with various androstenes, androstanes, oestratrienes and oestrenes in a dose of 100 μg/100 g b.w. on ventral prostate weights in castrated male rats are given in appendix paper 6. Effects of oestrogen, ACTH or dexamethasone administration on

weights of accessory sex organs in castrates have been presented in appendix papers 2 and 4 respectively. Testosterone, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol were more potent in this respect than 4-androstene-3,17-dione or 4-androstene-3β,17β-diol since daily injection of as little as 6.25 μg of these former androgens could prevent ventral prostate weights to decrease to castrate control values ($P < 0.01$) (Table 3.4.5.). Administration of 100 μg of either testosterone, 4-androstene-3β,17β-diol, 5α-dihydrotestosterone or 5α-androstane-3α,17β-diol maintained weights of seminal vesicles in castrates at values measured in normal controls ($P < 0.05$), but injection of 100 μg 4-androstene-3,17-dione did not show this effect and 5α-androstane-3β,17β-diol in the doses used had no effect on the weights of ventral prostates and seminal vesicles (Tables 3.4.5. and 3.4.6.). In addition to the data on weight of ventral prostate given in appendix paper 6, it was found that the weight changes of the seminal vesicles in general followed the pattern of weight changes of the ventral prostate subsequent to injection of various steroids in orchidectomized male rats. Administration of 100 μg of 5-androstene-3β,17β-diol but not of 5-androsten-3β-ol-17-one was able to keep weights of seminal vesicles in castrates at values encountered in normal animals, 5β-androstanes did not or only slightly affect weights of the seminal vesicles and prostates (appendix paper 6) in castrates. In contrast to 5β-androstanes, their 5α-epimers showed high activity in preventing weight decrease of seminal vesicles and ventral prostates (appendix paper 6) postgonadectomy. Given in a dose of 100 μg/100 g b.w./day for 7 days, 5α-androstane, 5α-androstan-3-one, 5α-androstane-3α,11β,17β-triol completely, and 5α-androstan-3α-ol-17-one, 5α-androstan-3β-ol, 5α-androstan-17β-ol, 5α-androstane-3,17-dione partly, prevented the decrease in weight of the seminal vesicles postcastration as compared with normal control rats. Administration of 5α-androstan-3α-ol, 5α-androstane-3α,16α,17β-triol, 5α-androstan-3β-ol-17-one or

TABLE 3.4.5.

Effects of various doses (µg/100 g b.w./day for 7 days s.c.) of different steroids on weights of ventral prostates (mg/100 g b.w.) in gonadectomized, mature male rats. Mean data ± S.D. ($n \geq 3$) are expressed as % of normal control values (=100%)

steroid / dose	4-androstene-3,17-dione	testosterone	4-androstene-3β,17β-diol	5α-dihydro-testosterone	5α-androstane-3α,17β-diol	5α-androstane-3β,17β-diol
oil	28 ± 3	28 ± 5	21 ± 3	24 ± 2	24 ± 2	28 ± 3
6.25	38 ± 9	66 ± 12	41 ± 14	59 ± 5	62 ± 9	24 ± 10
12.5	38 ± 15	73 ± 12	49 ± 4	61 ± 19	80 ± 11	23 ± 2
25.0	53 ± 10	81 ± 12	57 ± 27	64 ± 7	83 ± 12	26 ± 6
50.0	68 ± 12	85 ± 14	56 ± 16	75 ± 15	87 ± 13	26 ± 3
100.0	73 ± 14	96 ± 5	104 ± 10	117 ± 3	115 ± 31	35 ± 6
normal, oil	100 ± 19	100 ± 14	100 ± 25	100 ± 21	100 ± 21	100 ± 19

TABLE 3.4.6.

Effects of various doses (µg/100 g b.w./day for 7 days s.c.) of different steroids on weights of seminal vesicles (mg/100 g b.w.) in gonadectomized, mature male rats. Mean data ± S.D. ($n \geq 3$) are expressed as % of normal control values (=100%)

steroid / dose	4-androstene-3,17-dione	testosterone	4-androstene-3β,17β-diol	5α-dihydro-testosterone	5α-androstane-3α,17β-diol	5α-androstane-3β,17β-diol
oil	55 ± 3	43 ± 6	43 ± 9	59 ± 5	59 ± 5	55 ± 3
6.25	60 ± 17	60 ± 17	52 ± 5	76 ± 10	67 ± 8	51 ± 3
12.5	59 ± 6	63 ± 9	52 ± 20	79 ± 4	86 ± 4	55 ± 8
25.0	60 ± 17	71 ± 17	53 ± 6	74 ± 9	82 ± 20	61 ± 11
50.0	61 ± 16	74 ± 13	61 ± 22	84 ± 14	100 ± 8	57 ± 12
100.0	70 ± 6	82 ± 9	72 ± 18	105 ± 12	106 ± 6	59 ± 5
normal, oil	100 ± 18	100 ± 24	100 ± 15	100 ± 12	100 ± 12	100 ± 18

5α-androstane-3β,11β,17β-triol in the given dose could, however, not counteract this weight decrease following removal of the testes. Effects of oestrogen treatment on accessory reproductive organ weights are given in appendix paper 2. The oestratrienes and oestrenes tested did not affect the decrease of ventral prostate weights in castrates (appendix paper 6), but 5(10)-oestrene-3α,17β-diol, 1,3,5(10)-oestratrien-3-ol, 1,3,5(10)-oestratriene-3,16α, 17β-triol and 1,3,5(10)-oestratriene-3,17α-diol administered in a daily dose of 100 µg/100 g b.w. for 7 days could partly curb decrement in seminal vesicles weights postcastration, 4-oestren-17β-ol-3-one, 5(10)-oestren-17β-ol-3-one and 1,3,5(10)-oestratrien-3-ol-17-one given in the same dose had only a small effect. Furthermore, administration of 4-pregnene-11β,21-diol-3,20-dione, 5α-pregnane-17α, 21-diol-3,20-dione, 5α-pregnan-3-one, 5α-pregnane-3,20-dione and 5α-cholestan-3α-ol (100 µg/100 g b.w./day for 7 days) had either no or minor effects on weight decrease of the seminal vesicles in castrates. Only treatment with dexamethasone (1 mg/day for 7 days) (appendix paper 4) or with compounds of the oestratriene series in a daily dose of 100 µg/100 g b.w. during 7 days reduced total body weight in castrates with respectively 15 to 25% and 10% compared with body weights of castrates treated with vehicle only.

3.5 General discussion

A. hypothalamus-pituitary-testis system

Testicular steroid secretion

Biosynthesis and secretion of testicular steroids have recently been reviewed by Eik-Nes (1975). In the same review, Eik-Nes pointed out that most of the testicular steroids are delivered to the general circulation through the spermatic vein of the male gonad, since flow rate of the testicular lymph is very low, and the quantitative significance of lymphatic transport of androgens from the testis is small (Haltmeyer and Eik-Nes, 1974). Secretory mechanisms of the testis and influence of testicular nerve fibers on secretion are largely unknown. In addition to the steroids synthesized via 4-ene intermediates (prevailing in rat testis) or via 5-ene intermediates, catabolites of testosterone such as oestradiol-17β (Eik-Nes, 1967) and 5α-dihydrotestosterone (Folman et al. 1972) can also be secreted by the male gonad. Recently, the presence of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol was reported in the venous effluent of rabbit testes-epididymides perfused in vitro (Ewing et al. 1975). Mean secretion in spermatic vein blood of 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol by this preparation was 0.8, 0.15 and 0.5 µg/h respectively, which implied that testosterone constituted only 64% of the total mass of these four secreted androgens (Ewing et al. 1975). In dogs peripheral plasma concentrations of 5α-dihydrotestosterone varied from 7 to 16% of those of testosterone (Folman et al. 1972), while simultaneous measurement of plasma 5α-

reduced testosterone metabolites in rabbits showed that mean concentrations of 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were respectively 0.49, 0.17 and 0.24 ng per ml, which implies that in the peripheral blood of the rabbit testosterone accounts for only 53% of mean total mass of these four steroids (Schanbacher and Ewing, 1975). Testosterone production rate by the adult rat testes has been calculated to be in the order of 74 μg/day and testicular production of oestradiol-17β may be as little as 11 ng/day in the adult rat (de Jong et al. 1973), but only 20% of the minute circulating concentrations of oestradiol-17β originate from testicular secretion. Rat testicular tissue can convert radioactive testosterone to a variety of radioactive metabolites during incubations in vitro (Sowell et al. 1974; Lacroix et al. 1975), and contains measurable quantities of 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol (Podestá and Rivarola, 1974) and oestradiol-17β (de Jong et al. 1974).

Uptake and metabolism of steroids by the hypothalamic-pituitary system

Testicular steroids can pass the blood-brain barrier since retention of labelled testosterone (Resko et al. 1967), 5α-dihydrotestosterone (Pérez-Palacios et al. 1973) or oestradiol-17β (Green et al. 1969) in the hypothalamic-pituitary system can be recorded. Hypothalamic and hypophyseal tissue of adult male rats contain measurable quantities of testosterone and 5α-dihydrotestosterone (Robel et al. 1973). Moreover, it has been shown that the hypothalamus and the pituitary gland may contain specific steroid receptor macromolecules, which are generally thought to be a prerequisite for steroid action at the target organ (Jensen and De Sombre, 1972). In the hypothalamus and hypophysis of male rats specific macromolecular binding prin-

ciples have been demonstrated for oestradiol-17β (Kato et al. 1974; Kato, 1975), testosterone (Jouan et al. 1971, 1973) and 5α-dihydrotestosterone (Kato and Onouchi, 1973a,b). It has been shown that cytoplasmic binding principles for either androgens (Naess et al. 1975) or oestrogens (Vreeburg et al. 1975) are also present in other brain regions. Specificity of "specific" receptors is limited and competition by other steroids does occur. The oestradiol-17β receptor proteins from anterior hypothalamus or pituitary cytosols show affinity for oestriol, oestrone and also for 5α-androstane-3β,17β-diol (Vreeburg et al. 1975). Testosterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, oestradiol-17β and progesterone may compete for binding to a specific cytoplasmic dihydrotestosterone receptor from rat anterior hypophysis tissue (Thieulant et al. 1975).

Steroid metabolizing enzymes, such as 5α-steroid reductase (Rommerts and van der Molen, 1971; Massa et al. 1972), 5β-steroid reductase (Genot et al. 1975), 3α- and 17β-hydroxysteroid dehydrogenase (Rommerts and van der Molen, 1971) and 3β-hydroxysteroid dehydrogenase (Genot et al. 1975) are present in hypothalamus and pituitary tissue of male rats and the anterior hypothalamus contains steroid aromatizing activity (Naftolin et al. 1972). Steroid ring A reduction appears to be the predominant pathway for androgen metabolism in neuroendocrine cells rather than steroid ring A aromatization (Naftolin and Ryan, 1975). Oestradiol-17β is probably not metabolized in hypothalamus and hypophysis (Kato and Villee, 1967; Kato, 1975). Noma et al. (1975) have shown that also in the hypothalamus 5α-reduction of testosterone to 5α-dihydrotestosterone is irreversible, whereas reduction of this latter androgen to 5α-androstane-3α,17β-diol is reversible, and either 5α-androstane-3α,17β-diol or 5α-dihydrotestosterone cannot be converted to an oestrogen (Engel, 1973).

The prevailing androgen in the peripheral circulation of normal male animals is testosterone (Eik-Nes, 1972) and

hypothalamic and pituitary tissue of mature male rats contain higher concentrations of testosterone than of 5α-dihydrotestosterone (Robel et al. 1973). Testosterone is thus probably the main precursor for formation of 5α-reduced androstane metabolites and oestradiol-17β in the hypothalamic-pituitary system.

Effects of gonadectomy on steroids, gonadotrophins and GnRF

Within 2 h after castration of male animals, peripheral levels of testosterone decrease to less than 4% of those seen in intact rats while circulating concentrations of 5α-dihydrotestosterone are diminished to undetectable values at that interval following removal of the testes (Coyotupa et al. 1973). Testosterone, the prevailing androgen in systemic blood of male animals (Eik-Nes, 1972), is probably the main precursor for formation of 5α-reduced androstane metabolites and of oestradiol-17β. Hence, concentrations of these testosterone metabolites are likely to decrease in the peripheral circulation following gonadectomy. While serum testosterone levels decrease (appendix paper 3), serum gonadotrophin levels will increase following orchidectomy in adult male rats (appendix paper 2). These results are in agreement with previous observations in adult male rats that serum LH concentrations are drastically elevated as early as 8 h following gonadectomy and continue to increase over the ensuing days (Yamamoto et al. 1970). These changes in plasma levels may partly reflect pituitary LH concentrations which fall initially, reaching their lowest value 4 days following castration, but subsequently rise again to levels encountered in normal rats at 8 days following gonadectomy and then gradually increase over normal control values (Yamamoto et al. 1970).

Drastic increases in serum FSH can be observed 1 day after castration (Steinberger and Chowdhury, 1974) and up

to 3,5,7,10, (appendix paper 2) 14 and 21 (Amatayakul et al. 1971) days following gonadectomy these levels remain elevated but fairly constant. A progressive drop in pituitary FSH has been observed up to 72 h after castration (Steinberger and Chowdhury, 1974). Seven days following gonadectomy, pituitary FSH began to rise, to return to normal at 2 weeks after orchidectomy and to increase gradually thereafter (Steinberger and Duckett, 1966).

The effects of castration on LH and FSH levels may be mediated via hypothalamic GnRF. The first two days following gonadectomy serum and hypothalamic concentrations of GnRF appear to be raised, but a gradual decrease in hypothalamic GnRF content has been recorded from 5 to 13 days following castration, while serum GnRF levels returned to normal control values in that same time period (Shin and Howitt, 1975).

For hypertrophic pituitary glands from castrated male rats Mc Cann (1974) has calculated drastic increases in daily secretion rates of LH and FSH and decreased turnover times for LH and FSH. All these data may reflect that removal of male gonads results in an augmented release of gonadotrophins into the general circulation. In spite of the increased secretion rates of gonadotrophins, concentrations of gonadotrophins in pituitary tissue of male animals may still rise, and therefore, biosynthesis of pituitary gonadotrophins following gonadectomy must increase. This conclusion is supported by a report on augmented LH production in vitro by pituitary tissue from male castrates (Wakabayashi and Tamaoki, 1967).

B. effects of administration of steroids on serum gonadotrophins in castrates -negative feedback-

Since removal of the testes from male animals results in changed production and secretion rates of gonadotrophins

and gonadotrophin releasing material, administration of testicular steroids to gonadectomized animals would be expected to counteract these effects. Our approach has been to start injection of the various steroids immediately after orchidectomy and to determine whether the steroids injected could curb the hypersecretion of gonadotrophins into the peripheral circulation. A period of daily injections during 7 days was chosen, because we found both a rather stable plateau of serum LH and FSH levels at 3,5 and 7 days following castration (appendix paper 2) and an adequate weight decrement of the accessory reproductive organs at that time (appendix paper 2). Other investigators have studied effects of steroid injections on circulating gonadotrophins in long-term castrates. Disadvantages associated with work in long-term castrates have been discussed in chapter 2.

Experimental animal model

The experimental animal model used suffers certain drawbacks. In experiments with rats stress conditions are practically unavoidable. The standard conditions used in our experiments have inevitably involved stress stimuli such as handling and manipulations of the rats during daily injections, use of ether anaesthesia during surgery and sacrifice, and serial blood sampling in the GnRF experiments. These stress stimuli can influence circulating levels of LH and FSH (appendix paper 7; chapter 4), testosterone secretion (Bardin and Peterson, 1967) and testicular testosterone concentrations (Fariss et al. 1969). Using the present animal model, we could, however, not detect measurable changes in serum LH, FSH and testosterone levels in castrates whether the animals were killed immediately or following exposure to ether anaesthesia during 2 min before sacrifice (Table 3.4.1.). Furthermore, serum gonadotrophin

and testosterone concentrations in castrated rats treated with ACTH or dexamethasone (appendix paper 4) or corticosterone (appendix paper 6) and handled in the same standard fashion, were similar to those of control animals not treated with corticotrophin or one of these glucocorticoids during 7 days. Wasserman and Eik-Nes (1969) have shown, that the adrenal cortex of stressed dogs secreted substantial amounts of 4-androstene-3,17-dione, a metabolic precursor of testosterone and oestradiol-17β (Samuels and Eik-Nes, 1968). Over the range investigated 4-androstene-3,17-dione exhibits no effect on gonadotrophin levels in castrates (appendix paper 6, Tables 3.4.2. and 3.4.3.). Blood serum samples for determination of LH, FSH and testosterone were always obtained at the same time of the day in all our animals using the same stress conditions in order to withdraw this blood. We conclude from these data in our experimental animal model that the stress due to ether anaesthesia is not of paramount influence on circulating gonadotrophins, and that steroid secretion of the adrenal gland does not play a significant role as regulator for circulating gonadotrophin concentrations in the adult, male rat.

In the interpretation of the present results it has been assumed that: 1) the different steroids injected are released from the subcutaneous site of injection at the same rates and are undergoing metabolism at this site to the same (insignificant?) extent, 2) the various administered steroids are cleared with approximately the same speed; however, one daily subcutaneous injection with steroid may result in variable concentrations of steroid and gonadotrophins in the circulation over the ensuing period prior to the next injection (appendix paper 2; Hutchison and Goldman, 1975), 3) furthermore, we must assume that circulating gonadotrophins are true indicators of pituitary production and secretion of these glycoprotein hormones. Even more important, the gonadotrophins will reach their target

tissues through the systemic blood, and the concentrations of gonadotrophins in blood should play an essential role for the processes involved in tissue regulation (Eik-Nes, 1975).

Circulating gonadotrophin concentrations are determined by rate of secretion and rate of disappearance. It seems likely that the clearance of endogenous FSH will be unaffected by circulating gonadal steroids (Bogdanove and Gay, 1969), since castration or steroid treatment does not appear to alter the capacity of the male rat to remove and/or inactivate circulating exogenous FSH. Yamamoto et al. (1970) have concluded that the decay constant of LH is not appreciably influenced by removal of the gonads. Therefore, it may be assumed that steroids do not markedly influence the half-life of LH (approximately 30 min) and FSH (approximately 2 h) (Gay and Bogdanove, 1968). Bilateral nephrectomy of castrated, adult male rats resulted, however, in decreased rates of LH and FSH clearance accompanied by quantitatively similar increases in serum levels of LH and FSH (Gay, 1974). It is interesting to note that these data from Gay's laboratory seem to cast doubt on the existence of short-loop feedback regulation by circulating gonadotrophins at the hypothalamic-pituitary system in castrates (Gay, 1974).

Our data (appendix papers 2 and 3), and work done by Hutchison and Goldman (1975) show the same discrepancy between effects of administration of testosterone s.c. and testosterone propionate s.c. on serum gonadotrophins. The propionate derivative of testosterone given s.c. was more effective in suppressing serum gonadotrophins in castrates than "free" testosterone administered s.c. or i.v. (Hutchison and Goldman, 1975). Finally, neither continuous intravenous infusion (Hutchison and Goldman, 1975) nor subcutaneous injections of testicular steroids may adequately simulate the fluctuations in plasma testosterone levels which can be detected in blood samples from the same rat obtained at different times of the day (Bartke et al. 1973).

A periodic release of LH but not of FSH has also been detected in long-term castrates (Gay and Sheth, 1973).

In spite of these possible shortcomings of using "stressed" normal or castrated rats injected daily with different steroids, data from such experiments are of value on a comparative basis for an understanding of effects of steroids on regulation of circulating gonadotrophins.

Results

It is clear (Tables 3.4.2. and 3.4.3.; appendix papers 2, 3, 6 and 7; Verjans and Eik-Nes, 1976) that injection of ring A reduced or ring A aromatized testosterone metabolites is more effective in preventing the rise of circulating gonadotrophin concentrations following gonadectomy in male rats than testosterone or its metabolic precursors (Tables 3.4.2. and 3.4.3.; appendix papers 2, 3, 6 and 7; Verjans and Eik-Nes, 1976). Serum LH concentrations were more sensitive to steroid suppression than serum FSH concentrations. From the compounds tested oestradiol-17β (appendix paper 7) or oestradiol benzoate (appendix paper 2) exhibited the highest potency in this respect. Aromatization of the A ring in steroids appeared, however, not to be a prerequisite in order to affect gonadotrophin levels in serum (appendix paper 2). Some of the disubstituted 3 or 17 keto and/or 3α or 17β hydroxyl 5α-reduced testosterone metabolites investigated showed high activity in regulating serum gonadotrophin levels in castrates (appendix paper 6), however, 3β or 5β epimers of such compounds exhibited no activity in this respect (appendix paper 6). A possible contribution of adrenal corticosteroids, androgens and oestrogens was found to be of minor, if any, importance in male rats (appendix paper 4) for regulating circulating gonadotrophins. It was consistently

found that administration of low doses of androgens or oestrogens resulted in circulating gonadotrophin levels higher than those encountered in castrate control animals injected with vehicle only (appendix papers 2 and 3; Tables 3.4.2. and 3.4.3.). This effect was also observed following injection of relatively high doses of 3β-hydroxy and 17 disubstituted androstenes or androstanes (appendix paper 6; Tables 3.4.2., 3.4.3. and 3.4.4.).

Subcutaneous administration of testosterone to castrates resulting in serum testosterone levels not significantly different from those observed in intact rats, was not associated with the expected drop of the high serum LH and FSH concentrations to values encountered in normal rats (appendix paper 3). This finding was recently confirmed also for the immature, castrated male rat (Moger, 1975). This suggests that besides testosterone other testicular products are involved in pituitary gonadotrophin regulation. These data also suggest that peripheral metabolism of testosterone and metabolism of testosterone in androgen target organs outside the testis do not promote the needed concentrations of potent suppressors for circulating LH and FSH to prevent the postcastration rise of serum gonadotrophin concentrations. Therefore, it is most likely that in the normal rat secretion products of the testis, such as testosterone and especially 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol and oestradiol-17β, are the main factors required for regulation of circulating LH and FSH.

An "inhibin-like" factor isolated from bull seminal plasma has been shown to decrease selectively circulating FSH levels in castrates (Franchimont et al. 1975). We have indicated that such a testicular factor, probably of non-interstitial cell origin, may partly control serum FSH concentrations in normal male rats (Verjans and Eik-Nes, 1976; appendix paper 5; chapter 4).

The data discussed in this section may provide the biochemical base for a system where testicular products delivered into the general circulation affect the hypotha-

lamic-pituitary axis. It appears feasible that testosterone, in combination with its 5α-reduced and/or ring A aromatized metabolites and together with "inhibin" can maintain physiological balance between activities in the male gonad and activities in the hypothalamic-pituitary system.

C. effects of gonadotrophin releasing factor
-positive feedback-

The results on the effects of oestrogen and androgen on GnRF induced serum concentrations of gonadotrophins in castrates have been presented in appendix paper 7. Under the conditions of these experiments we have concluded that: 1) The androgen and oestrogen might have acted at different sites in the male hypothalamic-pituitary axis with respect to regulation of circulating gonadotrophins, LH being more sensitive to steroid treatment than FSH. However, the possibility of effects of steroids at higher brain centres cannot be excluded. Effects of oestrogen (Davidson, 1969) or 5α-dihydrotestosterone (Feder, 1971) or combined treatment with both steroids (Baum and Vreeburg, 1973) on mating behaviour in castrated male rats are well known. An animal model using systemic i.v. or s.c. steroid administration or steroid implants into the hypothalamic-pituitary area (Kingsley and Bogdanove, 1973; Smith and Davidson, 1974) will not provide decisive answers to this problem, because the administered or deposited steroid can reach many brain structures. 2) High doses of androgen administered to castrates will inhibit pituitary release of gonadotrophins, while injection of low doses of androgen may stimulate GnRF resulting in pituitary release of gonadotrophins and in higher serum gonadotrophin levels than those of castrate controls (positive feedback). Not only release but also the production of gonadotrophins can be affected by steroids in

castrates. Testosterone propionate administered in very high doses to castrated male rats increased pituitary FSH but not pituitary LH stores (Gay and Bogdanove, 1969). 3) Injection of low doses of oestradiol-17β to castrates may render the anterior pituitary gland more sensitive to GnRF, resulting in elevation of serum gonadotrophins over castrate control levels (positive feedback), while high doses of oestradiol-17β may inhibit hypothalamic production and/or secretion of GnRF. Very high doses of oestradiol benzoate are known to suppress LH and FSH concentrations in pituitaries of male castrate (Bogdanove et al. 1971) or normal rats (appendix paper 1). Positive feedback effects were also observed following administration of relatively high doses of 3β-hydroxy 17 disubstituted androstenes and androstanes to castrates (Table 3.4.4.). We are not aware of the presence of specific binding principles for these particular steroids in pituitary or hypothalamic tissues. However, we cannot exclude the possibility that these steroids exert their effects in castrates following metabolic conversion or by their affinity for binding sites of known "specific" receptors.

D. accessory reproductive organs and steroids

Discussion of results

Following removal of the male gonads the weights of accessory reproductive organs decreased concomitant with the decrease of circulating testosterone concentrations (appendix papers 2 and 6; Tables 3.4.5. and 3.4.6.). Administration of several steroids can prevent this weight decrease in castrates (appendix papers 2 and 6; Tables 3.4.5. and 3.4.6.). In appendix paper 6 effects of administration

of a relatively high dose (100 µg/100 g b.w./day during 7 days) of various steroids on weights of the ventral prostate in castrated, adult male rats have been compared and discussed. The daily injection of 100 µg/100 g b.w. of 3 or 3β and 17 or 17β disubstituted (keto or hydroxyl) androstenes could entirely or partly prevent weight decrement of the accessory reproductive organs postgonadectomy. The results indicate a discrepancy between the effects of different androgens on the hypothalamic-pituitary axis and the biological potency on the processes governing growth of accessory reproductive organs. Representative compounds of the 5α-androstane, oestratriene and oestrene series also showed a remarkable discrepancy in their biological activity as judged by effects on weights of ventral prostate and seminal vesicles and on suppression of serum gonadotrophins. Like for regulation of serum gonadotrophins in castrates, 5β-reduction of testosterone and 3β-reduction of 5α-dihydrotestosterone resulted in loss of biological activity in the accessory sex organs, while 3α-reduction of 5α-dihydrotestosterone did not alter the biological activity exhibited on the ventral prostate and seminal vesicles (Tables 3.4.5. and 3.4.6.). Low doses of testosterone, 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol, 4-androstene-3β, 17β-diol or 4-androstene-3,17-dione were more effective in curbing the decrease of prostate and seminal vesicles weights than in preventing the rise of serum gonadotrophins postorchiectomy. When circulating testosterone levels in castrated animals following s.c. injection of testosterone were comparable with those in normal rats, normal ventral prostate and seminal vesicles weights were observed in such animals, but these testosterone levels failed to suppress postcastration rise of serum LH and FSH (appendix paper 3; Tables 3.4.2. and 3.4.3.). We can, however, not exclude the possibility that this discrepancy in effects on gonadotrophins and accessory reproductive organ weights might be due to the use of daily injections of steroids, which may result in high peripheral steroid concentrations immediately

following injection with decreasing steroid levels prior to the next daily injection. The data obtained suggest that the effects of steroids on suppression of circulating gonadotrophins in castrates do not always correlate with the classical bioassay indicator for androgenicity, i.e. the effects of steroids on weight maintenance of seminal vesicles and ventral prostate in such animals (Dorfman and Shipley, 1956; Young and Corner, 1963).

