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MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 1 March 2002 .....
.....[redacted].....
for Sec. Research Graduate School Committee

ERRATA

p vii line 17: "Cotomegalovirus" for "Cytomegalovirus"
p vii line 24: "Dimethyl suplhoxide" for "Dimethyl sulphoxide"
p 18 line 5: "see Romerts, 1988" for "see Rommerts, 1988"

ADDENDUM

p ii line 5: *Comment*: It is not known whether the negative regulation of 5αR-1 is a direct action of testosterone on the Leydig cells or via an indirect mechanism (see p.189, 201, 202).

p vii line 11: *Comment*: Bovine growth hormone written as BGH and not bGH according to the manufacturers (see Invitrogen catalogue).

p ix line 1: *Comment*: "4-ene-3-oxosteroid 5α-oxidoreductase" was included to provide a description of the chemical structure of the cofactor.

Fig 1-3: *Comment*: This figure does not intend to show that LH and FSH are produced in different lobes, and the text on p 4 says that both LH and FSH are produced in the anterior pituitary, not in different lobes of the pituitary.

p 5 lines 3-4: delete "the release of LH, and inhibin inhibits the secretion of pituitary FSH" and read "LHRH release and consequently also LH-release from the pituitary"

p 18 line 19: delete "that"

p 18 line 18: *Comment:* The high concentration of testosterone normally found in the testis (~100 ng/ml) is due to its local production, however, the process of spermatogenesis can be maintained at much lower concentrations (12-15 ng/ml). Interestingly, the androgen requirement in peripheral tissues is lower (~3-4 ng/ml, Corpechot et al., 1981). Thus, there is a peculiar requirement for high concentrations of testosterone in the testis compared to other tissues. The reason for this in unknown, but may be due to the low level of 5αR in the testis compared to many androgen-dependent peripheral tissues, conversion of testosterone to estrogen via the aromatase enzyme, or different requirements of cofactors for the androgen receptor in the testis.

Fig 1-6: delete "3α-Androstanediol" and read "5α-androstane-3α, 17β-diol" and delete 2-way arrow from testosterone to estradiol and replace with 1-way arrow.

p 32 line 2: delete "an unexpected for" and read "an unexpected finding for"

p 34 line 13: delete "Overall, rat 5αR-1 shares 47% sequence identity with human 5αR-1, and rat 5αR-2 shares 77% identity with the human 5αR-2 enzyme. Therefore, at the amino acid level the 5αR-2" and read "Overall, rat 5αR-1 and 5αR-2 share 44% sequence identity and human 5αR-1 and 5αR-2 share 49% sequence identity (Normington and Russell, 1992). Therefore, at the amino acid level the 5αR-1 and 5αR-2"

p 34: *Comment:* Jenkins et al (1991) located a pseudogene for 5αR-1 on the X chromosome in humans that lacked introns and contained a premature translation termination codon. This pseudogene is not believed to encode a functional protein because of the nonsense codon in place of the specifying amino acid 147. This pseudogene was absent in mice and based on the following observations we have inferred that there is no 5αR-1 pseudogene in the rat: (i) Northern analysis indicated that only one rat 5αR-1 transcript (at 2.4 kb) was present in liver and prostate (Andersson et al., 1989) (ii) Primer extension analysis of rat 5αR-1 RNA indicated the existence of a single transcription start site (Andersson et al., 1989; Lopez-Solache et al., 1996)(iii) Southern blot analysis of rat genomic DNA indicated the existence of only one 5αR-1 gene (Andersson et al., 1989) and (iv) to allow us to assess whether contaminating DNA was amplified in the RNA samples, the 5αR-1 primers pairs were made to span an intron. There was no RNA-size 128 bp amplicon when rat genomic DNA was amplified with the 5αR-1 primers in the absence of reverse transcriptase, indicating that a 5αRI pseudogene was not present in the testis (the pseudogene lacks introns and thus would act as template for the amplification of a 128 bp amplicon). Furthermore, the melting curve analysis from the LightCycler showed a single T_m, and when the 5αR-1 product was run an agarose gel there was a single band. This, together with subsequent sequencing of the 5αR-1 products from the LightCycler, suggests that a single 5αR-1 transcript was produced in these studies. Thus, the 5αR-1 primers were measuring true levels of a single 5αR-1 transcript in our samples.

p 37: *Comment:* Based on the high K_m for rat 5αR-1 and its expression mainly in non-androgen dependent tissues, Normington and Russell (1992) suggested that 5αR-1 has a catabolic role in steroid metabolism, to inactivate

testosterone amplification by preventing conversion to DHT. This paragraph was included to represent the views of others even though I do not agree with this concept. Firstly, 5αR-1 is not the predominant isoform in all non-androgen dependent tissues, 5αR-1 is expressed at similar levels to 5αR-2 in the epididymis, ventral prostate and seminal vesicles, and 5αR-1 is the predominant isoform in the testis. Secondly, this thesis has shown that changes in the 5αR-1 isoform in the testis closely paralleled changes in the concentration of testicular 5α-reduced metabolites, suggesting that the role of 5αR-1 is not to inactivate testosterone metabolism, at least in the testis. Rather, it appears that 5αR-1 is almost solely responsible for 5α-reduction in the testis. Considering these findings, this concept was not discussed further but only briefly mentioned in the literature review.

p 63 line 25: *Comment:* Bromoergocryptine spelling is correct

p 64 line 2: delete "suppression (via bromoergocryptine-treatment)" and read "administration to bromoergocryptine-treated rat"

p 67 line 16: delete "the most common" and read "a common"

p 71 line 3: delete "and oral desogestrel (Fotherby and Caldwell, 1994; Kuhl, 1996)"

p 75 line 7: delete "and FSH, both acting" and read "and FSH acting"

p 81: *Comment: (A)* Section 2.2.3 b (p 81) described the procedure for preparing testicular supernatants for the measurement of 5αR activity. The method described under section 2.3.3 (p 84) described the procedure for measuring the intratesticular concentration of testosterone in the testis. In the past, the procedure of both these methods had been questioned due to possibility of continued metabolism within the testis. To overcome this problem, we snap froze the testis in liquid nitrogen immediately after the testis was excised and weighed, and all subsequent procedures upon thawing were performed on ice to prevent metabolism. Testes used for HPLC were immediately immersed in acetonitrile/TFA upon thawing before homogenisation. *(B)* There are various methods for measuring testicular androgen concentrations. Testosterone can be measured directly in unextracted testes/serum or specific androgens may be separated and measured. Solvents may be used to separate testosterone from binding proteins, however multiple extraction steps are required and these must be monitored. Many antisera that are used to measure testosterone also cross-react with 5α-reduced metabolites, which are structurally similar to testosterone. Thus, we chose to separate the androgens using HPLC and then measured each androgen separately using an RIA.

p 96 line 6: delete "(that is free of 5αR-1)" and read "(that is free of 5αR-2)"

p113 line 22: *Comment:* 230, 000 dpm ^{3}H added to the 5αR assay and ~200,000 dpm are recovered. ^{3}H-Testosterone is only converted to DHT and 3α-Adiol in this assay and no androstenedione is produced, as confirmed by undetectable radioactivity on the TLC plate.

p 113 line 16: *Comment:* There is no error in this formula, the SD has the same value as the mean. The variability in the backgrounds created a large SD because these were barely detectable (~0.1 % conversion). Thus, detection limits were set at two SD values away from the mean.

p 128 line 3: *Comment:* Various primers and reverse transcriptase enzymes were tested to obtain optimal conditions for synthesis of 5αR cDNA. These studies revealed that random primers were best suited for 5αR-1 since the desired amplicon was situated at the 5'end of the sequence, and oligo dT (hybridizes to the 3' end of the mRNA) was best suited for priming 5αR-2 because the desired amplicon was based at the 3'-end of the 5αR-2 sequence. The primer sets and reverse transcription procedure were chosen for optimal sensitivity and reproducibility rather than uniformity. Different reverse transcription methods will not affect subsequent analysis by the Lightcycler because the method used for quantitation was relative, not absolute, and was based on a given set of standards run within each analysis.

p 137 line 15: delete "(in yellow)" and read "(in blue)"

Fig 4-6 & 4-7: *Comment:* It is not possible to change the colours of the standards and samples in these figures because the Roche Software uses default settings that can't be changed.

Fig 4-4 & 4-9: *Comment:* Fig 4-4 represents melting curve analyses for both 5αR isoforms including blank controls, and Fig 4-9 shows melting curves for 5αR-1 samples and negative controls and their corresponding agarose gels, showing no amplification for negative controls.

p 139 para 2: *Comment:* PCR could not be used to quantitate ribosomal RNA because oligo dT was used to reverse transcribe 5αR-2. Thus, both 5αR-1 and 5αR-2 data were normalized against ribosomal RNA by electrophoresing equivalent amounts of total RNA on an agarose gel and quantitating the 28S ribosomal bands with the BioRad Gel Doc 2000 Documentation system. The density of the 28S band within a linear working range was obtained and the LightCycler data was normalised against the 28S band. The advantage of this method was that the integrity of the RNA samples could be verified by visualisation.

p 139 Discussion: *Comment:* When using the LightCycler, large amplicon lengths (> 700 bp) can reduce the amplification efficiency. Thus, to obtain optimal results it is recommended that amplicon lengths are kept at ~200 bp. I agree with the reviewer's comments that amplification of the short pieces of sequences does not equate with real absolute quantification of 5αR mRNA. However, it was not the intention of these studies to quantitate the absolute amounts of the 5αR but to measure the changes of the transcripts with different treatments (i.e. relative quantitiation of 5αR mRNA). Furthermore, the primer pairs designed to amplify these short amplicons provided specificity and could not recognise the other.

Fig 5-3: *Comment:* The Graphpad Prism software package determines whether there are one or two enzymes and gives you the K_m and the V_{max} of the Michaelis-Menten plot. The V_{max} corresponds to the x-intercept on the Eadie-Scatchard plot. The y-intercept of the Eadie-Scatchard plot can be determined from the formula: rise/run=K_m^{-1}, where run is the V_{max} value and K_m is given (ie. the y-intercept can be determined with this formula).

p 149 & 152: *Comment:* I agree with the reviewer that it is difficult to extrapolate from the V_{max}/ K_m ratio in vitro to the relative importance of the two isoforms in vivo. There have been reports (Thigpen et al., 1993a) to suggest that the 5αR-1 isoform may function at a neutral pH in vivo and that the acidic pH optimum seen in vitro is a consequence of the extraction process. The enzyme kinetic studies in this thesis for immature testis and epididymis were performed at pH 7.0, not at pH 5.0 as stated, and thus the V_{max} values and the V_{max}/K_m ratio obtained for these tissues I believe may be a reflection of the potential ability of the enzyme to function in vivo at a neutral pH.

p 153 line 5: delete "low, and thus" and read "low compared to adult testis, and thus"

p 162: *Comment: (A)* It can not be concluded that flutamide directly influenced 5αR levels, however testosterone suppression by flutamide administration stimulated 5αR-1 and testosterone restoration by hCG treatment suppressed 5αR, indicating that these effects on 5αR-1 were due to alterations in testosterone levels and not due to flutamide. *(B)* The endogenous testosterone levels in the testis did not influence the conversion rate of ^{3}H-testosterone as substrate in the 5αR assay as the concentration of testosterone (0.35 M) added into the activity assay was saturating (~320-fold higher than endogenous testosterone).

Fig 6-1 & 6-7: *Comment:* In the normal animal flutamide increases serum LH and testicular testosterone by blocking the negative feedback of androgen action in the pituitary (Gromoll et al., 1993). In these studies, flutamide administration to TE-treated rats partially restored serum LH and led to a 2-5 fold increase (not significant) in testicular testosterone over a 6-day period. There is evidence to suggest that flutamide may directly affect the synthesis of androgens as flutamide impairs rat testicular 17a-hydroxylase and 17,20-lyase activities, possibly preventing the restoration of testosterone in these studies (Ayub and Level, 1990, Clin Endocrinol, 32:329-339, and Ayub and Level, 1987, J Steroid Biochem, 28:521-531), so testosterone could be decreased directly by flutamide.

p 165: *Comment:* The regulation of 5αR-1 by testosterone at earlier ages is being currently studied by a fellow student in our laboratory and thus could not be presented in this thesis.

p 201 line 2: delete "localisation" and read "expression"

General Discussion: *Comment: (A)* 5αR-2 in the testis has not been extensively studied and its expression in the testis is controversial. This thesis has shown that 5αR-2 is present in the testis and is hormonally regulated. Our previous studies showed suppression of both 5αR isoforms in the testis suppressed spermatogenic restoration. Together these data suggest that 5αR-2 may play an important role in regulating spermatogenesis. Given the high affinity of 5αR-2 for testosterone, a possible function for 5αR-2 in the testis could be to convert limiting substrate concentrations (ie. at low testicular testosterone levels), such as during immaturity and during contraception. *(B)* It has been demonstrated that the half-lives of the 5αR proteins are not altered by 4-azasteroids inhibitors (Thigpen et al., 1993a) however it is not known whether testosterone directly affects the 5αR protein structure. Our study and others demonstrate that the 5αR isoforms are regulated at both the transcriptional and translational level (Viger and Robaire, 1995)

HORMONAL REGULATION OF 5α-REDUCTASE ISOFORMS IN THE RAT TESTIS

Kyriakos Pratis

Bachelor of Science (Honours)

Monash University, Clayton

Prince Henry's Institute of Medical Research

and

Obstetrics and Gynaecology, Monash University,

Clayton, Australia

Thesis submitted for

Doctor of Philosophy

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Summary

The process of spermatogenesis is dependent on follicle stimulating hormone (FSH) and testosterone. Testosterone can be metabolized via the 5α-reductase (5αR) enzyme to dihydrotestosterone (DHT), a more potent androgen that binds with greater affinity to the androgen receptor. Two 5αR enzymes have been cloned; 5αR type 1 (5αR-1) and type 2 (5αR-2). The 5αR-1 isoform has a low affinity for steroid substrates and is maximally active over a broad neutral pH range, whereas 5αR-2 is a high affinity enzyme with an acidic pH optimum. DHT is known to amplify androgen action and is crucial in androgen-dependent peripheral organs.

Studies in the rodent testis show that when testicular testosterone levels are low, such as during gonadotrophin suppression, 5α-reduction to DHT is important for androgen action on spermatogenesis. This observation is clinically relevant as studies in men receiving hormonal contraceptive treatments show that men whose sperm counts fall markedly, also have lower levels of 5αR, when compared to men whose sperm count do not adequately suppress. Thus, conversion to DHT in the testis may be an important factor in determining the degree of spermatogenic suppression during contraceptive treatment. Little is known about the physiology of testicular 5αR isoforms, particularly whether they are hormonally regulated.

This thesis aimed to investigate the hormonal regulation of the 5αR isoforms in the adult rat testis *in vivo*. Two different assay methods were developed, validated and used to measure 5αR isoform enzyme activity and mRNA levels. I*n vitro* assays were used to measure enzyme activity; 5αR-1 enzyme activity was measured at its optimal pH (7.0), however due to the overlap of 5αR-1 activity at pH 5.0, a new method was established to measure testicular 5αR-2 activity (Chapter 3). Quantitative PCR was used to measure the expression of 5αR-1 and 5αR-2 mRNAs, using the LightCycler PCR instrument (Chapter 4).

This thesis demonstrated that rat testis expressed both 5αR isoforms, that 5αR-1 was the predominant testicular 5αR, and that the activity of both isoforms declined with age (Chapter 5). Having characterized both 5αR isoforms in the testis, *in vivo* models of gonadotrophin suppression and replacement were used to investigate the regulation of the 5αR isoforms by testosterone and FSH in adult testis (Chapter 6 and 7). It was demonstrated that testosterone negatively regulated the 5αR-1 isoform, so that in the testosterone-suppressed testis the 5αR-1 isoform was up-regulated to increase DHT production. In contrast, the 5αR-2 isoform was not altered by testosterone. Both 5αR isoforms were shown to be positively regulated by FSH, at either the mRNA or activity level. These studies showed that 5αR-1 in the testis was increased by gonadotrophin suppression, and that the testicular 5αR isoforms were differentially regulated by the two main endocrine regulators of spermatogenesis.

In summary, this thesis has provided important information regarding the roles of FSH and testosterone in controlling testicular androgen biosynthesis. Further studies are required in rat, and in the longer term, primates and man, to elucidate the mechanism by which FSH and testosterone regulate the 5αR isoforms. Defining the regulation of the testicular 5αR isoforms will provide further knowledge on the control of these enzymes and will be useful for the design of new male contraceptive methods.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains neither material previously published or written by another person nor experimental data from another person's work except where due reference is made in the text of the thesis.

Kyriakos Pratis

Prince Henry's Institute of Medical Research

Monash Medical Centre

Clayton, Melbourne

Australia

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To all my friends, especially Theo, John, and Gerry, thank you for your support. To all my friends at PHIMR, you have been my extended family and support network for the last few years. I must individually thank Anna Baillie and Nick Cahir for their assistance in the lab, my fellow students Kara, Belinda, Rebecca and Pavel, and to Sarah Meachem for putting a smile on my face. Julie, you never failed to brighten up my day, you are truly one of a kind and I respect and admire you. I would like to acknowledge Peter Stanton and Fiona McLean for the testicular steroid analysis, Tanneale Stephenson for showing me the LH assay and for supplying reagents, and I would like to thank our graphic artist, Sue Panckridge, for help in preparing slides and figures.

I would like to thank my entire family for their support and encouragement throughout my studies. To mum and dad, I am in debt to you forever. Your sacrifices, love and sustained blessing for all my dreams and aspirations is touching. To Peter, Georgia and Sam, thanks for the help through 'thick and thin' and for always being there for me. To V and T, loving and thinking of you always. Zeus, thanks for getting me up bright and early every morning and for challenging me physically, as well as allowing my mind to escape from the pressures and stress of a PhD.

I dedicate this thesis to the grandparents I have not known, as a consequence of one of many sacrifices my parents made to provide their children with a better, educated life.