Mechanism of effect of androgens in accessory reproductive organs

5α-Dihydrotestosterone and 5α-androstane-3α,17β-diol are at least as potent as testosterone in preventing weight decrease of the accessory sex organs (Tables 3.4.5. and 3.4.6.), 5α-dihydrotestosterone propionate exhibited in this respect a higher potency than testosterone propionate (appendix paper 2). 4-Androstene-3,17-dione and 4-androstene-3β,17β-diol showed also high potencies (Tables 3.4.5. and 3.4.6.). Uptake (Hansson, 1971) and metabolism (Farnsworth and Brown, 1963) of androgens by accessory reproductive organs have been reported. The concentration of cytoplasmic androgen receptor in the rat prostate has been shown to decrease following orchiectomy (Bruchovsky and Craven, 1975) and there is also extensive androgen metabolism by the prostate (for review: Eik-Nes, 1976). Bruchovsky (1971) has shown that radioactive 5α-dihydrotestosterone is the major metabolite in prostatic nuclei following intravenous injection of either radioactive testosterone, 5α-dihydrotestosterone, 5α-androstan-3α-ol-17-one, 5α-androstane-3α,17β-diol, 5α-androstane-3,17-dione, 4-androstene-3,17-dione or 5-androsten-3β-ol-17-one in castrated male rats. Moreover, 5α-dihydrotestosterone is the principal steroid bound to androgen receptor in the rat prostate following injection of radioactive testosterone

(Bruchovsky and Wilson, 1968). Administration of 5α-dihydrotestosterone to castrates before prostatic atrophy had commenced, resulted in an increase of prostatic wet weight and stimulated secretory activity (Lesser and Bruchovsky, 1974). Recently, it has been shown that in rats and dogs the prostate receives blood directly from the deferential vein (Pierrepoint et al. 1975). This blood has a high concentration of androgens (Pierrepoint et al. 1975). Finally, in the rat prostate and seminal vesicles the mean concentration of 5α-dihydrotestosterone is higher than that of testosterone (Robel et al. 1973).

From these combined data it can be concluded that secretion of testicular steroids in the mature rat is of paramount importance for adequate functioning and maintenance of ventral prostate and seminal vesicles physiology and that 5α-dihydrotestosterone is the main androgen in these organs. 4-Androstene-3β,17β-diol could have exerted a possible effect on the weights of the ventral prostate and seminal vesicles through its affinity to known steroid receptors in these organs (Grover and Odell, 1975) or following conversion to testosterone or 5α-androstane-3β,17β-diol. The latter steroid has, however, no effect on these parameters in the doses tested (appendix paper 6; Tables 3.4.5. and 3.4.6.). Synergistic effects of prolactin and testosterone on weight gain in the ventral prostate and in the seminal vesicles have been observed (Keenan and Thomas, 1975). Castration is associated with unchanged prolactin secretion rates (Mc Cann, 1974), but we have not determined whether the steroid injections used in the current work will result in altered secretion rates of prolactin. Hence, it is not known whether prolactin has affected the effects of steroids on weights of accessory reproductive organs in our experiments.

Effects of other compounds on accessory reproductive organs

Several steroids from the pregnane, pregnene, cholestane and cholestene series do not affect the reduction of the weights of accessory reproductive organs in castrates (appendix paper 6). The C-21 metabolic precursors of testosterone also do not influence these parameters in hypophysectomized animals, indicating that conversion of these steroids to potent androgens is insignificant (Steinberger et al. 1975). Oestratrienes and oestrenes did not prevent the decrease of ventral prostate weight postorchiectomy but the oestratrienes tested could partly prevent the decrement of seminal vesicles weight in castrates (appendix paper 6). 1,4-Diphenyl-butane-2,3-diol, a testicular secretion product (Eik-Nes et al. 1967), followed in this respect the effects exhibited by the oestrogens on accessory sex organs (appendix paper 6). At variance with these findings is the fact that an oestradiol-17β receptor has been detected in the prostate of the rat but not in the seminal vesicles of this animal (van Beurden et al. 1974).

E. summary of conclusions

From the available information, it is difficult to conclude whether either 5α-dihydrotestosterone, or 5α-androstane-3α,17β-diol, or both are the biologically active component(s) in the hypothalamic-pituitary system and/or the accessory reproductive organs. More definite answers may be obtained via the use of proper inhibitors for the 3α-hydroxysteroid dehydrogenase enzyme activity, which in rat pituitary gland (Thien et al. 1975) and rat ventral prostate (Shimazaki et al. 1972) is mainly located in the cytosol fraction. Ratios between concentrations of testos-

terone and 5α-dihydrotestosterone in hypothalamus and anterior pituitary gland are much higher than for seminal vesicles and prostate in the normal mature rat (Robel et al. 1973). This might indicate that in accessory reproductive organs the metabolic conversion to the potent 5α-reduced testosterone metabolites is greater than in the hypothalamic-pituitary system. This difference might in part explain some of the discrepancies observed in gonadectomized male rats for the effects of steroids on LH and FSH in the peripheral circulation and on the weights of accessory reproductive organs.

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CHAPTER 4

EXPERIMENTS WITH NORMAL,ADULT MALE RATS

4.1 Introduction

The use of gonadectomized animals is frequently preferred in studies concerning possible effects of steroids on the gonadotrophic function of the male hypothalamic-pituitary axis, because the possible influence of endogenous steroids can be excluded as it has been shown that the contribution of adrenal steroids in castrated male animals (appendix paper 4) is of minor, if any, importance in this respect.

The suppressive effects of testosterone and oestradiol-17β on circulating levels of LH and FSH in intact, adult male rats are well known. Few data are available concerning effects of administration of other steroids on these parameters in normal male rats. We were also interested to investigate the effect of administration of low doses of steroids to intact male rats, since we had observed a positive feedback effect of low doses of androgens on circulating gonadotrophin concentrations in castrated rats (chapter 3). These studies also offered the opportunity to evaluate the significance of the results with steroids (particularly with respect to specificity and doses needed) in orchidectomized, adult male rats.

Furthermore, the effects of exposure to ether vapour during 2 min on serum LH, FSH and testosterone in normal rats were studied. The results of these experiments are discussed in light of data on intact, adult male rats pre-

sented in the appendix papers 1,3,4,5 and 7.

4.2 Animals

Adult, male Wistar rats (3 to 4 months old, body weights ranging from 230 to 275 g) were employed. The animals were kept under constant light (14 h light and 10 h darkness), temperature (19-21°C) and humidity conditions. Laboratory rat chow and tap water were provided ad libitum. The animals were used in experiments at least one week after arrival in the animal quarters.

4.3 Experimental animal model and hormone treatments

Daily subcutaneous injections with various doses of different steroids in sesame oil (0.04 ml oil/100 g body weight) were given for a period of 7 days between 1 and 2 p.m. Control animals received sesame oil only (0.04 ml/100 g b.w.). Steroids were purchased from Steraloids (Pawling, New York, U.S.A.) and used without purification. Four or 5 animals were employed in each of the different treatment groups. Twenty hours after the last injection, the animals were weighed, exposed to ether vapour in a jar for approximately 2 min and then immediately decapitated. Blood was collected from the trunk and allowed to clot overnight at 4°C. Resulting blood serum was stored at -20°C until analysed for hormones. Testes and accessory reproductive organs were removed immediately after sacrifice. After dissection, testis, seminal vesicles (emptied) and ventral prostate were weighed.

Treatment with ACTH or dexamethasone was as indicated

in appendix paper 4. Administration of gonadotrophin releasing factor (GnRF) to rats pretreated with steroids was as stated in appendix paper 7. Bilateral, local X-irradiation of the testes of normal rats was as described in appendix paper 5.

Significance of differences between data from the different treatment groups was determined using Student's t-test unless otherwise stated.

4.4 Results

Serum gonadotrophins and testosterone

Effects of exposure to ether vapour during 2 min on serum levels of LH, FSH and testosterone in normal male rats are shown in Table 4.3.1. One group of rats was

TABLE 4.3.1.

Effect of exposure to ether vapour during 2 min on mean (± S.D.) serum levels of LH, FSH and testosterone in normal, mature male rats (n=5) injected each day during 7 days with 0.04 ml sesame oil/100 g b.w.

Treatment	LH (ng/ml)	FSH (ng/ml)	testosterone (ng/ml)
ether	59 ± 18	447 ± 51*	3.6 ± 0.6
no ether	78 ± 18	355 ± 33	3.4 ± 0.5

*$P < 0.05$, compared with animals not exposed to ether

weighed, exposed to ether and decapitated immediately after removal from the ether jar, the other group was first weighed and then immediately decapitated. No statistically significant differences in mean circulating testosterone and LH concentrations were found between animals killed with or without this type of ether anaesthesia (Table 4.3.1.). Mean levels of serum FSH rose significantly ($P < 0.05$) in rats exposed to ether when compared with animals not exposed to this anaesthetic. In appendix paper 4 results of treatment with ACTH or dexamethasone on serum gonadotrophin and testosterone concentrations in normal, adult male rats are summarized and appendix paper 5 gives effects of local X-ray treatment of the testes on these

TABLE 4.3.2.

Effects of various steroids, administered s.c. in a daily dose of 50 µg/100 g b.w. during 7 days, on mean (± S.D.) serum gonadotrophin levels in normal, mature male rats (n=4)

	Serum gonadotrophin concentrations (ng/ml)	
Treatment	LH	FSH
progesterone	52 ± 19	480 ± 50
4-androstene-3,17-dione	83 ± 18	448 ± 103
4-androstene-3β,17β-diol	79 ± 22	513 ± 131
5-androstene-3β,17β-diol	78 ± 14	428 ± 111
5α-dihydrotestosterone	20 ± 6*	315 ± 46*
5α-androstane-3α,17β-diol	26 ± 7*	305 ± 59*
5α-androstane-3β,17β-diol	47 ± 10	331 ± 77
oestradiol-17α	16 ± 4*	320 ± 40*
sesame oil	54 ± 15	440 ± 61

*$P < 0.05$, compared with controls receiving vehicle only

parameters. Effects of oestrogen or testosterone administration on peripheral levels of blood serum testosterone and gonadotrophins in normal male rats are shown in appendix papers 1 and 3 respectively. Influence of injecting various steroids in a daily dose of 50 µg/100 g b.w. during 7 days on serum gonadotrophin levels in normal rats has been compared in Table 4.3.2. Significantly lower ($P < 0.05$) serum LH and FSH levels as compared with those of normal control rats were found following administration of 50 µg 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol or oestradiol-17α (Table 4.3.2.). Given in the same dose, progesterone, 4-androstene-3,17-dione, 4-androstene-3β,17β-diol, 5-androstene-3β,17β-diol or 5α-androstane-3β,17β-diol had no significant effect on blood serum LH and FSH (Table 4.3.2.). Effects of injection of 25, 12.5 or 6.25 µg/100 g b.w. of various androgens on mean serum LH and FSH concentrations in normal animals are summarized in Table 4.3.3. These doses did not have a significant effect on serum FSH concentrations, although some doses of 4-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol tended to augment serum FSH over control levels (Table 4.3.3.). Testosterone, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol, when given in a dose of 25 µg/100 g b.w. significantly suppressed ($P < 0.05$) serum LH, but only a dose of 12.5 µg/100 g b.w. 5α-dihydrotestosterone could induce statistically lower ($P < 0.05$) LH levels as compared with normal control animals injected with oil only (Table 4.3.3.). Injection of 4-androstene-3β,17β-diol tended to elevate mean serum LH levels, but this increase was not statistically significant (Table 4.3.3.).

Weights of reproductive organs

Data on the effects of administration of ACTH or dexamethasone on weights of testes, seminal vesicles and ven-

TABLE 4.3.3.

Effects of various doses (µg/100 g b.w./day for 7 days s.c.) of different steroids on mean (± S.D.) serum gonadotrophin levels (ng/ml) in normal, mature male rats ($n \geq 4$)

		Serum gonadotrophin concentrations (ng/ml)	
Steroid	Dose	LH	FSH
testosterone	25.0	35 ± 10*	349 ± 70
	12.5	61 ± 15	368 ± 46
	6.25	69 ± 14	394 ± 47
4-androstene-3β,17β-diol	25.0	94 ± 21	585 ± 148
	12.5	69 ± 15	523 ± 187
	6.25	90 ± 22	462 ± 72
5α-dihydrotestosterone	25.0	27 ± 7*	315 ± 74
	12.5	41 ± 2*	377 ± 102
	6.25	56 ± 20	420 ± 57
5α-androstane-3α,17β-diol	25.0	41 ± 5*	323 ± 32
	12.5	58 ± 25	527 ± 209
	6.25	66 ± 34	514 ± 215
5α-androstane-3β,17β-diol	25.0	64 ± 26	353 ± 112
	12.5	44 ± 15	387 ± 140
	6.25	76 ± 19	529 ± 133
Sesame oil		64 ± 14	397 ± 57

*$P < 0.05$, compared with controls receiving vehicle only

tral prostates in normal rats are given in appendix paper 4 and appendix paper 1 summarizes the influence of oestrogen treatment on these parameters. Changes in weights of reproductive organs induced by local X-irradiation of the male gonads are summarized in appendix paper 5. The doses of testosterone employed in the study of appendix paper 3 and the doses of 50, 25, 12.5 or 6.25 µg/100 g b.w. of the steroids tested in the present study (Tables 4.3.2. and 4.3.3.) did not significantly alter total body weight or weights of the testes, seminal vesicles and ventral prostates compared with normal control rats injected with sesame oil only.

4.5 General discussion

Discussion of results

Our data demonstrate that several steroids can lower circulating LH and FSH concentrations in normal male rats when given subcutaneously for several days. Ring A 5α-reduced or ring A aromatized metabolites of testosterone had a high potency in this respect (Tables 4.3.3. and 4.3.2.; appendix papers 1 and 7), which is compatible with the findings reported for gonadectomized rats (chapter 3). For depression of circulating gonadotrophins in normal male rats lower doses of androgens were required than in castrates, whereas oestrogens were more effective in castrated than in normal male rats with regard to lowering blood serum gonadotrophins (appendix paper 7). In both type of animals serum LH appeared more sensitive to steroid treatment than serum FSH. Of all steroids tested, oestrogen exhibited the highest potency in suppressing circulating LH and FSH levels (appendix papers 1 and 7). In appendix

paper 7, the effects of oestradiol-17β or 5α-dihydrotestosterone pretreatment on GnRF induced serum levels of LH and FSH in normal rats have been described and discussed. It was concluded, that under our experimental conditions oestradiol-17β exerts its effects on the hypothalamic-pituitary axis in a way different from that of the non-aromatizable 5α-dihydrotestosterone.

Discussion of experimental animal model

Our intact animal model suffers the same experimental shortcomings (i.e. handling, manipulation, anaesthesia, daily s.c. steroid injections) as the castrate animal model. Furthermore, effects of ether anaesthesia appear to be more serious in normal than in orchidectomized rats. Exposure to ether during 2 min induced a slight, but significant elevation of serum FSH levels, but did not significantly affect serum levels of testosterone and LH (Table 4.3.1.). Depressive effects of anaesthesia (Eik-Nes, 1962; Bardin and Peterson, 1967) on testicular androgen secretion are well known and a drastic decrease in testicular testosterone concentrations has been reported for rats sacrificed 2 min following ether anaesthesia (Fariss et al. 1969). Increased serum LH and FSH levels have been found 10 min following short exposure to ether vapour (Krulich et al. 1974). These data on the effects of ether anaesthesia point to direct, rapid effects of this anaesthetic on the male gonad. We have sampled blood for measurement of serum testosterone, LH and FSH using the same stress condition in all our animals. Moreover, administration of ACTH did not result in significant changes in serum testosterone, LH and FSH levels in normal male rats (appendix paper 4). If the stress condition connected with our experimental set up were of paramount importance for circulating gonadotrophins, one would expect that ACTH treated rats, subjected already

to the stress stimuli of the standard procedure of handling and ether anaesthesia, should have shown different concentrations of such trophins in blood serum compared to rats not subjected to ACTH injections. Dexamethasone treatment of normal mature rats had no influence on circulating testosterone and LH concentrations (appendix paper 4), and adrenalectomy does not significantly affect plasma testosterone levels in such animals (Frankel et al. 1975) indicating that the adrenal cortex does not significantly contribute to peripheral testosterone concentrations. We, therefore, do not believe that in our experiments effects of the stress conditions form a serious complication for comparing effects of different treatments on circulating LH and FSH levels in normal animals. Furthermore, under these conditions secretions from the adrenal gland do not appear to contribute appreciably to secretions from the testis in regulating circulating gonadotrophins (appendix paper 4).

Regulation of FSH

Dexamethasone injection during 7 days resulted in slightly elevated serum FSH levels in normal rats. This might be due to a deleterious effect of this corticoid on the germinal epithelium in the seminiferous tubules of the testes as has been reported for cortisone (Albert, 1961).

Depletion of germinal epithelium in seminiferous tubules of rats by chemical agents (Gomes et al. 1973; Debeljuk et al. 1973) or local testicular X-irradiation (Verjans and Eik-Nes, 1976; appendix paper 5) results in an elevation of serum FSH levels. It is believed, that a factor of non-interstitial cell origin may participate in the regulation of FSH in addition to circulating steroids from testicular origin (Verjans and Eik-Nes, 1976). "FSH depressing activity" is present in ram rete testis fluid (Setchell, 1974), in bull seminal plasma (Franchimont et al. 1975a) and bull

spermatozoa (Lugaro et al. 1974) and it is speculated that this FSH suppressing material, called "inhibin", may originate from the germinal epithelium in the testis (Franchimont et al. 1975b). To which cell type(s) in the germinal epithelium "inhibin" is related and whether it can be delivered into the peripheral blood is not yet clear. Effects of bilateral testicular X-irradiation on serum LH and testosterone levels have been discussed in appendix paper 5 (Verjans and Eik-Nes, 1976a) and effects of this treatment on testis histology have recently been reviewed by Oakberg (1975). Finally, data on GnRF induced serum LH and FSH levels in X-ray treated rats suggest that "inhibin" possibly acts on the anterior hypophysis by modulating pituitary release of FSH (appendix papers 5 and 7).

Mechanism of action of administered steroids in normal rats

It was already discussed in chapter 3, that testicular 5α-reduced testosterone metabolites in addition to "inhibin", testosterone and possibly oestradiol-17β are essential for maintaining the endocrine balance between testis and hypothalamic-pituitary axis by inhibiting pituitary release of gonadotrophins. Therefore, the total output of testicular secretion products delivered through the spermatic vein blood to the target organs can in all likelihood account for the lower levels of gonadotrophins in normal, adult male rats as compared with the high levels of such trophins in castrates. In addition, we found that removal of one testis from mature male rats resulted in slightly increased serum levels of LH and FSH and slightly elevated weights of the remaining testis at 7 and 14 days after hemigonadectomy. However, at 21 and 28 days after this operation, these parameters were indistinguishable from those of normal control animals of the same age. Other parameters investigated like serum testosterone concentrations, total

body weight, ventral prostate and seminal vesicles weights were not significantly changed at 7, 14, 21 and 28 days following hemi-castration when compared with control animals of the same age (Verjans and Eik-Nes, 1976b). These data indicate that eventually one testis is able to maintain the endocrine balance in the hypothalamic-pituitary-testicular system. Synergism of the steroids injected and the potent testicular secretion products present in the peripheral circulation may explain the suppression of circulating gonadotrophins in normal rats as observed in the present study (Tables 4.3.2. and 4.3.3.). These data provide additional evidence for the specificity of the effects observed with these compounds in castrates (chapter 3), and biochemical support for these data with regard to possible metabolic conversion and binding to "specific" receptor protein, has been quoted (chapter 3). Furthermore, increases in serum testosterone (appendix paper 3) and serum oestradiol-17β (de Jong et al. 1975) concentrations have been found following administration of testosterone and oestradiol-17β respectively to normal rats. In normal rats, no distinct positive feedback effects of androgens in the doses tested were observed in contrast to castrated rats. It might be that the suppressive effect of circulating testicular products on gonadotrophins in normal rats has prevailed over possible positive feedback effects of the administered androgens.

Effects of steroids of non-testicular origin in normal and castrated male rats may result either from metabolic conversion to potent steroids in the circulation or in the target organ(s), or these compounds could act per se by binding to known receptors for the potent testicular steroids. This latter affinity has been shown for the oestradiol-17β and 5α-dihydrotestosterone receptor from the hypophysis (chapter 3) and also 4-androstene-3β,17β-diol and 5-androstene-3β,17β-diol can compete in this respect with 5α-dihydrotestosterone in the prostate (Grover and Odell, 1975).

Effects of steroids administered to normal rats

Dose dependent effects of administered androgen on spermatogenesis are well known. Low doses of testosterone suppress circulating gonadotrophin concentrations (appendix paper 3) and androgen production by the Leydig cells, resulting in atrophy of the germinal epithelium. It has been shown that treatment of normal men with moderate doses of testosterone propionate decreased testicular testosterone concentrations, while plasma LH levels were reduced and plasma testosterone levels were elevated (Morse et al. 1973). Administration of very high doses of testosterone can, however, maintain high intratesticular testosterone levels and hence prevent atrophy of the germinal epithelium in spite of suppressed gonadotrophin levels. Concomitant with these dose dependent effects of testosterone on spermatogenesis, dose dependent effects of testosterone can be observed on ventral prostate weight (Walsh and Swerdloff, 1973), testicular weight and testicular ABP content (Weddington et al. 1976).

Oestrogen exhibits in this respect a pattern different from testosterone or its 5α-reduced metabolites. Oestrogen depresses pituitary and serum levels of LH and FSH and testicular and serum testosterone concentrations (appendix paper 1), which causes arrest of spermatogenesis and atrophy of accessory reproductive organs. In addition to hypothalamic-hypophyseal mediated effects of oestrogens in male animals, direct effects of oestrogen on testicular testosterone formation can be recorded (Samuels et al. 1969). Moreover, it has been observed that the interstitial tissue of the testis contains a specific oestradiol-17β binding principle (Brinkmann et al. 1972). Very low doses of oestrogen, administered to normal rats, tended to elevate serum FSH over control levels (appendix paper 1). It might be that this effect is mediated by the same mechanism as proposed for augmented FSH levels in castrated males given low doses

of oestrogen (appendix paper 7). One may, however, argue whether the minute concentrations of circulating and testicular oestradiol-17β *per se* can serve a physiological purpose in male animals. Interestingly, it has been reported that 5α-dihydrotestosterone can inhibit the initial rate of formation of the oestradiol-17β-receptor complex in the anterior pituitary gland (Korach and Muldoon, 1975), probably indicating that in the normal animal many complex regulatory effects - combinations of different hormones and receptors at the target organ level - are in operation.

Summary

It has been discussed that 5α-dihydrotestosterone is a true androgen and may be the principal androgen in the androgen target organs studied (Verjans and Eik-Nes, 1976a). Whether the androgenicity exhibited by 5α-androstane-3α,17β-diol can be ascribed to its conversion to 5α-dihydrotestosterone is not yet solved, but De Moor *et al.* (1975) have indicated that this metabolic conversion at the target tissue level may be an important factor in the responsiveness of several organs to 5α-androstane-3α,17β-diol. The data presented emphasize that 5α-steroid reductase and 3α-hydroxysteroid dehydrogenase activities are of paramount importance in the target organs investigated in the present study, and it is clear that 5α-reduced ring A metabolites of testosterone exert major functions in androgen sensitive target organs. Surprisingly, 3β-reduction of 5α-dihydrotestosterone results in inactivation at both target organ levels tested. Finally, one may ask about the possible role of testosterone in androgen target organs. Is it merely required as a prehormone for the active metabolites?

4.6 References

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SUMMARY AND CONCLUSIONS

The endocrine and spermatogenic functions of the testes in the adult male animal are regulated and maintained by the gonadotrophins LH (luteinizing hormone) and FSH (follicle stimulating hormone) which are secreted by the anterior pituitary gland under the influence of GnRF (gonadotrophin releasing factor) from the hypothalamus. Products from the testis seem to control the gonadotrophic function of the hypothalamic-pituitary unit since removal of the male gonads results in increased peripheral concentrations of LH and FSH.

The current thesis deals with a study concerning the effects of steroids, particularly those of testicular origin, on blood serum parameters reflecting the gonadotrophic functions of the hypothalamic-pituitary axis and on the endocrine function of the testis in the adult male rat. The introduction of sensitive radioimmunoassay techniques for the measurement of serum concentrations of LH, FSH and testosterone (chapter 2) made this investigation possible. The following aspects have been investigated:

I. Effects of steroids on circulating levels of LH, FSH and testosterone in normal (appendix papers 1 and 3; chapter 4) or orchidectomized (appendix papers 2, 3 and 6; chapter 3) adult male rats. The latter animal model offers the possibility to investigate specific effects of steroids without interference from endogenous testicular factors.

II. The influence of testicular secretion products on GnRF induced serum levels of LH and FSH in the normal intact or castrated animal (appendix papers 2 and 7).

III. The possible involvement of adrenal steroid hormones in the regulation of the hypothalamic-pituitary-testicular system (appendix paper 4).

IV. The effect of local X-irradiation of the male gonads on parameters reflecting activity of the hypothalamic-pituitary-testicular system (appendix paper 5).

From the experiments on effects of s.c. administered steroids on circulating concentrations of LH, FSH and testosterone in normal or gonadectomized, adult male rats (chapters 3 and 4; appendix papers 1, 2, 3 and 6) and on the basis of published data on testicular steroid secretion and on uptake and metabolism of steroids in the hypothalamic-pituitary unit, it has been concluded that:

1. The level of circulating testosterone per se cannot account for the concentrations of serum gonadotrophins in normal, mature male rats as compared with castrated, mature male rats. Other testicular factors must be involved in maintaining the endocrine balance between the mature male gonad and the hypothalamic-pituitary unit.
2. 5α-Reduced or ring A aromatized metabolites of testosterone are more effective than testosterone or its metabolic precursors in preventing the rise of serum gonadotrophin levels postgonadectomy, circulating LH being more sensitive to steroid treatment than FSH. Steroid specific properties have been observed in this respect, since only the 5α-, but not 5β-reduced disubstituted metabolites of testosterone are active. Moreover, the orientation of the hydroxyl group at C-3 in 5α-reduced testosterone metabolites influences the activity of the steroid in suppressing circulating gonadotrophins.
3. Aromatization of ring A in steroids is not a prerequisite for affecting circulating gonadotrophins in the male rat.
4. Testicular secretion products can cause the depressed serum gonadotrophin levels in normal male rats as compared to levels in orchidectomized animals. Data obtained from hemi-gonadectomized, adult male rats indicate that the secretory activity of only one testis is sufficient to maintain the endocrine balance in the hypotha-

lamic-pituitary-testicular system in adult male rats.

5. Administration of low doses of steroids can result in elevated serum gonadotrophin levels in castrated rats (positive feedback).
6. There is not always a close correlation between the effects of steroids on processes at the hypothalamic-pituitary and the accessory reproductive organ level. 5α-Dihydrotestosterone, however, may be the principal androgen in these different androgen target organs.

From the experiments on the effects of steroids on GnRF induced serum gonadotrophin levels in castrated or normal, adult male rats, it has been concluded that:

7. Steroids can alter pituitary sensitivity to GnRF. Androgens inhibit and oestradiol-17β enhances pituitary release of gonadotrophins in response to exogenous GnRF.
8. Oestrogens and androgens may act at different sites in the male hypothalamic-pituitary unit with respect to regulation of pituitary release of gonadotrophins. Depending on the dose of administered oestrogens or androgens this unit may be affected in different ways.

From the experiments concerning the potential involvement of adrenocortical steroids in the control of pituitary gonadotrophins, it has been concluded that:

9. Secretions from the adrenal gland do not appreciably influence the effects of secretions from the testis on the control of pituitary release of gonadotrophins in adult male rats.

Experiments with rats in which the germinal epithelium in the seminiferous tubules was depleted by local testicular X-irradiation, indicated that:

10. Serum FSH concentrations in the mature male rat are partly regulated by testicular steroids and partly by an unknown factor ("inhibin") from seminiferous tubules in the testis.

SAMENVATTING EN CONCLUSIES

De endocriene en spermatogene functies van de testikels in de volwassen mannelijke rat worden gereguleerd en instandgehouden door de gonadotrofe hormonen LH (luteïniserend hormoon) en FSH (follikel stimulerend hormoon), welke door de hypofyse-voorkwab worden gesecerneerd onder invloed van GnRF (gonadotrofine releasing factor). Steroidhormonen gesecerneerd door de testikels blijken op hun beurt deze gonadotrofe hypothalamus-hypofyse activiteit te kunnen beinvloeden, aangezien verwijdering van de gonaden resulteert in sterk verhoogde serumspiegels van LH en FSH.

In dit proefschrift worden de resultaten beschreven van een onderzoek over de invloed van steroiden, en voornamelijk die welke door de volwassen testikel worden gesecerneerd, op serum LH en FSH concentraties, als parameters voor de gonadotrofe activiteit van het hypothalamus-hypofyse systeem, en op serum testosteron concentraties, als parameter voor de endocriene functie van de testikels. Voor de bepaling van de lage concentraties van de betreffende hormonen, was het noodzakelijk om nieuwe gevoelige radioimmunologische technieken te evalueren (hoofdstuk 2). De volgende aspecten van de interactie tussen steroiden en het hypothalamus-hypofyse-testis systeem in volwassen mannelijke ratten zijn bestudeerd:

I. De invloed van steroiden op serum concentraties van LH, FSH en testosteron bij zowel normale (appendix artikelen 1 en 3; hoofdstuk 4) als gecastreerde (appendix artikelen 2, 3 en 6; hoofdstuk 3) volwassen mannelijke ratten. Laatstgenoemd model biedt het voordeel de effecten van de steroiden te kunnen bestuderen zonder invloed van endogene testikulaire producten.

II. De invloed van testikulaire steroiden op serum concentraties van LH en FSH na intraveneuze injectie van

GnRF in intacte of gecastreerde proefdieren (appendix artikelen 2 en 7).

III. De invloed van bijnierschorssteroiden op de regulatie van het hypothalamus-hypofyse-testis systeem (appendix artikel 4).

IV. Het effect van röntgenbestraling van de mannelijke gonaden op de activiteit van het hypothalamus-hypofyse-testis systeem (appendix artikel 5).

De volgende conclusies zijn gebaseerd op de resultaten van de experimenten beschreven in de hoofdstukken 3 en 4 en in de appendix artikelen 1, 2, 3 en 6 en op grond van gepubliceerde gegevens over steroid secretie door de testikel, en over opname en metabolisme van steroiden in het hypothalamus-hypofyse systeem:

1. De concentratie van testosteron in de perifere circulatie kan niet alleen verantwoordelijk zijn voor de verlaagde concentraties van LH en FSH in perifeer bloed van volwassen mannelijke ratten vergeleken met gecastreerde volwassen mannelijke ratten. Naast testosteron moeten andere factoren uit de testis betrokken zijn bij de hormonale balans tussen de testis en het hypothalamus-hypofyse systeem van de volwassen rat.
2. De toename van de concentraties van gonadotrofinen in serum na verwijdering van de gonaden wordt effectiever geremd door subcutane injectie van 5α-gereduceerde of ring A gearomatizeerde metabolieten van testosteron dan door testosteron of metabole precursors van testosteron. Serum LH spiegels tonen een grotere gevoeligheid voor steroidtoediening dan serum FSH spiegels. Het effect van de toegediende steroiden op gonadotrofine spiegels in bloed wordt beïnvloed door specifieke isomere eigenschappen van de steroiden; in dit verband is gebleken, dat alleen de 5α-, maar niet de 5β-gereduceerde digesubstitueerde metabolieten van testosteron een negatieve terugkoppeling uitoefenen op de gonadotrofinen. Bovendien heeft de orientatie van de hydroxylgroep in de C-3 posi-

tie van androstaan steroiden een invloed op de activiteit in dit opzicht.

3. Aromatizering van de A-ring in steroiden is niet noodzakelijk voor het effect op LH en FSH.
4. Door de testikels uitgescheiden producten zijn verantwoordelijk voor de verlaagde serum LH en FSH spiegels in normale ratten vergeleken met gecastreerde ratten. De secretie van slechts één testis is voldoende om het hormonale evenwicht in het hypothalamus-hypofyse-testis systeem te handhaven.
5. Naast een negatieve terugkoppeling kunnen steroiden, in lage doseringen, ook een positieve terugkoppeling uitoefenen op de hypofysaire secretie van gonadotrofinen in gecastreerde mannelijke ratten.
6. Fysiologische effecten van steroiden op de secundaire mannelijke geslachtsorganen correleren niet altijd met die op de terugkoppeling in het hypothalamus-hypofyse systeem. Het is evenwel mogelijk dat 5α-dihydrotestosteron het voornaamste androgeen is in deze androgeengevoelige doelwitorganen.

Op grond van de resultaten van de experimenten betreffende de invloed van GnRF op serum LH en FSH concentraties in proefdieren voorbehandeld met steroiden van testikulaire oorsprong, werden de volgende conclusies getrokken:

7. Steroidhormonen kunnen de gevoeligheid van de hypofyse voor exogeen GnRF beïnvloeden. Androgenen remmen en oestradiol-17β bevordert de hypofysaire secretie van gonadotrofinen in dit opzicht.
8. Oestrogenen en androgenen kunnen hun effect op de regulatie van gonadotrofinen wellicht uitoefenen op meer dan één plaats in het hypothalamus-hypofyse systeem. Afhankelijk van de dosering beïnvloeden ze dit systeem wellicht op verschillende wijze.

Uit de resultaten van de experimenten over de mogelijke rol van bijnierschorssteroiden bij de regulatie van het hypo-

thalamus-hypofyse-testis systeem is de volgende conclusie getrokken:

9. Secretieproducten van de bijnier hebben geen significante invloed op het effect van testikulaire secretieproducten op de regulatie van de hypofysaire secretie van gonadotrofinen in de volwassen mannelijke rat.

Uit de resultaten van de experimenten met ratten, waarbij het kiemepitheel in de seminifere tubuli ernstig werd beschadigd door middel van röntgenbestraling van de gonaden, is geconcludeerd:

10. Serum FSH spiegels in de volwassen mannelijke rat worden behalve door testikulaire steroiden gedeeltelijk ook gereguleerd door een (onbekende) factor ("inhibine") uit de seminifere tubuli in de testikel.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 8 juni 1949 geboren te Maastricht. In 1967 behaalde hij het getuigschrift gymnasium-β aan het Henric van Veldeke College te Maastricht en begon hij de studie scheikunde aan de Rijksuniversiteit te Utrecht. Hij legde het kandidaatsexamen af in 1970 en het doctoraalexamen met hoofdvak analytische chemie en bijvak klinische chemie in 1972.

Van oktober 1972 tot augustus 1973 was hij als wetenschappelijk medewerker verbonden aan de afdeling Chemische Endocrinologie (Instituut Biochemie II) van de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam.

Sinds september 1973 was hij als wetenschappelijk assistent verbonden aan Institutt for Biofysikk, Norges Tekniske Høgskole, Universitetet i Trondheim, Trondheim, Noorwegen; in deze functie was hij betrokken bij het theoretisch en practisch onderwijs in de biochemie en fysiologie en begeleidde hij verschillende studenten bij hun werkzaamheden voor hun M.Sc.-thesis.

APPENDIX PAPERS

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EFFECT OF OESTRADIOL BENZOATE ON PITUITARY AND TESTIS FUNCTION IN THE NORMAL ADULT MALE RAT

By

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*H. J. van der Molen*** and *K. B. Eik-Nes**

ABSTRACT

The effects of subcutaneous administration of oestradiol benzoate (EB) on the weights of sex organs and on levels of testosterone and gonadotrophins in normal adult male rats have been studied. Doses of EB varied from 0.01 to 100 μg and were administered daily for seven days. Administration of 100 μg EB resulted in suppression of pituitary LH, while serum LH levels were already decreased after treatment with EB $\geqq$ 1 μg. Pituitary and serum FSH levels were suppressed after administration of 100 and 10 μg EB respectively. Testicular tissue and serum levels of testosterone decreased after treatment with amounts of EB $\geqq$ 1 μg. These decreased androgen levels were also reflected in a concomitant decrease in the weights of the ventral prostate and seminal vesicles. A decrease in the weight of the testes was obtained following a dose of EB $\geqq$ 10 μg.

It is now established, that oestradiol-17β is synthesized and secreted by human, monkey, dog and rat testes (*Kelch et al.* 1972; *Eik-Nes* 1967; *de Jong et al.* 1973). However, its physiological role in the male is still uncertain, although it has been proposed as a regulator of pituitary gonadotrophin secretion. The influence of oestradiol-17β on the hypothalamic-pituitary axis in the male rat has mainly been studied following gonadectomy. In order to suppress the high

levels of circulating gonadotrophins after gonadectomy to those found in normal adult male rats (*Gay & Dever* 1971; *Swerdloff & Walsh* 1973; *Swerdloff et al.* 1973; *Kalra et al.* 1973; *Verjans et al.* 1974) lower daily doses of oestradiol-17β or oestradiol benzoate were required than of androgens. These observations might suggest a role for circulating oestradiol-17β in the feedback regulation of pituitary gonadotrophins (*Walsh et al.* 1973).

Recently, however, there have been several reports showing that administration of oestradiol-17β to intact male rats caused a decrease in circulating testosterone levels but not, as might be expected, a decrease in circulating LH and FSH levels (*Danutra et al.* 1973; *Mallampati & Johnson* 1973).

These observations can be explained if there were a direct effect of oestradiol-17β on testicular testosterone secretion or if, besides LH or FSH, other factors regulate the secretion of testicular testosterone. Since most of these studies have been carried out with supraphysiological amounts of administered oestradiol-17β, the present study was undertaken to determine the lowest dose of administered oestradiol benzoate that could bring about changes in levels of testosterone in serum and in testis tissue of adult male rats. In addition pituitary and serum LH and FSH have been measured together with the weights of seminal vesicles, ventral prostates and testes.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (3–4 months old) with a body weight of 225–250 g were used in this study. The animals were kept under controlled light (14 hours light and 10 hours darkness) and temperature (20–22°C) conditions. Laboratory chow and tap water were provided *ad libitum.*

Daily subcutaneous injections with various doses of EB in 0.1 ml sesame oil were given for a period of seven days. Control animals received 0.1 ml vehicle only. Six animals were used in each of the different treatment groups. After decapitation of the animals on day 8 blood samples were collected and allowed to clot overnight at 4°C. Serum was stored at –20°C until assayed for testosterone and gonadotrophins. Testes, ventral prostate and seminal vesicles were dissected and weighed. Testes were stored frozen at –20°C until further processed. The pituitary glands were removed and kept frozen at –20°C in 1 ml 0.9 % sodium chloride solution until thawed and homogenized. The homogenate was diluted to 4 ml with saline and centrifuged at 3000 rpm for 25 min. The supernatant was further diluted with saline and assayed for gonadotrophins.

Radioimmunoassay methods

Gonadotrophin levels were measured using double antibody radioimmunoassays. Antisera against ovine LH and ovine FSH were obtained by immunizing rabbits with NIH-LH-S17 and NIH-FSH-S9. For both radioimmunoassays the procedures as described by *Niswender et al.* (1968) were followed. Sensitivity, accuracy and specificity of both radioimmunoassays have been published (*Welschen et al.* 1974, in press). Serum

Table 1.

Mean levels (± SD) of serum and pituitary gonadotrophins in adult male rats treated with various doses of EB per day for seven days.

Treatment	Serum LH (ng*/ml)	Serum FSH (ng**/ml)	Pituitary LH (mg*/pituitary)	Pituitary FSH (mg**/pituitary)
Control, sesame oil	23.8 ± 12.9 (6)	288 ± 47 (6)	0.271 ± 0.062 (6)	0.140 ± 0.020 (6)
0.01 μg EB	11.0 ± 3.4 (6)	322 ± 83 (6)	0.274 ± 0.088 (6)	0.148 ± 0.038 (6)
0.1 μg EB	19.4 ± 6.4 (6)	336 ± 51 (6)	0.291 ± 0.100 (6)	0.145 ± 0.024 (6)
1 μg EB	< 7.5 (4)***	278 ± 80 (4)	0.201 ± 0.045 (5)	0.111 ± 0.023 (5)
10 μg EB	< 7.5 (5)***	163 ± 23 (5)***	0.237 ± 0.070 (5)	0.102 ± 0.029 (5)***
100 μg EB	< 7.5 (6)***	155 ± 11 (6)***	0.190 ± 0.058 (5)***	0.084 ± 0.011 (5)***

Number of rats is given in parentheses.

* NIAMD rat-LH RP-1.

** NIAMD rat-FSH RP-1.

*** $P < 0.05$, compared with controls.

samples were assayed in duplicate at two levels per assay and serum LH and FSH levels were expressed on basis of reference preparations NIAMD rat-LH RP-1 and NIAMD rat-FSH RP-1 respectively. Pituitary gland extracts were assayed in duplicate at two dilutions per assay and contents of gonadotrophins were expressed as mg of the respective reference preparation per hypophysis. Radioimmunoassay of testosterone in blood serum and testis tissue was performed as described previously (*Verjans et al.* 1973).

Determinations of total protein in testicular tissue were carried out according to the method of *Lowry et al.* (1951). Statistical significance of the obtained data was determined by Student's *t*-test.

RESULTS

Doses of 1, 10 and 100 μg EB per day respectively, significantly ($P < 0.05$) suppressed serum levels of LH compared with rats receiving vehicle only, while hypophyseal LH levels were decreased after daily administration of 100 μg EB (Table 1). The levels of FSH in both serum and pituitary gland exhibited a significant decrease ($P < 0.05$) in animals treated daily with 10 or 100 μg EB for 7 days.

Table 2 shows effects of daily, subcutaneous administration of various doses of EB for 7 days on testosterone levels in serum and testicular tissue in normal adult male rats. Compared with the control group, a dose of 1 μg per day caused a significant lowering of serum as well as of testis tissue testosterone ($P < 0.05$). Serum and testicular tissue testosterone levels decreased further with increasing doses of EB. The effects of various doses of EB on weights of male sex organs are summarized in Table 3. Administration of

Table 2.

Mean levels (± SD) of serum and testis tissue testosterone in adult male rats treated with various doses of EB per day for seven days.

Treatment	Number of rats	Serum testosterone (ng/ml)	Testis tissue testosterone (ng/mg protein)
Control, sesame oil	6	2.3 ± 0.9	1.00 ± 0.35
0.01 μg EB	6	2.4 ± 2.6	0.68 ± 0.56
0.1 μg EB	6	3.3 ± 1.3	0.97 ± 0.40
1 μg EB	5	0.1 ± 0.2*	0.22 ± 0.12*
10 μg EB	5	< 0.01*	0.06 ± 0.01*
100 μg EB	6	< 0.01*	0.08 ± 0.01*

* Significantly different ($P < 0.05$) from controls.

Table 3.
Mean weights (± SD) of sex organs of adult male rats treated with various doses of EB per day for seven days.

Treatment	Testis (g)	Seminal vesicle (mg)	Ventral prostate (mg)
Control, sesame oil	1.50 ± 0.10 (12)	112 ± 20 (12)	312 ± 34 (6)
0.01 μg EB	1.47 ± 0.12 (12)	98 ± 19 (12)	251 ± 28 (6)*
0.1 μg EB	1.56 ± 0.10 (12)	117 ± 18 (12)	332 ± 52 (6)
1 μg EB	1.49 ± 0.17 (10)	72 ± 4 (10)*	166 ± 30 (5)*
10 μg EB	1.40 ± 0.06 (10)*	59 ± 8 (10)*	94 ± 30 (5)*
100 μg EB	1.30 ± 0.06 (10)*	68 ± 15 (12)*	99 ± 26 (5)*

Number of organs is given in parentheses.
* $P < 0.05$, compared with controls.

10 or 100 μg EB per day during 7 days resulted in a significant reduction in testicular weight ($P < 0.05$) compared with the control group and the seminal vesicle and ventral prostate weights were decreased using a daily dose of 1, 10 or 100 μg EB.

DISCUSSION

It is apparent from the present study, that administration of 1 μg per day of oestradiol benzoate decreases levels of both testosterone (Table 2) and LH (Table 1) in serum. No effect on either hormone was observed with smaller amounts of EB. Thus it is possible that EB acts directly on the hypothalamic-pituitary axis causing a decreased secretion of LH and consequently lower circulating levels of testosterone. FSH serum levels (Table 1) were also lowered by the administration of EB, but compared with the smallest doses required to suppress LH and testosterone, 10 times more or at least 10 μg daily, were needed. Serum and pituitary FSH levels were suppressed by a daily dose of 10 μg EB for 7 days (Table 1), which probably reflects that production and release of FSH are more or less equally sensitive to EB administration. *Mallampati & Johnson* (1973) found, however, a lowering of pituitary FSH levels in adult male rats following a daily dose of only 0.5 μg EB for 4 days, while serum FSH levels did not differ significantly from control levels with this dose of EB. *Debeljuk et al.* (1972) reported suppression of serum FSH levels in intact adult male rats even following a single, subcutaneous injection of

20 μg EB. A significant depression of pituitary LH levels in the present investigation was observed following daily administration of 100 μg EB for 7 days, while serum LH levels were already suppressed by one hundredth of this dose (Table 1). This might indicate that the pituitary release of LH is more sensitive to oestrogen than the pituitary production of this hormone. *Danutra et al.* (1973) found, however, no significant change in plasma LH levels in adult, male rats after daily treatment with 100 μg oestradiol-17β for 10 days. Our data are also in disagreement with those reported by *Mallampati & Johnson* (1973), who found unaltered serum LH levels after a daily dose of 50 μg EB for 4 days, but depressed pituitary LH levels by as little as 0.5 μg EB. Continuous intravenous infusion of oestradiol-17β for 4 days in men suppressed equally both plasma LH and FSH levels (*Sherins & Loriaux* 1973).

A daily dose of 1 μg EB, which significantly lowered testosterone levels in serum as well as in testis tissue, did not affect total testicular weight (Table 3), but 10 μg EB for 7 days resulted in a significant weight decrease of the male gonad (Table 3). Changes in testicular weight parallel changes in serum FSH. Thus, the processes leading to production and secretion of testosterone are more sensitive to EB administration than those promoting growth of the testicular tissue. *Mallampati & Johnson* (1973) observed no reduction in either testicular weights or serum FSH levels of adult male rats following injection of a daily dose of 50 μg EB for 4 days. This discrepancy could be due to differences in duration of the EB treatment. Compared with weights of control organs, the relative weight decrease of the seminal vesicle after treatment with daily doses of 1, 10 or 100 μg EB for 7 days was less than that of the ventral prostate after such treatments (Table 3). This may reflect that the latter organ is more sensitive to the possible "anti-androgenic" effect of oestrogen and/or the decrease in circulating testosterone levels than the former.

The interaction of testicular and adrenal oestrogens and androgens at the level of the hypothalamic-pituitary-gonadal axis for regulation of production and secretion of gonadotrophins via hypothalamic releasing factor(s), and the subsequent secretion pattern of steroids, is still not clear and will require further exploration.

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EFFECTS OF TESTOSTERONE PROPIONATE, 5α-DIHYDROTESTOSTERONE PROPIONATE AND OESTRADIOL BENZOATE ON SERUM LEVELS OF LH AND FSH IN THE CASTRATED ADULT MALE RAT

By

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*F. J. M. Vels*** and *H. J. van der Molen****

ABSTRACT

The influence of treatment with various doses of testosterone propionate, 5α-dihydrotestosterone propionate or oestradiol benzoate on serum levels of LH and FSH (measured by radioimmunoassay) and on weights of ventral prostates and seminal vesicles was investigated in castrated, adult, male rats. For depression of the high, castrate levels of serum gonadotrophins with either of these steroid esters, the inhibition curves were different for LH and for FSH. Serum LH was kept at levels encountered in intact, adult, male rats by lower doses of steroid ester than was serum FSH. Oestradiol benzoate was the most potent suppressor of the serum gonadotrophins among the steroid esters tested, testosterone propionate the least. Treatment with low doses of oestradiol benzoate, however, resulted in serum FSH levels significantly above those of castrates treated with vehicle only. Finally, administration of a synthetic LH-releasing factor to testosterone propionate, 5α-dihydrotestosterone propionate or oestradiol benzoate treated, castrated, adult, male rats resulted in a further release of both LH and FSH. The latter effect was more pronounced in oestradiol benzoate treated castrates than in testosterone propionate or 5α-dihydrotestosterone propionate treated castrates.

Following gonadectomy in male rats, serum levels of LH and FSH will increase rapidly (*Gay & Bogdanove* 1969; *Gay & Dever* 1971; *Swerdloff et al.* 1972, 1973; *Swerdloff & Walsh* 1973; *Dufy-Barbe & Franchimont* 1972; *Kalra et al.* 1973). Administration of testicular steroids to such animals may be used to suppress serum levels of these gonadotrophins (*Gay & Bogdanove* 1969; *Gay & Dever* 1971; *Swerdloff et al.* 1972, 1973; *Swerdloff & Walsh* 1973; *Dufy-Barbe & Franchimont* 1972; *Kalra et al.* 1973). Testosterone is in this respect a potent suppressor, but little is known about the potency of other steroids, such as 5α-dihydrotestosterone and oestradiol which are also secreted by the testis. In order to study the relative effects of testicular steroids on serum levels of LH and FSH, we have examined the doses of testosterone propionate (TP), 5α-dihydrotestosterone propionate (5α-dhTP) or oestradiol benzoate (EB) to keep circulating LH and FSH in the castrated, adult, male rat at levels comparable with those found in intact male rats of the same age. Furthermore, we have investigated the effect of such substitution doses of testicular steroids on weights of accessory sex organs.

The release of LH and FSH from the pituitary gland is mediated via the hypothalamus which secretes gonadotrophin releasing factor(s). It was suggested (*Naftolin et al.* 1972) that androgens act on the hypothalamus after being converted to oestradiol-17β. *Swerdloff et al.* (1972), however, showed that 5α-dihydrotestosterone, which cannot be converted to oestradiol-17β, also inhibits LH and FSH secretion in castrated, adult, male rats. These investigators concluded that the androgens may act directly on the hypothalamic-pituitary axis. It has been reported (*Debeljuk et al.* 1972) that in normal, adult, male rats treated with relatively high doses of TP the circulating levels of LH and FSH are lower than in EB treated animals following administration of a LH-releasing factor. Since no data exist on effects of steroids on LH and FSH release after injection of gonadotrophic releasing factor to castrated rats, serum levels of LH and FSH were determined in TP, 5α-dhTP or EB treated castrated rats subsequent to administration of a synthetic LH-releasing factor.

MATERIALS AND METHODS

Animals

Four months old male rats (R × U strain) weighing 350–450 g were used throughout the experiments. The animals were kept under controlled lighting (14 hours light and 10 hours darkness) and temperature (20–22°C) conditions. Laboratory chow and tap water were provided *ad libitum*.

Experiment I. – Animals were gonadectomized under light ether anaesthesia. Daily, subcutaneous injections of TP, 5α-dhTP or EB in sesame oil (0.04 ml/100 g b. w.)

started immediately after operation and were continued for the next six days. These steroids were injected between 2 and 4 p. m. Control animals received sesame oil only (0.04 ml/100 g b. w.). Steroid esters were purchased from Steraloids, Pawling, New York, and were used without further purification. Twenty-four hours after the last injection of steroids, blood samples were drawn under ether anaesthesia by puncturing the ophthalmic venous plexus. If used 500 ng synthetic LH-releasing factor (Beckman, Palo Alto, California), dissolved in 0.1 ml 0.9 % sodium chloride solution, was administered via the jugular vein immediately thereafter. Control animals received 0.1 ml 0.9 % sodium chloride solution only via this route and 15 and 45 min after the injection of LH-releasing factor or saline, the animals were bled again by puncturing the ophthalmic venous plexus under light ether anaesthesia. The animals were then sacrificed with chloroform, the ventral prostate and seminal vesicles were dissected free and weighed.