Publications and Presentations

Pratis K., O'Donnell L., Ooi G.T., McLachlan R.I., Robertson D.M (2000) Enzyme assay for 5α-reductase Type 2 activity in the presence of 5α-reductase Type 1 activity in rat testis. Journal of Steroid Biochemistry and Molecular Biology, 75:75-82.

Kyriakos Pratis, Liza O'Donnell L, Guck T Ooi, Robert I McLachlan and David M Robertson (2000) Differential regulation of 5α-reductase type 1 and type 2 activity in rat testis. 11th International Congress of Endocrinology. ICE 2000, Sydney, Australia.

Kyriakos Pratis, Liza O'Donnell L, Guck T Ooi, Robert I McLachlan and David M Robertson (1999) Rat testicular 5α-reductase isoforms: Differential regulation by testosterone and FSH. 42nd Annual Scientific Meeting of the Endocrine Society, Melbourne, Australia.

Kyriakos Pratis, Liza O'Donnell L, Guck T Ooi, Robert I McLachlan and David M Robertson (1999) Hormonal regulation of type 1 and type 2 5α-reductase activity in rat testis. 81st Annual Meeting of The Endocrine Society (US ENDO 99) San Diego, CA.

Pratis K, O'Donnell L, Robertson DM, and McLachlan RI (1998) *In vivo* hormonal regulation of testicular 5α-reductase activity in adult male rats. 41st Annual Scientific Meeting of the Endocrine Society, Perth, Australia.

O'Donnell L, Pratis K, Stanton PG., Robertson DM, and McLachlan RI (1997) Variable androgen requirements for rat spermatogenic cell maturation. 40th Annual Scientific Meeting of the Endocrine Society, Canberra, Australia.

List of Abbreviations

❶	see Appendix 1
❷	see Appendix 2
3α-Adiol	3α-Androstanediol (5α-androstane-3α, 17β-diol)
5αR	5α-Reductase
5αR-1	5αR type 1
5αR-2	5αR type 2
ABP	Androgen binding protein
AR	Androgen receptor
ARE	Androgen response elements
β-Gal	β-Galactosidase
BGH	Bovine growth hormone
BPH	Benign Prostatic Hyperplasia
cDNA	Complimentary DNA
cAMP	Cyclic adenosine 3', 5'-monophosphate
CHO	Chinese Hamster Ovary
CO_2	Carbon dioxide
CMV	Cotomegalovirus
C_t	Threshold cycle
CV	Coefficient of Variation
DAI	Double stranded RNA-activated inhibitor
DEPC	Diethyl pyrocarbonate-treated water
DHT	Dihydrotestosterone (17β-hydroxy-5α-androsten-3-one)
DMPA	Depot medroxyprogesterone acetate
DMSO	Dimethyl suplhoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
dsRNA	Double stranded RNA
EDS	Ethane dimethane sulphonate
EGF	Epidermal growth factor
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
FSH*Ab*	FSH antibody
G	Gauge
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GFP	Green fluorescent protein
GnRH	Gonadotrophin-Releasing Hormone
H_2O	Water
hCG	Human chorionic gonadotrophin
HPLC	High performance liquid chromatography
IFMA	Immunofluorometric assay
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IU	International Units
LH	Luteinising hormone
LB	Luria-Bertaini
$Media^{+FCS}$	Media containing fetal calf serum
$Media^{-FCS}$	Media without fetal calf serum
MENT	7-alpha-methyl-19-nortestosterone
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
N_2	Nitrogen

NADPH	nicotinamide dinucleotide adenine phosphate (4-ene-3-oxosteroid 5α-oxidoreductase)
NaOH	Sodium hydroxide
NCad	Neural cadherin
NETE	norethisterone enanthate
NRS	Normal Rat Serum
OD	Optical density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
QC	Quality control
rhFSH	Recombinant human FSH
RIA	Radioimmunoassay
RNAi	RNA interference
RNA	Ribonucleic acid
rpm	Revolutions per minute
SSG1	Steroid sensitive gene-1
SV40	Simian virus 40
TE	3cm testosterone and 0.4cm oestradiol implants
TFA	Trifluoroacetic acid
TGFα	Transforming growth factors α
TGFβ	Transforming growth factors β
TLC	Thin Layer Chromatography
T_m	Melting temperature
U	Units
x-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

0.4cm-*E.imp*	0.4cm oestradiol silastic implant
3cm-*T.imp*	3cm testosterone silastic implant
6cm-*T.imp*	6cm testosterone silastic implant
10cm-*T.imp*	10cm testosterone silastic implant
24cm-*T.imp*	24cm testosterone silastic implant (i.e. 3 x 8cm implants)

Chapter 1

Review of Literature

1.1 INTRODUCTION

Spermatogenesis is the process by which immature germ cells undergo a series of complex events to differentiate into mature spermatids. The role of the testis in spermatogenesis is to provide the physical structures and the appropriate conditions for germ cell development. The Sertoli cells provide the structure of the testis to nurture germ cells throughout their development. Leydig cells produce androgens that regulate the spermatogenic process and are also required for development of male secondary sexual characteristics.

Hypothalamic gonadotrophin-releasing hormone (GnRH) stimulates the synthesis and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. FSH acts directly on the seminiferous tubules and LH acts indirectly via stimulation of Leydig cells to produce androgens, particularly testosterone. Although FSH and testosterone are known to be the major regulators of spermatogenesis, their relative roles and mechanism of action remain controversial.

Knowledge of the hormonal control of spermatogenesis is vital to our understanding of male infertility as well as for the development of contraceptive strategies. The hormonal control of spermatogenesis involves a complex array of autocrine, paracrine, and endocrine factors that act on cells within the testis, namely the Sertoli, Leydig, peritubular and germ cells. The majority of this review will concentrate on spermatogenesis in the rat, an animal model that has been used extensively over many years to study the regulation of spermatogenesis.

The main aim of this chapter is to review the regulation of spermatogenesis and the involvement of 5α-reduced androgens in this process. The role of androgens in spermatogenesis has been well characterized, but until recently testosterone was thought to be the only androgen involved. However, accumulating evidence supports a role for 5α-reduced androgens in this process. First, an overview of the process of spermatogenesis and its regulation by hormones and paracrine factors will be given. Then 5α-reductase (5αR) will be introduced, and its role in testosterone metabolism and androgen physiology will be reviewed. Testicular 5αR and its

possible roles and regulation will be presented, and finally the relevance of 5αR in spermatogenesis, and its role in human male contraception, will be discussed.

1.2 SPERMATOGENESIS

1.2.1 Compartments of the Testis

A dense connective tissue, called the tunica albuginea, surrounds the testis, which itself is composed of two internal compartments (*Figure 1-1*), the seminiferous tubules and the interstitial tissue (Steinberger and Steinberger, 1975).

The seminiferous tubules are convoluted tubules within the testis and are the site of germ cell development (Russell *et al.*, 1990). Within the tubules are the complex somatic cells, called Sertoli cells, that nurture the developing germ cells (Russell, 1993). Each Sertoli cell is connected to neighboring Sertoli cells by junctional complexes such as gap junctions and tight junctions, to form the blood testis barrier (Vitale *et al.*, 1973). The external wall of the tubules is composed of the basement membrane, myoid and peritubular cells (Dym and Fawcett, 1970) and lymphatic endothelium (Fawcett *et al.*, 1973).

The interstitial tissue, or interstitium, surrounds the seminiferous tubules and contains numerous cell types. The Leydig cell is the most common cell type in the interstitium and is primarily involved in the production and secretion of androgens (Christensen, 1975). Other cell-types include the macrophages, lymphocytes, and blood and lymphatic vessels, which transport nutrients and hormones into and out of the testis (Pollanen and Maddocks, 1988).

1.2.2 The Process of Spermatogenesis

Spermatogenesis is a complex process whereby immature germ cells develop into mature spermatids. This process takes place within the seminiferous tubules and is regulated by a variety of factors. The most primitive germ cells are situated at the basement membrane, and as they

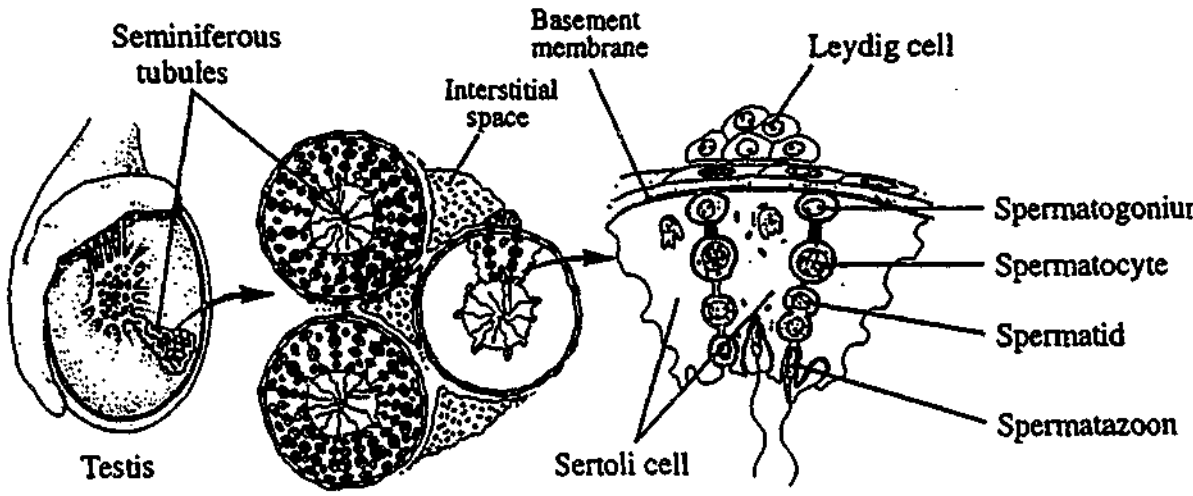


Figure 1-1: The testis and its two major compartments, the seminiferous tubules and the interstitial tissue. The seminiferous tubules are highly convoluted and account for the bulk of the testis. Within the tubules are precursor germ cells that undergo the process of spermatogenesis to form spermatozoa. The interstitial tissue lies between the tubules, and consists of Leydig cells that are responsible for androgen production.

divide and mature, later cell types move further away from the basement membrane and are eventually released into the tubule lumen as mature spermatids. There are three main phases during the process of spermatogenesis:

(1) *Spermatogonial proliferation and development:* spermatogonia are the stem cells of the testis (Clermont and Bustos-Obregon, 1968) and are situated between the Sertoli cells and the basement membrane (Russell *et al.*, 1990). Spermatogonia either divide to renew their own population or become committed to differentiation. These cells undergo multiple mitoses to provide a continuous supply of germ cells, which further differentiate into spermatocytes. In the rat, the last mitosis of spermatogonia (type B spermatogonia) yields preleptotene spermatocytes (Russell *et al.*, 1990).

(2) *Meiosis:* two meiotic divisions occur during spermatogenesis whereby germ cell chromosomes are recombined and the genetic material in each cell is halved (Clermont, 1972). The first meiotic division yields secondary spermatocytes, and the second meiotic division yields haploid round spermatids.

(3) *Spermiogenesis:* is the process by which round spermatids undergo a series of cytological transformations to give rise to mature elongated spermatids, without any cell division. Nineteen different steps of rat spermiogenesis have been defined based on morphological appearance (Leblond and Clermont, 1952a). The extent of spermatid acrosomal development and position of the nucleus are two characteristics used to differentiate the spermiogenic steps during stereological analysis (Hess, 1990; Russell *et al.*, 1990). Spermiation is the release of mature sperm from the Sertoli cell into the tubule lumen at the end of spermiogenesis, a process which involves the loss of junctional complexes (Russell, 1991). After sperm are released from the seminiferous epithelium they are transported through the rete testis and efferent ductules and then are further matured and stored in the epididymis.

1.2.3 Cycle of the Seminiferous Epithelium

The different types of germ cells are arranged in strictly defined cellular associations called stages (*Figure 1-2*), and the rat spermatogenic process is divided into 14 different stages (Leblond and Clermont, 1952b). Each stage is designated a Roman numeral and the stages follow one another in an ordered fashion along the length of the tubules. A fixed association of germ cells defines each stage and each stage contains one or two generations of spermatogonia, spermatocytes and spermatids. One stage will be seen in a single cross section of the seminiferous epithelium in the rat, however in the human two or three stages are visible (Russell, 1991).

The 'cycle' of the seminiferous epithelium is defined by the completion of all 14 stages of the spermatogenic process (Russell *et al.*, 1990). The duration of the cycle of the seminiferous epithelium is the time taken to complete one cycle (Parvinen, 1982). In the rat, four cycles (51.6 days) are required for the development of mature sperm from type A spermatogonia (Clermont and Harvey, 1965; Steinberger and Steinberger, 1975). Each stage of the cycle differs in duration, so the longer the duration of a stage, the more frequent that stage will appear in a cross-section (Clermont and Harvey, 1965).

1.3 REGULATION OF SPERMATOGENESIS

1.3.1 Hypothalamic-Pituitary-Gonadal Axis

Normal reproductive function is dependent on the coordinated release of hormones in the hypothalamic-pituitary-gonadal axis (*Figure 1-3*). Neuroendocrine cells in the hypothalamus intermittently release GnRH into the pituitary portal blood, stimulating the anterior pituitary to synthesize and release two glycoproteins, FSH and LH (see for review Loosfelt *et al.*, 1989; Sprengel *et al.*, 1990).

The peptide hormones FSH and LH act to maintain gonadal function in the male, including germ cell formation and production of Sertoli cell substances such as activin, inhibin, and

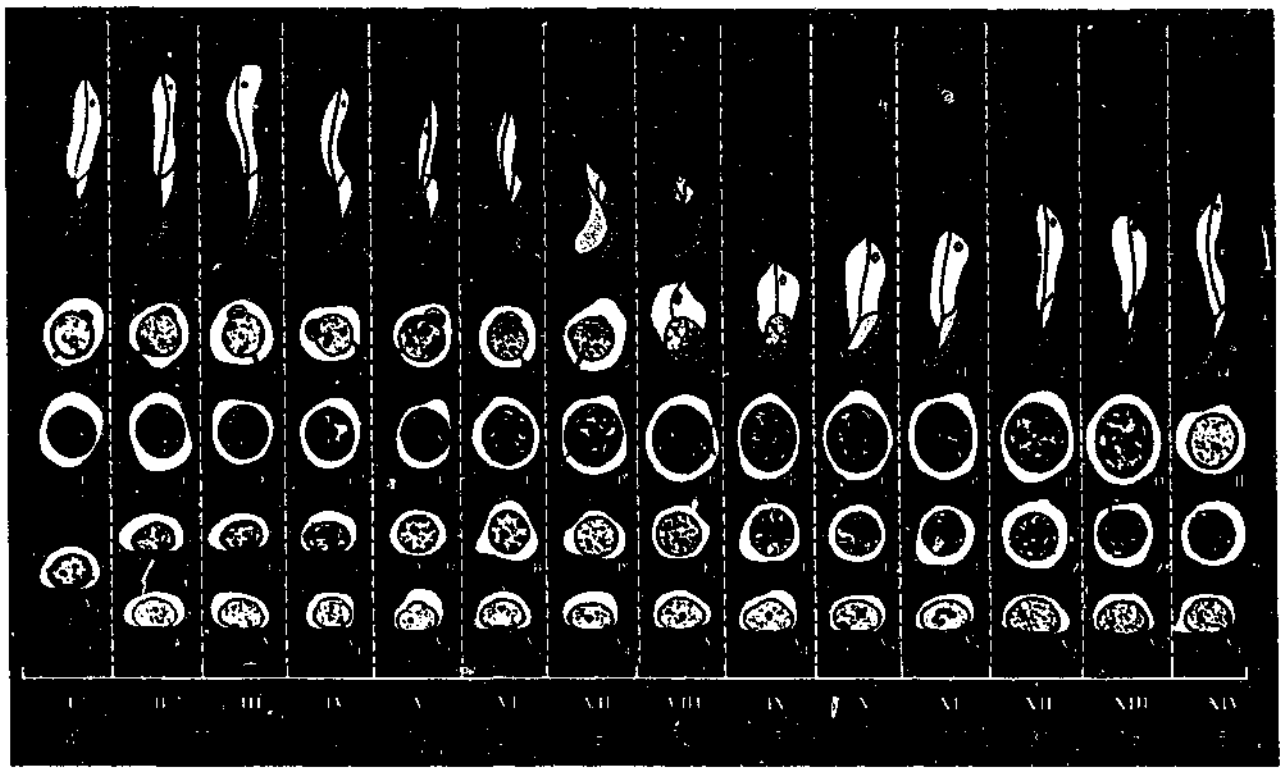


Figure 1-2: The spermatogenic cycle of the rat illustrating the fourteen germ cell associations (denoted in roman numerals below each column) present in the seminiferous epithelium at each stage of the cycle. The time of each stage in hours is also represented below each column. During spermatogenesis each germ cell develops through successive spermatogonial stages (type A spermatogonia, intermediate [In], and type B spermatogonia [B]), primary spermatocyte stages (preleptotene [Pl], leptotene [L], zygotene [z], pachytene [P], diplotene [Di]), before completing meisosis when secondary spermatocytes give rise to step 1 round spermatids. Round spermatids differentiate through 19 steps, to eventually be released into the tubule lumen (modified from Russell *et al.*, 1990).