Experiment II. – In one group of gonadectomized animals treated with 5 μg 5α-dhTP/100 g b. w./day for ten days, blood samples were taken under light ether anaesthesia between 2 and 3 p.m. three days after castration. Daily treatment with 5α-dhTP was then continued and blood samples were drawn on day 5, 7 and 10 after removal of the gonads. As controls served castrated animals subjected to the same type of blood withdrawal but treated with sesame oil only (0.04 ml/100 g b. w.). In each of these treatment groups four animals were used. Blood samples from experiments I and II were allowed to clot overnight at 4°C. Serum was stored at –20°C until assayed for gonadotrophins.

Radioimmunoassay

Serum levels of LH and FSH were measured using double antibody radioimmunoassays. Antisera against ovine LH and ovine FSH were obtained by immunizing rabbits with NIH-LH-S17 or NIH-FSH-S9 (Drs. Uilenbroek and Dullaart, Departments of Endocrinology, Growth and Reproduction and Anatomy, Erasmus University, Rotterdam). For both radioimmunoassays the procedures as described by *Niswender et al.* (1968) were followed. Sensitivity, accuracy and specificity of both radioimmunoassays have been described previously (*Welschen et al.* 1974, in press). In the radioimmunoassay of rat LH, NIAMD rat LH 1-1 was used for the preparation of the iodinated derivative (*Greenwood et al.* 1963). The biological activity of this preparation was 1.0 unit NIH-LH-S1/mg as measured by the ovarian ascorbic acid depletion test and the FSH contamination was less than 0.04 unit NIH-FSH-S1/mg as measured by the human chorionic gonadotrophin augmentation test. Iodination was performed with ^{125}I (Philips-Duphar, Petten, The Netherlands). Serum LH levels were expressed on basis of a reference standard preparation (NIAMD rat LH RP-1) which had a biological acticity of 0.03 unit NIH-LH-S1/mg and FSH contamination of 0.56 unit NIH-FSH-S1/mg. NIAMD rat FSH 1-1 with a biological acticity of 100 unit NIH-FSH-S1/mg and a LH contamination of less than 0.002 unit NIH-LH-S1/mg was used for iodination with ^{125}I (*Greenwood et al.* 1963) in the radioimmunoassay of rat FSH. Serum FSH levels were expressed on basis of a reference preparation NIAMD rat FSH RP-1 with a biological activity of 2.1 unit NIH-FSH-S1/mg and a LH contamination of 0.02 unit NIH-LH-S1/mg. All serum samples from one single experiment were assayed in duplicate at two levels per assay in order to eliminate inter-assay variations. Statistical significance of the obtained data was determined by Student's *t*-test.

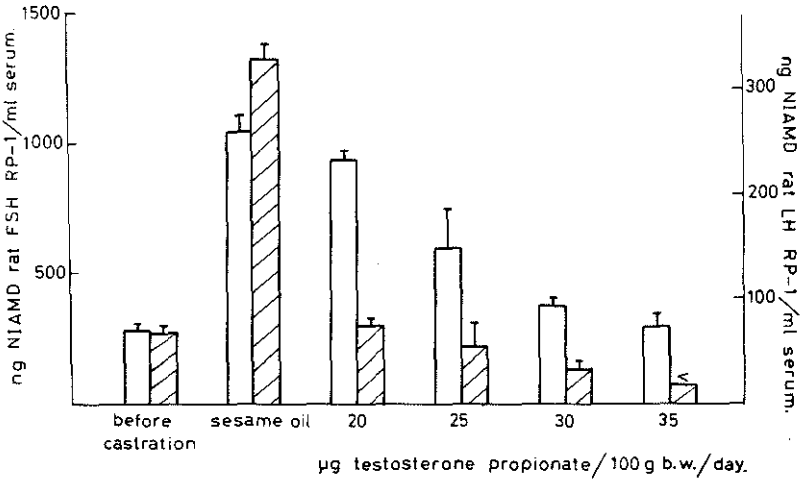

Fig. 1.

Effect of treatment with various doses of *testosterone propionate*/100 g b. w./day during seven days on levels of serum FSH and LH in castrated, adult, male rats. Mean data ± SEM from three animals are shown. Open bars: FSH; Cross bars: LH.

RESULTS

Effect of treatment with steroid esters on serum levels of LH and FSH

The effects of daily administration of various doses of TP, 5α-dhTP or EB on serum levels of LH and FSH in castrated, adult, male rats are depicted in

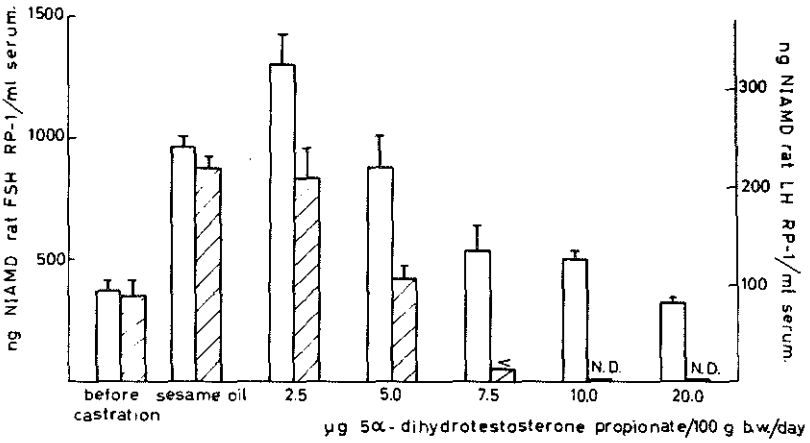

Fig. 2.

Effect of treatment with various doses of *5α-dihydrotestosterone propionate*/100 g b. w./day during seven days on levels of serum FSH and LH in castrated, adult, male rats. Mean data ± SEM from three animals are shown. Open bars: FSH; Cross bars: LH. N. D.: not detectable.

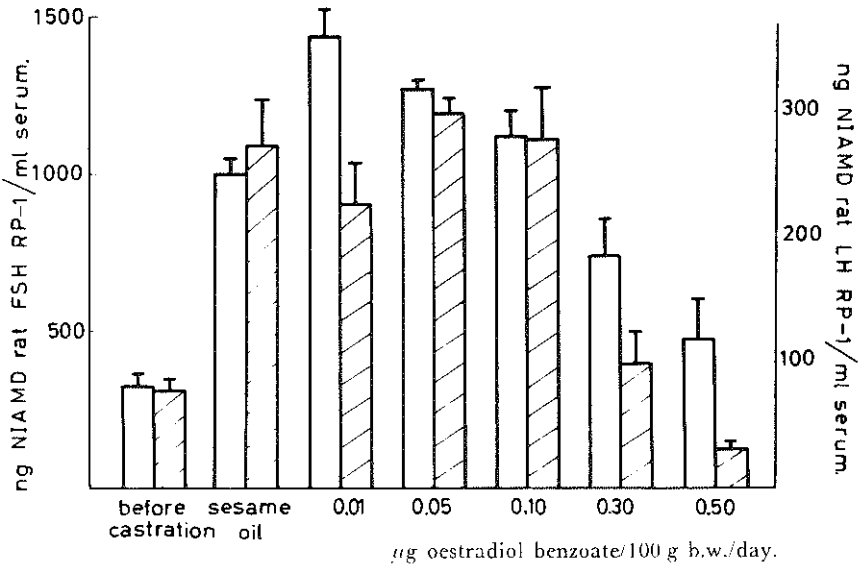

Fig. 3.

Effect of treatment with various doses of *oestradiol benzoate*/100 g b. w./day during seven days on levels of serum FSH and LH in castrated, adult, male rats. Mean data ± SEM from three or more animals are shown. Open bars: FSH; Cross bars: LH.

Figs. 1–3. Treatment with 20 μg TP during 7 days gave suppression of circulating LH to levels below those found in castrated controls ($P < 0.001$), but not significantly different from the concentrations found in intact controls. For suppression of FSH to levels not significantly different from those of intact control animals, 35 μg TP was required. 5α-dhTP in a dose of 5μg gave significant suppression of LH below castrate levels ($P < 0.005$) with no significant lowering of FSH. Twice this dose of 5α-dhTP gave FSH levels significantly lower than those of castrates ($P < 0.001$) and not significantly higher than those of intact controls ($P < 0.10$). A dose of 2.5 μg 5α-dhTP caused no significant elevation in serum FSH over castrate control levels ($P < 0.10$).

Treatment with 0.3 μg EB lowered serum LH levels to those of intact control rats and a dose of 0.5 μg EB suppressed serum LH significantly ($P < 0.01$) compared to intact control animals. Serum FSH, however, was not significantly higher in the former group of rats than in intact controls ($P > 0.10$). Administration of 0.01 or 0.05 μg EB elevated serum FSH significantly above castrate control levels ($P < 0.001$ and $P < 0.005$ respectively). Figs. 4 and 5 depict LH and FSH serum levels respectively in a longitudinal study on castrated, adult, male rats given daily injections of sesame oil containing 5 μg 5α-dhTP.

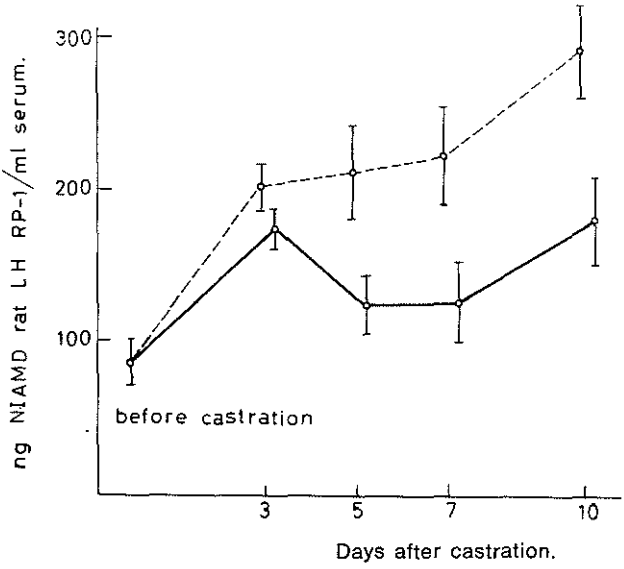

Fig. 4.

Levels of serum LH in castrated, adult, male rats in a longitudinal study. Mean data ± SEM from four animals are shown. Solid line: castrated animals treated daily with 5 μg 5α-dihydrotestosterone propionate/100 g b. w. (subcutaneous) for ten days; dotted line: castrated animals treated daily with sesame oil (subcutaneous) only for ten days.

Table 1.

Effect of different doses of *testosterone propionate* (μg/100 g b. w./day for seven days) on organ weights (mg/100 g b. w.) in castrated, adult, male rats (n = 3).

Treatment	Ventral prostate (mean ± SD)	Seminal vesicles (mean ± SD)
Intact, controls	73.7 ± 6.4x	82.2 ± 5.9x
Castrate, sesame oil	21.7 ± 4.0*	40.3 ± 1.5*
Castrate, 25 μg TP	74.6 ± 12.0x	62.4 ± 7.5x*
Castrate, 30 μg TP	82.5 ± 5.8x	81.6 ± 17.8x
Castrate, 35 μg TP	88.0 ± 10.9x	98.1 ± 4.4x*

* Mean significantly different ($P < 0.05$ one sided) from mean of intact controls.

x Mean significantly different ($P < 0.02$) from mean of castrated animals treated with sesame oil.

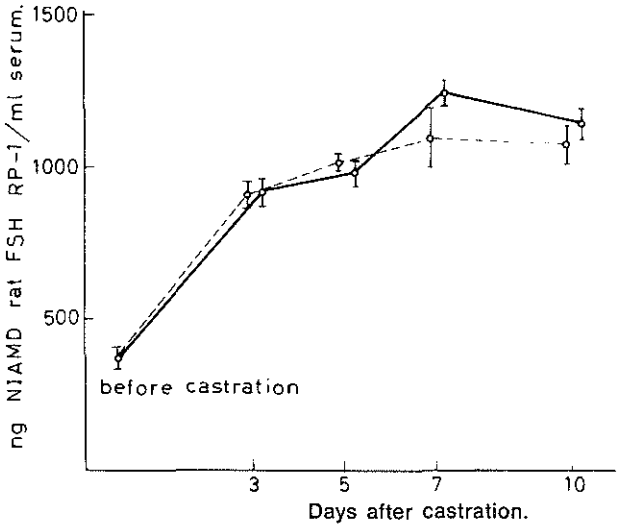

Fig. 5.

Levels of serum FSH in castrated, adult, male rats in a longitudinal study. Mean data ± SEM from four animals are shown. Solid line: castrated animals treated daily with 5 μg 5α-dihydrotestosterone propionate/100 g b. w. (subcutaneous) for ten days; dotted line: castrated animals treated daily with sesame oil (subcutaneous) only for ten days.

Table 2.

Effect of different doses of *5α-dihydrotestosterone propionate* (μg/100 g b. w./day for seven days) on organ weights (mg/100 g b. w.) in castrated, adult, male rats (n = 3).

Treatment	Ventral prostate (mean ± SD)	Seminal vesicles (mean ± SD)
Intact, controls	68.1 ± 7.3×	73.9 ± 7.5×
Castrate, sesame oil	26.0 ± 5.7*	42.2 ± 4.0*
Castrate, 2.5 μg 5α-dhTP	41.3 ± 6.1×*	51.5 ± 6.4*
Castrate, 5 μg 5α-dhTP	41.7 ± 7.9*	58.6 ± 7.3×
Castrate, 7.5 μg 5α-dhTP	51.5 ± 9.4×	67.8 ± 9.1×
Castrate, 10 μg 5α-dhTP	72.9 ± 7.2×	74.3 ± 17.6×
Castrate, 20 μg 5α-dhTP	58.0 ± 8.3×	81.7 ± 11.0×
Castrate, 30 μg 5α-dhTP	69.5 ± 2.2×	77.3 ± 7.9×

* Mean significantly different ($P < 0.05$ one sided) from mean of intact controls.

× Mean significantly different ($P < 0.05$) from mean of castrated animals treated with sesame oil.

Table 3.

Effect of different doses of *oestradiol benzoate* (μg/100 g b. w./day for seven days) on organ weights (mg/100 g b. w.) in castrated, adult, male rats (n = 3).

Treatment	Ventral prostate (mean ± SD)	Seminal vesicles (mean ± SD)
Intact, controls	68.1 ± 7.3x	73.9 ± 7.5x
Castrate, sesame oil	26.0 ± 5.7*	42.2 ± 4.0*
Castrate, 0.01 μg EB	24.2 ± 5.4*	54.8 ± 2.5x*
Castrate, 0.05 μg EB	25.5 ± 2.5*	56.1 ± 3.0x*
Castrate, 0.10 μg EB	24.2 ± 2.8*	60.7 ± 6.4x
Castrate, 0.50 μg EB	28.6 ± 6.4*	65.0 ± 3.5x

* Mean significantly different ($P < 0.05$ one sided) from mean of intact controls.

x Mean significantly different ($P < 0.01$) from mean of castrated animals treated with sesame oil.

Five days after castration there was a significant difference in serum LH between castrated animals receiving sesame oil and 5 μg 5α-dhTP in this vehicle ($P < 0.05$), while the level of serum LH in the castrates after treatment with 5 μg 5α-dhTP was not significantly higher than the intact control level ($P > 0.10$). The FSH levels were not significantly different between castrates administered sesame oil or 5 μg 5α-dhTP in sesame oil during this longitudinal study.

Effects of steroid esters on weights of accessory sex organs

The effects of various doses of TP, 5α-dhTP or EB on ventral prostate and seminal vesicles weights are summarized in Tables 1–3. When given by daily injections, less 5α-dhTP than TP was required to maintain normal ventral prostate and seminal vesicles weights in castrated, adult, male rats. Administration of $\geqq 0.01$ μg EB prevented the weight decrease of the seminal vesicles as observed in castrated rats, although the weights were slightly decreased when compared to intact controls. The weights of the ventral prostate in animals treated with either amount of EB, even with 0.5 μg, were however, not significantly different from those of control castrates.

Effects of synthetic LH-releasing factor in TP, 5α-dhTP, or EB treated, castrated, adult, male rats

Table 4 shows levels of serum LH and FSH following intravenous injection of 500 ng synthetic LH-releasing factor in castrated rats treated during the 7 preceding days with 40 μg TP, 10 μg 5α-dhTP or 1 μg EB per day. 15 min

Table 4.

Effect of 500 ng synthetic LH-releasing factor (LH-RF) on serum LH and FSH levels in intact, adult, male rats and castrated, adult, male rats, treated for seven days with either sesame oil, 40 μg testosterone propionate/100 g b. w./day, 10 μg 5α-dihydrotestosterone propionate/100 g b. w./day or 1 μg oestradiol benzoate/100 g b. w./day. A single injection of LH-RF was given on day 8 at 3 p. m. Effect of injecting saline in intact, adult, male rats is also shown. Blood samples were drawn immediately before and 15 and 45 min after injection of saline or saline containing LH-RF. Three or more animals were used per experiment.

Treatment	ng NIAMD rat LH RP-1/ml serum (mean ± SD)	ng NIAMD rat FSH RP-1/ml serum (mean ± SD)
Intact controls	80 ± 19	378 ± 42
15 min after 500 ng LH-RF	209 ± 38	463 ± 42
45 min after 500 ng LH-RF	108 ± 37	487 ± 49
15 min after saline	73 ± 4	378 ± 9
45 min after saline	64 ± 17	377 ± 13
Control castrates, sesame oil	220 ± 15	958 ± 67
15 min after 500 ng LH-RF	364 ± 184	1107 ± 99
45 min after 500 ng LH-RF	303 ± 113	1189 ± 123
Castrates, 40 μg TP	31 ± 5	406 ± 78
15 min after 500 ng LH-RF	89 ± 19	464 ± 151
45 min after 500 ng LH-RF	59 ± 13	465 ± 50
Castrates, 10 μg 5α-dhTP	n. d.*	508 ± 64
15 min after 500 ng LH-RF	111 ± 19	642 ± 20
45 min after 500 ng LH-RF	48 ± 24	673 ± 91
Castrates, 1 μg EB	21 ± 2	470 ± 63
15 min after 500 ng LH-RF	399 ± 23	992 ± 115
45 min after 500 ng LH-RF	242 ± 11	843 ± 295

* n. d.: not detectable.

after injection of this LH-releasing factor (dissolved in saline) there was a significant increase ($P < 0.025$) in serum FSH and LH levels, compared with the levels found before injections of this LH-releasing factor. However, no significant increase occurred in serum LH and FSH in castrates given saline only and in serum FSH in castrates treated with 40 μg TP for the 7 previous days.

DISCUSSION

The data presented show the effects of daily injections of TP, 5α-dhTP or EB on serum levels of LH and FSH and on weights of ventral prostates and

seminal vesicles in castrated, adult, male rats. Castration resulted in elevated gonadotrophin levels (Figs. 1–3). To prevent this rise of serum LH levels, 20 μg TP, 5 μg 5α-dhTP or 0.3 μg EB per 100 g body weight had to be administered subcutaneously in daily injections for 7 days (Figs. 1–3). Larger doses of these steroids had to be given in order to curb the rise of serum FSH: 35 μg TP, 20 μg 5α-dhTP or 0.5 μg EB (Figs. 1–3). The doses of TP and 5α-dhTP required to maintain normal weights of ventral prostates and seminal vesicles after castration were slightly higher than those effecting normal LH after castration (Tables 1 and 2, Figs. 1 and 2). If the same amount of androgen reaches the target organs following daily administration of either TP or 5α-dhTP in sesame oil, 5α-dhTP appears to be more potent than TP in inhibiting secretion of gonadotrophins after castration and in keeping ventral prostate and seminal vesicles weights of castrated rats equal to those of intact controls (Figs. 1 and 2, Tables 1 and 2). However, the suppression curves for serum LH and FSH by steroid esters are not parallel (Figs. 1–3). Other investigators (*Gay & Bogdanove* 1969; *Swerdloff et al.* 1972, 1973; *Swerdloff & Walsh* 1973; *Dufy-Barbe & Franchimont* 1972; *Kalra et al.* 1973; *Mallampati & Johnson* 1973) who have studied the problem of steroid effects on the pituitary-hypothalamic system have used other time schedules with regard to duration of steroid treatment and interval after castration such treatment was started. The results obtained in the current work are in agreement with those previously reported, although smaller doses of steroid esters were required in our investigation to keep normal serum LH and FSH levels in castrates. Since 5α-dihydrotestosterone cannot be converted to oestradiol-17β, the current work and also the data of *Swerdloff et al.* (1972) indicate that the androgens must not necessarily be metabolized to oestrogens in order to inhibit pituitary production of gonadotrophins. The doses of 5α-dhTP or 5α-dihydrotestosterone used in the present investigation and by *Swerdloff et al.* (1972) are high, however, compared to the production *in vitro* of 5α-dihydrotestosterone by the rat testis (*Folman et al.* 1972). Moreover, in neither investigation the proof has been delivered that 5α-dihydrotestosterone is the "active form" of this androgen at the tissue organization tested. Low doses of EB gave a significant increase in serum FSH over castrate levels possibly indicating a stimulatory feedback mechanism, if circulating gonadotrophins are true indications of pituitary production and secretion of these hormones. However, it must be kept in mind that the clearance rates may differ for the different steroids, employed in our work and that the use of one daily, subcutaneous injection of a steroid ester may result in a variable concentration of that steroid in circulating blood over the ensuing 24 hours. This is indeed the case when TP is administered subcutaneously to castrated rats (unpublished observations). Therefore, we have used a strict time scheme of daily steroid injections and in the longitudinal study (Figs. 4 and 5) levels of serum LH and FSH on day 5.

7 and 10 after castration in the adult, male rats treated with 5 μg 5α-dhTP are rather similar.

Administration of LH-releasing factor caused an increase in serum LH and FSH levels in castrated, adult, male rats treated with steroid esters (Table 4). Using the same dose of this LH-releasing principle higher serum levels of FSH and LH were obtained in castrates treated with EB than in castrates treated with either TP or 5α-dhTP (Table 4). In normal, male rats treated with relatively high doses of TP the circulating levels of LH and FSH are lower than in EB treated animals following administration of a LH-releasing factor (*Debeljuk et al.* 1972). Circulating levels of LH and FSH in the castrates are affected in a different way by the steroid treatments, which might have influenced the hypothalamic secretion of releasing factor(s) and/or modified the sensitivity of the pituitary gland to hypothalamic regulators. The nature and the dose of the steroid employed may play an important role in both aspects. The sensitivity to administration of LH releasing factor appeared to be higher in EB treated castrates than in TP or 5α-dhTP treated castrates.

The oestrogen-androgen interaction at the level of the hypothalamic-pituitary axis may still remain the topic of much debate. Since it is known that C_{19}-steroid 5α-reductase activity will increase in the adrenal gland (*Maynard & Cameron* 1973) and in the hypophysis (*Kniewald & Milković* 1973) following castration, it is of interest to measure circulating levels of LH and FSH in adrenalectomized, gonadectomized male rats.

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RELATION BETWEEN CIRCULATING LEVELS OF TESTOSTERONE, LH AND FSH IN INTACT AND CASTRATED, ADULT, MALE RATS AFTER TESTOSTERONE ADMINISTRATION

By

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ABSTRACT

Serum levels of LH, FSH and testosterone were measured by radioimmunoassay in intact and castrated, adult, male rats after testosterone was administered subcutaneously for seven days in doses ranging from 25 to 200 μg per 100 g body weight per day. Such treatment increased circulating testosterone both in intact and castrated rats, but its effects on serum gonadotrophins were different in these animal groups. All doses of testosterone suppressed serum LH and FSH in the normal rat. In the castrates, treatment with the lowest dose of testosterone resulted in serum LH levels significantly above the high castrate levels, while serum FSH tended to drop. Administration of the highest doses of testosterone did not depress serum LH and FSH in the castrates to those of intact, normal animals, though serum testosterone in these castrates was much higher than in normal, male rats. It is concluded, that the sensitivity of the hypothalamic-pituitary system for daily, subcutaneous testosterone administration during seven days is not the same in the intact and castrated, adult, male rat and that testicular factors different from testosterone may play a role in regulating production and/or secretion of gonadotrophins by the hypophysis in male animals.

A relationship between testosterone and circulating gonadotrophins is rather well documented in the adult, male rat. Following gonadectomy, circulating testosterone will decrease to very low, almost undetectable values (*Coyotupa et al.* 1973), while circulating LH and FSH will increase (*Gay & Bogdanove* 1969; *Swerdloff et al.* 1972; *Kalra et al.* 1973; *Verjans et al.* 1974*b*). Administration of supraphysiological doses of testosterone or testosterone propionate to such animals prevents the rise of serum gonadotrophins (*Gay & Bogdanove* 1969; *Swerdloff et al.* 1972; *Kalra et al.* 1973; *Verjans et al.* 1974*b*). It is, however, unclear at what site of the hypothalamic-pituitary axis testosterone or its metabolites exert this action. Recently, *Kingsley & Bogdanove* (1973) demonstrated that androgenic steroids can exert direct intrapituitary effects *in vivo* in orchidectomized rats. Using an animal model (gonadectomized, adult, male rat) high doses of 5α-dihydrotestosterone, 5α-dihydrotestosterone propionate or of oestradiol-17β or oestradiol benzoate appeared to be even more potent than testosterone or testosterone propionate in preventing the increase in serum gonadotrophins following castration (*Swerdloff & Walsh* 1973; *Kalra et al.* 1973; *Verjans et al.* 1974*b*). Data on the dependency between circulating testosterone and circulating gonadotrophins following exogenous administration of testosterone are, however, lacking. The availability of radioimmunoassay techniques for testosterone and the gonadotrophins prompted us to study this dependency in normal and castrated rats.

MATERIAL AND METHODS

Animals

Adult, male Wistar rats (3–4 months old) were used. The animals were kept under controlled lighting (14 h light and 10 h darkness) and temperature (19–21°C) conditions. Laboratory chow and tap water were provided *ad libitum*. One group of animals (body weight ranging from 210 to 265 g) was gonadectomized under light ether anaesthesia, another group (body weight ranging from 290 to 330 g) was employed as intact animals. Daily subcutaneous injections with various doses of testosterone (Steraloids, Pawling, New York) in sesame oil (0.04 ml/100 g b. w.) were given for a period of seven days between 1 and 2 p. m. Steroid treatment of the gonadectomized animals started immediately after operation.

Twenty hours after the last testosterone injection the rats were decapitated under light ether anaesthesia, blood samples were collected and allowed to clot overnight at 4°C. Serum was stored at –20°C until assayed for testosterone and gonadotrophins.