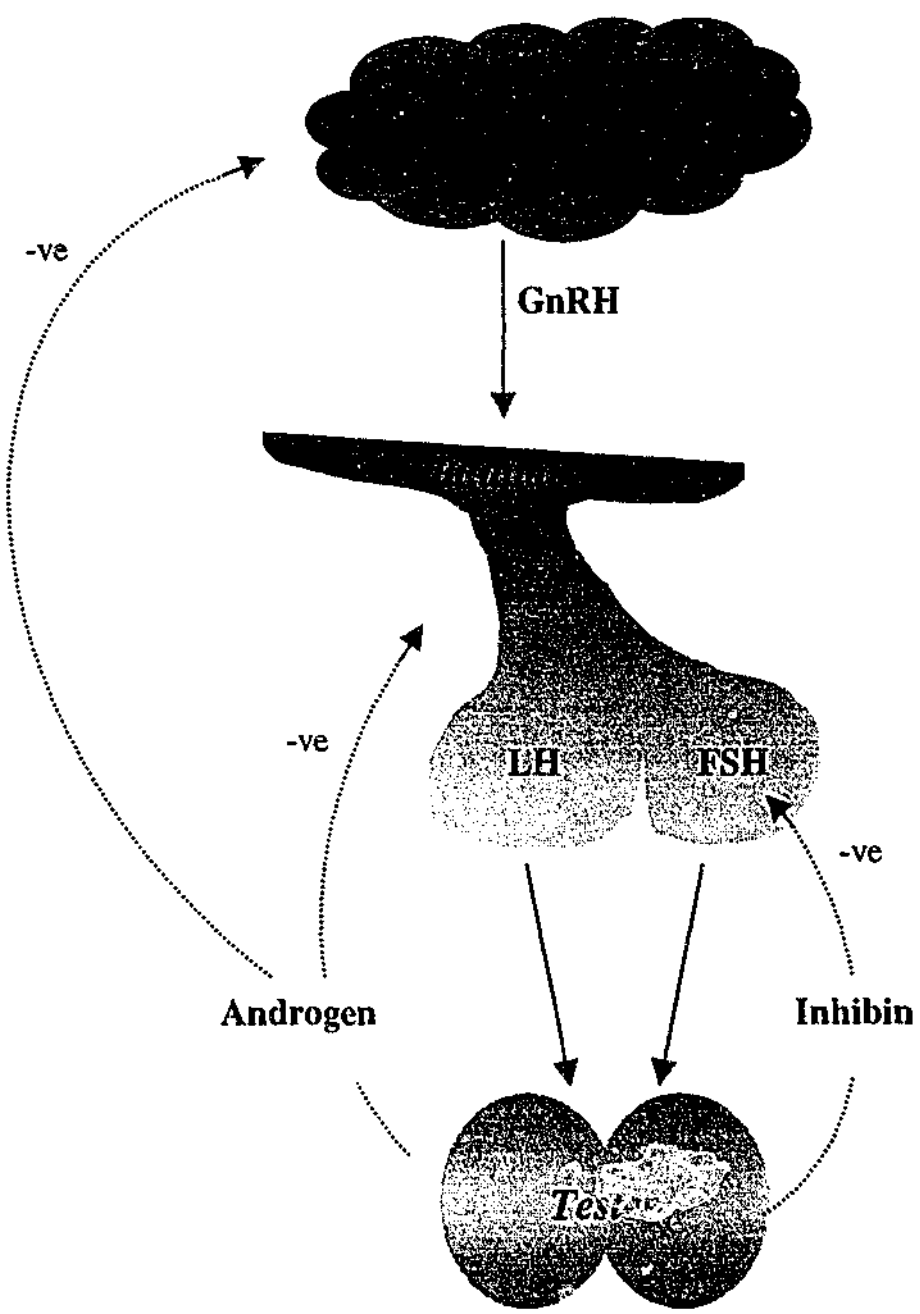


Figure 1-3: The hypothalamic-pituitary gonadal axis showing the negative feedback sites of steroids and peptides on gonadotrophic hormones.

androgen binding protein (ABP) (for review see Griswold, 1993). This control of GnRH secretion is maintained by steroid and peptide feedback at both the hypothalamic and pituitary level. For example, testosterone exerts a negative feedback effect on the hypothalamus to prevent the release of LH, and inhibin inhibits the secretion of pituitary FSH (see *Figure 1-3*). Some of this feedback results from estradiol via aromatisation of testosterone.

Steroid modulation of the gonadotrophin axis has been extensively studied in rodent models following gonadectomy, which causes a dramatic rise in serum LH and FSH (Shupnik and Schreihofer, 1997). These changes may result from removal of negative feedback at the level of the pituitary or, indirectly, at the level of the hypothalamus. Changes in sex steroid levels result in variations in GnRH pulse frequency and amplitude, which alter gonadotrophin secretion and subunit mRNA levels. At the level of the pituitary, hormonal modulation may occur by changes in GnRH or steroid receptor number or subtype, to alter or enhance pituitary responsiveness to hormones, or by direct modification of gonadotrophin gene activity.

1.3.2 Models Used to Study The Hormonal Regulation of Spermatogenesis

Many different types of *in vivo* and *in vitro* model systems have been used to investigate the hormonal regulation of mammalian spermatogenesis. The different types of models, treatment regimens, and end points that have been utilized to assess the complex process of spermatogenesis makes the interpretation of data difficult. Despite the large amount of data available, there is still considerable controversy regarding the actions of many of the autocrine, endocrine and paracrine factors that regulate germ cell development.

Due to the different models and treatments used to study spermatogenesis, a number of distinctions need to be made when assessing these studies:

(1) The phase of spermatogenesis examined: (a) *initiation* is the first completion of spermatogenesis during puberty (b) *maintenance* is the requirement(s) for continued spermatogenesis, and (c) *restoration* is the requirements(s) for the commencement of spermatogenesis after the process has been interrupted (usually in the adult).

(2) The mode of analysis: (a) *qualitatively normal spermatogenesis* refers to the presence of all germ cell types but in reduced numbers, and (b) *quantitatively normal spermatogenesis* refers to the presence of all germ cell types in normal numbers. Quantitatively normal spermatogenesis can only be assessed using appropriate cell counting techniques.

(3) The mode of assessment refers to the endpoints used to assess the role of a given factor in spermatogenesis. Some methods include; counting the number of elongated spermatids in tissue homogenates which are resistant to Triton x 100 (Robb *et al.*, 1978), stereological methods for counting different germ cell types (see Wreford, 1995), and immunocytochemical markers for assessing the proliferative and apoptotic activity of cells (Sinha-Hikim *et al.*, 1997).

Most *in vivo* models used to study the hormonal control of spermatogenesis in rodents involve the withdrawal (suppression phase) and then the replacement (restoration phase) of hormone(s). Thus the role of substances that regulate spermatogenesis can be investigated during the restoration of spermatogenesis after its suppression. There are several different *in vivo* models that have been utilized, each with their own distinct advantages and disadvantages.

Hypophysectomy is a surgical model whereby the pituitary is removed, thus eliminating all pituitary hormones (see Steinberger and Steinberger, 1974) and abolishing sperm production (Clermont and Harvey, 1965). Administration of testosterone after hypophysectomy allows the role of androgens during the maintenance (Buhl *et al.*, 1982; Santulli *et al.*, 1990) and restoration (Huang *et al.*, 1987) of spermatogenesis to be investigated. The duration of hypophysectomy determines the severity of germ cell loss, and the degree of spermatogenic restoration by testosterone depends on the delay in testosterone replacement after hypophysectomy (Muffly *et al.*, 1993, 1994). These studies have shown that testosterone alone cannot quantitatively maintain spermatogenesis during the restoration (Huang *et al.*, 1987; Muffly *et al.*, 1993, 1994) or maintenance (Santulli *et al.*, 1990) of spermatogenesis, indicating that both FSH and testosterone

are necessary for the quantitative maintenance of spermatogenesis in this model (Bartlett *et al.*, 1989). Hypophysectomy is the classical model of gonadotrophin withdrawal, however, interpretation of data from this model is complicated because all pituitary hormones are removed, making it difficult to assess the role of individual hormones. Two other *in vivo* models that have been used more recently will be discussed in detail below.

(a) GnRH Withdrawal Model

GnRH withdrawal is achieved by active immunization against GnRH (Awoniyi *et al.*, 1989b, 1992a; McLachlan *et al.*, 1994b) or by treatment with GnRH analogues (Sinha-Hikim and Swerdloff, 1993) to abolish GnRH release (Awoniyi *et al.*, 1989b) and selectively suppress FSH and LH (*Figure 1-4*). Production of testicular testosterone is suppressed to ~1% of normal (McLachlan *et al.*, 1994a) resulting in the total loss of elongated spermatids in rats (Awoniyi *et al.*, 1989b; McLachlan *et al.*, 1994b).

This model of gonadotrophin suppression in rats is similar to human contraception, whereby both testosterone and FSH are inhibited following administration of androgens to induce spermatogenic suppression (WHO, 1990, 1996). Zhengwei *et al* (1998b) showed that in response to testosterone-induced gonadotrophin withdrawal in men, type B spermatogonia fell and later germ cell types decreased markedly, whereas the number of type A spermatogonia were unchanged. Thus, the degree of germ cell arrest is similar, though not identical, between rodents and men, in response to gonadotrophin withdrawal. In humans the suppression of spermatogonial number is more severe than in rats (type B spermatogonia in man decline by ~90% compared with ~50% decline in all spermatogonia in rats).

Following GnRH-immunization, spermatogenesis can be restored to normal (Awoniyi *et al.*, 1989b) or near normal levels (McLachlan *et al.*, 1994a) by partially restoring testicular testosterone via treatment with 24 cm testosterone silastic implant (24 cm-*T.imp*). A feature of the GnRH withdrawal model in rodents is that testosterone replacement following GnRH-immunization (McLachlan *et al.*, 1994b) or GnRH-antagonist treatment (Arslan *et al.*, 1989;

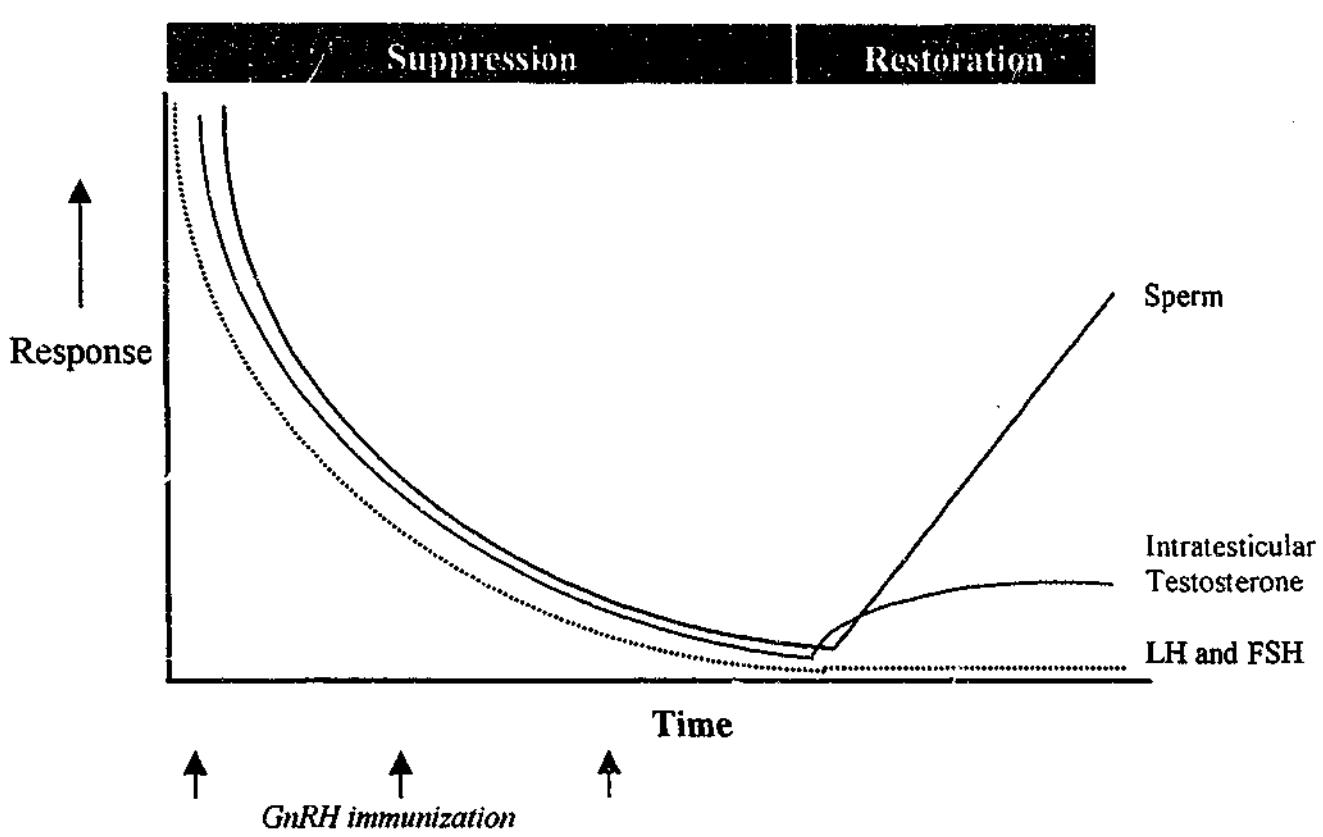


Figure 1-4: The GnRH-Immunization Model: During the suppression phase, animals are actively immunized against GnRH, causing suppression of both testosterone and FSH, and subsequently spermatogenesis. Spermatogenesis can be restored during the restoration phase by increasing testicular testosterone levels via testosterone silastic implants or by human chorionic gonadotropin (hCG) administration. Thus, this model is one of LH/testosterone and FSH deficiency.

Sharma *et al.*, 1990) also restores serum FSH levels, an effect that is peculiar to rodents. The concomitant rise in FSH with testosterone treatment is believed to involve a mechanism that is independent of GnRH (Drouin and Labrie, 1976; Kitahara *et al.*, 1991), since testosterone can stimulate pituitary levels of FSHβ mRNA after GnRH antagonist treatment (Wierman and Wang, 1990). A GnRH independent action of testosterone on FSH has also been shown in rodent pituitary cell cultures (Kitahara *et al.*, 1991). This concomitant rise in FSH precludes the investigation of the role of testosterone alone in this model. However, passive immunization with an FSH antibody (FSH*Ab*) can be used (see below).

The testosterone-dependent restoration of spermatogenesis can be studied after FSH withdrawal that is achieved by passive immunoneutralization of FSH. As mentioned above, testosterone administration during GnRH-immunization causes a concomitant rise in FSH. However, if FSH antibodies are co-administrated with testosterone, the action of FSH is blocked, allowing the role of testosterone alone in the restoration of spermatogenesis to be explored. Meachem *et al* (1998) showed that >90% of serum FSH was immuno-neutralized by FSH*Ab* treatment (2 mg/ml) for 7 days. Only the short term effect of FSH withdrawal can be examined because rats develop neutralizing antibodies within 8 days when passively injected with heterologous antibodies (Sharpe, 1989; Meachem *et al.*, 1998).

(b) TE-Treatment Model

Testosterone administration has been used to investigate the role of hormones in the regulation of rat spermatogenesis. This model is unique in that testosterone can both inhibit and stimulate the spermatogenic process *in vivo* depending on the dose of testosterone administered (Walsh and Swerdloff, 1973; Berndston *et al.*, 1974; Huang and Boccabella, 1988; Sun *et al.*, 1989; Zirkin *et al.*, 1989). Thus, testosterone has a biphasic action allowing the suppression and restoration of spermatogenesis to be explored (*Figure 1-5*).

Administration of low doses of exogenous testosterone (3 cm testosterone silastic implant; 3 cm-*T.imp*) produce supraphysiological serum testosterone levels that inhibit the release of pituitary

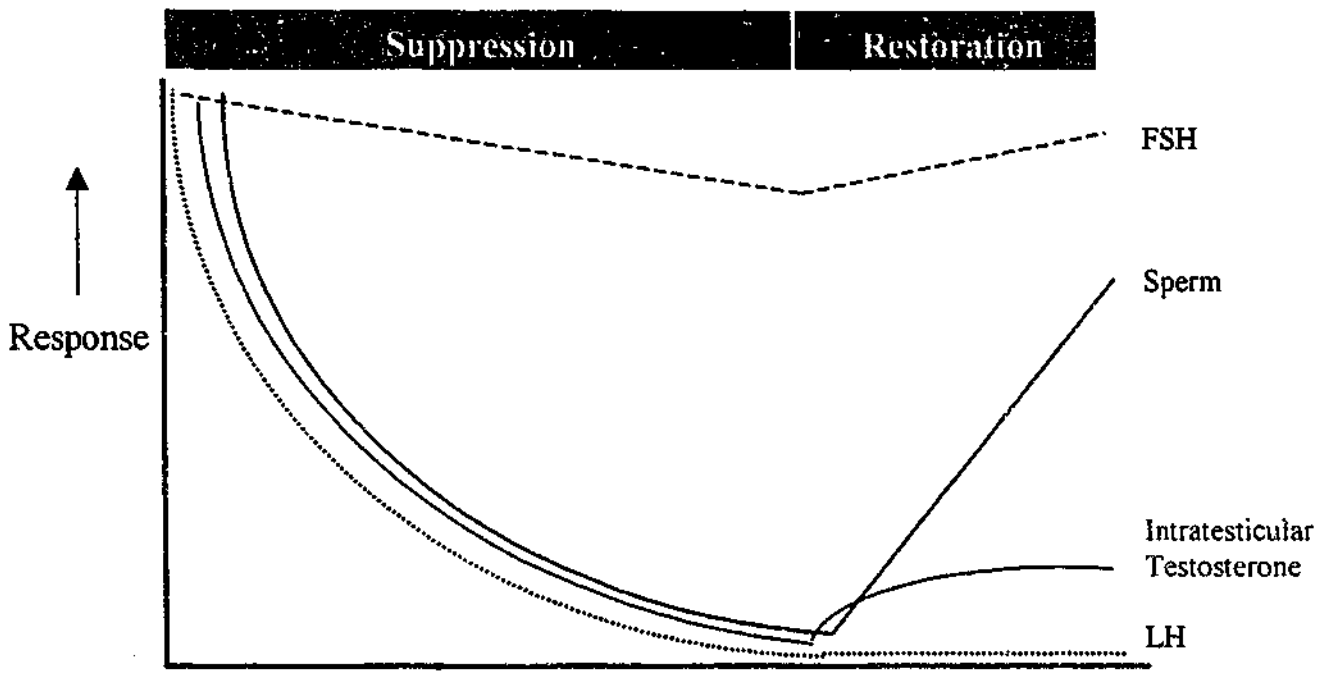


Figure 1-5: The TE-Treatment Model: During the Suppression Phase, low doses of testosterone in combination with estrogen (TE-treatment) are administered via silastic implants to slightly elevate serum testosterone and selectively suppresses pituitary LH/testicular testosterone, and consequently spermatogenesis. During the Restoration Phase, the testosterone and estradiol implants are replaced with higher doses of testosterone. Intratesticular testosterone levels are partially restored and sperm production can be normalized, depending on the dose of testosterone. Thus, this model is one of LH/testosterone deficiency but near-normal FSH levels.

LH via negative feedback effects. The suppression of serum LH results in the inhibition of the production of testosterone by Leydig cells, so that testicular testosterone concentrations fall to ~3% of control, and sperm production is suppressed (McLachlan *et al.*, 1994). Co-administration of estradiol (0.4 cm estradiol silastic implant; 0.4 cm-*E.imp*) with testosterone is thought to more profoundly suppress LH (Ewing *et al.*, 1977; Robaire *et al.*, 1979) to ensure a consistent and uniform suppression of spermatogenesis (Awoniyi *et al.*, 1989a, 1990; McLachan *et al.*, 1994a; O'Donnell *et al.*, 1994, 1996a, b, 1999). Administration of 3 cm testosterone and 0.4 cm estradiol implants (TE) causes only a slight suppression of serum FSH levels (Robaire *et al.*, 1979; Sun *et al.*, 1989; Awoniyi *et al.*, 1989a, 1990; McLachan *et al.*, 1994a; O'Donnell *et al.*, 1996a, b, 1999). Thus, the TE model for spermatogenic suppression in the rat is primarily one of LH and testosterone deficiency in a setting of near-normal FSH.

Following TE-suppression, the restoration of spermatogenesis can be examined by removing TE implants and administering higher doses of testosterone. Pituitary LH and testicular testosterone production remain suppressed with high dose testosterone treatment, but the concentration of testosterone in the testis is partially restored via the exogenous testosterone. High concentrations of testosterone (>3 cm testosterone implants) result in testicular testosterone levels that are several fold higher, but still significantly less, than normal (O'Donnell *et al.*, 1996b, 1999). The degree to which testosterone is restored in the testis depends upon the concentration of exogenous testosterone (i.e. length of the testosterone implant). Elongated spermatid numbers can be restored to control levels (Awoniyi *et al.*, 1989a, 1990) or near control (McLachlan *et al.*, 1994a) when intratesticular testosterone levels are restored to ~12% of control with 24 cm-*T.Imp*, indicating that spermatogenesis can be restored even at reduced intratesticular testosterone levels.

(c) Other *In Vivo* Models

As well as using *in vivo* models to suppress hormone levels to study the regulation of spermatogenesis, other treatments can also be administered to manipulate hormonal levels. For example, residual levels of testosterone following TE-treatment can be blocked at the receptor

level or by using agents that directly affect testosterone production. Anti-androgens such as flutamide (Peets *et al.*, 1974; Chandolia *et al.*, 1991b) and cyproterone acetate (Russell *et al.*, 1981) have been used to study the effects of androgen blockade on spermatogenesis. Flutamide has been used in combination with GnRH-antagonists (Chandolia *et al.*, 1991a, c; Kangasniemi *et al.*, 1995) or TE treatment (Meachem *et al.*, 1997; O'Donnell *et al.*, 1999) to markedly suppress testicular function, by blocking the action of residual testosterone levels.

Ethane dimethane sulphonate (EDS) is a specific Leydig cell cytotoxin that destroys Leydig cells in the testis within 3 days (Kerr *et al.*, 1985) and reduces serum and testicular testosterone to undetectable levels (Bartlett *et al.*, 1986). After 10-12 days, Leydig cells begin to regenerate, and by 42 days are restored to normal numbers (Kerr *et al.*, 1987). This method has been used to study Leydig cell development (Kerr *et al.*, 1985) and the effects of androgen suppression on the testis (Kerr *et al.*, 1993).

(d) *In Vitro* Models

In vitro cultures have also been used to study the hormonal regulation of spermatogenesis. An advantage of *in vitro* models over *in vivo* models is that they allow the investigation of a direct action of hormones and other factors on cells without the complexities of the whole organism. The disadvantage of these models is that *in vitro* effects may not truly reflect what occurs *in vivo*.

Seminiferous tubules can be isolated and cultured *in vitro*. *In vitro* culture of tubule segments has allowed the investigation of stage-specific secretion of ABP (Ritzen *et al.*, 1982), inhibin (Allenby *et al.*, 1991), and androgen-regulated proteins (Sharpe *et al.*, 1992).

Sertoli cell cultures can be derived from immature (Steinberger and Jakubowiak, 1993; Perryman *et al.*, 1996) and adult (Lampa *et al.*, 1999) rats. Mature Sertoli cell cultures are technically more difficult partly because of the presence of large numbers of germ cells and their associated Sertoli cell junctions. Sertoli-germ cell co-cultures can be used to study both the junctional and biochemical interactions between germ cells and Sertoli cells. Recently, *in vitro* cultures of Sertoli-germ cells have proven invaluable in studying the hormonal regulation of round

spermatid binding to Sertoli cells (Cameron and Muffly, 1991; Cameron *et al.*, 1993b; Perryman *et al.*, 1996).

Leydig cell cultures can be derived from immature (Murono and Washburn, 1990; Murono *et al.*, 1994) and adult (Vreeburg *et al.*, 1988; Murono *et al.*, 1994) rats. In addition to Sertoli and Leydig primary cell cultures, there are also cell lines derived from adult Sertoli cells in the rat (Roberts *et al.*, 1995) and immature mouse Sertoli (TM4) cell lines (Mather, 1980; Zwain *et al.*, 1991) and Leydig (TM3) cell lines (Mather, 1980).

1.3.3 Regulation of Spermatogenesis by FSH

Many studies have contributed to our knowledge of the hormonal control of spermatogenesis. It is well established that testosterone and FSH are the major regulators of spermatogenesis, and that they exert their biological effects directly via the Sertoli cell (for review see Sharpe, 1989, 1994; de Kretser *et al.*, 1992; Weinbauer and Nieschlag, 1993; Zirkin *et al.*, 1994). In the male, FSH is generally considered essential for the pubertal initiation of spermatogenesis and maintenance of quantitatively normal sperm production in adults (Matsumoto *et al*, 1986; Sharpe, 1994; Zirkin *et al*, 1994). However, the relative contributions of FSH and testosterone to the overall process of spermatogenesis and the molecular mechanisms involved are less clear (for review see Sharpe, 1994; Zirkin *et al.*, 1994; McLachlan *et al.*, 1996).

Many studies have focused their attention on the regulation of spermatogenesis by FSH in the immature animal. FSH is the major factor that regulates Sertoli cell proliferation both *in vitro* and *in vivo* (Steinberger and Steinberger, 1971; Orth, 1984; Orth *et al.*, 1984), and FSH plays a vital role in the proliferation of Sertoli cells in early life (Orth *et a.l*, 1984; Meachem *et al*, 1996). Meachem *et al* (1996) showed that administration of FSH during early postnatal life results in an increase in the number of Sertoli cells, with an accompanying increase in the number of germ cells. These effects were carried through to adulthood to significantly increase testicular weight and Sertoli cell and spermatid numbers.

Withdrawal of FSH in immature rats by treatment with FSH antibody impairs spermatogenesis (Raj and Dym, 1976), suggesting a role for FSH in pubertal spermatogenesis. Administration of FSH to immature rats reduces the number of degenerating cells (Russell *et al.*, 1987) and prevents apoptotic cell death (Tapanainen *et al.*, 1993). Russell and colleagues demonstrated the ability of FSH to prevent germ cell degeneration is potentiated in the presence of LH (Russell *et al.*, 1987), and postulated a role for FSH as a germ cell survival factor and synergism between FSH and LH to prevent germ cell degeneration in immature animals. FSH may also have a role in the metabolism of testosterone (see chapter 1.7.5) to its 5α-reduced metabolites (Oshima *et al.*, 1970; Folman *et al*, 1973) and may regulate the number of ARs on the Sertoli cell (Buzek and Sanborn., 1990).

The role of FSH in spermatogenesis has been studied recently using hypophysectomy, in both immature and adult rats by Russell and colleagues. El Shennawy (1998) demonstrated that FSH and testosterone were capable of preventing germ cell loss, and that FSH could partially compensate for low testosterone levels by maintaining germ-cell viability, suggesting an important role for FSH action at low testosterone levels. Russell *et al* (1998) showed that FSH restoration in pubertal hypophysectomized rats stimulated cells beyond, but not inclusive, of type A spermatogonia. These cell types were also sensitive to testosterone, suggesting that testosterone and FSH have a common goal, and work together to support spermatogenesis and to affect the survival of the same cell types. Franca *et al* (1998) showed that elimination of residual androgen, via administration of the androgen receptor (AR) antagonist flutamide, resulted in marked reduction in germ-cell numbers in the long-term hypophysectomized rat, suggesting that low levels of androgens can enhance germ cell survival. In summary, these findings suggest that FSH can prevent germ cell loss and that FSH and testosterone can act synergistically and may substitute for each other to maintain germ cell viability, especially during the early stages of spermatogenesis.

The regulation of spermatogenesis by FSH in the adult has not been as extensively studied as that in the immature animal (for review see Zirkin *et al.*, 1994). Shetty *et al* (1996) reported that immunoneutralization of FSH increased spermatogonial and spermatocyte apoptosis and Ghraf *et al* (1997) reported a reduction in sperm number in adult rats immunized against the FSH receptor. The role of FSH in the maintenance of adult spermatogenesis has been studied in GnRH antagonist-treated (Chandolia *et al.*, 1991c; Sinha-Hikim and Swerdloff, 1995) and hypophysectomized (Bartlett *et al.*, 1989; Kerr *et al.*, 1992; Russell *et al.*, 1993a) models. These studies show that FSH supports germ cell development up to the round spermatid stage, but completion of spermiogenesis can only be achieved in the presence of testosterone (Bartlett *et al.*, 1989; Kerr *et al.*, 1992).

Experiments examining the restoration of adult rat spermatogenesis by FSH have shown that recombinant human FSH (rhFSH) administration increases the number of germ cells up to and including the round spermatid stage, however elongated spermatids remain at less that 1% of normal (McLachlan *et al.*, 1995). This suggests that an additional factor, presumably testosterone, is required to restore elongated spermatids. Another study showed that following long-term regression by hypophysectomy, testosterone alone (Huang *et al.*, 1987) could not restore sperm output, suggesting that an additional factor (e.g. FSH) is required.

In vivo studies by Cameron, Muffly and colleagues have indicated an important role for FSH in maintaining and restoring Sertoli cell structural features during mid-spermiogenesis. They showed that FSH *in vivo* was crucial for Sertoli cell cytoskeletal structures that are required for round spermatid binding, whereas testosterone induced the actual binding (Cameron and Muffly, 1991; Cameron *et al.*, 1993b). Furthermore, testosterone could maintain binding capacity of the Sertoli cell in the absence of gonadotrophins, but could not restore binding competency after prolonged hormonal absence (Muffly *et al.*, 1993). Therefore, testosterone maintains elongated spermatid numbers but could not fully restore elongated spermatids following the prolonged absence of hormones. A further study showed that FSH could restore the cytoskeletal arrangement of the Sertoli cell proteins, actin and vinculin, thus enabling testosterone to restore elongated

spermatid numbers (Muffly *et al.*, 1994). Therefore, it appears that one of the actions of FSH during normal spermiogenesis is for the arrangement of Sertoli cell cytoskeletal proteins.

Meachem *et al* (1998) demonstrated that the ability of exogenous testosterone to reinitiate spermatogenesis is profoundly impaired in the absence of FSH. They showed a major role for FSH in the facilitation of spermatogenic restoration by testosterone, particularly in the restoration of spermatogonial number and spermatocyte maturation, and demonstrated synergy between testosterone and FSH in the restoration of spermatocyte and round spermatid populations in the context of short-term FSH withdrawal. A further study showed a time-dependent decrease in spermatogonial and preleptotene spermatocyte number following acute withdrawal of FSH by immunoneutralization in normal adult rats, and that FSH withdrawal resulted in an increase in the percentage of stage XIV-III tubules containing TUNEL-positive cells (Meachem *et al.*, 1999). In conclusion, FSH is essential for qualitatively normal adult rat spermatogenesis, and it appears that FSH promotes germ cell survival.

Although FSH is not essential for spermatogenesis, the general consensus to date is that FSH has a role in maintaining quantitatively normal spermatogenesis (Sharpe, 1989; Zirkin *et al.*, 1994; McLachlan *et al.*, 1996). This is not surprising since the seminiferous tubules are the only target in the male for FSH (Griswold, 1993).

There is however some evidence to suggest that FSH is not essential for normal adult rat spermatogenesis. Dym *et al* (1979) suggested that FSH was not required for spermatogenesis since FSH immunoneutralization in normal adult rats for 14 days had no significant effects on spermatogenesis. Buhl *et al* (1982) showed that testosterone alone could maintain spermatogenesis in adult rats, and Sharpe *et al* (1988) reported that testosterone alone could quantitatively maintain spermatogenesis and fertility for a 10 week period, despite serum FSH levels being subnormal. Awoniyi *et al* (1992a) reported that testosterone alone, administered via silastic implants, can quantitatively maintain sperm output in GnRH-immunized rats in the absence of detectable levels of FSH, although others have showed that the regime used by these authors restores FSH (McLachlan *et al*, 1994a).

Singh *et al* (1995) argues against an essential role for FSH in the initiation of spermatogenesis in mice, from studies in the *hpg* mouse (lacks a functional GnRH gene and therefore is congenitally deficient in gonadotrophins). These studies have shown that quantitatively complete spermatogenesis can be initiated by androgens in *hpg* mice.

Transgenic mice possessing targeted disruptions of the FSH receptor gene (Dierich *et al.*, 1998) or the FSHβ-subunit gene (Kumar *et al.*, 1997) have been constructed to determine whether FSH plays an important role in spermatogenesis. Both transgenic models produce fertile mice which display all stages of germ cell development, as are the androgen-treated hpg mice (Singh *et al.*, 1995), suggesting that FSH is not an absolute requirement for fertility. However, in all cases the testes are smaller, and less sperm are produced (Singh *et al.*, 1995; Kumar *et al.*, 1997; Dierech *et al.*, 1998), due to the requirement for FSH during the neonatal period of Sertoli cell division (Singh *et al.*, 1996a, b). More recent quantitative studies on FSH receptor knockouts also demonstrated defects in sperm development, leading to the production of poor quality sperm (Krishnamurthy *et al.*, 2000). Thus, while FSH is not essential for spermatogenesis, it is clearly essential for quantitatively normal spermatogenesis and fertility.

FSH exerts its biological effect on the testis via FSH receptors (see Griswold *et al.*, 1995 for review) present on the plasma membrane of Sertoli cell, the gene for which is likely to be expressed only in the Sertoli cells (Heckert and Griswold, 1993). However, one report suggests that FSH receptors may be present on spermatogonia (Orth and Christensen, 1978). FSH binds to a G protein coupled receptor on the plasma membrane of the Sertoli cell, promoting cyclic adenosine 3', 5'-monophosphate (cAMP) mediated increase in protein kinase A (PKA) (Casey and Gilman, 1988). PKA phosphorylates proteins to either up-regulate or down-regulate their activity. Binding of FSH to its receptor may lead to changes in the structure/function of proteins such as regulatory factors and enzymes (see Griswold, 1993). The biochemical actions of FSH on the Sertoli cell can be mimicked by the addition of cAMP derivatives (Monaco *et al.*, 1995) highlighting the importance of the cAMP-mediated pathway in FSH action. Other pathways of

FSH signal transduction include the phospolipase C or protein kinase C (PKC) and calcium pathway (Rudge *et al.*, 1995; Gorczynska *et al.*, 1996).

In summary, it is clear that FSH is important for establishing the number of Sertoli cells during early testicular development, which determines the capacity of sperm output in the adult. It seems that FSH is not essential for testicular development as suggested in congenitally deficient mice, however FSH is important for quantitatively normal spermatogenesis. While FSH has a role in the development of the immature testis, controversy persists as to whether FSH is important for the maintenance of adult spermatogenesis. FSH appears to effect adult spermatogenesis by possibly modulating the numbers of spermatogonia and by regulating the structure of the Sertoli cell. FSH also probably exerts major effects on spermatogenesis when acting synergistically with testosterone.

1.3.4 Regulation of Spermatogenesis by Testosterone

The role of testosterone and the concentration of testosterone needed for qualitatively and quantitatively normal spermatogenesis has been the subject of considerable research (for review, see de Kretser *et al.*, 1992; Weinbauer and Neischlag, 1993; Sharpe, 1994; McLachlan *et al.*, 1996). More recently, the role of testosterone in contraception has been clinically investigated as a male contraceptive (WHO, 1990, 1996). These studies have shown that suppression of testicular testosterone to very low levels, induced by gonadotrophin suppression, yields an effective and reversible contraceptive.

(a) Effects of Testosterone on Spermatogenesis

The role of androgens in the initiation of spermatogenesis has been studied in congenitally gonadotrophin-deficient mice (Singh *et al.*, 1995). This study reported normalization of spermatogenesis by androgens in the absence of measurable FSH, suggesting that androgens alone can initiate spermatogenesis in mice. In the rat, testosterone is involved in the completion of the

first wave of spermatogenesis during puberty (Cameron *et al.*, 1993a), and a single injection of EDS to immature rats causes a transient reduction in elongated spermatid numbers.

There is no doubt that testosterone has an essential role in normal adult spermatogenesis (de Kretser *et al.*, 1992; Sharpe, 1994; McLachlan *et al.*, 1996). Testosterone alone can maintain at least qualitatively normal spermatogenesis in hypophysectomized (Chowdhurry, 1979; Buhl *et al.*, 1982; Bartlett *et al.*, 1989; Sun *et al.*, 1989; Huang *et al.*, 1991; Muffly *et al.*, 1993), GnRH antagonist-treated (Pogach *et al.*, 1993), EDS-treated (Kerr *et al.*, 1992) and intact rats (Walsh and Swerdloff, 1973; Huang and Bocabella, 1988; Sun *et al.*, 1990). Quantitatively normal spermatogenesis can be maintained by testosterone alone in GnRH-immunized rats (Awoniyi *et al.*, 1989b, 1992a) and in intact rats (Zirkin *et al.*, 1989). However, testosterone administration to rats is unable to maintain normal elongated spermatid numbers, suggesting a need for FSH or other factors (Santulli *et al.*, 1990).