Radioimmunoassay methods

Radioimmunoassay of testosterone in serum was performed as described previously for plasma (*Verjans et al.* 1973), and serum LH and FSH were measured using double antibody radioimmunoassays (*Welschen et al.* 1975, in press). For both radioimmunoassays the procedures as published by *Niswender et al.* (1968) were followed. Iodination with ^{125}I (Institut for Atomenergic, Kjeller, Norway) was performed according to

Greenwood et al. (1963). Serum LH and FSH concentrations were expressed on the basis of the reference preparations NIAMD rat-LH RP-1 and NIAMD rat-FSH RP-1 respectively. Statistical significance of differences between data from the different animal groups was determined using Student's *t*-test.

RESULTS

Circulating testosterone was very low in castrated, adult, male rats seven days following castration (Table 1), and varied from zero to 0.37 ng per ml. Daily, subcutaneous administration of testosterone during seven days to intact and castrated, adult, male rats resulted in increased serum testosterone (Tables 1 and 2). In intact animals injected with testosterone the concentration of this hormone in systemic blood seemed to depend on the amount of testosterone injected. Variation in circulating testosterone among the individual animals was, however, large, especially in castrates treated with large amounts of testosterone.

All doses of testosterone used suppressed serum LH and FSH in intact animals compared with the control group receiving vehicle only (Table 2),

Table 1.
Mean levels (± standard error of the mean) of serum testosterone and serum gonadotrophins in *castrated,* adult, male rats treated with various doses of testosterone/100 g b. w./day for seven days.

Treatment	Number of rats	Serum testosterone (ng/ml)	Serum LH (ng/ml)	Serum FSH (ng/ml)
Sesame oil	9	0.09 ± 0.03	249 ± 41	1153 ± 87
25 μg testosterone	5	1.42 ± 0.34*	427 ± 60*	1099 ± 67
50 μg testosterone	5	3.99 ± 1.52*	360 ± 31	793 ± 91*
100 μg testosterone	5	42.24 ± 34.40	172 ± 43	776 ± 80*
200 μg testosterone	5	35.90 ± 21.79	137 ± 44	547 ± 80*

* $P < 0.05$, compared with castrated rats receiving sesame oil only.

Table 2.
Mean levels (± standard error of the mean) of serum testosterone and serum gonadotrophins in *intact*, adult, male rats treated with various doses of testosterone/100 g b. w./day for seven days.

Treatment	Number of rats	Serum testosterone (ng/ml)	Serum LH (ng/ml)	Serum FSH (ng/ml)
Sesame oil	9	4.14 ± 0.60	37 ± 4	317 ± 31
25 μg testosterone	4	4.68 ± 0.79	< 10*	245 ± 6
50 μg testosterone	4	9.59 ± 4.70	< 10*	198 ± 14*
100 μg testosterone	4	20.89 ± 9.87*	< 10*	192 ± 39*

* $P < 0.05$, compared with intact rats receiving sesame oil only.

though the dose of 25 μg testosterone gave no significant depression of serum FSH ($P > 0.10$). Daily treatment with 25 μg testosterone for seven days significantly ($P < 0.01$) increased serum LH in the castrates, but higher doses of testosterone did not significantly ($P > 0.10$) affect serum LH (Table 1). For circulating FSH levels to be significantly ($P < 0.05$) below those of castrate control, 50 μg testosterone was sufficient in gonadectomized animals (Table 1). Testosterone treatment of the castrates with a daily dose of 50 μg for seven days resulted in circulating testosterone concentrations comparable with those encountered in intact, normal animals receiving vehicle only. Circulating gonadotrophins in the castrate group receiving this dose of testosterone were, however, still highly elevated compared with intact, control rats (Tables 1 and 2).

DISCUSSION

Circulating levels of testosterone in adult, male rats have been published before (*Miyachi et al.* 1973; *Bartke et al.* 1973; *Robel et al.* 1973; *Kinson & Liu* 1973) and after gonadectomy (*Coyotupa et al.* 1973) and our data are in adequate agreement with those of other investigators. Daily subcutaneous administration of testosterone for seven days resulted in elevation of serum levels of this testicular androgen in intact and castrated, adult, male rats

(Tables 1 and 2), but only the dose of 25 μg testosterone produced significantly different ($P < 0.01$) serum testosterone levels between intact and castrated animals (Tables 1 and 2). The effects of testosterone administration on circulating gonadotrophins depended on the type of animal used (Tables 1 and 2). In intact rats all the doses of testosterone employed suppressed circulating LH and FSH (Table 2). It has been shown that treatment of normal men with testosterone propionate decreased testicular testosterone concentrations while plasma levels of this hormone were elevated and plasma LH levels were reduced (*Morse et al.* 1973). Continuous intravenous infusion of testosterone for four days in men resulted in increased plasma testosterone and oestradiol-17β and decreased plasma LH and FSH (*Sherins & Loriaux* 1973). Thus, it is possible that depression of serum gonadotrophins in normal, adult, males following treatment with testosterone might be due both to an elevation of circulating testosterone, and of oestradiol-17β. Since administration of oestradiol-17β or oestradiol benzoate will suppress both serum gonadotrophins and testosterone in men (*Sherins & Loriaux* 1973) and in intact, adult, male rats (*Verjans et al.* 1974*a*), circulating oestrogens are probably of significance for the regulation of circulating gonadotrophins in male animals.

Gonadectomy of adult, male rats gave marked reduction of serum testosterone levels seven days following operation (Table 1). *Coyotupa et al.* (1973) observed a decrease of circulating testosterone and a decline of circulating 5α-dihydrotestosterone to undetectable values within 24 hours after removal of the testes in rats. In the presence of decreased circulating testosterone, and probably also of 5α-dihydrotestosterone, serum LH and FSH in the castrates were increased (Table 1). Both testosterone and 5α-dihydrotestosterone or their respective propionate esters are suppressors of circulating LH and FSH in intact and castrated adult, male rats (*Swerdloff et al.* 1972; *Naftolin & Feder* 1973; *Zanisi et al.* 1973; *Verjans et al.* 1974*b*), but oestradiol-17β or oestradiol benzoate, however, appear to be even more potent suppressors (*Dufy-Barbe & Franchimont* 1972; *Swerdloff et al.* 1972; *Swerdloff & Walsh* 1973; *Kalra et al.* 1973; *Verjans et al.* 1974*b*). A possible lack of circulating oestradiol-17β in castrated rats may contribute to the rise of serum gonadotrophins following gonadectomy, since the rat testis secretes both oestradiol-17β (*de Jong et al.* 1973) and the precursors for this hormone. Circulating oestradiol-17β is, however, in the low picogram range in normal, male rats and only 20 % of circulating oestradiol-17β stems from testicular secretion (*de Jong et al.* 1973).

Daily, subcutaneous administration of 25 μg testosterone to the castrates during seven days gave a significant increase in serum LH levels over castrate control levels (Table 1). *Swerdloff & Walsh* (1973) observed similar effects administering low doses of androgens or oestrogens to castrated, adult, male rats. In a previous publication (*Verjans et al.* 1974*b*) we demonstrated that testosterone propionate in a daily dose of 20 μg per 100 g body weight for

seven days could prevent the rise of serum LH levels following castration. Using the subcutaneous route of administration the clearance rate of the ester compounds compared to that of the free compounds may differ, moreover, different strains of animals were utilized in these studies. The apparent discrepancy in these data and the fact that low doses of testosterone *can* augment serum LH in castrated, adult rats warrant, however, further investigations.

In castrated rats 50 μg testosterone per day during seven days produced circulating levels of testosterone comparable with those of intact, adult, male rats (Tables 1 and 2), although circulating gonadotrophin concentrations were still markedly elevated compared with intact control values (Tables 1 and 2). Circulating testosterone levels in castrated, adult, male, rats which were maintained by daily administration of testosterone during seven days (starting steroid treatment immediately after removal of the testes), apparently failed to affect serum gonadotrophin levels in the same way as comparable steroid levels after such steroid treatment in intact rats. It is therefore suggested that the sensitivity of the hypothalamic-pituitary system producing LH and FSH differs for daily, subcutaneous testosterone administration during seven days between intact and castrated, adult, male rats. Thus, in addition to testosterone other testicular factors can be involved in regulation of production and secretion of LH and FSH by the pituitary gland in male rats.

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HYPOTHALAMIC-PITUITARY-TESTICULAR SYSTEM AND ADRENOCORTICAL FUNCTION

By

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ABSTRACT

Effect of intramuscular administration of ACTH or dexamethasone on blood serum levels of testosterone, LH and FSH was examined in intact and castrated, adult, male rats. Six IU ACTH or 1 mg dexamethasone were given daily for 7 days. Corticotrophin treatment had no influence on circulating testosterone, LH and FSH in intact or castrated male rats. Dexamethasone administration resulted in a slight elevation of serum FSH in intact animals but not in castrates. LH and testosterone remained normal in both intact and castrated animals injected with dexamethasone. Under our conditions of study the secretions from the adrenal gland appear to be insignificant for the regulation of pituitary secretion of gonadotrophins in the male rat.

Testosterone, 5α-dihydrotestosterone and oestradiol-17β are known secretory products of the testis having regulatory effects on the hypothalamic-pituitary axis in adult male rats. Gonadectomy in such animals results in very low circulating levels of testosterone and 5α-dihydrotestosterone (*Coyotupa et al.* 1973), while circulating LH and FSH will increase markedly (*Gay & Bogdanove* 1969; *Gay & Dever* 1971; *Swerdloff et al.* 1972, 1973; *Dufy-Barbe & Franchimont* 1972; *Kalra et al.* 1973). Administration of these testicular steroids (or their propionate and benzoate ester derivatives) can prevent the rise of serum gonadotrophins following castration. In this respect, oestradiol-17β and 5α-dihydrotestosterone are very potent (*Swerdloff et al.* 1973; *Verjans et al.* 1974). In addition to the testis, the adrenal cortex is a source of androgenic and oestrogenic steroids in various animal species (*Dorfman & Ungar* 1965). The contribution

of androgenic and oestrogenic steroids from the adrenal gland as potential regulators of circulating gonadotrophins has scarcely been investigated. Furthermore, the possible effect of 11-oxygenated and 11-deoxygenated corticosteroids is unknown in this respect. We have, therefore, studied effects of adrenal suppression and adrenal stimulation on the hypothalamic-pituitary-testicular system in intact and castrated, adult, male rats by determining circulating testosterone, LH and FSH levels in such animals by appropriate radioimmunoassay techniques.

MATERIAL AND METHODS

Animals

Adult, male, Wistar rats (3–4 months old) were used in the experiments. The animals were kept under controlled light (14 hours light and 10 hours darkness) and temperature (19–21°C) conditions. Rat chow and tap water were provided *ad libitum*.

Experiment I. – From a group of 18 animals with body weights varying from 225–250 g, 9 animals were gonadectomized under light ether anaesthesia and the rest was used as intact animals. Five gonadectomized and 5 intact animals were injected with 1 mg dexamethasone sodium phosphate (Decadron® phosphate, Merck Sharp & Dohme B. V., Haarlem, The Netherlands) in 0.25 ml buffer while 4 animals of each group were injected with 0.25 ml 0.9 % sodium chloride solution only. Daily, intramuscular injections were given for a period of 7 days. Treatment of the gonadectomized animals started immediately following surgery.

Experiment II. – From a group of 16 animals with body weights ranging from 380–400 g, 8 animals were gonadectomized under light ether anaesthesia and the rest was employed as intact animals. Four gonadectomized and 4 intact animals were injected with 6 IU corticotrophin (Frederiksberg Chemiske Fabriker A/S, København, Denmark) in 0.1 ml aqua dest. while 4 animals of each group were injected with 0.1 ml water only. Daily, intramuscular injections were given for a period of 7 days. Treatment of the gonadectomized animals started immediately following operation. Twenty hours after the last injection blood samples from experiment I and II were collected following decapitation of the animals under light ether anaesthesia. The blood was allowed to clot overnight at 4°C. Resulting blood serum was then stored at –20°C until analysed for gonadotrophins and testosterone. Adrenal glands, testes, ventral prostate and seminal vesicles were dissected and weighed shortly after the animals were sacrificed.

Radioimmunoassays

Serum LH and FSH levels were measured using double antibody radioimmunoassays (*Welschen et al.* 1975). For both radioimmunoassays the procedures as described by *Niswender et al.* (1968) were followed. Iodination with ^{125}I (Institut for Atomenergi, Kjeller, Norway) was performed according to the method of *Greenwood et al.* (1963). Serum samples from all experiments were assayed as duplicates in one assay and serum LH and FSH concentrations were expressed on the basis of reference preparations NIAMD RAT LH RP-1 and NIAMD RAT FSH RP-1 respectively. Radioimmunoassay of testosterone in serum was performed as described previously for plasma (*Verjans et al.* 1973). Statistical significance of the differences between data from the different animal groups was determined using Student's *t*-test.

Table 1.

Mean (± SD) organ weights (g) of intact and castrated, adult, male rats treated with 1 mg Decadron® (Dex.) per day for 7 days.

Treatment	No. of rats	Adrenals	Testis	Ventral Prostate	Seminal vesicles
Intact, 1 mg Dex.	5	0.026 ± 0.003*	1.284 ± 0.070	0.139 ± 0.014	0.062 ± 0.009
Intact, saline	4	0.059 ± 0.008	1.394 ± 0.089	0.120 ± 0.010	0.075 ± 0.013
Castrate, 1 mg Dex.	5	0.026 ± 0.004**		0.040 ± 0.010	0.037 ± 0.004
Castrate, saline	4	0.056 ± 0.006		0.054 ± 0.008	0.035 ± 0.010

* $P < 0.001$, compared with intact controls.
** $P < 0.001$, compared with castrate controls.

RESULTS

Effects of treatment with ACTH or dexamethasone on organ weights

The effects of daily, intramuscular administration of 1 mg dexamethasone or 6 IU ACTH during 7 days on the weights of the adrenal glands, testis, ventral prostate and seminal vesicles in intact and castrated, adult, male rats have been summarized in Tables 1 and 2 respectively. Compared with control rats ACTH treatment during 7 days had no influence on body weight of either castrated or intact animals. Treatment with dexamethasone for 7 days caused, however, reduction of body weight (15–25 %) in both intact and castrated animals. We have, therefore expressed the organ weights as absolute values rather than relative to total body weight. Following castration the weights of the

Table 2.

Mean (± SD) organ weights (g) of intact and castrated, adult, male rats treated with 6 IU ACTH per day for 7 days.

Treatment	No. of rats	Adrenals	Testis	Ventral Prostate	Seminal Vesicles
Intact, 6 IU ACTH	4	0.094 ± 0.010	1.640 ± 0.221	0.257 ± 0.026	0.232 ± 0.018
Intact, aqua dest.	4	0.069 ± 0.016	1.591 ± 0.109	0.246 ± 0.034	0.216 ± 0.018
Castrate, 6 IU ACTH	4	0.091 ± 0.012		0.091 ± 0.023	0.111 ± 0.014
Castrate, aqua dest.	4	0.081 ± 0.008		0.108 ± 0.029	0.131 ± 0.022

accessory reproductive organs in the control animals fell, but no significant change was observed in the weights of the adrenal glands in these animals (Tables 1 and 2). Administration of 1 mg dexamethasone per day during 7 days resulted in a profound reduction in adrenal weight in intact ($P < 0.001$) and castrated ($P < 0.001$) animals (Table 1). Testicular weight, the weights of the ventral prostate and the seminal vesicles remained unchanged in intact animals following 7 days treatment with dexamethasone (Table 1). The weights of the latter two organs in castrated animals exhibited no change following dexamethasone treatment (Table 1). Corticotrophin treated intact and castrated, adult, male rats gained adrenal weight, these gains were, however, not statistically significant for intact ($0.05 < P < 0.10$) or castrated ($0.20 < P < 0.25$) animals compared with respective control rats (Table 2). Testicular weight and the weights of the accessory reproductive organs in both type of animals were not influenced by the treatment with ACTH (Table 2).

Effects of treatment with ACTH or dexamethasone on serum levels of testosterone, LH and FSH

The effects of daily, intramuscular administration of 6 IU ACTH or 1 mg dexamethasone for 7 days on serum levels of testosterone in intact and castrated, adult, male rats are depicted in Figs. 1 and 2 respectively. Seven days following castration circulating testosterone decreased to very low values, while circulat-

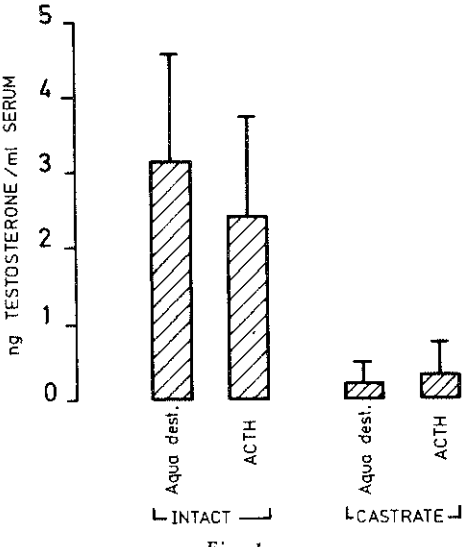

Fig. 1.
Effect of daily treatment with 6 IU ACTH for seven days on levels of serum *testosterone* in intact and castrated, adult, male rats. Mean data ± SD from four animals are shown.

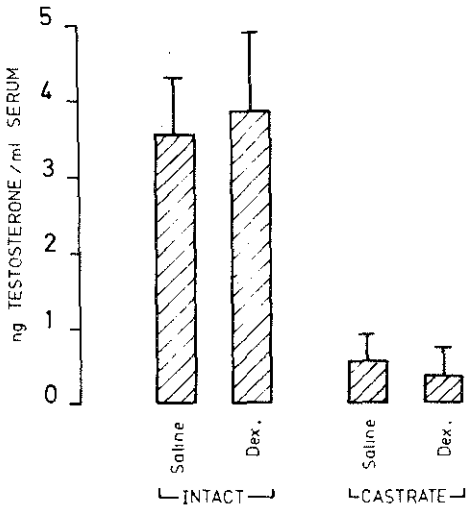

Fig. 2.
Effect of daily treatment with 1 mg Decadron® (Dex.) for seven days on levels of serum *testosterone* in intact and castrated, adult, male rats. Mean data ± SD from four or more animals are shown.

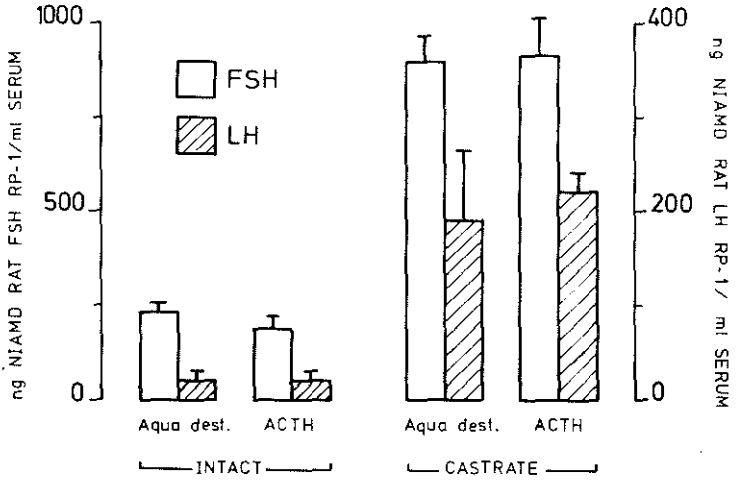

Fig. 3.
Serum levels of LH and FSH in intact and castrated, adult, male rats treated with 6 IU ACTH per day for seven days. Mean data ± SD from four animals are shown.

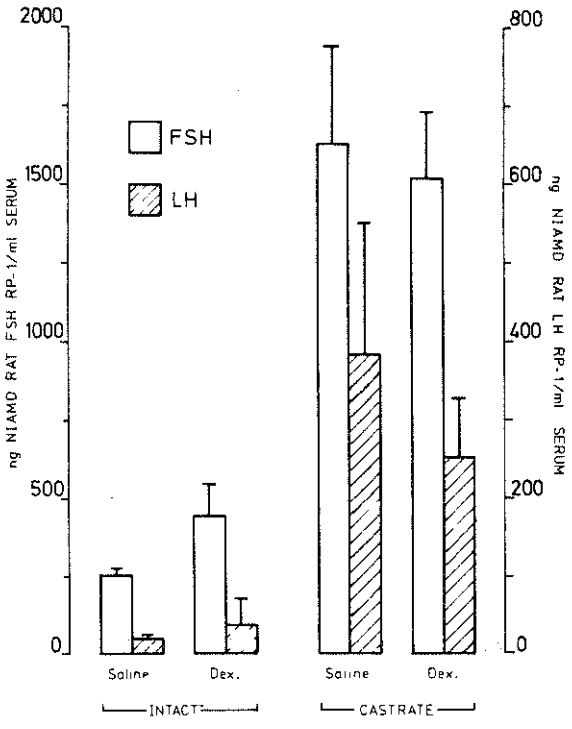

Fig. 4.
Serum levels of LH and FSH in intact and castrated, adult, male rats treated with 1 mg Decadron® (Dex.) per day for seven days. Mean data ± SD from four or more animals are shown.

ing LH and FSH (Figs. 3 and 4) showed a remarkable increment. Daily, intramuscular corticotrophin treatment during 7 days did not bring about changes in serum levels of testosterone, LH and FSH in intact or castrated, adult, male rats in comparison with respective control animals receiving water only (Figs. 1 and 3). Incubation of 6 IU ACTH with diluted antiserum, however, revealed a slight cross-reaction of the ACTH preparation used with both the LH and the FSH antibodies. In experiment I serum FSH levels were somewhat higher than in experiment II (Figs. 3 and 4), which might be due to interassay-variations in the radioimmunoassay of serum FSH or to biological variation. Treatment with 1 mg dexamethasone per day for 7 days resulted in an increase in serum FSH levels in intact animals ($P < 0.01$) compared with the controls receiving saline only (Fig. 4). Such elevation was not observed in castrates following the same dose of dexamethasone (Fig. 4). The other serum parameters investigated were not influenced by dexamethasone treatment in either normal or castrated rats (Figs. 2 and 4).

DISCUSSION

Seven days following gonadectomy low testosterone (Figs. 1 and 2) and elevated gonadotrophin levels (Figs. 3 and 4) were found in blood serum of adult male rats. These data compare well with those of others (*Coyotupa et al.* 1973; *Gay & Bogdanove* 1969; *Gay & Dever* 1971; *Swerdloff et al.* 1971) and with data previously reported by our laboratory (*Verjans et al.* 1974, 1975). Daily, intramuscular administration of ACTH for 7 days was followed by adrenocortical hypertrophy in normal and castrated rats though this was not of significant nature (Table 2). Corticotrophin treatment for 7 days did not influence the hypothalamic-pituitary-testicular system investigated (Figs. 1 and 3). This is consistent with the observation that the weights of the androgen-sensitive, male, accessory organs remained unchanged following ACTH treatment in both intact and castrated animals compared with respective controls (Table 2). It has been reported (*Hudson et al.* 1965) that the administration of ACTH to normal men will not change plasma testosterone levels. *Beitins et al.* (1973) found, however, significantly lower testosterone and 5α-dihydrotestosterone concentrations in plasma of normal young men with no change in serum LH levels after exogenous ACTH. *Gay & Dever* (1971) showed that adrenalectomy in orchidectomized, mature, male rats produced no additional rise in serum levels of LH and FSH. Thus, the present data favour the suggestion that steroids of adrenocortical origin do not play a significant role as regulators for gonadotrophin secretion in male rats. Moreover, the amount of adrenocortical androgens and oestrogens secreted in castrated male rats cannot substitute for the testicular steroids in their feedback action on the hypothalamic-pituitary axis. Finally, if the major effect of a continuous "stress" situation is via pituitary secretion of ACTH, the possibility is remote that such a "stress" form will influence the testicular system.

Dexamethasone has a high glucocorticoid activity and will suppress ACTH secretion at a pituitary and/or hypothalamic level (*Zimmermann & Critchlow* 1969). Suppression of adrenocortical function in our investigation was evident by the decreased adrenal weights of dexamethasone treated normal and castrated rats (Table 1). In the castrates, 1 mg dexamethasone per day for 7 days had no measurable effects on circulating testosterone (Fig. 2), LH or FSH (Fig. 4), nor did this glucocorticoid affect the falling ventral prostate and seminal vesicles weights (Table 1). Thus, in the present study the endocrine changes induced by castration were not influenced by the use of a large dose of a potent glucocorticoid. It has been shown that administration of a daily dose of 3 mg cortisone for 15 days will cause a small weight decrease of the ventral prostate in castrated rats while a daily dose of 9 mg cortisone administered over the same period of time will not do so, both doses of cortisone were, however, found to induce growth of the seminal vesicles in such animals (*Tisell* 1970). Cortisone, having

a weak androgenic effect when administered alone, can, however, counteract partially the growth of the male, accessory, reproductive organs induced by testosterone propionate administration in castrated, adrenalectomized rats (*Tisell* 1972). *Tveter & Aakvaag* (1969) reported that corticosterone will reduce the uptake of [^{3}H]testosterone by the prostatic lobes and the seminal vesicles. Simultaneous administration of cortisol augments the androgenic activity of testosterone propionate as measured by ability to increase the weights of the seminal vesicles and the ventral prostate in immature castrates (*Klaiber et al.* 1968). These discrepancies could be due to differences in doses and duration of glucocorticoid treatment.

In the normal rat, dexamethasone treatment for 7 days did not influence circulating testosterone and LH (Figs. 2 and 4). This was also reflected by unchanged weights of the ventral prostate, seminal vesicles and testis in these animals (Table 1). Circulating FSH, however, exhibited a slight but significant elevation (Fig. 4) in the intact rat following dexamethasone treatment. It has been reported that cortisone exerts a deleterious effect on the germinal epithelium of rats and mice (*Albert* 1961). Thus, in the doses given, dexamethasone could have a direct effect on the testicular tissue and change the production in this tissue of factors responsible for regulated secretion of FSH. *Setchell & Sirinathsinghji* (1972) have published on FSH depressing activity in rete testis fluid and *Van Thiel et al.* (1972) have suggested that factors produced in the germinal epithelium are involved in FSH feedback control in men. Our data could indicate that dexamethasone will lower testicular production of an unknown factor controlling specifically FSH secretion in the male rat. Thus, a direct effect of large doses of dexamethasone on possible testicular factors, regulating serum FSH concentrations in normal rats, is possible. No data are, however, available on the effects of long-term treatment with dexamethasone on the pituitary-testicular system. *Faiman & Winter* (1971) have shown that administration of a total dose of 3 mg dexamethasone to normal men will elevate plasma LH levels but not those of plasma FSH and testosterone. *Judd et al.* (1973) reported, however, that a single dose of 2 mg dexamethasone did not block nocturnal rise of plasma testosterone in normal male subjects, a probable LH function. A total dose of 0.6 mg dexamethasone given on late dioestrus and early pro-oestrus will block ovulation in female rats, probably by inhibiting pituitary release of LH (*Baldwin & Sawyer* 1974).