Testosterone can restore spermatogenesis to normal or near normal levels in rats given high doses of testosterone (Elkington and Blackshaw, 1974; Huang *et al.*, 1987). In the TE-suppressed model, where FSH levels are not significantly reduced, testosterone can restore elongated spermatids to normal (Awoniyi *et al.*, 1989a, 1990) or near normal levels (McLachlan *et al.*, 1994a). In GnRH-immunized rats, spermatogenesis can be restored by testosterone (Awoniyi *et al.*, 1989b, 1992b; McLachlan *et al.*, 1994b), although a concomitant restoration of FSH levels in these experiments may have contributed to this restoration (McLachlan *et al.*, 1994).

Meachem *et al* (1997) showed that TE-suppression reduced type A spermatogonia and type B spermatogonia/preleptotene spermatocyte numbers per testis to 60% and 77% of control, respectively. Neither testosterone nor human chorionic gonadotrophin (hCG) treatment, which restored both androgen action and FSH levels, could restore early germ cell numbers, whereas elongated spermatids numbers were restored. This study also examined the effects of residual levels of testosterone following TE-suppression, and found that blocking residual androgens via flutamide administration resulted in a further inhibition of spermatocyte development and meiosis. Therefore, partial restoration of testicular testosterone following TE-suppression restores

spermatid maturation but not spermatogonial number, and residual levels of testosterone in the TE-suppression model have clear persisting effects on spermatogenesis, particularly on spermatocytes and on spermiogenesis.

There is still debate as to whether high intratesticular testosterone concentrations are essential for the maintenance of spermatogenesis (see Romerts, 1988). Numerous studies (Cunningham and Huckins, 1979; Marshall *et al.*, 1984) have reported that spermatogenesis can be maintained at 10-20% of normal testicular testosterone levels, with subnormal levels of sperm. In GnRH antagonist treated rats given testosterone implants, quantitatively normal spermatogenesis can be maintained at 15% of normal intratesticular testosterone (Rea *et al.*, 1986).

The maintenance of spermatogenesis by testosterone occurs over a narrow dose range, so that very small changes in testicular testosterone levels result in large changes in sperm output (Awoniyi *et al.*, 1989a, 1990, 1992a; Sun *et al.*, 1989, 1990; Zirkin *et al.*, 1989, McLachlan *et al.*, 1994b). In the TE model, spermiogenesis (specifically the conversion of stage VII to VIII round spermatids) is normalized with testicular testosterone concentrations at 12% of control but not at 5% of control (O'Donnell *et al.*, 1996a). Therefore it seems that the spermatogenic process is extremely sensitive to small changes in testosterone levels. The high levels of testosterone in the testis may simply be a consequence of local production aimed at maintaining serum testosterone levels needed to supply peripheral tissues with testosterone or its 5α-reduced metabolites.

Maintenance studies of spermatogenesis that have looked at the effects of administering increasing doses of testosterone (Huang and Boccabella, 1988; Rommerts, 1988; Sun *et al.*, 1989). These studies have demonstrated that spermatogenesis can be maintained when intratesticular testosterone levels are significantly lower than normal (Zirkin *et al.*, 1989), thus the normally high levels of testosterone in the testis are not essential for the maintenance of qualitatively normal spermatogenesis.

(b) Stage-Specific Effects of Testosterone on Spermatogenesis

The acute withdrawal of testosterone by EDS treatment is associated with stage-specific degeneration of germ cells, leading to a disruption of spermatogenesis (Bartlett *et al.*, 1986; Kerr *et al.*, 1992, 1993). Following EDS treatment, pachytene spermatocytes and round spermatids in stage VII of spermatogenesis degenerate (Bartlett *et al.*, 1986). Stage VII of the spermatogenesis cycle is the most sensitive stage to hormonal withdrawal (Russell *et al.*, 1981, 1987; Sharpe, 1994). Intratesticular testosterone suppression by TE-treatment suppresses spermatogonial and spermatocyte numbers to 60% of normal, and partial restoration of testicular testosterone by high doses of exogenous testosterone, restores sperm output to 85% of normal, suggesting a role for testosterone in the maintenance of spermatogenesis (McLachlan *et al.*, 1994a).

The major role of testosterone appears to be in the conversion of round to elongated spermatids during spermiogenesis (Sun *et al.*, 1990; McLachlan *et al.*, 1994a, 1996). Administration of 3 cm-*T.imp* to intact rats suppressed the conversion of round to elongated spermatids, whereas the conversion was maintained by 24 cm-*T.imp* (Sun *et al.*, 1990; McLachlan *et al.*, 1994a; O'Donnell *et al.*, 1994, 1999). TE treatment in adult rats suppressed round spermatids between steps 1-8 to 29-45% of control and abolished elongated spermatids, whereas high dose testosterone treatment restored round and elongated spermatid numbers to 80% of control (McLachlan *et al.*, 1994a; O'Donnell *et al.*, 1994, 1996, 1999).

The role of testosterone in spermiogenesis has been extensively studied in our laboratory. The TE model of spermatogenic suppression together with stereological techniques have been used to identify the germ cells during spermiogenesis that are sensitive to testosterone (O'Donnell *et al.*, 1994). We have shown that TE-treatment suppressed intratesticular testosterone levels and round spermatids in stages I-VII (~30% of control), however, round spermatids in stage VIII were more markedly (5% of control) suppressed. High dose testosterone treatment (24 cm-*T.imp* for 4 days) did not significantly increase the number of stage I-VII round spermatids, but significantly increased stage VIII round spermatids. The conversion of round spermatids between stages I-III, IV-VI and VII were not altered during TE treatment or during the recovery phase. In contrast, the

conversion of stages VII and VIII round spermatids was reduced to 16% of control by TE-treatment and was normalized (95% of control) by 24 cm-*T.imp* for 4 days (testosterone levels restored to 12% of control). Thus, the conversion of round spermatids between stages VII-VIII of the spermatogenic cycle is a testosterone-dependent event, and is consistent with the classic view that these stages are androgen dependent (Russell *et al.*, 1981).

In a further study, the testosterone-dependent loss of round spermatids during stages VII and VIII was investigated (O'Donnell *et al.*, 1996a). Sections of the cauda epididymis revealed no round spermatids in control animals, but the epididymis was filled with many degenerating round spermatids after 6 weeks of TE-treatment, suggesting round spermatids that fail to undergo the transition from stages VII to VIII become detached from the seminiferous epithelium and proceed to the epididymis to degenerate. A subsequent study showed that the Sertoli cell ectoplasmic specialization was normal in TE-suppressed rats, suggesting that the loss of round spermatids from stages VII to VIII was not caused by the loss of the ectoplasmic specialization (O'Donnell *et al.*, 2000).

In summary, these studies (O'Donnell *et al.*, 1994, 1996a, b) have demonstrated that the location at which testosterone effects spermatogenesis to promote the progression of round to elongated spermatids is between stages VII and VIII. Round spermatids are unable to proceed through this conversion because there is a loss of testosterone-dependent attachment to the seminiferous tubule. This is consistent with the findings of Cameron and Muffly (1991) who reported sloughing of step 8 round spermatids after EDS-induced testosterone withdrawal in pubertal and adult rats (Muffly *et al.*, 1994).

While it is known that testosterone is essential for adult spermatogenesis, the mechanisms by which testosterone regulates germ cell development are largely unknown. Testosterone effects on spermatogenesis are generally stage-specific, with stages VII and VIII regarded as the primary androgen-dependent stages (see Sharpe, 1994 for review). Therefore, although testosterone is the major regulator of adult spermatogenesis (see Weinbauer and Neischlag, 1993; Sharpe, 1994;

McLachlan *et al.*, 1996) little is known about specific genes and proteins that may be androgen-dependent. The AR will be discussed later (section 1.4.8).

1.3.5 Regulation of Spermatogenesis by 5α-Reduced Androgens

Testosterone can be converted via the 5αR enzyme to the 5α-reduced metabolites, dihydrotestosterone (DHT) and 3α-androstanediol (3α-Adiol) (see section 1.4.1). DHT is a more potent androgen than testosterone and is extremely important in androgen-dependent peripheral tissues where local testosterone concentrations are low (Wilson, 1975; Grino *et al.*, 1990; Chen *et al.*, 1994). 3α-Adiol has also been suggested to have androgenic effects in some systems although it is unclear whether this is due to back-conversion to DHT, a direct interaction with the AR, or via a non-AR mediated mechanism (Nakhla *et al.*, 1995).

The role of DHT in the testis however is unclear. It is speculated that the low levels of testosterone during puberty require metabolism to DHT to amplify the androgenic effect. From birth to puberty, the concentrations of the 5α-reduced metabolites are greater than the concentration of testosterone (Corpechot *et al.*, 1981), presumably due to the high levels of 5αR enzyme activity (Matsumoto and Yamada, 1973; Dorrington and Fritz, 1975a; van der Molen *et al.*, 1981), suggesting that 5α-reduced metabolites may be important for the first wave of spermatogenesis. Therefore, 5α-reduction of the low levels of testosterone that are present in the pubertal testis may be important for androgen action on spermatogenesis at this time.

From maturity to adulthood, the concentration of 5α-reduced metabolites are significantly lower than the concentration of testosterone (Corpechot *et al.*, 1981). Thus, testosterone is quantitatively the predominant testicular androgen, and testosterone rather than DHT is the major androgen bound in nuclei isolated from adult rat seminiferous tubules (Wright and Frankel, 1979). Despite the fact that 5αR activity (Rivarola and Podesta, 1972a; Folman *et al.*, 1972, 1973; Matsumoto and Yamada, 1973; Sowell *et al.*, 1974; Dorrington and Fritz, 1975) and mRNA

(Normington and Russell, 1992; Viger and Robaire, 1995) is present in the adult rat testis (see section 1.6.1), the role of 5αR in adult spermatogenesis is unclear.

5α-Reduced androgens are able to initiate or maintain spermatogenesis similarly to testosterone (Chowdhury and Steinberger, 1975), and DHT can maintain spermatogenesis at a concentration that is half the concentration of testosterone needed for quantitative maintenance (Chen *et al.*, 1994). This demonstrates that DHT is a more potent androgen that testosterone.

Our group is interested in investigating the role of 5α-reduced metabolites in the regulation of adult spermatogenesis. We are especially interested in the effects of 5α-R on spermatogenesis when testicular testosterone levels are experimentally reduced (i.e. contraception, see section 1.7). In this experimental setting, such as in the TE-treated rodents (see section 1.3.2 b) or testosterone-treated men (see section 1.7.1), the concentration of testosterone in the testis is reduced to concentrations that are similar to the concentration of testosterone in androgen-dependent peripheral tissues, which are critically dependent on 5α-reduction of testosterone.

The role of 5α-reduced androgens in spermatogenesis was investigated by suppressing sperm production via TE-treatment, and then using the androgen-dependent conversion of round spermatids between stages VII and VIII to assess the restoration of spermiogenesis (O'Donnell *et al.*, 1996b). Spermiogenesis was restored by increasing doses of testosterone for 4 days, and inhibition of 5αR using a non-competitive 5αR enzyme inhibitor suppressed the conversion of round spermatids between stages VII and VIII at low doses of testosterone (3 cm-*T.imp* and 6 cm testosterone silastic implants; 6 cm-*T.imp*). This data supports a role for 5α-reduced androgens in the restoration of spermiogenesis at low testosterone doses. When higher doses (10 cm testosterone silastic implant; 10 cm-*T.imp* and 24 cm-*T.imp*) of testosterone were administered, the inhibitor had no effect on the restoration of spermiogenesis, as seen previously with low testosterone doses. The inability of the inhibitor to have an effect at high testosterone doses could be due to either the competitive nature of the inhibitor (L685,273, a gift from Merck, Sharp and Dohme● [see Appendix 1]), or the testosterone levels associated with 10 cm-*T.imp* and 24 cm-

T.imp may be sufficient to permit testosterone to interact with the AR without requiring conversion to the more potent androgen, DHT.

A subsequent study (O'Donnell *et al.*, 1999) looked at the suppression of spermatogenesis by 5αR inhibition in the short (4 days) and long-term (6 weeks), and also examined the effect of androgen blockade on the restoration of spermatogenesis. 5αR inhibition suppressed the restoration of round spermatid conversion between stages VII and VIII in the short and long term and suppressed the number of elongated spermatids in the long term. This study confirmed and extended the previous results (O'Donnell *et al.*, 1996b), showing that testicular 5αR is involved in the restoration and maintenance of low levels of sperm production in a hormonally based contraceptive setting between stages VII and VIII of the spermatogenic cycle (O'Donnell *et al.*, 1999). A surprising finding in this study was that flutamide treatment elevated testicular levels of DHT (3- to 6-fold) and 3α-Adiol (2- to 8-fold), despite no change in serum LH or testicular testosterone levels. This data suggested that 5αR activity in the testis may be up regulated in the absence of androgens. Therefore, the data reported by O'Donnell and colleagues (1996b, 1999) supports the proposition that 5α-reduction of testosterone may be important in determining the extent of spermatogenic suppression induced by exogenous testosterone-based contraceptives. The concepts presented in this section will be expanded later (section 1.7.2), where the relevance of 5αR to spermatogenesis during male contraception will be discussed.

A role for 5α-reduced androgens in the regulation of GnRH release has been suggested. Studies have demonstrated that 5α-reduced metabolites do play an important role in the feedback regulation of LH release at the pituitary and hypothalamic levels in male rats (Purvis *et al.*, 1977; Celotti *et al.*, 1992). More recently, Yokoi *et al* (1996) showed that the inhibitory effect of testosterone on gonadotrophins is probably partly due to its conversion to DHT. Furthermore, an effect of DHT on gonadotrophin release may be exerted at the pituitary gland itself since flutamide administration blocks the DHT suppression of serum FSH and LH in the pituitaries of gonadectomized male rats (Ghraf *et al.*, 1982).

Naturally occurring mutations of the 5αR-2 gene in males, termed 5αR-2 deficiency or male pseudohermaphroditism, impairs virilization of the external reproductive organs and prostate in men, indicating that this isoform synthesizes the DHT required for virilization of the male urogenital tract (Wilson *et al.*, 1993). Naturally occurring mutations in the 5αR-1 gene have not been identified. Therefore, to determine the physiological role of 5αR-1, homologous recombination in mouse embryonic stem cells was used to produce mice with a disruption (null allele) in the 5αR-1 gene (Mahendroo *et al.*, 1996). Mutation of the 5αR-1 gene in male mice did not affect virilization, but in the female caused defects in parturition and fecundity. Failure to 5α-reduce androgens in these 5αR-1 deficient mice lead to the conversion of testosterone to estrogens, causing fetal death in midgestation as a result of estrogen toxicity (Mahendroo *et al.*, 1997). It was hypothesized that mutations in 5αR-1 did not effect males because wild type male mice express 5αR-2 mRNA in a wider range of tissues than do female mice, and 5αR-2 mRNA levels are markedly higher in the adrenal gland of male mice. Thus, the fact that males contain more 5αR-2 than do females may explain why a disruption of the 5αR-1 gene appears to affect only females. It was further suggested that a disruption of the 5αR-2 gene in male mice might have more severe consequences.

Further experiments showed that mutations in the 5αR-2 gene had no affect on the phenotype of female mice (Mahendroo *et al.*, 1997), however, in this report, there was no mention of 5αR-2-deficient male mice. However, in a recent review by the same authors (Mahendroo and Russell, 1999) it was stated that male mice without the 5αR-2 gene failed to virilize properly, although the effect was not as severe as the genetic disease in human males.

There are no knockout models available for the rat, instead 5αR inhibitors have been administered to rats to determine the role of 5αR in testicular androgen action. Brooks *et al* (1982) administered the 5αR inhibitor 4-MA to pregnant female rats, and showed that inhibition of 5αR during male sexual differentiation caused feminization of male fetuses, and prevented growth of

the ventral prostate and seminal vesicles. However, when the inhibitor was administered to mature male rats (for 67 days) they remained fertile and were able to mate. Given that the 4-MA compound is a competitive inhibitor, it is not surprising that 5αR inhibition did not have an effect on adult spermatogenesis. The dose of inhibitor was probably not sufficient to compete with the exceedingly high concentration of testosterone normally present in the adult testis. Higher doses of the inhibitor were administered to immature rats, who have lower testicular testosterone levels than adults, but this dose of inhibitor was lethal.

To investigate the role of DHT in postnatal androgen physiology, George *et al* (1989) administered the 5αR inhibitor, finasteride, to male rats from birth through the onset of puberty. Treatment with finasteride for 7 weeks significantly suppressed testicular DHT levels and elevated testosterone by 6-fold. Sperm production and testicular histology were similar in inhibitor-treated and control groups, whereas there was impaired growth of the epididymis, seminal vesicles, prostate, and penis. These authors suggested that DHT does not play a critical role in spermatogenesis, but had a critical role in the formation of secondary sexual characteristics. Despite the authors conclusions that spermatogenesis was not dependent on intratesticular DHT formation, DHT was only modestly suppressed (~25%). Furthermore, elevated testosterone (3-fold) levels in this study could have compensated for the decrease in DHT.

1.3.6 Synergistic effects of FSH and Testosterone on Spermatogenesis

It is quite clear that both FSH and testosterone have effects on adult rat spermatogenesis, since either hormone can at least qualitatively maintain spermatogenesis. However, when the two hormones are given in combination, the effects on spermatogenesis are greater that the response expected if their individual effects were simply additive (Elkington and Blackshaw, 1974; Bartlett *et al.*, 1989b; Kerr *et al.*, 1992). There are many reports suggesting that FSH and testosterone act co-operatively and that a lower dose of either is equally effective when the other is present (Bartlett *et al.*, 1989; Sun *et al.*, 1989; Chandolia *et al.*, 1991; Sinha-Hikim and Swerdloff, 1994; El Shennawy *et al.*, 1998; Franca *et al.*, 1998; Russell *et al*, 1998). Thus it seems that the two

hormones probably act in synergy to promote normal germ cell development (see Weinbauer and Neischlag, 1993; Sharpe, 1994; McLachlan *et al.*, 1996).

Testosterone and FSH have been suggested to synergize by exerting effects at different stages of the spermatogenic cycle, acting together to allow completion of germ cell development. There is a preferential action of FSH on stages XIII-V of the spermatogenic cycle (Parvinen, 1982), and FSH has been implicated in maintaining earlier germ cell populations such as spermatogonia and preleptotene spermatocytes (McLachlan *et al.*, 1995; Sinha-Hikim and Swerdloff, 1995). In contrast, stages VII and VIII of the cycle appear to be predominantly androgen-dependent (for review, see Sharpe, 1994), especially during the later stages of spermiogenesis (Bartlett *et al.*, 1989b; Sun *et al.*, 1990; McLachlan *et al.*, 1994a; O'Donnell *et al.*, 1994, 1996a, b).

FSH and testosterone may both control the same basic metabolic functions of the Sertoli cells that are essential for spermatogenesis. This overlap in function may explain why in some species either hormone may qualitatively complete spermatogenesis though the quantitative extent to which this can be achieved is probably determined by the specific roles of FSH and testosterone.

It appears that FSH potentiates testosterone action since androgen requirements for spermatogenesis are much lower in the presence of FSH (Sun *et al.*, 1989, 1990; Zirkin *et al.*, 1989; Awoniyi *et al.*, 1990; Santulli *et al.*, 1990; Kerr *et al.*, 1992). Rats receiving both testosterone and FSH have significantly higher intratesticular testosterone concentrations than animals receiving testosterone alone, despite the fact that both groups have identical serum testosterone levels (Huang *et al.*, 1991). Thus, one way that FSH may lower the concentration of testosterone required for spermatogenesis may be by modulating the availability of testicular testosterone via FSH-stimulation of ABP. In immature rats at least, FSH appears to stimulate the AR (Verhoeven and Cailleau, 1988). The presence of testosterone may facilitate FSH action on spermatogenesis, since the effect of FSH on the maintenance of germ cells populations in GnRH-antagonist treated rats was diminished when Leydig cells were destroyed by EDS treatment (Spiteri-Grach *et al.*, 1993) or when the anti-androgen flutamide was administered (Chandolia *et al.*, 1991c).

Another site of FSH and testosterone co-operativity appears to be during spermiogenesis. The binding of round spermatids to Sertoli cells *in vitro* is dose-responsive to testosterone only when FSH is present (Cameron and Muffly, 1991; Cameron *et al.*, 1993b). Similarly, *in vivo* experiments show that following prolonged absence of gonadotrophins, testosterone is only able to restore spermiogenesis if previously exposed to FSH (Muffly *et al.*, 1993, 1994). Furthermore, binding of round spermatids to Sertoli cells has been shown to involve the cell adhesion molecule neural cadherin (NCad) (Perryman *et al.*, 1996), and maximal NCad production by Sertoli cells *in vitro* is dependent on both FSH and testosterone. Testosterone can maintain Sertoli cell cytoskeletal structures that are needed for round spermatids to adhere to Sertoli cells, whereas only FSH can restore these structures. Perryman *et al* (1996) showed that N-cadherin production was dependent on testosterone and FSH in round spermatid-Sertoli cell adhesion studies *in vitro*, suggesting that N-cadherin may subserve the androgen-dependent process of round to elongated maturation.

More recently, a synergistic effect of testosterone and FSH has been demonstrated during spermiation *in vivo*. Saito *et al* (2000) showed that suppression of FSH or testosterone for 1 week caused 11% and 14% of spermatids to fail to spermiate, whereas 50% of the spermatids in the testis failed to spermiate after combined suppression of both FSH and testosterone. Thus, it appears that FSH and testosterone act synergistically to support spermiation.

1.3.7 Other Factors that Regulate Spermatogenesis

Although FSH and testosterone are clearly the major hormonal regulators of spermatogenesis, numerous other paracrine factors are involved in normal germ cell development in the testis. Although the roles of paracrine and autocrine factors in spermatogenesis are largely undefined, there is evidence to suggest that these factors may play a role in coordinating spermatogenesis. Only a few examples of such factors are presented here, and the reader is directed to other reviews on this subject.

Inhibins and activins have been reported to have paracrine activity in the testis (see Moore *et al.*, 1994). The major known role for inhibin is its feedback effect on the pituitary to inhibit FSH release (Robertson *et al.*, 1991). However, administration of inhibin *in vivo* to mice and hamsters reduces spermatogonial numbers independent of FSH suppression (van Dissel-Emiliani *et al.*, 1989), suggesting a local testicular action. A paracrine role of activin in the testis has been suggested since activin binds to germ cells in a stage-specific manner (Woodruff *et al.*, 1992), and activin receptor mRNA has been localized in the testis (de Winter *et al.*, 1992).

Growth factors have also been implicated in the paracrine regulation of spermatogenesis. Transforming growth factors α and β (TGFα and TGFβ) have been suggested to be involved in some aspects of testicular function, although their roles remain relatively unclear (Spiteri-Grech and Nieschlag, 1993). TGFα is expressed and secreted by Sertoli cells (Skinner *et al.*, 1989) and the TGFα receptor is localized to the Sertoli cells (Suarez-Quian *et al.*, 1989). TGFα has also been localized to the Leydig cells in pubertal rat testis (Teerds *et al.*, 1990) where it is believed to be involved in the development of immature Leydig cells (Kahn *et al.*, 1992). TGFβ may act as a growth inhibitor to control prepubertal spermatogonial growth and to terminate growth of maturing Sertoli cells (see Skinner, 1993).

Other factors believed to be involved in the regulation of spermatogenesis include the interleukins (see Gerard *et al.*, 1991 and Hakovirta *et al.*, 1995), the *c-kit* proto-oncogene (see Chabot *et al.*, 1988 and Manova *et al.*, 1990), the insulin-like growth factors (see Spiteri-Grech and Nieschlag, 1993) and fibroblast growth factor and nerve growth factor (see Skinner, 1993).

1.4 5α-REDUCTASE ISOFORMS

1.4.1 5α-Reduction of Testosterone

The 5αR enzyme (3-oxo-5α-steroid:Δ^4-oxidoreductase, EC 1.3.99.5) was initially characterized in rat liver (Schneider and Horstmann, 1951) and was shown to be a membrane-

bound protein that catalyzes the reduction of $\Delta^{4,5}$ double bonds in a variety of steroid substrates (Tomkins, 1957; Wilson, 1975; for review see Russell and Wilson, 1994).

Testosterone (17β-hydroxy-4-androsten-3-one) is the major androgen secreted by the testis and is quantitatively the most important androgen in the testis and in the circulation. Testosterone serves as the precursor for the formation of two other hormones, 5α-reduced androgens and estradiol (*Figure 1-6*). Testosterone is irreversibly metabolized to DHT (17β-hydroxy-5α-androsten-3-one) via the membrane-bound microsomal steroid enzyme 5αR (Wilson, 1975), and DHT can undergo reversible metabolism to 3α-Adiol (5α-androstane-3α, 17β-diol) and 3β-Adiol (5α-androstane-3β, 17β-diol) via the hydroxysteroid dehydrogenase enzyme. Alternatively, testosterone may also undergo irreversible metabolism to estradiol via the aromatase enzyme (Wilson, 1975).

The 5α-reduction of testosterone to DHT is critically dependent on the cofactor nicotinamide adenine dinucleotide phosphate (NADPH, 4-ene-3-oxosteroid 5α-oxidoreductase, EC 1.3.99.5; Tomkins, 1957; McGuire and Tomkins, 1960; McGuire *et al.*, 1960, Moore and Wilson, 1972). Voigt *et al* (1970) showed that liver 5αR activity was stimulated by NADPH but not by NADH, and Frederiksen and Wilson (1971) showed that 5αR required NADPH as the source of reducing hydrogen. Addition of NADPH significantly increases the amount of testicular 5α-reduced metabolites formed in immature and adult rats (Sowell *et al.*, 1974). The apparent K_m for NADPH cofactor is in the low micromolar range (~5 μM) for 5αR (Thigpen *et al.*, 1993a).

1.4.2 Importance of 5α-Reductase in Androgen Physiology

In the early 1970's, the 5αR enzyme was shown to be present in the nuclei of androgen target tissues. Within a few minutes, when radioactive testosterone was administered to castrated male rats, the predominant hormone recovered from the ventral prostate was DHT (Bruchovsky and Wilson, 1968a; Bruchovsky, 1971). Furthermore, DHT was the predominant radiolabelled hormone actually bound to nuclear proteins (Bruchovsky and Wilson, 1968b). Another study

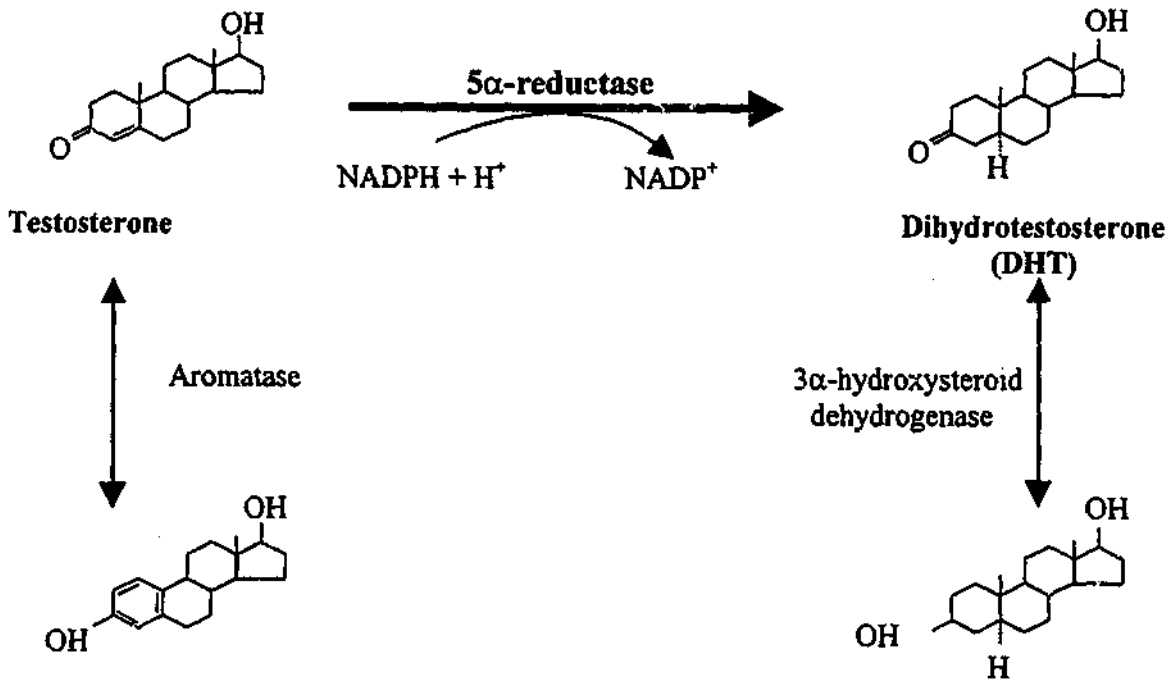


Figure 1-6: The NADPH-dependent metabolism of testosterone to dihydrosterone (DHT) via the steroid enzyme 5α-reductase. Alternatively, testosterone can be converted to estradiol via the aromatase enzyme.

showed that when rat ventral prostate was incubated with radiolabelled testosterone *in vitro*, there was a time-dependent accumulation of DHT in the nuclei of ventral prostate cells (Anderson and Liao, 1968). These studies collectively showed that DHT was a potent androgen with distinct physiological roles from testosterone.

Further evidence was obtained over the next few years to suggest that DHT was an important intracellular mediator of androgen action. Bruchovsky (1968b) showed that the 5α-reduced metabolite 3α-Adiol exerted its biological effects via conversion to DHT, and Wilson (1975) demonstrated that 5αR could not catalyze the back reaction (dehydrogenation) of 5α-reduced steroids, implying that 5αR may be a regulatory step. This data indicated that the 5α-reduction of testosterone to DHT was a crucial step in androgen action, and this attracted the attention of many scientists and clinicians who now focused their attention on 5αR and its role in androgen physiology (Russell and Wilson, 1994)

Definitive evidence for a key role of 5αR in androgen action was obtained from two lines of evidence. Developmental studies showed that testosterone itself mediated virilization of the Wolffian ducts, whereas DHT was responsible for the formation of the male external genitalia (Wilson and Lasnitzki, 1971; Wilson, 1972). Formal genetic proof of the crucial role of DHT in androgen action came from studies examining 5αR-2 deficiency, an inborn error of male phenotypic sexual differentiation (Imperato-McGinley, 1974). This genetic deficiency of DHT production causes a condition of intersex in which virilization of genetic males was impaired, leaving patients with normal internal reproductive structures but developmental defects in the formation of the prostate and external genitalia, such that they resemble those of female external genitalia (see section 1.5.3 for further details).

1.4.3 Purification of 5α-Reductase

All attempts to purify 5αR from both rat and human non-gonadal tissues (Frederiksen and Wilson, 1971, 1975; Moore and Wilson, 1972; Scheer & Robaire, 1983; Ichihara and Tanaka,

1987; Levy *et al.*, 1990a) have been unsuccessful. Two major obstacles hampering the isolation of 5αR were the profound insolubility of the protein and the low levels of 5αR enzyme activity expressed in most tissues (Moore and Wilson, 1972; Wilson, 1975).

Some attempts were successful in partially purifying 5αR from rat sources, such as the prostate, and showed that 5αR was an integral membrane protein of the endoplasmic reticulum or nuclear membrane (Moore and Wilson, 1972). With the exception of Ichihara and Tanaka (1989) who were successful in solubilizing and partially purifying 5αR from rat testicular microsomes, there are no other reports on solubilized 5αR in the testis. Advances in the study of 5αR and the existence of multiple 5αR enzymes was made possible by the isolation of cDNAs and genes that encoded this enzyme.

1.4.4 Expression Cloning of 5α-Reductase cDNAs

Andersson and colleagues (1989) used expression cloning in *Xenopus* oocytes to clone and sequence a cDNA encoding rat liver and prostate 5αR. Nucleic acid hybridization experiments demonstrated that the 5αR mRNA and gene expressed in prostate and liver were identical. The full-length cDNA for rat liver 5αR was cloned from female rat liver mRNA, because unlike most tissues (Yates *et al.*, 1958; Moore & Wilson, 1972; Liang *et al.*, 1983; Levy *et al.*, 1990a) female rat liver expressed large amounts of 5αR activity (Andersson *et al.*, 1989; Normington and Russell, 1992). The 2.5 kb mRNA isolated from female rat liver was injected into *Xenopus* oocytes, to synthesize a hydrophobic protein of 29 kDa that metabolized testosterone to DHT.

The full length cDNA encoding rat liver 5αR was subsequently used to isolate a human homolog of the rat cDNA by cross-hybridization of a prostate cDNA library (Andersson and Russell, 1990). The human cDNA identified was 2.1 kb, and DNA sequence analysis showed that the enzyme was a hydrophobic protein of 259 amino acids with a predicted molecular weight of 29 kDa. Transfected human and rat cDNAs in COS cells produced proteins showing similar substrate specificities, but markedly different sensitivities to 5αR inhibition by 4-azasteroids. Another

puzzling feature was that rat and human 5αR both showed maximal activity at neutral pH. This was an unexpected for the human enzyme, which had been previously reported to operate at an acidic pH (5.0-5.5) optimum in prostate (Liang *et al.*, 1985), genital skin fibroblast (Moore *et al.*, 1975) and epididymal (Fisher *et al.*, 1978) cell homogenates. Thus, the relationship between the protein encoded by the human cDNA and the acidic- and alkaline-optimum enzymes was puzzling.

A possible explanation for this puzzling observation was that there might be two different 5αR isoforms. The only evidence for the existence of multiple 5αR enzymes was from studies with cultured human fibroblasts, in which separate 5αR activities with acidic and basic pH optimas were detected, where normal genital skin was highly active at an acidic pH (5.5) whereas non-genital fibroblasts displayed a broad neutral pH range (7.0-9.0) (Moore *et al.*, 1975). Based on the difference of pH optima, it was postulated that there may in fact be two different 5αR enzymes, one with an acidic pH optimum and one with a broad neutral pH range.

Jenkins and colleagues (1992) showed that the enzyme activity in fibroblasts biopsied from 5αR deficient patients was very low to undetectable. Southern blots revealed several features between the isolated 5αR cDNA and its encoded enzyme in affected patients: (a) there were no mutations in the coding sequence of the gene despite the altered biochemical properties of 5αR in 5αR deficient individuals, (2) the cDNA-encoded enzyme was maximally active over a broad neutral pH range (6.0-8.5) whereas the predominant enzyme activity present in human prostate showed a narrow acidic pH optimum at pH 5.0, and (c) the 4-azasteroid inhibitor finasteride was a weak inhibitor (K_i ~300-600 nM) of the cDNA-encoded enzyme but a potent inhibitor (K_i ~ 10 nM) of prostatic 5αR activity. This data strongly supported the existence of a second human 5αR gene.

A cDNA encoding a second putative 5αR enzyme was finally isolated from a cDNA library from human prostate mRNA using expression cloning and polymerase chain reaction (PCR) (Andersson *et al.*, 1991). Similar to the major enzyme in genital skin fibroblasts (Moore *et al.*,

1975) and prostate (Liang *et al.*, 1985), expression cloning showed that the enzyme activity encoded by the second cDNA had an acidic pH optimum, unlike the first cDNA which produced maximal enzyme activity over a basic pH range. Along with the cloning studies, PCR experiments using cDNA reverse transcribed from human prostate ribonucleic acid (RNA) as a template generated a product whose DNA sequence was 57% identical to the corresponding region of the human cDNA. This product was used to screen a pool of prostate cDNAs, and DNA sequence analysis of a positive clone indicated that both cloning and PCR approaches had identified the same cDNA. The DNA sequence of the 2.43 kb cDNA insert was encoded by a hydrophobic protein of 254 amino acids and contained a long 3'-untranslated region. This cDNA-encoded a second enzyme that was 50% identical to the first enzyme isolated from human and 46% identical to the rat. Previously it was shown that finasteride markedly inhibited human prostate 5αR (Liang *et al.*, 1985; Jenkins *et al.*, 1992) but was a poor inhibitor of the first 5αR cDNA cloned (Griffin and Wilson, 1989). Expression of the second cDNA produced an enzyme that was markedly inhibited by finasteride (IC_{50} = 30 nM) compared to the first cDNA (IC_{50} = 900 mM).