The present work using stimulation or suppression of adrenocortical function in gonadectomized, adult, male rats with long-term administration of supra-pharmacological doses of ACTH or dexamethasone and the work of *Gay & Dever* (1971) using adrenalectomized, orchidectomized, mature, male rats reveal that the steroids of the adrenal cortex do not significantly affect circulating gonadotrophin concentrations in such animals. The contribution of adrenal corticosteroids, androgens and oestrogens to the testicular steroids in regulating

circulating gonadotrophin concentrations is likely to be of minor importance. Thus, only secretory products of the testes reaching target organs in the brain via the general circulation seem to be responsible for control of the gonadotrophic function of the hypophyseal-hypothalamic system in adult male rats.

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HYPOTHALAMIC - PITUITARY - TESTICULAR SYSTEM FOLLOWING TESTICULAR X-IRRADIATION*

By

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* A short summary of some of the data in this paper will be published elsewhere (Verjans and Eik-Nes, 1975).

A B S T R A C T

Testes of adult, male rats were exposed to a total dose of 1,500 R of X-irradiation. Testicular weight decreased from day 8 after X-ray treatment. This decrease was, however, preceded by an increment of the testis weight on day 4 following treatment. X-ray treatment of testes was associated with significant increases in serum FSH. Testicular irradiation had, however, no effect on ventral prostate and seminal vesicles weights. Serum testosterone increased only on day 1, 2 and 4 after irradiation, while serum LH levels tended to increase from day 8 postirradiation. These changes were not significant, however, when compared with non-irradiated controls. At 7,13 and 20 days following 1,500 R of bilateral, testicular X-irradiation, the hypothalamic-pituitary unit was still capable of responding to exogenous gonadotrophin releasing factor. Serum FSH may in male rats be regulated at least partly by circulating steroids of testicular origin and partly by an unknown factor of non-interstitial cell nature.

I N T R O D U C T I O N

Following gonadectomy circulating testosterone concentrations will decrease in adult, male rats (Coyotupa et al. 1973; Verjans et al. 1975) while circulating LH and FSH concentrations will increase (Gay and Bogdanove 1969; Gay and Dever 1971; Swerdloff et al. 1972; Verjans et al. 1974). Testosterone is considered being the hormone directing a feedback system between the hypothalamic-pituitary unit and the male gonad. Recent work, however, points out that metabolites of testosterone like 5α-dihydrotestoste-

rone and 5α-androstane-3α,17β-diol are more potent than testosterone in curbing circulating LH and FSH concentrations in gonadectomized, adult, male rats (Swerdloff et al. 1972; Verjans et al. 1974; Eik-Nes 1974). Moreover, higher doses of 5α-reduced metabolites of testosterone are required for suppression of circulating FSH than for suppressing circulating LH in such rats (Verjans et al. 1974; Eik-Nes 1974). The specific role and contribution of the different testis cell compartments in regulating production and/or secretion of gonadotrophins by the pituitary gland remain to be clarified. Local, testicular X-irradiation will destroy the germinal cells of the seminiferous tubules, leaving Sertoli and interstitial cells relatively unaffected (Fogg and Cowing, 1951; Shaver 1953; Schoen 1964). In order to study effect of the absence of proliferative germinal epithelium on the hypothalamic-pituitary-testicular system, we have measured serum testosterone, LH and FSH levels in adult, male rats at different time intervals following local testicular X-irradiation with a dose of 1,500 R. Furthermore, we have investigated effect of administering a gonadotrophin releasing factor on circulating LH and FSH in rats exposed to testicular irradiation.

MATERIALS AND METHODS

Animals

Adult, male Wistar rats (3-4 months old) with body weights ranging from 300 to 350 g were used. The animals were kept under controlled light (14 hrs light, 10 hrs darkness) and temperature (19-21^{o} C) conditions. Laboratory chow and tap water were provided ad libitum.

Experiment I. - Rats were anaesthesized with ether and without shielding the rest of the body, the scrotal area was exposed to irradiation by means of a medical X-ray machine operating at 200 kV with a setting of 20 mA and a filter of 0.5 mm Cu. The animals were subjected to a total dose of 1,500 R directed over a period of 10 min. At 1,2,4, 8,13,20,26 and 52 days following irradiation, three or more animals were decapitated under light ether anaesthesia at 9 a.m. Control animals of the same age were anaesthesized with ether for a period of 10 min and 4,8,13,20,26 and 52 days following this treatment, three or more animals were also decapitated at 9 a.m. Blood samples of irradiated and control animals were collected and allowed to clot overnight at 4° C. Serum was stored at -20° C until analysed for testosterone and gonadotrophins. Testes, ventral prostate and seminal vesicles were dissected and weighed.

Experiment II. - Twelve animals were irradiated as stated under experiment I. At 7, 13 and 20 days following irradiation, blood samples were drawn under light ether anaesthesia by puncturing the ophthalmic venous plexus. 500 ng synthetic gonadotrophin releasing factor (GnRF, Abbott Laboratories, North Chicago, Illinois, U.S.A.) dissolved in 0.1 ml 0.9% sodium chloride solution were then administered via the jugular vein. Blood was obtained 15 and 60 min after this injection from the ophthalmic venous plexus. Control, non-irradiated animals were treated in the same fashion but received 0.1 ml saline or 0.1 ml saline containing 500 ng GnRF via the jugular vein. Blood samples were withdrawn 15 and 60 min afterwards, and all blood specimens were handled as dicussed in experiment I.

Radioimmunoassay methods

Serum gonadotrophins were measured by double antibody radioimmunoassays employing antisera raised against ovine

LH and ovine FSH by immunizing rabbits with NIH-LH-S17 and NIH-FSH-S9 respectively. NIAMD rat LH I-1 and NIAMD rat FSH I-1 were used for preparation of iodinated derivatives. Iodination was performed with ^{125}I (Institutt for Atomenergi, Kjeller, Norway) according to Greenwood et al. (1963). For both radioimmunoassays the procedures described by Niswender et al. (1968) were followed. Sensitivity, accuracy and specificity of both radioimmunoassays have been published (Welschen et al. 1975). Serum samples from individual rats were assayed in duplicate in one assay and serum LH and FSH levels expressed on the basis of standard reference preparations NIAMD rat LH RP-1 and NIAMD rat FSH RP-1 respectively. Serum testosterone levels were measured using a radioimmunoassay technique described previously (Verjans et al. 1973). Statistical significance of the differences between data from the different animal groups was determined by Student's t-test.

R E S U L T S

Relative weights of testes and accessory, reproductive organs

Relative weight (weight of organ/100 g of total body weight) of testes and accessory, reproductive organs at different days following exposure of testes to X-ray is depicted in Figs. 1 and 2 respectively. At 4 days after irradiation the relative testicular weight of irradiated animals was significantly higher ($P<0.01$) than testis weight of controls. A progressive decrease ($P<0.01$) of testicular weight and size was observed from 8 to 52 days postirradiation when relative testis weight was only 32% of that of control rats. From 4 to 52 days after testicular X-irradiation we observed some changes in the relative

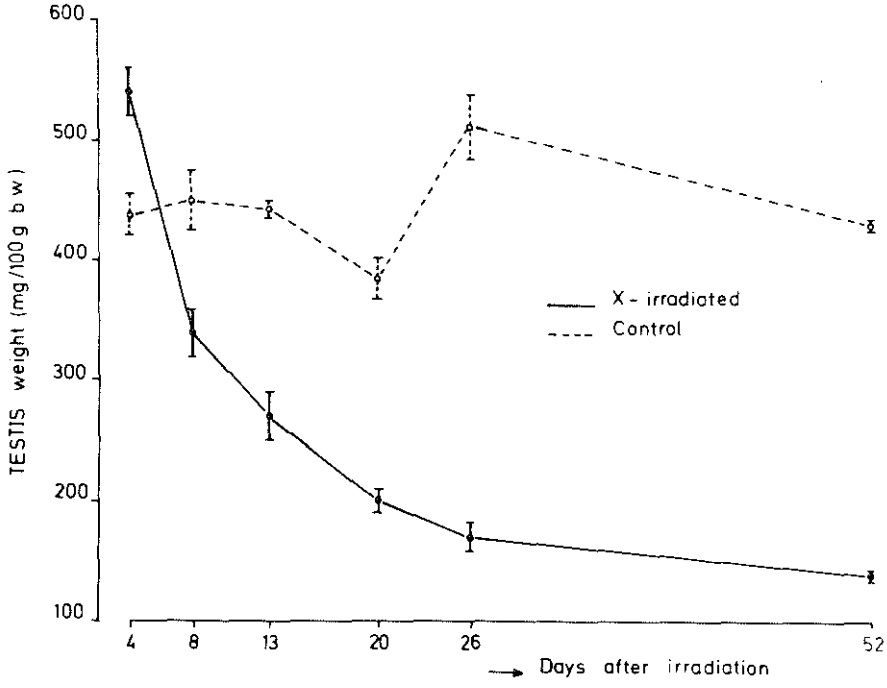

Fig. 1.
Relative testis weight (mg/100 g b.w.) in adult, male rats at different time intervals following testicular X-irradiation with 1,500 R. Mean data ± S.E.M. ($n \geq 6$) are presented.

weights of the male, accessory, reproductive organs (Fig. 2). These changes, however, were not of statistically significant nature (data not shown).

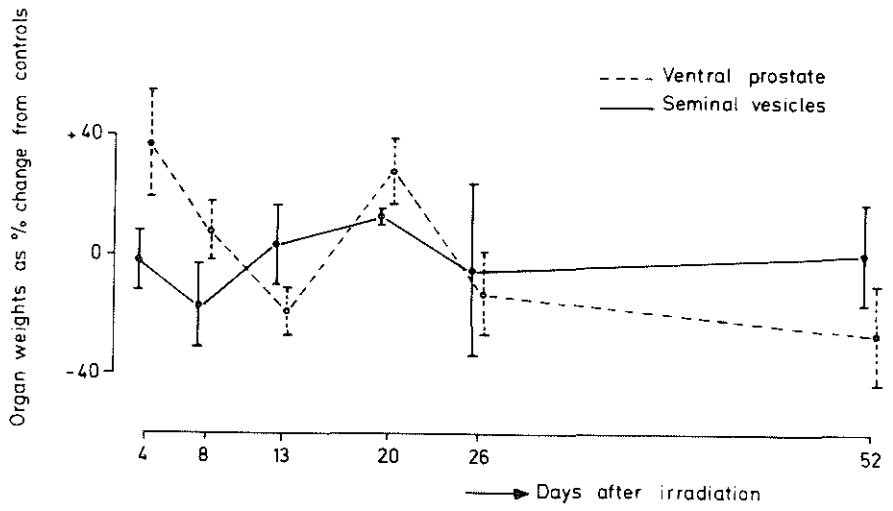

Fig. 2.
Relative weights of accessory, reproductive organs (mg/ 100 g b.w.) in adult, male rats at different time intervals following testicular X-irradiation with 1,500 R. Mean data $\pm$ S.D. ($n \geq 3$) are expressed as % change from control rats.

Serum levels of testosterone, LH and FSH

Fig. 3 shows serum testosterone levels on various days after X-irradiation of testes. The first days after treatment serum testosterone appeared slightly elevated compared to the other days. This elevation and the differences in serum testosterone between irradiated and non-irradiated animals from day 4 onwards were, however, not statistically significant. Circulating LH in irradiated and control animals did not change significantly throughout the period of study (Fig. 4), but serum FSH (Fig. 5) started to increase as early as 4 days postirradiation. Serum FSH levels in X-ray treated animals were significantly higher ($P<0.05$)

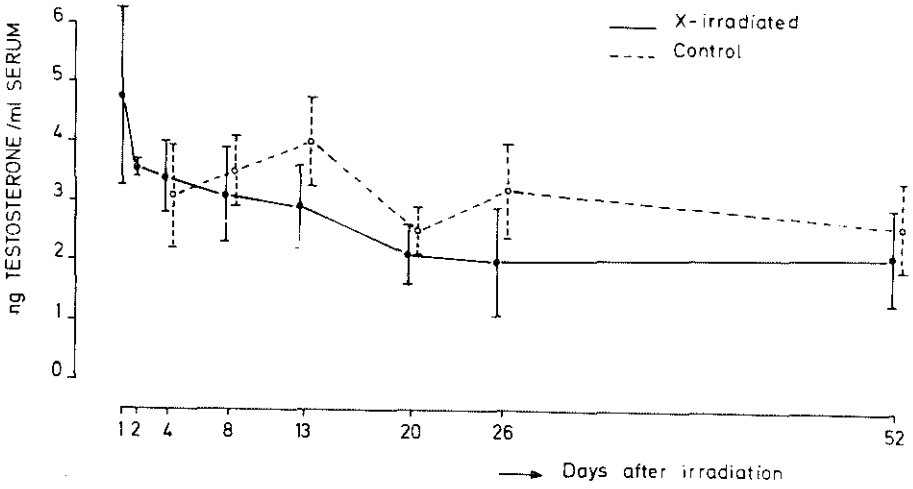

Fig. 3.

Serum levels of testosterone in adult, male rats at different time intervals following testicular X-irradiation with 1,500 R. Mean data ± S.D. from three or more animals are shown.

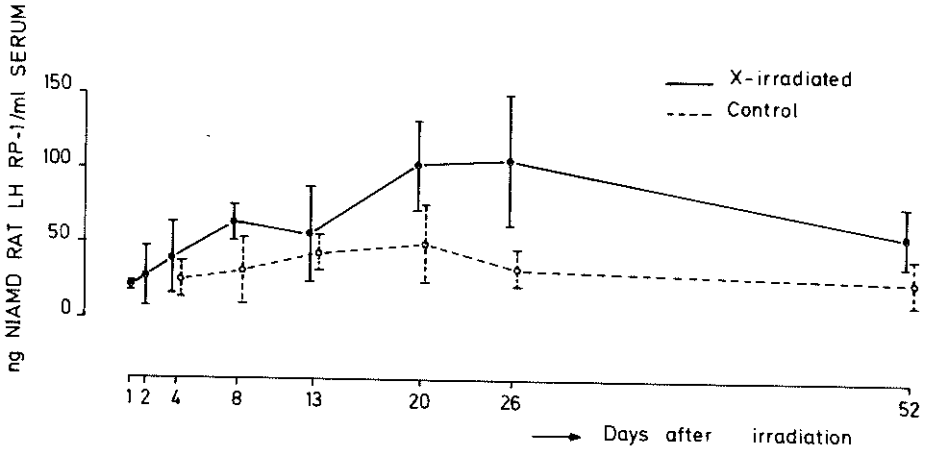

Fig. 4.

Serum levels of LH in adult, male rats at different time intervals following testicular X-irradiation with 1,500 R. Mean data ± S.D. from three or more animals are shown.

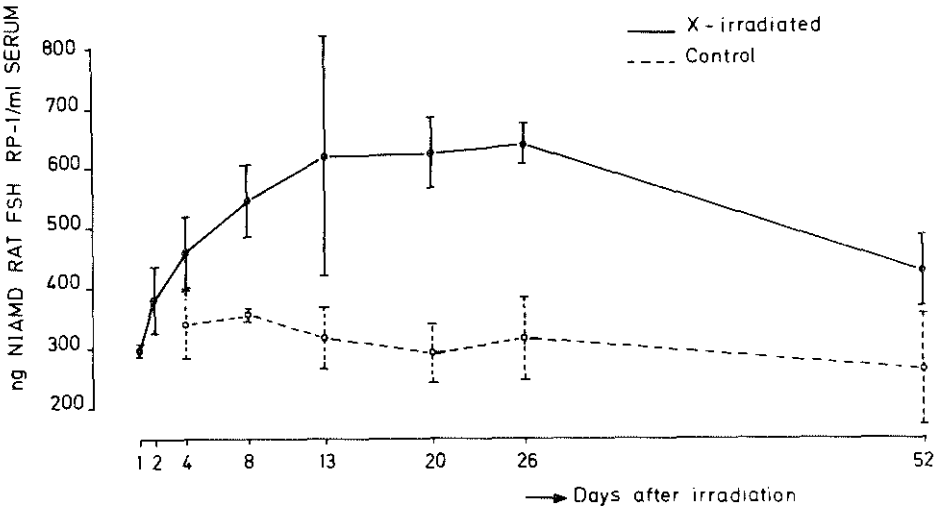

Fig. 5.
Serum levels of FSH in adult, male rats at different time intervals following testicular X-irradiation with 1,500 R. Mean data ± S.D. from three or more animals are shown.

than in non-irradiated animals on day 8,13,20 and 26 after X-ray exposure. Circulating FSH levels on day 52 post-irradiation were not significantly different from the levels in control animals.

Effect of GnRF in X-ray treated adult, male rats

Effects of intravenous administration of 500 ng synthetic GnRF on circulating LH and FSH in control rats and rats at different intervals following testicular X-irradiation are summarized in Figs. 6 and 7. 15 min after injection of GnRF serum LH and FSH levels in non-irradiated control ani-

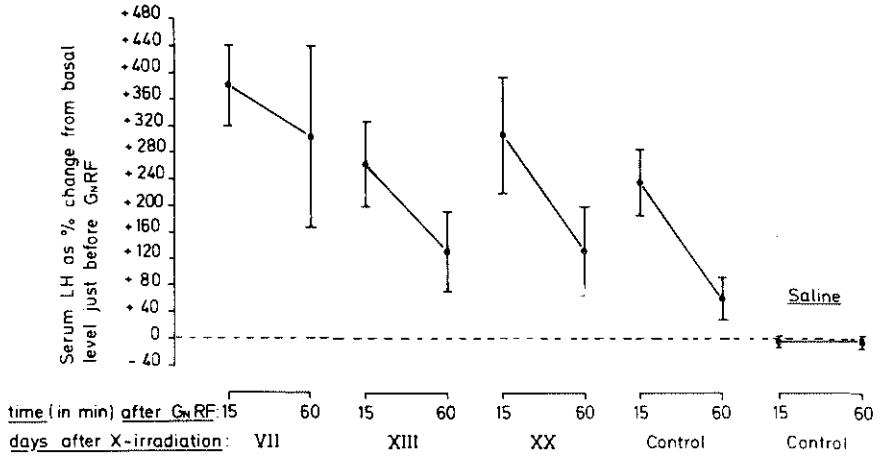

Fig. 6.

Effect of intravenous injection of 500 ng GnRF on serum LH levels in adult, male rats at different days after testicular X-irradiation with 1,500 R. Mean data ± S.E.M. ($n \geq 3$) 15 and 60 min following administration of GnRF (dissolved in saline) are expressed as % change from the basal level measured just before injection of GnRF. LH response in normal rats of the same age injected with 500 ng GnRF in saline or with saline only is also shown.

mals were higher than the basal levels before administration of GnRF, and 60 min after GnRF, mean serum LH and FSH levels had returned to the preinjection range in these rats. 15 min following administration of the standard dose of 500 ng GnRF to rats exposed to testicular irradiation 7,13 or 20 days before, serum LH levels were increased (Fig. 6), but 60 min subsequent to GnRF injection these levels were those of normal animals. In these rats the elevated serum

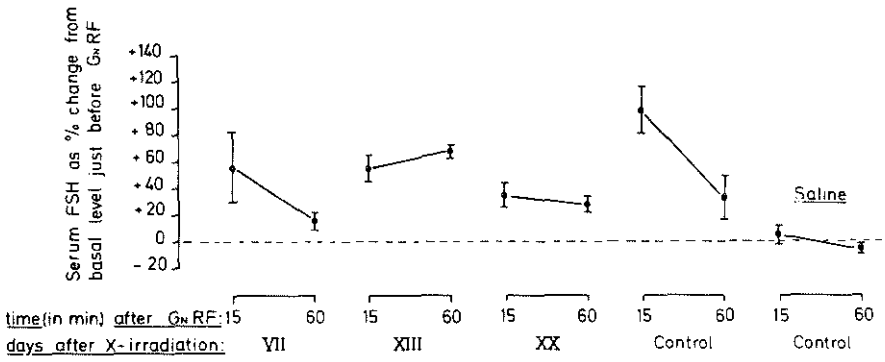

Fig. 7.

Effect of intravenous injection of 500 ng GnRF on serum FSH levels in adult, male rats at different days after testicular X-irradiation with 1,500 R. Mean data ± S.E.M. ($n \geq 3$) 15 and 60 min following administration of GnRF (dissolved in saline) are expressed as % change from the basal level measured just before injection of GnRF. FSH response in normal rats of the same age injected with 500 ng GnRF in saline or with saline only is also shown.

FSH concentrations (Fig. 5) underwent further elevation 15 min following 500 ng GnRF (Fig. 7). Compared to normal rats given the same dose of GnRF (Fig. 7), the FSH response to GnRF injection in rats 13 and 20 days after irradiation of the testes, showed either no decline or a slow decline between 15 and 60 min after GnRF (Fig. 7).

D I S C U S S I O N

Damage of testicular tissue following total body or local, testicular exposure to ionizing X-irradiation has been described by many workers (Fogg and Cowing 1951; Shaver 1953; Oakberg 1955; Schoen 1964; Ellis and Van Kampen 1971). Interstitial cells and Sertoli cells are known to remain intact by histologic criteria following bilateral, testicular X-irradiation. Moreover, by such criteria the germinal epithelium of the seminiferous tubules is not radioresistant and will be destroyed. The most radiosensitive cells in the tubular elements are thought to be type A_1-A_4 spermatogonia, while type A_0 spermatogonia will survive following 300 R of X-irradiation (Dym and Clermont 1970). When the supply of germinal cells is exhausted following X-irradiation, mature sperm cells will disappear. The cycle of sperm maturation will thus be interrupted until regeneration occurs via type A spermatogonia which are resistant to X-ray destruction. This will lead to repopulation of the germinal epithelium in the seminiferous tubules. Schoen (1964) reported that bilateral, testicular X-irradiation decreased the tubule diameter and resulted in increased number of interstitial cells per tubule when evaluated 14 days post X-ray treatment. Our histologic findings (not shown) were in adequate agreement with the above observations. Moreover, decrease of size and weight of the male gonad (Fig. 1) was recorded from day 8 postirradiation. The first days after bilateral, testicular X-irradiation, circulating testosterone concentrations (Fig. 3) were slightly elevated compared to those measured later on. This was also reflected by an augmented testicular weight on day 4 preceding the decrease in testicular weight seen from day 8 postirradiation (Fig. 1). The changes in the relative weights of the accessory, reproductive organs of X-ray treated rats (Fig. 2) appeared largely to follow the pattern observed for circulating

testosterone postirradiation (Fig. 3). Concomitantly with the decrease of circulating testosterone, circulating LH concentrations tended to increase (Fig. 4). None of the changes observed in the weights of the accessory, reproductive organs, circulating testosterone or circulating LH concentrations, however, were statistically significant when compared with non-irradiated, control animals. Our data (Fig. 3) are compatible with the observation that X-irradiation of mice will initially increase testicular androgen production from radioactive precursors in vitro, but is followed later by diminished ability to synthesize such androgens (Ellis and Berliner 1963). Circulating levels of 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol after irradiation were not measured in our work. 5α-Steroid reductase activity is present in seminiferous tubules of adult rats (Rivarola and Podestá 1972; Folman et al. 1973). X-ray induced changes in these cells may have altered circulating concentrations of the 5α-reduced metabolites of testosterone exerting potent activity on androgen target tissues. The slight elevation of circulating LH concentrations post X-ray (Fig. 4) may result from testes failing to produce required amounts of androgens to keep circulating LH within normal range. The data presented (Figs. 2, 3 and 4), however, are indicative of that a functional, interstitial cell compartment exists following testicular X-irradiation.

X-ray treatment of rat testes resulted in elevation of circulating FSH in these animals (Fig. 5). Swerdloff et al. (1971) showed that the relatively high levels of circulating FSH in immature, male rats will fall with appearance of mature sperm in seminiferous tubules and that circulating FSH will further decrease in sexually mature animals with normal spermatogenesis. In Klinefelter's syndrome, azoospermia and hypogonadism (Franchimont 1973), cryptorchidism (Swerdloff et al. 1971) and following treatment with antispermatogenic chemical agents (Gomes et al. 1973), graded degree of germinal epithelium damage is associated

with elevated circulating concentrations of FSH. Paulsen (1968) found that urinary FSH levels will increase following X-irradiation of the testis prior to decrement in sperm count, and urinary FSH will decrease again to normal levels before the germinal epithelium is fully recovered. Recent reports demonstrate FSH depressing activity present in rete testis fluid (Setchell and Sirinathsinghji 1972) or associated with germinal epithelium of seminiferous tubules (Van Thiel et al. 1972). In the present study serum FSH levels were not as high as observed in castrated, adult, male rats (Verjans et al. 1974). Daily administration of testosterone for 7 days to castrated, adult, male rats, resulting in circulating testosterone concentrations found in normal, adult, male rats, will prevent serum FSH levels to rise to those of untreated castrates (Verjans et al. 1975). Also 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol injections will suppress serum FSH levels in castrated rats (Verjans et al. 1974, Eik-Nes 1974).

Therefore circulating FSH concentrations in the adult, male rat might be regulated at least partly by circulating androgen concentrations and partly by an unknown factor, probably produced by spermatogonial cells and/or Sertoli cells supporting such spermatogonia. From our data it is difficult to conclude whether increased circulating FSH following testicular X-irradiation has affected testicular function in the animals. Intravenous administration of FSH will not change testosterone concentrations in testicular, venous plasma of normal rats (de Jong et al. 1973). Furthermore, Sertoli cell production of an androgen binding protein (Hagenäs et al. 1974 ; Vernon et al. 1974) appears to depend on FSH (Hansson et al. 1973). The high serum FSH concentrations in our animals (Fig. 5) might have maintained proper levels of testosterone and/or 5α-reduced testosterone metabolite concentrations in the depleted tubules by augmenting androgen binding protein concentrations in these cells.