The rat homolog of the second human 5αR cDNA was cloned from total rat prostate RNA (Normington and Russell, 1992). The RNA was isolated from animals that had been castrated for 7 days and then administered testosterone propionate for 3 days since previous findings showed that readministration of androgens to castrated rats causes rapid regeneration of the ventral prostate and a dramatic induction of 5αR enzyme activity (Moore and Wilson, 1973) and mRNA (Andersson *et al.*, 1989). The PCR yielded a second cDNA with a novel sequence that was 58% identical to the corresponding region of the first 5αR cDNA. An oligonucleotide derived from this novel sequence was used to screen a rat testis cDNA library, and a positive clone containing a 1.8 kb insert was isolated and sequenced. The cDNA encompassed the entire coding region of the mRNA but not the complete copy of the molecule as no poly(A) sequence was present at the 3'end of the insert.

Therefore, there are two 5αR genes encoded by different proteins that catalyze the same biochemical reaction. These isoforms of 5αR were referred to as 5αR type 1 (5αR-1) and 5αR type 2 (5αR-2), according to the order in which their respective cDNAs were cloned. These are hydrophobic proteins composed of 254-260 amino acids with predicted molecular weights of 28,000-29,000 (Andersson *et al.*, 1989; Thigpen *et al.*, 1993a, b). An average of 37% of the residues have side chains buried in the hydrophobic interior of globular proteins, and these hydrophobic amino acids suggest that the 5αR isoforms are intrinsic membrane proteins deeply embedded in the lipid bilayer.

Amino acids sequence identities between the 5αR isoforms in rat and human indicate a sequence identity of 60% between 5αR-1 isoforms and 77% between 5αR-2 isoforms. Identical sequences of amino acids among all of the 5αR isoforms has allowed the production of oligonucleotide primers which have been proven useful in cDNA cloning of 5αR using PCR (Andersson *et al.*, 1991). Overall, rat 5αR-1 shares 47% sequence identity with human 5αR-1, and rat 5αR-2 shares 77% identity with the human 5αR-2 enzyme. Therefore, at the amino acid level the 5αR-2 isoforms of the rat and human are more closely related to each other than they are to their 5αR counterparts.

Both human 5αR genes contain five exons separated by four introns (Jenkins *et al.*, 1991; Thigpen *et al.*, 1992b), and the position of the introns are identical in both genes. Although the gene structure is shared, the two genes are located on separate chromosomes in both human and mouse (Jenkins *et al.*, 1991; Thigpen *et al.*, 1992b). The gene encoding human 5αR-1 (gene symbol SRD5A1) is located on the distal short arm of chromosome 5 (band p15), whereas 5αR-2 (gene symbol SRD5A2) is located in band p23 of chromosome 2 (Thigpen *et al.*, 1992b).

1.4.5 pH Optima

The 5αR isoforms have remarkably different pharmacological properties, including pH optima, affinity for steroid substrates and sensitivity to 4-aza steroid inhibitors (Andersson and Russell, 1990; Normington and Russell, 1992).

5αR-1 has a broad neutral pH range whereas 5αR-2 has a sharp acidic pH optimum (see *Table 1*). The alkaline and acidic pH optima are thus diagnostic for the 5αR-1 and 5αR-2 isoforms, respectively, and this feature is often used to assign a 5αR activity in a tissue to a particular isoform. For example, one would predict that 5αR-1 is responsible for the alkaline pH optimum activity in non-genital skin fibroblasts (Moore and Wilson, 1976) and that 5αR-2 is responsible for the acidic pH optimum activity in cultured human genital skin fibroblasts (Moore *et al.*, 1975). These predictions for 5αR isoform activity, based on pH optimum, can and have been confirmed by other experiments, such as RNA blotting (Thigpen *et al.*, 1993b).

Table1: pH optimum for rat and human 5αR-1 and 5αR-2 isoforms.

	pH Optimum		Reference
	Neutral (5.0-9.5)		Andersson & Russell, 1990 Normington and Russell, 1992
	Acidic (5.0)		Andersson & Russell, 1990 Normington and Russell, 1992

The biochemical basis of the acidic pH optimum of 5αR-2 is puzzling, and was originally thought to be a reflection of its location within an acidic subcellular compartment (e.g. endosomes or lysosomes). However, recently it was shown that the activity of 5αR-2 in transfected Chinese hamster ovary (CHO) cells is not affected by agents that neutralize the pH of acidic subcellular compartments (Thigpen *et al.*, 1993a). Furthermore, both 5αR isoforms were present in the endoplasmic reticulum of CHO cells, a compartment with a neutral pH (Thigpen *et al.*, 1993a). It

is believed that 5αR-2 may have a neutral pH optimum in its native state (Thigpen *et al.*, 1993a). Thus, 5αR-2 would appear to function at a neutral pH range within the cell and to shift to a pH 5.0 active form upon cell lysis. The shift in pH requirement may reflect a conformational change in the isoform.

1.4.6 Affinities of 5α-Reductase Isoforms for Steroid Substrates

An analysis of the kinetic properties of the rat 5αRs revealed that 5αR-2 has a lower apparent K_m for testosterone, androstenedione, progesterone, and corticosterone than does 5αR-1 (Normington and Russell, 1992). Transfecting rat or human 5αR-1 produces an enzyme activity in cell lysates with a micromolar affinity for steroid substrates, whereas rat and human 5αR-2 produces an enzyme activity with a nanomolar affinity for steroid substrates (see Table 2). The K_m values for 5αR-2 in *Table 2* were conducted at pH 5.0, and surprisingly when these experiments are repeated at pH 7.0 in cell lysates, permealized cells, and intact cells, the K_m for 5αR-2 is still in the nanomolar range (40-50 nM, Thigpen *et al.*, 1993a; Faller *et al.*, 1993).

Table2: Affinity of transfected rat and human 5αR-1 and 5αR-2 isoform for testosterone.

	Source	Reference
	Rat (COS cells)	Andersson & Russell, 1990
	Rat (293 cells)	Normington and Russell, 1992
	Human (COS cells)	Andersson & Russell, 1990
	Human (CHO cells)	Thigpen *et al.*, 1993a
	Rat (293 cells)	Normington and Russell, 1992
	Human (CHO cells)	Thigpen *et al.*, 1993a

The significant differences in apparent K_m values exhibited by the two isoforms suggests that they may have different physiological functions. The low K_m for rat 5αR-2, when considered with

the low serum concentration of testosterone (~50 nM), indicates that this enzyme might be mainly responsible for the paracrine and autocrine generation of DHT in androgen-target tissues. This is supported by the tissue distribution of 5αR-2 mRNA and protein in adult rats, which demonstrate that 5αR-2 is found mainly in reproductive tissues (Normington and Russell, 1992). In contrast, the high K_m for rat 5αR-1 and its tissue distribution (mainly in non-androgen target tissue; Normington and Russell, 1992) suggests that the role of 5αR-1 may be to inactivate testosterone amplification, by preventing conversion to DHT, in non-androgen target tissues (see Russell and Wilson, 1994 for review).

1.4.7 5α-Reductase Inhibitors

With the recognition that DHT plays important roles in androgen action came the notion that inhibitors of 5αR might have therapeutic value (Wilson, 1972). This attracted the attention of pharmaceutical research laboratories and has led to the development of several potent inhibitors. Numerous steroidal and non-steroidal inhibitors have been designed and synthesized as competitive and uncompetitive inhibitors of 5αR.

Table3: Inhibitors of rat and human 5αR-1 and 5αR-2 isoforms and their corresponding K_i values.

Inhibitor/species	Reference
Finasteride (rat)	Normington and Russell, 1992
4-MA (rat)	Normington and Russell, 1992
L6852'8 (rat)	Normington and Russell, 1992
LY191,704 (human)	Hirsh *et al.*, 1998; Liang *et al.*, 1995
Epristeride (human)	Levy *et al.*, 1994
Finasteride (rat)	Normington and Russell, 1992
4-MA (rat)	Normington and Russell, 1992
L6852'8 (rat)	Normington and Russell, 1992
Epristeride (human)	Levy *et al.*, 1994

Inhibitors can be steroid derivatives such as the 4-azasteroids, 4-MA and finasteride (*Table 3*). Finasteride is a potent inhibitor of 5αR-2, however in pharmacological doses it inhibits both isoforms (Andersson and Russell, 1990). The difference in sensitivity of finasteride has been traced to four-amino acids in the amino terminus of 5αR-1 (Thigpen and Russell, 1992c).

Non-steroidal inhibitors include certain benzoylaminophenoxybutanoic acid derivatives, such as ONO-3805, a potent inhibitor (IC_{50} = 120 nM) of rat and human prostatic 5αR activity (Holt, 1993a). The other class of non-steroidal inhibitors are the benzoquinolines, such as LY191704, which are potent nonsteroidal, noncompetitive selective inhibitors of human 5αR-1 (see *Table 3*).

Epristeride is a potent uncompetitive inhibitor that selectively inhibits 5αR-2 enzyme activity and is a weak inhibitor of human 5αR-1 (*Table 3*; Levy *et al.*, 1994), and is being evaluated as a treatment of benign prostatic hyperplasia (Audet *et al.*, 1994).

Development of highly specific and potent 5αR inhibitors which have no affinity for ARs have allowed the discrimination between DHT-mediated and direct testosterone effects in animal models (Rasmusson *et al.*, 1986). In the future, the use of isoform-selective inhibitors *in vivo* will prove invaluable in assigning physiological roles to a given 5αR.

In summary, the differences in the biochemical properties between the 5αR enables these isoforms to be distinguished by their distinct pH optima's and substrate affinities. At the pharmacological level, the two 5αR isoforms have distinguishing features:

1) 5αR-1 has a broad neutral pH range whereas 5αR-2 has a sharp acidic pH optimum,
2) 5αR-2 has a higher affinity (i.e. lower K_m) for steroid substrates compared to 5αR-1, and
3) 5αR-2 is highly sensitive to 4-azasteroid inhibitors.

1.4.8 The Androgen Receptor

Steroids such as androgens are small hydrophobic molecules which are thought to cross the plasma membrane and bind to nuclear receptors to initiate steroid-dependent transcription (see

Kallio *et al.*, 1996 for review). The actions of testosterone and DHT are mediated through the AR, a member of the steroid hormone receptor family.

Binding of an androgen to the AR promotes dissociation of heat shock proteins (see Landers and Spelsberg, 1991) and promotes transformation in which the ligand-receptor complex acquires the ability to bind DNA (Kovacs *et al.*, 1983). Ligand binding initiates a conformational change, causing an increase in receptor stability (Kemppainen *et al.*, 1982). The testosterone-AR complexes dimerise (Kallio *et al.*, 1994) and then bind to androgen-response elements (AREs) in specific genes to regulate androgen-dependent transcription (Evans, 1988; Brann *et al.*, 1994). The AR is phosphorylated in the absence of hormone and undergoes hormone-stimulated additional phosphorylation (Kuiper *et al.*, 1993). The phosphorylation sites are generally located in the N-terminal region of the receptor (Kuiper *et al.*, 1993) which is the region of the receptor important for transcriptional activity. Therefore, it is possible that hormone-induced phosphorylation of the AR is important in the regulation of androgen-dependent transcription.

It is commonly accepted that both testosterone and DHT act via a single cytoplasmic receptor protein (Kovacs *et al.*, 1984). The notion that both steroids act through the same AR gene is also supported by the observation that mutations in the AR gene on the X chromosome lead to developmental abnormalities in both testosterone- and DHT-dependent processes (Griffin and Wilson., 1989). Even though genetic and biochemical evidence (Deslypere *et al.*, 1992) indicates that testosterone and DHT act through the same receptor protein, other possibilities such as translational or post-translational modification of the receptor to generate a molecule that preferentially interacts with one of the androgens, cannot be excluded.

The AR has been localized to Sertoli cells, peritubular myoid cells (Bremner *et al.*, 1994; Van Roijen *et al.*, 1997) and Leydig cells (Bremner *et al.*, 1994). The nuclei of Sertoli cells are predominantly immunostained and show stage-specific staining, with peak staining observed at stage VII of the spermatogenic cycle (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). This stage is considered to be more androgen-dependent than later spermatogenic stages. Most data suggest that the AR is not present in germ cells (Bremner *et al.*, 1994), which is in agreement with biochemical

data showing the absence of androgen binding in spermatocytes and spermatids (Grootegoed *et al.*, 1977). However, there is some immunocytochemical evidence to suggest the presence of AR in spermatids (Vornberger *et al.*, 1994). In the normal testis the AR would appear to be constantly saturated since testicular testosterone levels are in the micromolar range (Sharpe *et al.*, 1988) and the K_d of the AR for testosterone is ~3 nM (Isomaa *et al.*, 1985).

1.4.9 Affinities of Androgens for the Androgen Receptor

It was originally speculated that the conversion of testosterone to DHT amplified the androgenic signal because DHT could not be aromatised to estrogen, whereas the action of testosterone was the sum of its own effects plus those of the 5α-reduced and estrogenic metabolites (Wilson, 1975). Liao and Fang (1969) proposed that the more planar DHT molecule fits more tightly than testosterone into the critical site on the hormone-binding domain of the AR. The mechanism by which DHT is a more potent than testosterone has been the subject of a number of studies.

Grino and colleagues (1990) demonstrated that high concentrations of testosterone were needed to produce the same effects as lower concentrations of DHT. At equal concentrations of testosterone and DHT, testosterone exhibited a five-fold faster dissociation rate from the AR than did DHT, and DHT accounted for the majority (80%) of the bound steroid to the receptor. Equal binding of androgens was only observed when the concentration of testosterone was approximately 4-fold higher than that of DHT. At saturating steroid concentrations for the AR, turnover of the AR was slower when the binding ligand was DHT, suggesting that DHT was more effective than testosterone in promoting up-regulation of the receptor. Therefore, binding of DHT to the AR is favored even when DHT was present at lower concentrations, and the weaker interaction of testosterone with the AR could be compensated for by higher testosterone concentrations.

The fact that DHT forms a more stable complex with the AR is important since Kemppainen *et al* (1992) showed that when COS cells were transfected with recombinant AR, the localization

and degradation of ARs was effected by androgens. In the absence of androgens or with the antiandrogen flutamide, the AR immunolocalized to the cytoplasm and was rapidly degraded ($t_{½}$ = 1 h). However, in the presence of androgens, the AR showed strong nuclear immunostaining and androgen binding caused a 6-fold increase in receptor stabilization ($t_{½}$ = 6 h). Therefore DHT, which has a slower dissociation rate than testosterone (Grino *et al.*, 1990) will more efficiently prevent the AR from degradation than testosterone. The faster dissociation rate of testosterone from the AR compared to DHT, and the finding that testosterone was less effective in stabilizing the AR was confirmed by Zhou *et al* (1995) in recombinantly expressed wild-type and mutant AR in COS cells. They also showed that the NH_2-terminal domain of the AR was responsible for stabilizing the receptor by slowing the rate of ligand dissociation and AR degradation.

Thus, it is clear that DHT has a greater affinity for the AR than does testosterone. However, it is not clear whether this binding phenomenon was the basis for the differential action of androgens on the AR. The first study to examine the mechanisms by which testosterone and DHT act to regulate androgen-responsive genes was performed on MMTV-CAT, an androgen-responsive gene (Deslypere *et al.*, 1992). This study suggested that testosterone bound to the AR was 10-fold less potent than the DHT-AR complex in activating expression of the MMTV-promoter-reporter gene construct. This finding suggests that the differential effects of testosterone and DHT can be explained as a consequence of different binding affinities to the receptor, and is in keeping with the hypothesis that DHT formation is required in peripheral target-tissues for androgen action, whereas in tissues such as testes and Wolffian ducts, which have high concentrations of testosterone, DHT formation may not be crucial.

There may be other factors that explain why testosterone and DHT exert different physiological actions via the same receptor. For example, some genes may be regulated differently by testosterone and DHT. To date, there are two studies examining this possibility. Lin and Chang (1997) reported that testosterone repressed the androgen target gene, TDD5, more so than DHT, suggesting that genes may in fact be differentially regulated by testosterone and DHT. A second more recent study by Avila *et al* (1998) identified genes in the prostate that were modulated

differently by the 5αR inhibitor, finasteride, at the messenger ribonucleic acid (mRNA) level, by showing that some genes were up-regulated only in intact rats and other genes were up-regulated only with finasteride treatment. This suggests that some genes expressed in rat prostate may be regulated in fundamentally different ways in response to testosterone and DHT.

In summary, the enhanced binding affinity of DHT for the receptor is mainly due to a decreased rate of dissociation of the DHT-receptor complex (Grino *et al.*, 1990). The net consequence of similar association rates, but markedly different dissociation rates, is that DHT will be the predominant androgen bound to the AR under steady state conditions, even when testosterone is quantitatively the predominant hormone (Grino *et al.*, 1990). Despite these clear differences in receptor interactions, testosterone in high concentrations can promote maximal transcription of reporter genes that are linked to androgen-response elements (Deslyere *et al.*, 1992).

1.5 5α-REDUCTASE ISOFORMS IN REPRODUCTIVE AND NON-REPRODUCTIVE TISSUES

1.5.1 Subcellular Localization

The subcellular localization of 5αR isoforms has been shown to differ depending on the tissue source of the enzyme. Enzyme activity sediments with the nuclear fraction of rat prostate cells (Frederiksen and Wilson, 1971; Moore and Wilson, 1972) but with the endoplasmic reticulum fraction of liver cells (Moore and Wilson, 1972, 1973). Savory *et al* (1995) has shown that 5αR-1 in rat liver is an integral membrane protein present in rough endoplasmic reticulum and outer nuclear membrane. This is consistent with subfractionation studies of an endoplasmic reticulum association for 5αR in rat liver, in contrast to a nuclear membrane association of prostate 5αR (Moore and Wilson, 1972). The reason for the different subcellular localization of 5αR in liver and prostate is not known.