Circulating LH and FSH following injection of a standard

dose of GnRF should evaluate the reserve capacity of the pituitary gland to secrete these trophins. Administration of GnRF (Figs. 6 and 7) causes a larger increase in circulating LH than in circulating FSH both in normal and X-irradiated rats. Moreover, in spite of the high control levels of both trophins before GnRF injection in the X-ray treated rats (Figs. 4 and 5), pituitary secretion of either trophin was not maximal since additional secretion of these trophins was promoted by exogenous hypothalamic releasing factor. The increment in circulating FSH and LH is smaller in male rats following X-ray treatment of the testes than following removal of these organs (Verjans et al. 1974). Thus, the effects of gonadectomy and of X-ray treatment of the testes on the feedback control at the hypothalamic-pituitary level are different. The LH response measured 15 and 60 min after GnRF injection appears normal in the X-ray treated rats (Fig. 6). The response of FSH to GnRF in rats 13 and 20 days after irradiation of the testis shows a slower decline than in normal rats over a 45 min observation period (Fig. 7). The slower decrease of circulating FSH levels following injection of GnRF in rats 13 and 20 days posttesticular irradiation, could be caused by pituitary tissue producing increased amounts of FSH in the absence of a physiological regulator for the secretion of this hormone.

A C K N O W L E D G M E N T S

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EFFECTS OF ANDROSTENES, 5α-ANDROSTANES, 5β-ANDROSTANES, OESTRENES AND OESTRATRIENES ON SERUM GONADOTROPHIN LEVELS AND VENTRAL PROSTATE WEIGHTS IN GONADECTOMIZED, ADULT MALE RATS

By

H.L. Verjans and K.B. Eik-Nes

A B S T R A C T

The effects of subcutaneous administration of steroids from the androstene, 5α-androstane, 5β-androstane, oestrene and oestratriene series on serum levels of LH and FSH and on ventral prostate weights were investigated in gonadectomized, adult male rats. Each steroid was administered for seven days in a standard dose of 100 µg per 100 g body weight per day. 3 Or 3α (but not 3β) and 17 or 17β disubstituted 5α-androstanes were effective in suppressing either one or both serum gonadotrophins. 5β-Androstanes and androstenes exhibited very little inhibitory effect in this respect. Potent suppression of serum gonadotrophins was observed following treatment with oestratrienes, the presence of only a phenolic A-ring in these oestrogens appeared sufficient for such suppression. 3 Or 3β and 17 or 17β disubstituted androstenes could entirely or partly prevent ventral prostate weight decrease postgonadectomy 5α-Androstane-3α,17β-diol, 5α-androstane-3α,11β,17β-triol and 5α-androstan-17β-ol-3-one were most potent, while 5β-androstanes and oestratrienes had no effect on this decrement. Regulatory processes by steroids at the hypothalamic-pituitary and at the ventral prostate level were not parallel.

The results indicate, however, that 3 or 3α and 17 disubstituted (keto or hydroxyl) 5α-reduced testosterone metabolites, of which 5α-androstan-17β-ol-3-one might be the principal one, are the main steroids directing androgenic regulation at both organ systems tested in the mature male rat.

I N T R O D U C T I O N

Gonadectomy in mature male rats results in very low circulating concentrations of 4-androsten-17β-ol-3-one (testosterone) (Coyotupa et al. 1973; Verjans et al. 1975a) and 5α-androstan-17β-ol-3-one (5α-dihydrotestosterone) (Coyotupa et al. 1973) and elevated circulating concentrations of the gonadotrophins LH and FSH (Gay and Bogdanove 1969; Swerdloff et al. 1972; Dufy-Barbe and Franchimont 1972; Verjans et al. 1974a). The accessory reproductive organs are known to undergo atrophy following castration. Secretory products of the testis are therefore believed to regulate circulating gonadotrophins and to maintain function of accessory reproductive organs. Administration of known testicular secretion products like testosterone or 5α-dihydrotestosterone to gonadectomized mature, male rats most effectively prevents the rise of serum gonadotrophins and the weight decrement of the accessory reproductive organs (Swerdloff et al. 1973; Verjans et al. 1974a). Oestrogen treatment of castrated rats inhibits circulating gonadotrophin concentrations (Kalra et al. 1973; Swerdloff et al. 1973; Verjans et al. 1974a), and promotes growth of the seminal vesicles but not of the ventral prostate (Verjans et al. 1974a). It has moreover been demonstrated that 5α-dihydrotestosterone is more potent than testosterone in curbing serum gonadotrophins in adult male rats (Swerdloff et al. 1972; Verjans et al. 1974a) and in preventing weight decrement of the accessory reproductive organs following gonadectomy (Verjans et al. 1974a). Testosterone the prevailing androgen in systemic blood is metabolized extensively and 5α-reduction of this hormone might be necessary for androgen regulation of the hypothalamic-pituitary system and for maintaining weight of accessory reproductive organs. These tissues contain 5α-steroid reductase activity (Bruchovsky and Wilson 1968; Rommerts and

van der Molen 1971; Massa et al. 1972). Metabolism of 5α-reduced testosterone may, however, occur in these organs. Furthermore, 5α-reduction of testosterone is not the only metabolic route for this steroid. In light of these possibilities and in order to assess steroid structure needed for "androgenic activity" in the hypothalamic-pituitary and the ventral prostate systems, we have studied the effect of administering a standard dose of various compounds from the androstene, 5α-androstane, 5β-androstane, oestrene and oestratriene series on serum levels of gonadotrophins and on weights of the ventral prostate in gonadectomized, mature male rats.

MATERIALS AND METHODS

Adult male Wistar rats (3-4 months old) with body weights ranging from 250 to 320 g were used. The animals were kept under controlled light (14 hrs light, 10 hrs darkness) and temperature (19-21°C) conditions. Rat chow and tap water were provided ad libitum.

The animals were gonadectomized under light ether anaesthesia. Daily subcutaneous injections of the different compounds in sesame oil (0.04 ml/100 g b.w.) in a standard dose of 100 µg/100 g b.w. were started immediately following operation and continued for the next six days. Control intact and castrated animals received sesame oil only. Intact control animals were exposed to 10 min of light ether anaesthesia prior to the first injection with sesame oil. Injections were given between 11 a.m. and 1 p.m. 4-Androstene-7α,17β-diol-3-one and 4-androstene-17β,19-diol-3-one were a gift from Merck, Sharp & Dohme.

The other steroids used were purchased from Steraloids. (±)1,4-Diphenyl-butane-2,3-diol was a gift of Dr. R. Neher. All compounds were used without purification. Twenty hours

after the last injection the rats were weighed and then decapitated under ether anaesthesia. Blood samples were collected and allowed to clot overnight at 4°C. Resulting blood serum was stored at -20°C until analysed for serum LH and FSH levels by double antibody radioimmunoassay techniques as previously described (Verjans et al. 1974a; Welschen et al. 1975). Serum LH and FSH concentrations were expressed on the basis of the reference preparations NIAMD rat LH RP-1 and NIAMD rat FSH RP-1 respectively. Only sample values from a single assay have been compared. Ventral prostates were dissected and weighed shortly after the animals were sacrificed.

R E S U L T S

Effects of steroid treatments on serum gonadotrophin levels

Gonadectomy results in a substantial rise of serum LH and FSH levels seven days post surgery (Tables 1-4). Table 1 shows effects of steroids from the androstene series on serum gonadotrophin levels. In the tested dose of 100 µg/100 g b.w./day for seven days only 4-androsten-17β-ol-3-one (testosterone) suppressed serum FSH below castrate control levels, while serum LH also tended to drop following this treatment. Neither of the trophins reached intact control levels employing this dose of testosterone (Table 1).

Treatment with 4-androstene-3β,17β-diol or 4-androsten-17α-ol-3-one augmented serum LH levels over castrate controls given vehicle only, but did not affect circulating FSH. In Table 2 effects of different 5α-androstanes on serum gonadotrophin levels in gonadectomized male rats are summarized. Serum LH levels in castrates were depressed by 5α-androstan-3α-ol-17-one, 5α-androstane-3α,17β-diol and

Table 1

Effect of various androstenes (100 µg/100 g b.w./day for seven days s.c.) on ventral prostate weight (mg/100 g b.w.) and serum gonadotrophin levels (ng/ml) in gonadectomized, mature male rats. Mean data ± S.D. ($n \geq 3$) are expressed as % of castrate control values (=100%)

Compound	Ventral prostate	Serum LH	Serum FSH
4-androstene-3β,17α-diol	97 ± 27	114 ± 31	128 ± 13
4-androstene-3β,17β-diol	363 ± 35	165 ± 35	96 ± 12
4-androstene-3β,6β,17β-triol	100 ± 10	104 ± 31	104 ± 24
4-androstene-3β,6β-diol-17-one	75 ± 30	121 ± 21	122 ± 10
4-androstene-6β,17β-diol-3-one	117 ± 22	95 ± 39	93 ± 11
4-androstene-7α,17β-diol-3-one	121 ± 17	99 ± 7	107 ± 15
4-androstene-11β,17β-diol-3-one	194 ± 70	86 ± 40	98 ± 12
4-androsten-17α-ol-3-one	149 ± 24	138 ± 9	109 ± 42
4-androsten-17β-ol-3-one	375 ± 30	60 ± 33	58 ± 13
4-androstene-17β,19-diol-3-one	78 ± 32	130 ± 22	90 ± 10
4-androstene-3,17-dione	272 ± 50	134 ± 19	105 ± 9
5-androstene-3β,17β-diol	230 ± 10	120 ± 47	114 ± 15
5-androsten-3β-ol-17-one	172 ± 13	133 ± 82	112 ± 32
intact, sesame oil	350 ± 12	20 ± 5	27 ± 6
castrate, sesame oil	100 ± 26	100 ± 18	100 ± 17

Table 2

Effect of various 5α-androstanes (100 µg/100 g b.w./day for seven days s.c.) on ventral prostate weight (mg/100 g b.w.) and serum gonadotrophin levels (ng/ml) in gonadectomized, mature male rats. Mean data ± S.D. ($n \geq 3$) are expressed as % of castrate control values (=100%)

Compound	Ventral prostate	Serum LH	Serum FSH
5α-androstane	218 ± 48	124 ± 3	90 ± 23
5α-androstan-3α-ol	118 ± 3	87 ± 36	91 ± 18
5α-androstan-3α-ol-17-one	260 ± 5	58 ± 9	84 ± 13
5α-androstane-3α,17β-diol	363 ± 99	< 5	23 ± 3
5α-androstane-3α,11β,17β-triol	314 ± 83	107 ± 69	109 ± 18
5α-androstane-3α,16α,17β-triol	81 ± 30	143 ± 30	111 ± 7
5α-androstan-3β-ol	146 ± 18	115 ± 26	118 ± 18
5α-androstan-3β-ol-17-one	189 ± 28	96 ± 18	96 ± 4
5α-androstane-3β,17β-diol	110 ± 18	83 ± 35	70 ± 8
5α-androstane-3β,11β,17β-triol	116 ± 32	117 ± 59	108 ± 9
5α-androstan-17β-ol	205 ± 45	86 ± 33	84 ± 7
5α-androstan-17β-ol-3-one	369 ± 10	< 5	36 ± 10
5α-androstan-3-one	143 ± 27	115 ± 27	117 ± 12
5α-androstane-3,17-dione	343 ± 32	40 ± 5	98 ± 16
intact, sesame oil	360 ± 15	22 ± 6	31 ± 5
castrate, sesame oil	100 ± 23	100 ± 19	100 ± 18

Table 3

Effect of various 5β-androstanes (100 µg/100 g b.w./day for seven days s.c.) on ventral prostate weight (mg/100 g b.w.) and serum gonadotrophin levels (ng/ml) in gonadectomized, mature male rats. Mean data ± S.D. ($n \geq 3$) are expressed as % of castrate control values (=100%)

Compound	Ventral prostate	Serum LH	Serum FSH
5β-androstane	108 ± 4	108 ± 35	98 ± 25
5β-androstan-3α-ol	145 ± 29	109 ± 49	89 ± 9
5β-androstan-3α-ol-17-one	121 ± 28	80 ± 25	98 ± 8
5β-androstane-3α,17β-diol	90 ± 29	86 ± 30	70 ± 15
5β-androstan-17β-ol	87 ± 25	140 ± 21	94 ± 6
5β-androstan-17β-ol-3-one	149 ± 25	104 ± 12	95 ± 9
5β-androstan-17-one	137 ± 26	114 ± 22	91 ± 10
intact, sesame oil	381 ± 40	25 ± 7	33 ± 5
castrate, sesame oil	100 ± 23	100 ± 23	100 ± 18

Table 4

Effect of various oestrenes and oestratrienes (100 µg/100 g b.w./day for seven days s.c.) on ventral prostate weight (mg/100 g b.w.) and serum gonadotrophin levels (ng/ml) in gonadectomized, mature male rats. Mean data ± S.D. ($n \geq 3$) are expressed as % of castrate control values (=100%)

Compound	Ventral prostate	Serum LH	Serum FSH
4-oestren-17β-ol-3-one	177 ± 55	88 ± 21	107 ± 15
5(10)-oestrene-3α,17β-diol	138 ± 18	146 ± 10	162 ± 21
5(10)-oestren-17β-ol-3-one	140 ± 27	19 ± 11	84 ± 17
1,3,5(10)-oestratrien-3-ol	129 ± 35	24 ± 5	37 ± 7
1,3,5(10)-oestratrien-3-ol-17-one	108 ± 6	14 ± 6	55 ± 6
1,3,5(10)-oestratriene-3,16α,17β-triol	141 ± 12	14 ± 1	36 ± 4
1,3,5(10)-oestratriene-3,17α-diol	94 ± 39	26 ± 9	42 ± 19
intact, sesame oil	350 ± 12	25 ± 7	31 ± 5
castrate, sesame oil	100 ± 25	100 ± 23	100 ± 18

5α-dihydrotestosterone. The steroids used from the 5β-androstane series had no effect on circulating gonadotrophins in this animal preparation at the dose used (Table 3). Table 4 demonstrates that all oestratrienes tested suppressed serum gonadotrophins to levels comparable to those of intact, control animals, except for the 1,3,5(10)-oestratrien-3-ol-17-one effect on serum FSH. Of the oestrenes tried (Table 4) 4-oestren-17β-ol-3-one exhibited no influence on the high serum gonadotrophin levels of gonadectomized rats. The postcastration rise of serum LH, but not of serum FSH was prevented by 5(10)-oestren-17β-ol-3-one injections. Administration of 5(10)-oestrene-3α,17β-diol resulted in elevation of both serum LH and FSH over castrate control levels (Table 4). In addition, treatment with a standard dose of 100 µg/100 g b.w./day for seven days of 4-pregnene-11β,21-diol-3,20-dione, 5α-pregnane-17α,21-diol-3,20-dione, 5α-pregnan-3-one, 5α-pregnane-3,20-dione, 5α-cholestan-3α-ol, 5β-cholestan-3α-ol or 1,4-diphenyl-butane-2,3-diol did not depress serum LH and FSH levels in gonadectomized, adult male rats (data not shown).

Effects of steroid treatments on ventral prostate weights

Significant decrement of ventral prostate weights could be recorded seven days postgonadectomy (Tables 1-4). When administered in the daily standard dose of 100 µg/100 g b.w. during seven days testosterone and 4-androstene-3β, 17β-diol most adequately prevented this weight decrease (Table 1), and maintained prostatic weight at levels encountered in intact animals (Table 1). The 17α-epimers or the 6β-hydroxylated derivatives of these steroids, however, showed no androgenic potency in this respect (Table 1). Of the compounds tested in this series (Table 1), some androgenic activity on the ventral prostate was exerted by 4-androstene-3,17-dione, 5-androstene-3β,17β-diol and 5-androsten-3β-ol-17-one. 5α-Androstane-3α,17β-diol, 5α-

androstane-3α,11β,17β-triol, 5α-dihydrotestosterone and 5α-androstane-3,17-dione maintained ventral prostate weights following castration at values found in intact animals, while 5α-androstane, 5α-androstan-3α-ol-17-one, 5α-androstan-3β-ol-17-one and 5α-androstan-17β-ol were less effective (Table 2). Daily subcutaneous treatment (100 µg/100 g b.w. for seven days) with 5α-androstan-3α-ol, 5α-androstane-3α,16α,17β-triol, 5α-androstan-3β-ol, 5α-androstane-3β,17β-diol, 5α-androstane-3β,11β,17β-triol or 5α-androstan-3-one had no effect on the falling ventral prostate weights in castrated animals (Table 2). Compounds from the 5β-androstane series showed no androgenic action as evaluated by this bioassay method (Table 3). This was also the case for steroids of the oestrene and oestratriene series (Table 4). In addition, administration of 4-pregnene-11β,21-diol-3,20-dione, 5α-pregnane-17α,21-diol-3,20-dione, 5α-pregnan-3-one, 5α-pregnane-3,20-dione, 5α-cholestan-3α-ol, 5β-cholestan-3α-ol or 1,4-diphenyl-butane-2,3-diol (100 µg/100 g b.w./day for seven days) could not counteract the decrement in ventral prostate weight following castration (data not shown).

D I S C U S S I O N

Provided that the steroids injected are released from the injection site at the same rate and are undergoing metabolism at this site to the same extent, it is clear that 6β-hydroxylation, 7α-hydroxylation, 11β-hydroxylation, 19-hydroxylation or 17 epimerization of 4-androsten-17β-ol-3-one (testosterone) are associated with loss of androgenic activity at the hypothalamic-pituitary axis and in the ventral prostate (Table 1). Noteworthy is the fact that 4-androstene-3β,17β-diol appears to elevate serum LH, but not serum FSH over castrate control levels (Table 1). This

positive feedback effect, affecting serum FSH levels too, can also be obtained employing lower doses of this compound (unpublished data). 4-Androstene-3β,17β-diol exerts, however, potent androgenicity as evaluated by preventing weight decrement of the ventral prostate in castrated animals (Table 1). If this steroid were predominantly converted to testosterone in our experiments in vivo, one would expect to measure depression of circulating gonadotrophins (Table 1). If it were converted to 5α-androstane-3β,17β-diol one would not expect to measure gain in ventral prostatic weight (Table 2). We have, however, observed that administration of lower doses of 5α-androstane-3β,17β-diol than used in this work, results in positive feedback effects on circulating gonadotrophins in castrated male rats (unpublished data). Whether 4-androstene-3β,17β-diol has any physiological importance in regulating male reproductive function is unknown, but the results obtained warrant further exploration. As for testosterone, 17 epimerization or 6β-hydroxylation of 4-androstene-3β,17β-diol are associated with loss of activity on the prostate (Table 1). Precursors of testosterone, such as 4-androstene-3,17-dione, 5-androstene-3β,17β-diol or 5-androsten-3β-ol-17-one had no effect on serum gonadotrophins in the dose tested but the former two compounds were rather potent in preventing weight decrease of the ventral prostate postcastration (Table 1). Such data might indicate that the processes involved in regulating the hypothalamic-pituitary unit and accessory reproductive organs by steroids are not parallel. Whether this difference resides at a receptor or at an organ metabolic level remains for future work to decide.

Steroids from the 5α-androstane series substituted with a 3α-hydroxyl or a 3-keto but containing a 17β-hydroxyl or a 17-keto group most effectively suppressed serum LH levels in gonadectomized rats and with the exception of 5α-androstane-3,17-dione and 5α-androstan-3α-ol-17-one, also serum FSH levels (Table 2). In previously published work we have demonstrated that lower doses of 5α-dihydrotestosterone

or 5α-androstane-3α,17β-diol are required for suppression of serum LH than for suppression of serum FSH in castrated, mature male rats (Verjans and Eik-Nes 1975b). Compounds of the 5α-androstane series without substitution or with only one substituent group in 3, 3α, 3β or 17β position and 3β-hydroxyl 17-disubstituted 5α-androstanes were unable to influence serum gonadotrophins in castrated rats (Table 2). Of some interest is the observation that 11β- or 16α-hydroxylation of 5α-androstane-3α,17β-diol renders this compound inactive pertinent to gonadotrophin regulation (Table 2). Since the respective 5β-epimers of the potent 5α-androstanes failed to affect serum gonadotrophins (Table 3), 5α-reduced testosterone metabolites substituted in 3 or 3α and 17 or 17β position are probably of importance for regulating circulating gonadotrophins. Rat brain tissue of males contains 5α-steroid reductase as well as 3α- and 17β-hydroxysteroid dehydrogenase activity (Rommerts and van der Molen 1971).

It is therefore possible that 5α-androstane-3α,17β-diol, 5α-androstane-3,17-dione and 5α-androstan-3α-ol-17-one exert biological effects after being converted to 5α-dihydrotestosterone. The slightly higher potency of 5α-androstane-3,17-dione than that of 5α-androstan-3α-ol-17-one in suppressing circulating LH levels might favor this suggestion. Aromatization of ring A in steroids seems therefore not to be required in order to obtain such regulation, since 5α-androstanes cannot be converted to oestrogens in vertebrate animals. At the dose employed 3β-hydroxylation of 5α-dihydrotestosterone is associated with no androgenic activity (Table 2), 3β-hydroxysteroid dehydrogenase activity appears, however, to be low, because 5α-androstane-3β,17β-diol did not show negative feedback effects on circulating gonadotrophins in the dose administered (Table 2).

5α-Androstane-3α,17β-diol, 5α-androstane-3α,11β,17β-triol, 5α-androstane-3,17-dione and 5α-dihydrotestosterone exerted potent androgenicity as evaluated by weight of the ventral prostate (Table 2). The presence of only one sub-

stituent group in the 5α-androstane molecule, either in 3, 3α or 3β position, decreased the androgenic activity as compared to 5α-androstane. Additional 17-keto substitution, however, increased this potency (Table 2). The influence of only a 17β-hydroxy group of 5α-androstane appeared to be larger than substitution with only a 3α- or a 3β-hydroxyl group (Table 2). 3β-Epimerization of 5α-androstane-3α,17β-diol and of 5α-androstane-3α,11β,17β-triol rendered these compounds completely inactive in maintaining ventral prostate weights (Table 2). And, unlike 11β-hydroxylation, 16α-hydroxylation of 5α-androstane-3α,17β-diol drastically decreased the potency of the compound in this respect (Table 2). These observations might indicate differences in the regulatory processes for the two organs evaluated. The 5β-androstanes tested lost the biological activity exhibited by their respective 5α-epimers (Table 3). Bruchovsky (1971) showed that radioactive 5α-dihydrotestosterone was the major metabolite in prostatic nuclei following intravenous injection of radioactive testosterone, 5α-dihydrotestosterone, 5α-androstan-3α-ol-17-one, 5α-androstane-3α,17β-diol, 5α-androstane-3,17-dione, 4-androstene-3,17-dione or 5-androsten-3β-ol-17-one in castrated male rats. The experiments of Bruchovsky (1971) show that all relevant enzyme activities for conversion of these compounds to 5α-dihydrotestosterone are present in the rat. Furthermore, 5α-dihydrotestosterone was the principal steroid bound to androgen receptor in the rat prostate following administration of radioactive testosterone (Bruchovsky and Wilson 1968). Our data (Tables 1 and 2) corroborate these findings since in no case significant biological activity of 5β-androstanes (Table 3) was observed.

Evaluating the effects of the oestratrienes (Table 4), we extended our previous findings that oestrogen will exert potent influence on circulating LH and FSH levels in intact and castrated, adult male rats (Verjans et al. 1974a, 1974b). The current results indicate that the presence of only a phenolic A-ring is sufficient to afflict serum gona-

dotrophin levels in gonadectomized animals, which seems to reduce the importance of 17α- or 17β-hydroxyl substitution in such molecules in order to be effective.

In addition, we found that administration of 5,6,7,8-tetra-hydro-β-naphthol (100 µg/100 g b.w./day given s.c. for seven days) did not mimic the effects exerted by oestrogens on serum gonadotrophins (unpublished data). This could indicate that the presence of the C- and D-ring in the oestrogens is necessary for depressing circulating gonadotrophins in the animal preparation used. The oestratrienes and oestrenes did not affect the falling ventral prostate weights in castrated male rats (Table 4), but 1,3,5(10)-oestratrien-3-ol, 1,3,5(10)-oestratriene-3,16α,17β-triol, 1,3,5(10)-oestratriene-3,17α-diol and 5(10)-oestrene-3α, 17β-diol could partly prevent the decrement of seminal vesicles weights in such animals (unpublished data). Unlike 4-oestren-17β-ol-3-one, 5(10)-oestren-17β-ol-3-one exerted potent activity at least on serum LH (Table 4). It might be that this shift in the double binding creates a structure more resembling the aromatic structure in the A-ring of physiological oestrogens. It should be noted that 5(10)-oestrene-3α,17β-diol has a positive feedback action on both serum gonadotrophins (Table 4). Treatment with pregnanes and cholestanes had no effect on the parameters investigated, while 1,4-diphenyl-butane-2,3-diol, which is present in the testis from mature rats (Neher 1963) and is also secreted into the spermatic venous blood of the gonadotrophic stimulated dog (Eik-Nes et al. 1967), exhibited only some activity in preventing the seminal vesicles weight decrease in castrates (unpublished data). The failure of 4-pregnene-11β,21-diol-3,20-dione (corticosterone) to affect serum gonadotrophins and the falling ventral prostate weight in castrates is in accordance with previously published work (Verjans and Eik-Nes 1975c) in which we showed that dexamethasone treatment did not affect these parameters in castrates. Thus adrenocortical steroids are not of major importance in regulating circulating gonado-

trophin levels and ventral prostate growth.

A C K N O W L E D G M E N T S

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SERUM LH AND FSH LEVELS FOLLOWING INTRAVENOUS INJECTION OF A GONADOTROPHIN RELEASING PRINCIPLE IN NORMAL AND GONADECTOMIZED, ADULT MALE RATS TREATED WITH OESTRADIOL-17β OR 5α-DIHYDROTESTOSTERONE

By

H.L. Verjans and K.B. Eik-Nes

A B S T R A C T

Effect of intravenous administration of a synthetic gonadotrophin releasing factor (GnRF) on circulating LH and FSH concentrations was investigated in normal and gonadectomized, adult male rats injected subcutaneously each day during seven days with various doses of oestradiol-17β or 5α-dihydrotestosterone in sesame oil. Higher increase in serum LH and FSH levels subsequent to intravenously administered GnRF was observed in castrated control animals than in intact control animals, though this increment was not of significant nature for serum FSH. Pretreatment of normal and gonadectomized rats with oestradiol-17β resulted in an augmented response of serum LH and FSH concentrations to GnRF i.v. Pretreatment of normal and gonadectomized rats with 5α-dihydrotestosterone diminished serum LH and FSH response following administration of the same amount of GnRF. For these steroids to affect the response pattern of serum LH and FSH to GnRF i.v., higher doses were required in normal than in gonadectomized animals. The data indicate that oestrogen and androgen may act at different sites in the male hypothalamic-pituitary axis with respect to regulation of pituitary gonadotrophins.

I N T R O D U C T I O N

Synthesis and release of the gonadotrophins LH and FSH from the anterior pituitary gland are mediated by releasing material produced in the hypothalamus (for review: Schally et al. 1973a) and circulating gonadotrophins regulate and maintain testicular functions in the adult male animal. Testicular steroids have, in turn, been shown to influence

circulating concentrations of the gonadotrophins in normal and gonadectomized, adult male rats. Oestradiol-17β, 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol exhibit higher potency than testosterone in preventing the drastic increase of serum LH and FSH concentrations following gonadectomy in adult male rats (Verjans and Eik-Nes 1975a). In these experiments circulating LH appeared more sensitive to steroid treatment than circulating FSH. It has been demonstrated that testicular steroids can modulate the response in vivo to exogenous GnRF in intact (Debeljuk et al. 1972; Debeljuk et al. 1974; Hopkinson et al. 1974; Galloway et al. 1974) and orchidectomized, adult male animals (Debeljuk et al. 1973; Debeljuk et al. 1974; Pelletier 1974; Smith and Davidson 1974; Verjans et al. 1974a). Modulatory effects of steroid hormones on GnRF induced release of gonadotrophins in vitro by pituitary tissue from male animals have also been reported (Schally et al. 1973b; Spona 1974; Kao and Weisz 1975). We have studied the effect of treatment with an oestrogen or a non-aromatizable androgen on GnRF induced levels of LH and FSH in the blood serum of normal and gonadectomized, adult male rats in order to assess the modulatory effect of these steroids on gonadotrophin response in an experimental animal model which has been characterized in previously published work (Verjans et al. 1974a,b; Verjans et al. 1975; Verjans and Eik-Nes 1975a,b).

MATERIALS AND METHODS

Animals

Adult, male Wistar rats (3-4 months old) were used. The animals were kept under controlled light (14 hrs light and 10 hrs darkness) and temperature (19-21^{o}C) conditions.

Laboratory chow and tap water were provided ad libitum.

Experiment I. - From fifteen rats of the same age (body weights ranging from 260 to 290 g) blood samples (1.2 - 1.5 ml) were drawn around 9 a.m. by puncturing the ophthalmic venous plexus under ether anaesthesia. Synthetic gonadotrophin releasing factor (GnRF, Abbott Laboratories, North Chicago, Illinois, U.S.A.) in doses of 1,000, 500, 250 or 50 ng, dissolved in 0.1 ml 0.9% sodium chloride solution, was then injected via the jugular vein under ether anaesthesia. Control animals received 0.1 ml saline only via this route. Fifteen min after such injection, approximately 1.5 ml blood was removed by puncturing the ophthalmic venous plexus under ether anaesthesia. All injections were given to groups of three rats.

Experiment II. - One group of animals with body weights varying from 310 to 370 g was gonadectomized (Verjans et al. 1974a), another group of the same age with body weights ranging from 335 to 380 g was employed as intact animals. Daily subcutaneous injections with various doses of oestradiol-17β (Steraloids, Pawling, New York, U.S.A.) in sesame oil (0.04 ml oil/100 g b.w.) were given for a period of seven days between 1 and 2 p.m. to both animal groups. Control intact and control castrate animals received sesame oil only (0.04 ml/100 g b.w./day for seven days). All intact animals were exposed to 10 min of ether anaesthesia prior to the first injection with sesame oil or sesame oil containing oestradiol-17β. Oestrogen treatment of the orchidectomized rats started immediately following surgery. Twenty hours after the last injection, blood samples (1.2 - 1.5 ml) were obtained at 9 a.m. by puncturing the ophthalmic venous plexus under ether anaesthesia. Then 500 ng GnRF in 0.1 ml saline was administered via the jugular vein (see above). Intact and gonadectomized animals, injected with sesame oil for the seven preceding days, received as control 0.1 ml saline only via this route.

Fifteen min following administration of saline or saline containing 500 ng GnRF, all animals were bled again (approximately 1.5 ml) by puncturing the ophthalmic venous plexus under ether anaesthesia. Three or more rats were used for each treatment group.

Experiment III. - One group of animals with body weights ranging from 260 to 290 g was castrated, another group of rats of the same age with body weights varying from 260 to 275 g was employed as intact animals. Daily subcutaneous treatment of intact and orchidectomized animals with various doses of 5α-dihydrotestosterone (Steraloids, Pawling, New York, U.S.A.) in sesame oil or with this vehicle only and injection of GnRF followed the protocol used in experiment II. Three or more rats were used for each treatment group.

Radioimmunoassay methods for blood serum LH and FSH concentrations

All blood samples from the three experiments were allowed to clot overnight at 4°C. Resulting blood serum was stored at -20°C until analysed for serum LH and FSH by double antibody radioimmunoassay techniques as previously described (Verjans et al. 1974a; Welschen et al. 1975). Serum LH and FSH concentrations were expressed on the basis of the reference preparations NIAMD rat LH RP-1 and NIAMD rat FSH RP-1 respectively. Serum samples from each experiment were measured in duplicate at the same volume level in a single assay and only sample values thus obtained have been compared. Significance of differences between the gonadotrophin responses following intravenous administration of GnRF in the different animal groups was determined using Duncan's new multiple-range test (Duncan 1955). In all experiments changes in blood serum levels of LH and FSH were calculated by subtracting the resting gonadotrophin levels

(measured just before administration of GnRF or saline) from the gonadotrophin levels determined 15 min after injection of GnRF or saline. Whenever circulating LH concentrations were depressed to undetectable values following steroid treatment, we used an LH level of 7 ng/ml serum (the detection limit of the radioimmunoassay employed) in order to calculate the LH response following GnRF i.v.

R E S U L T S

Normal rats

Effects of intravenous administration of increasing doses of synthetic GnRF on circulating levels of LH and FSH in intact, adult male rats measured 15 min following intravenous injection of the gonadotrophin releasing principle are shown in Table 1. GnRF augmented both serum LH and FSH in a dose-dependent way, 500 ng GnRF i.v. appeared to give adequate stimulation of both trophins (Table 1) and was used as the standard dose for the rest of the study. Intravenous injection of saline tended to reduce the resting gonadotrophin levels (Table 1).

Oestradiol treated rats

Daily treatment of intact rats with 5 µg or 0.5 µg oestradiol/100 g b.w. for seven days depressed circulating LH to undetectable levels and mean circulating FSH to respectively 64% ± 17 (S.D.) and 92% ± 39 (S.D.) of intact control levels (100% ± 9 (S.D.)). Administration of 0.05 µg oestradiol resulted in circulating FSH levels slightly higher than those of intact control animals injected with sesame oil only. In gonadectomized rats treatment with 5 µg oestradiol reduced circulating LH to undetectable levels,

Table 1

Effect of various doses of intravenously administered synthetic GnRF on serum LH and FSH levels in intact, adult male rats (n=3)

Treatment	Difference (mean ± S.D.) between serum levels of LH and FSH measured before and 15 min after injection of 0.1 ml saline or 0.1 ml saline containing GnRF	
	LH[x]	FSH[xx]
1,000 ng GnRF	+ 587 ± 181	+ 303 ± 43
500 ng GnRF	+ 488 ± 144	+ 233 ± 12
250 ng GnRF	+ 258 ± 151	+ 142 ± 39
50 ng GnRF	+ 96 ± 21	+ 79 ± 8
saline	- 15 ± 14	- 10 ± 52

[x] expressed as ng NIAMD rat LH RP-1/ml serum

[xx] expressed as ng NIAMD rat FSH RP-1/ml serum

while the doses of 0.5 µg or of 0.05 µg oestradiol suppressed mean circulating LH levels to values respectively 11% ± 2 (S.D.) and 81% ± 9 (S.D.) of castrate control levels (100% ± 14 (S.D.)). Mean circulating FSH in the castrates decreased to 28% ± 1 (S.D.) and 62% ± 12 (S.D.) of castrate control levels (100% ± 6 (S.D.)) following daily administration of 5 or 0.5 µg oestradiol respectively. The dose of 0.05 µg oestradiol slightly augmented circulating FSH levels over FSH levels encountered in castrate controls receiving oil only.

The effects of treatment with oestradiol on GnRF induced serum concentrations of LH and FSH in intact and gonadecto-

Table 2

Effect of treatment with various doses of oestradiol-17β (μg/100 g b.w./day for seven days s.c.) on increment of serum gonadotrophins following 500 ng intravenously administered synthetic GnRF in intact and gonadectomized, adult male rats ($n \geq 3$)

Treatment	Increment (mean ± S.D.) in serum LH and FSH 15 min after injection of 500 ng GnRF in 0.1 ml saline	
	LH^{x}	FSH^{xx}
Intact, 5 μg oestradiol-17β	785 ± 318^{xxx}	596 ± 38^{xxx}
intact, 0.5 μg oestradiol-17β	357 ± 106	405 ± 228
intact, 0.05 μg oestradiol-17β	352 ± 91	233 ± 10
intact, sesame oil	338 ± 12	251 ± 133
Castrate, 5 μg oestradiol-17β	2,057 ± 403^{xxxx}	1,087 ± 415^{xxxx}
castrate, 0.5 μg oestradiol-17β	1,856 ± 99^{xxxx}	960 ± 254^{xxxx}
castrate, 0.05 μg oestradiol-17β	1,038 ± 234	643 ± 232
castrate, sesame oil	958 ± 145	330 ± 135

x expressed as ng NIAMD rat LH RP-1/ml serum

xx expressed as ng NIAMD rat FSH RP-1/ml serum

xxx $P < 0.05$, compared with mean increment in sesame oil treated intact animals following GnRF

xxxx $P < 0.05$, compared with mean increment in sesame oil treated castrated animals following GnRF

mized male rats are summarized in Table 2. GnRF i.v. in a dose of 500 ng resulted in elevated levels of LH and FSH in castrates treated with sesame oil (Tables 2 and 3). Intravenous injection of saline in intact and castrated rats treated for seven days with sesame oil only gave slightly lower circulating gonadotrophin levels than those measured just before saline i.v. (data not shown). The increment in serum LH following GnRF i.v. in castrate controls was significantly higher ($P < 0.01$) than in intact rats treated for seven days with sesame oil (Tables 2 and 3). Augmentation in serum FSH in castrated control rats was, however, not significantly higher compared with intact control rats given the same amount of GnRF i.v. (Tables 2 and 3). A higher ($P < 0.05$) LH and FSH increase was measured in intact rats treated with 5 µg oestradiol compared with the increases observed in circulating gonadotrophins in oil treated intact rats 15 min subsequent to injection of 500 ng GnRF i.v. (Table 2). Treatment with doses of 0.5 µg and 0.05 µg oestradiol had no influence on GnRF induced serum concentrations of LH and FSH in intact animals (Table 2). Augmented LH and FSH response ($P < 0.05$) to GnRF i.v. was, however, observed in castrates treated with 5 µg or 0.5 µg oestradiol, while treatment with the dose of 0.05 µg oestradiol did not affect gonadotrophin response in castrates following administration of GnRF i.v. (Table 2).

5α-Dihydrotestosterone treated rats

Circulating concentrations of LH in intact rats were depressed to undetectable values and to 56% ± 8 (S.D.) of intact control levels (100% ± 34 (S.D.)) following daily administration of 5α-dihydrotestosterone in doses of 100 or 10 µg/100 g b.w. for seven days respectively. Treatment with 1 µg 5α-dihydrotestosterone did not affect circulating LH levels and doses of 1 µg and 10 µg of this androgen were without effect on circulating FSH levels compared with

Table 3

Effect of treatment with various doses of 5α-dihydrotestosterone (μg/100 g b.w./day for seven days s.c.) on increment of serum gonadotrophins following 500 ng intravenously administered synthetic GnRF in intact and gonadectomized, adult male rats ($n \geq 3$)

Treatment	Increment (mean ± S.D.) in serum LH and FSH 15 min after injection of 500 ng GnRF in 0.1 ml saline	
	LH[x]	FSH[xx]
Intact, 100 μg 5α-dihydrotestosterone	84 ± 6[xxx]	121 ± 21[xxx]
intact, 10 μg 5α-dihydrotestosterone	240 ± 62	265 ± 42
intact, 1 μg 5α-dihydrotestosterone	297 ± 137	166 ± 117
intact, sesame oil	465 ± 178	262 ± 59
Castrate, 100 μg 5α-dihydrotestosterone	182 ± 61[xxxx]	218 ± 60[xxxx]
castrate, 10 μg 5α-dihydrotestosterone	587 ± 58[xxxx]	268 ± 72
castrate, 1 μg 5α-dihydrotestosterone	1,132 ± 516	438 ± 192
castrate, sesame oil	1,152 ± 310	397 ± 129

[x] expressed as ng NIAMD rat LH RP-1/ml serum

[xx] expressed as ng NIAMD rat FSH RP-1/ml serum

[xxx] $P < 0.05$, compared with mean increment in sesame oil treated intact animals following GnRF

[xxxx] $P < 0.05$, compared with mean increment in sesame oil treated castrated animals following GnRF

intact controls receiving vehicle only. Only treatment with the dose of 100 µg 5α-dihydrotestosterone resulted in lower average serum FSH levels (69% ± 3 (S.D.)) compared with mean FSH levels in intact control rats injected with sesame oil (100% ± 17 (S.D.)).

In castrates, 100 µg 5α-dihydrotestosterone lowered circulating LH to undetectable values and mean circulating FSH to 34% ± 4 (S.D.) of castrate control levels (100% ± 21 (S.D.)). Daily administration of 10 or 1 µg 5α-dihydrotestosterone/100 g b.w. for seven days did not change serum LH and FSH levels compared with castrate controls.

Table 3 shows effects of intravenous administration of 500 ng GnRF on serum levels of LH and FSH in normal and gonadectomized, adult male rats treated with various doses of 5α-dihydrotestosterone during the seven preceding days. Treatment of intact rats with 100 µg 5α-dihydrotestosterone resulted in a lower LH and FSH response ($P < 0.05$) to GnRF i.v. when compared with intact rats treated with sesame oil only (Table 3). Pretreatment of intact rats with 10 µg or 1 µg 5α-dihydrotestosterone had no significant effect on LH and FSH response to GnRF (Table 3). In castrates injected with 100 µg or 10 µg 5α-dihydrotestosterone, LH increment following GnRF i.v. was depressed ($P < 0.05$) as compared with oil treated control rats, while treatment with the dose of 1 µg 5α-dihydrotestosterone was without effect on the LH response (Table 3). FSH response was inhibited ($P < 0.05$) in castrates pretreated with 100 µg 5α-dihydrotestosterone, but treatment with doses of 10 µg or 1 µg of this androgen did not give such effects.

D I S C U S S I O N

Intravenous administration of GnRF resulted in elevated serum levels of LH and FSH in intact (Tables 1, 2 and 3)

and castrated (Tables 2 and 3) adult male rats. Furthermore, this gonadotrophin response was more pronounced in gonadectomized than in normal animals 15 min after i.v. injection of the gonadotrophin releasing principle (Tables 2 and 3). Percentage increase relative to resting gonadotrophin levels was greater for serum LH than for serum FSH as measured 15 min following the same dose of GnRF. The synthetic preparation of GnRF used exhibited higher LH and FSH releasing capacity than the one employed in a previous study (Verjans et al. 1974a). In that study, however, a different strain of rats was used. Both studies suffer certain shortcomings: handling of the animals, use of ether anaesthesia and frequent blood sampling. These conditions will influence circulating levels of LH and FSH (Krulich et al. 1974; Euker et al. 1975) and ether anaesthesia is known to depress testosterone secretion in rats (Bardin and Peterson 1967). Decapitation of intact rats exposed to ether vapour for 2 min results in slightly decreased LH, significantly increased FSH and unchanged testosterone serum concentrations compared with animals decapitated without ether anaesthesia (unpublished data). Such effects are not observed in castrated animals (unpublished data). The slightly lowered levels of LH and FSH found 15 min after 0.1 ml saline i.v. might therefore be caused by the standard procedure of animal treatment (Table 1). One may, moreover, question whether intact and short-term castrated rats really can be compared with regard to circulating gonadotrophin concentrations, owing to pleomorphism of rat FSH as observed in long-term castrated male rats (Bogdanove et al. 1974). At any rate, the circulating concentrations of the gonadotrophins are the ones reaching the target tissue(s).

Provided that peripheral gonadotrophin concentrations are true indicators of pituitary production and secretion of these hormones and that clearance rates of these hormones are not afflicted by the different treatments, it is abundantly clear from the present study that treatment with

high doses of 5α-dihydrotestosterone or oestradiol-17β, which will depress circulating LH and FSH concentrations in normal (Verjans et al. 1974b; Result section) and orchidectomized (Swerdloff et al. 1972; Kalra et al. 1973; Verjans et al. 1974a; Result section) rats, will alter serum gonadotrophin response to the same high dose of the same gonadotrophin releasing principle (Tables 2 and 3). Serum levels of LH and FSH following GnRF i.v. are, however, different in rats after seven days treatment with either a non-aromatizable androgen or an oestrogen. Exposure of intact and castrated male rats to high doses of 5α-dihydrotestosterone for seven days was associated with inhibited serum gonadotrophin response to exogenous GnRF i.v. (Table 3), while exposure to high doses of oestradiol-17β during the same period of time markedly enhanced this response in both type of animals (Table 2).

It is now well established that rat testis tissue contains measurable quantities of oestradiol-17β (de Jong et al. 1974) and of 5α-dihydrotestosterone (Folman et al. 1972). The male gonad can also secrete these steroid hormones and in the adult male rat mean plasma levels of oestradiol-17β and 5α-dihydrotestosterone are respectively 2 (de Jong et al. 1973) and 350 (Coyotupa et al. 1973) pg per ml. Testicular steroids can pass the blood-brain barrier since retention of testosterone (Resko et al. 1967), 5α-dihydrotestosterone (Pérez-Palacios et al. 1973) or oestradiol-17β (Green et al. 1969) in the hypothalamic-pituitary system can be recorded. Robel et al. (1973) showed that 5α-dihydrotestosterone is present in measurable quantities in hypothalamic and hypophyseal tissue of normal, adult male rats. Metabolism of radioactive androgens will occur in these tissues (Rommerts and van der Molen 1971; Massa et al. 1972; Naftolin et al. 1972; Genot et al. 1975). 5α-Reduction appears to be the predominant pathway for androgen metabolism in neuroendocrine cells (Naftolin and Ryan 1975) and oestradiol-17β is not likely to undergo biotransformation in the hypophysis and the hypothalamus

(Kato and Villee 1967; Kato 1975). 5α-Reduction of testosterone to 5α-dihydrotestosterone is irreversible, but reduction of 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol is reversible (Noma et al. 1975). 5α-Dihydrotestosterone cannot be converted to an oestrogen (for review: Engel 1973). In addition, it has been demonstrated that the hypophysis and hypothalamus of male rats contain specific binding principles for oestradiol-17β (Kato et al. 1974; Kato 1975), testosterone (Jouan et al. 1971, 1973) and 5α-dihydrotestosterone (Kato and Onouchi 1973 a,b). Testosterone is, however, quantitatively the main androgen in the peripheral circulation and thus probably the main precursor for 5α-dihydrotestosterone and oestradiol-17β formation in the hypothalamic-pituitary system. Higher concentrations of testosterone than of 5α-dihydrotestosterone are found in male hypothalamic and hypophyseal tissue (Robel et al. 1973). The possibility must, however, not be overlooked that 5α-dihydrotestosterone, its 3α- or 3β-reduced metabolites, or oestradiol-17β, mediated by their specific receptor binding molecules in the hypophysis and the hypothalamus (Kato and Onouchi 1973 a,b; Kato et al. 1974; Kato 1975), may direct physiological regulatory events at these sites.

Verjans et al. (1974a) reported decreased serum gonadotrophin response to GnRF i.v. in castrates treated with the propionates of testosterone or 5α-dihydrotestosterone compared with castrates treated with oestradiol benzoate. Debeljuk et al. (1972) found inhibitory effects of testosterone propionate injection on serum LH and FSH response following GnRF i.v. in intact animals, while 5α-dihydrotestosterone administration suppressed only serum LH response following GnRF i.v. in normal and orchidectomized rats (Debeljuk et al. 1974). Schally et al. (1973b) observed that testosterone or 5α-dihydrotestosterone in vitro inhibited LH and FSH discharge from male pituitary tissue following GnRF addition in vitro. Administration of 100 µg 5α-androstane-3α,17β-diol/100 g b.w./day during seven days

to castrated male rats resulted in a diminished serum gonadotrophin response to 500 ng GnRF i.v., while administration of the same dose of 4-androstene-3β,17β-diol to castrates did not mimic this effect (unpublished data). Furthermore, we have shown previously that 5α-androstane-3α, 17β-diol is at least as potent as 5α-dihydrotestosterone in curbing serum LH and FSH concentrations in orchidectomized, adult male rats (Verjans and Eik-Nes 1975a).

It is therefore possible that testosterone, most likely after conversion to its potent 5α-reduced androstane metabolites, modifies pituitary responsiveness to GnRF. This may mean that the anterior pituitary gland is the possible site of androgen regulatory effects on the hypothalamic-pituitary axis, a view also shared by Mittler (1972), Schally et al. (1973b) and Kingsley and Bogdanove (1973). We can, however, not exclude the possibility that in our experiments seven days treatment with this potent 5α-reduced testosterone metabolite may act on endogenous GnRF production and/or secretion. Altered endogenous GnRF could have changed pituitary sensitivity to a high dose of exogenous GnRF i.v. Low serum GnRF concentrations are found in adult male rats subsequent to hypophysectomy (Shin et al. 1974) and no increase in such levels can be determined five or eight days following gonadectomy (Shin and Howitt 1975). Stimulating effects of testosterone on GnRF in castrates have been observed (Shin et al. 1974) indicating that in addition to the anterior pituitary gland, the hypothalamus is also involved in the action of androgens. We are apt to conclude from these data that the high circulating gonadotrophin levels following orchidectomy may be induced by lack of testicular secretion products influencing release of LH and FSH by the hypophysis. Low doses of androgen injected in castrates may stimulate GnRF release resulting in elevation of serum gonadotrophin concentrations over castrate control levels (positive feedback)(Walsh et al. 1973; Verjans et al. 1975). Injection of high androgen doses will inhibit pituitary release of gonadotrophins, and

for both - high and low doses of androgen - LH release is more sensitive than that of FSH. In this respect it is interesting to note that Franchimont et al. (1975) observed a diminished response of serum FSH, but not of serum LH, to a gonadotrophin releasing preparation in intact and castrated, adult male rats pretreated with an inhibin-like factor isolated from bull seminal plasma. In X-ray treated male rats with significant increase of serum FSH levels only, an elevated response of serum FSH, but not of serum LH, can be observed 60 min after GnRF i.v. (Verjans and Eik-Nes 1975c). These data might indicate that inhibin, which may partly regulate FSH, possibly acts directly on the anterior hypophysis by modulating pituitary release of FSH only.

In contrast to the androgen treatment used, exposure during seven days to high doses of oestradiol-17β, which also depressed basal gonadotrophin levels in normal and castrated rats, enhanced serum gonadotrophin response to GnRF i.v. in normal and gonadectomized rats (Table 2). In order to induce this potentiating effect, lower doses of oestradiol-17β were required in castrate than in normal animals (Table 2). Furthermore, it is known that treatment with oestradiol-17β results in decreased testicular and peripheral concentrations of testosterone in intact male rats (Verjans et al. 1974b). This latter effect may not only be caused by suppressed LH levels, but also by a direct testicular influence of oestradiol-17β. It has been demonstrated that oestrogen depresses testicular testosterone formation by affecting enzymes involved in androgen biosynthesis (Samuels et al. 1969; Yanaihara et al. 1972) and that testicular interstitial tissue contains a specific receptor for oestradiol-17β (Brinkmann et al. 1972). In normal rats oestrogen treatment should result in low peripheral concentrations of testosterone, thus removing androgen inhibition of the hypothalamic-pituitary axis. The potentiating effect of oestrogen on serum gonadotrophin response following GnRF i.v. is, however, also observed in the castrate animal (Table 2). Augmentation of serum gonado-

trophin response to GnRF i.v. in male castrates subjected to high doses of oestrogen has been reported before (Debeljuk et al. 1973; Verjans et al. 1974a). Eshkol et al. (1975) observed that exposure of male pituitary glands in vitro to low doses of oestradiol-17β caused higher LH and FSH response to GnRF. When, however, higher doses of oestradiol-17β were employed in these studies, inhibited LH and FSH response to GnRF occurred thus confirming the work in vitro by Schally et al. (1973b). It is difficult to compare data obtained in vivo with data obtained in vitro, but the anterior pituitary gland could be a possible feedback site of action of oestradiol-17β and it is also likely that oestradiol-17β can alter pituitary sensitivity to GnRF and thereby change pituitary release of gonadotrophins. In our experiments changes in endogenous GnRF production and/or secretion following seven days exposure to high doses of oestradiol-17β could have occurred. This may alter pituitary responsiveness to a high dose of exogenous GnRF i.v. The fact that treatment with high doses of oestradiol-17β during seven days suppressed basal gonadotrophin concentrations in the peripheral circulation, but enhanced circulating gonadotrophin response to exogenous GnRF i.v. points to dual regulatory sites of oestrogen action in the hypothalamic-pituitary axis. A hypothalamic site of inhibitory action of oestradiol-17β on GnRF production and/or secretion is in this respect rather possible, though convincing evidence on this point is lacking. It is known that low doses of oestradiol-17β exert positive feedback effects on peripheral gonadotrophin levels in male castrates (Walsh et al. 1973; Kalra et al. 1973; Verjans et al. 1974a). Thus, low doses of oestradiol-17β administered to male castrates may render the anterior pituitary gland more sensitive to endogenous GnRF without affecting release or production of this material and may result in elevation of serum LH and FSH levels over castrate control levels (positive feedback). High doses of oestradiol-17β administered to castrated and normal animals may inhibit hypothalamic production and/or

release of GnRF. Such inhibition could render the anterior hypophysis more sensitive to high concentrations of exogenous GnRF. It is a matter of speculation, however, whether a major physiological role can be assigned to the minute circulating oestradiol-17β concentrations in normal male rats with respect to regulation of pituitary gonadotrophins (Gay and Dever 1971; de Jong et al. 1975).

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