Yeast cells expressing the rat 5αR isoforms showed that 5αR-1 activity was maximal in fractions containing nuclei, whereas maximal 5αR-2 activity was found in fractions sedimented at higher speeds (≥ 20,000 g) (Poletti *et al.*, 1996). In rat pituitary, 5αR-1 has been immunolocalized mainly on the membrane of the rough endoplasmic reticulum (Yokoi *et al.*, 1996), and CHO cells expressing the human 5αR isoforms demonstrate that both isoforms are associated with the endoplasmic reticulum, presumably in the lipid bilayer (Thigpen *et al.*, 1993a). Further studies in transfected CHO cells using pulse-chase experiments showed that both human 5αR isoforms have long half-lives (20-30 h) and are not altered by the presence of micromolar concentrations of 4-azasteroid inhibitors (Russell and Wilson, 1994).

When subcellular fractions were prepared from immature rat testis, the largest proportion of 5αR activity (i.e. at pH 7.0, therefore 5αR-1 activity) was present in the microsomes (Oshima *et al.*, 1970; Yoshizaki *et al.*, 1978). The smaller amounts of activity in the mitochondria were found to be due to microsomal contamination (Yoshizaki *et al.*, 1978). The microsomal fraction isolated from immature rat testis has been used to partially solubilize 5αR (Ichihara and Tanaka, 1989). In summary, both 5αR isoforms are membrane-bound and can be identified primarily in two subcellular compartments, the microsomal and nuclear fractions.

1.5.2 Tissue Distribution of Steroid 5α-Reductase Isoforms

The types of 5αR genes expressed in some rat tissues is controversial. The majority of studies examining 5αR expression have focused on the liver, ventral prostate, and epididymis. These are tissues that express relatively high levels of 5αR activity and rely on 5αR activity for normal androgenic function. The localization and expression of 5αR isoform(s) in these tissues will be briefly discussed.

Female rat liver expresses the highest amount of 5αR activity and mRNA compared to all other tissues (Normington and Russell, 1992). Some studies report the presence of both 5αR isoforms (McGuire and Tomkins, 1960; McGuire *et al.*, 1960) in female rat liver microsomes,

whereas others have demonstrated only the 5αR-1 isoform (Moore & Wilson, 1973; Normington and Russell, 1992). Berman and Russell (1993) showed 5αR-1 mRNA expression, but no 5αR-2 mRNA, in normal male liver. This study, however, was unable to demonstrate 5αR-1 mRNA by in situ hybridization, in either normal or castrated ventral prostate sections, a tissue that clearly contains 5αR-1. Therefore, the level of 5αR expression in a particular tissue and the sensitivity of the method used for detection of mRNA, activity, or protein must be kept in mind when interpreting results. Savory *et al* (1995) generated antisera for both 5αR isoforms to show by Western blots that male rat liver contained 5αR-1, however, as neither of the antisera was specific for 5αR-2, they could not rule out the existence of 5αR-2 isoform in the liver.

Berthaut and colleagues (1997) showed by Northern blot analysis, RT-PCR, and enzyme assays that human prostate cells in culture express both 5αR isoforms, a finding that is supported by in situ hybridization in human ventral prostate biopsies (Pelletier *et al.*, 1998). The major isoform in human prostate is 5αR-2 as demonstrated by enzyme assays (Thigpen *et al.*, 1993b), Western blot analysis (Thigpen *et al.*, 1993b), and immunohistochemical analysis (Silver *et al.*, 1994a). 5αR-1 is undetectable by enzyme assays, however low levels of 5αR-1 mRNA have been detected (Andersson and Russell, 1992). The majority of 5αR-1 activity in rat ventral prostate is found in the nuclear fraction (Shimazaki *et al.*, 1971; Span *et al.*, 1996a) whereas 5αR-2 has a predominantly microsomal localization (Span *et al.*, 1996a). In human ventral prostate, 5αR-1 activity is also localized in the nucleus (Houston *et al.*, 1985) and immunostaining has localized 5αR-1 to the basal portion of basal and epithelial cells (Patel *et al.*, 1996). Other reports however do not observe 5αR-1 immunostaining in human prostate (Silver *et al.*, 1994).

Both 5αR mRNA's exhibit a gradient of isoform expression in the epididymis, being highest in the initial segment and caput and lowest in the cauda (Normington and Russell, 1992). Whereas different cell types express different 5αR isoforms in rat ventral prostate, the epithelial cells of rat epididymis express both isoforms (Berman and Russell, 1993). Epididymal epithelial cells proximal to the testis express high amounts of activity and mRNA, whereas those distal to the

testis express lower levels. This gradient of expression was first reported by Viger and Robaire (1991, 1992) for 5αR-1, and was subsequently demonstrated for the 5αR-2 isoform (Normington and Russell, 1992).

Recently, 5αR in human scalp has received considerable attention, as it is believed that inhibitors of 5αR may have therapeutic value for male pattern baldness since bald scalp skin contains increased amounts of 5α-reduced metabolites (Bingham and Shaw, 1973). The major 5αR activity in human scalp is 5αR-1 (Harris *et al.*, 1992; Thigpen *et al.*, 1993b; Amichai *et al.*, 1997; Bayne *et al.*, 1999) and Patel *et al* (1996) immunolocalized 5αR-1 to the sebaceous glands. 5αR-1 is present in all regions of the balding scalp whereas 5αR-2 is not detectable in any region of the balding scalp (Thigpen *et al.*, 1993b).

Normington and Russell (1992) used RNA blot hybridization to determine the relative steady state levels of rat 5αR-1 and 5αR-2 mRNAs in a variety of rat (49 days) tissues. 5αR-1 mRNA was predominantly expressed in peripheral tissues and was abundantly expressed in the liver. In contrast, 5αR-2 mRNA was the predominant isoform in male reproductive tissues (epididymis, testis, and vas deferens). Interestingly, two androgen-dependent tissues, the seminal vesicles and ventral prostate (George *et al.*, 1989), expressed equal levels of both 5αR mRNAs. The size of the mRNA for 5αR-1 varied between different tissues, two 5αR transcripts were detected in the adrenal gland (2.5 and 2.6 kb) whereas brain and colon only contained one of these forms (2.6 and 2.5 kb transcript). Similarly, the 5αR-2 mRNA in the kidney was smaller (3.2 kb) than that detected in the intestine (3.6 kb). To substantiate the RNA blotting results, 5αR enzyme activity in rat epididymis and ventral prostate from rats (~65 days) were examined (Normington and Russell, 1992). These studies showed that essentially all of the 5αR activity in the epididymis could be attributed to 5αR-2 based on its acidic pH optimum and activity at low substrate concentrations, whereas both isoforms contributed equally to total enzyme activity in the ventral prostate.

Several tissues in the human express 5αR-1 mRNA but do not appear to have detectable protein (Jenkins *et al.*, 1992; Thigpen *et al.*, 1993b). It is not known whether this finding is indicative of translational regulation or an unusual mRNA structure that precludes translation, or instability of the protein. The sequences of the 5'-untranslated regions of the 5αR isoform mRNAs are different, and these mRNAs may therefore be translated with different efficiencies. As mentioned previously, there are tissue-specific differences in the lengths of rat 5αR mRNAs (Normington and Russell, 1992) and multiple mRNAs are detected for all human and rat isoforms in blot hybridization experiments (Normington and Russell, 1992; Thigpen *et al.*, 1993b). The molecular basis or physiological reason for these differences has not yet been elucidated.

1.5.3 Human Steroid 5α-Reductase Type 2 Deficiency

Human 5αR deficiency is an inborn error of metabolism that impairs the conversion of testosterone to DHT, causing a distinct form of male pseudohermaphroditism in which genetic males differentiate predominantly as phenotypic females (Imperato-McGinley *et al.*, 1974; Wilson, 1975; Wilson *et al.*, 1993).

When genital skin fibroblasts were grown from affected individuals, 5αR activity with an acidic pH optimum was absent (Moore *et al.*, 1975) whereas activity at an alkaline pH, in both genital skin fibroblasts and non-genital fibroblasts, was normal (Moore and Wilson, 1976). The genetic basis for 5αR deficiency was found when it was demonstrated that the gene encoding 5αR-1 cDNA was normal in subjects with 5αR deficiency (Andersson *et al.*, 1991; Jenkins *et al.*, 1992), whereas mutations of the 5αR-2 gene were observed in 5αR deficient patients (Russell and Wilson, 1994). Twenty-nine different mutations in the 5αR-2 gene have been identified, including two deletion mutations, two nonsense mutations, one splicing defect, and 24 missense mutations (Andersson *et al.*, 1991; Thigpen *et al.*, 1992a, b). There are twelve mutations that completely inactivate the enzyme, ten mutations that impair enzyme activity, two of these affect the ability of the enzyme to bind testosterone whereas the other eight decrease the affinity of the enzyme for

NADPH, and all but one mutation alter the pH optimum of 5αR-2 (Wigley *et al.*, 1994). Normally, 5αR-2 protein has a half-life of 20-30 h in transfected 293 cells, whereas mutations can lead to a normal protein half-life or a reduced half-life of 1-2 h or 5-10 h (Wigley *et al.*, 1994). It seems that most mutations directly inactivate the enzyme since there is no correlation between protein half-life and enzyme activity.

In all individuals with 5αR deficiency, including those who do not synthesize a functional 5αR-2 gene, DHT is demonstratable in small amounts (Wilson *et al.*, 1993). In humans, the synthesis of 5αR-1 is induced at or near puberty in the skin and scalp, and is continuous thereafter (Thigpen *et al.*, 1993b). 5αR type 2 deficient patients do undergo varying degrees of virilization during puberty, an effect this is believed to be due to normal expression of 5αR-1 in other non-reproductive tissues (Thigpen *et al.*, 1993b). Spermatogenesis may be absent or profoundly impaired in 5αR deficient patients, and these varying degrees of fertility are probably related to the failure of the testes to descend (i.e. cryptorchidism) during puberty (Johnson *et al.*, 1986).

1.5.4 The Role of 5α-Reductase in Androgen Physiology

An important role for DHT has been demonstrated during male sexual development. There are three main phases during sexual development in mammals. First there is establishment of chromosomal sex at fertilization, followed by establishment of gonadal sex when the indifferent gonad develops into a testis or an ovary (Wilson, 1978: Wilson *et al.*, 1993). The genetic determinants that induce the gonad to develop into a testis are present on the Y chromosome and are termed the SRY gene (Sinclair *et al.*, 1990). Expression of SRY transforms the bipotential gonad into the fetal testis capable of synthesizing testosterone and other hormones required for the establishment of phenotypic sex. During this phase, the internal and external genitalia are converted into female or male as a direct consequence of the type of gonad formed and the endocrine secretions of the fetal testis. The testis produces the hormones Mullerian inhibiting substance and testosterone; Mullerian inhibiting substance causes regression of the Mullerian

ducts, which normally form the female internal genitalia (Wilson, 1978), whereas androgens mediate development of the male urogenital tract (internal male reproductive tract) and male external genitalia (Wilson, 1978, 1984).

The androgen-dependent development of male genitalia can be divided into events requiring testosterone and those requiring DHT (Wilson, 1984). Testosterone initiates the formation of the internal male reproductive structures, inducing the virilization of the Wolffian ducts into epididymes, vas deferens, and seminal vesicles. Development of the external organs is not mediated by testosterone, but rather by the more potent androgen DHT. In the target tissues of the urogenital tract that differentiate to form the male external genitalia, testosterone is converted into DHT by 5αR, which in turn binds to the AR and drives the differentiation of the external genitalia (penis and scrotum) and growth of the prostate (Wilson, 1978).

An example of the effect of a lack of 5αR in the developing male reproductive tract is in male pseudohermaphroditism induced by 5αR deficiency (Imperato-McGinley *et al.*, 1974; Wilson *et al.*, 1993). Recently, it was shown that two 46, XY siblings with partial androgen insensitivity syndrome showing very different phenotypes, one raised as a girl and the other as a boy, was due to the lack of 5αR-2 activity in the genital skin fibroblasts from the phenotypic female patient (Boehmer *et al.*, 2001). This further emphasizes the importance of DHT during embryonic sex differentiation. Conversely, overexpression of 5αR can lead to androgenic-related disorders in both males and females. Abnormally high 5αR activity in humans resulting in excessively high DHT levels in peripheral tissues is implicit in benign prostatic hyperplasia (BPH), male pattern baldness, and female hirsutism (Li *et al.*, 1995).

Carcinoma of the prostate is the most frequently diagnosed malignancy and the second leading cause of death as a result of cancer in men in many Western countries. Human prostate carcinomas are often androgen sensitive and react to hormonal therapy by temporary remission, followed by relapse to an androgen-insensitive state. These well established features of prostate cancer strongly suggest that steroid hormones, particularly androgens, play a major role in human

prostate cancer, but the precise mechanisms by which androgens affect this process are unknown (Metcalf *et al.*, 1989; Bosland, 2000).

BPH is a condition where the prostate enlarges with age to cause obstruction to the urinary tract and/or rectum (Wilson, 1980). Development of BPH is partly under androgenic control, and DHT accumulation within the gland serves as the hormonal mediator for hyperplasia (Walsh, 1986). This disorder develops only in the presence of intact testes and regresses following castration. The importance of DHT in the human prostate is highlighted in 5αR deficient patients who have an underdeveloped prostate (Imperato-McGinley *et al.*, 1992).

Male pattern baldness, the most common type of hair loss in men and women, is a DHT-dependent process and occurs with varying severity and age of onset. The predominant 5αR isoform in human scalp is 5αR-1 (Thigpen *et al.*, 1993b; Amichai *et al.*, 1997; Bayne *et al.*, 1999) and is immunolocalized to the sebaceous glands (Bayne *et al.*, 1999). No 5αR-1 is present in hair follicles (Bayne *et al.*, 1999) whereas 5αR-2 plays a central role within hair follicles to convert testosterone to DHT (Hoffman and Happle, 2000). Although 5αR-1 is the predominant 5αR isoform in scalp, male 5αR-2 deficient patients do not suffer from male-pattern baldness (Griffin and Wilson, 1989) and finasteride decreases scalp DHT concentrations and promotes hair growth in men with male pattern baldness (Bayne *et al.*, 1999; Hogan and Chamberlain, 2000).

Hirsutism is a clinical feature of disordered 5αR physiology in women (Mowszowicz *et al.*, 1983) and is characterized by the presence of hair in females in a male-like pattern, affecting 5-10% of women (Azziz *et al.*, 2000). Androgens are the most important sex steroids in determining the type and distribution of hair growth over the human body, and hirsutism is caused by excess DHT as a result of high 5αR activity (Mowszowicz *et al.*, 1983). It is not known which 5αR isoform is responsible for hirsutism, but this disorder can be treated by anti-androgens and 5αR inhibitors (Falsetti and Gambera, 1999; Muderris *et al.*, 2000).

1.5.5 Regulation of Non-Gonadal 5α-Reductase Isoforms

The regulation of 5αR isoforms has been examined in a variety of tissues, however interpretation of data before 1990 is complicated, since 5αR-2 had not yet been cloned. Therefore in studies performed before 1990, the methods of each experiment were carefully analyzed to determine which 5αR isoform was examined (e.g. enzyme activity assays performed at neutral pH would be judged as investigating the regulation of 5αR-1).

Following the identification of a second 5αR isoform, the regulation of 5αR has been re-examined using gene-specific probes and isoform-selective antibodies. Current studies have focused on establishing which tissues and cell types express a given 5αR and the impact of hormonal manipulation on expression. 5αR expression is regulated by androgens in a number of tissues and species. Androgen treatment can enhance 5αR activity in some tissues (prostate, epididymis, and skin) but reduce activity and mRNA in others (liver, adrenal cortex, and pituitary) (Robaire *et al.*, 1981; Mowszowicz *et al.*, 1983; Andersson *et al.*, 1989; George *et al.*, 1991; Lephart *et al.*, 1991).

Many studies have focused on the regulation of 5αR in rat ventral prostate. Castration of male rats caused a marked regression in the size and weight of the ventral prostate, and administration of testosterone propionate after castration significantly increased 5αR activity (Moore and Wilson, 1973). Treatment of intact adult rats with the 5αR inhibitor 4-MA caused the prostate to regress, an effect that was overcome by DHT administration (Brooks *et al.*, 1981). The induction of 5αR-1 enzyme activity in the ventral prostate of castrated rats given testosterone was accompanied by a large increase in the 5αR-1 mRNA (Andersson *et al.*, 1989; Normington and Russell, 1992). Administration of testosterone to normal rats did not affect the expression of mRNA in the prostate, however, testosterone administration after castration caused a significant increase in mRNA (Andersson *et al.*, 1989). It is not known whether the increase in steady-state mRNA levels was a consequence of mRNA stabilization or an increase in the transcription of the 5αR genes. Thus, 5αR-1 in the prostate is positively regulated by androgens (Moore and Wilson,

1973), and this regulation is mediated at the level of expression of 5αR-1 mRNA (Andersson *et al.*, 1989).

Induction of 5αR-1 activity and mRNA in the prostate of intact rats was inhibited by blocking 5αR was the inhibitor finasteride, whereas finasteride had no affect on DHT-mediated increases in 5αR-1 activity or mRNA (George *et al.*, 1991). This requirement for DHT results in an unusual situation in which the product of the enzyme is responsible for up-regulating the expression of the gene that encodes the enzyme. This so-called feed-forward regulation (George *et al.*, 1991) is the exact opposite of the usual feedback regulation in which the product of an enzyme negatively regulates expression of the gene.

Normington and Russell (1992) castrated rats (10 weeks old) for 7 days and then over a 3 day period administered testosterone propionate ± finasteride, or DHT ± finasteride. Castration caused a 2-fold increase in the steady state level of 5αR-2 mRNA in the regressing prostate, and testosterone or DHT administration caused a significant increase in 5αR-2 mRNA. Co-administration of finasteride with testosterone significantly reduced the level of 5αR-2 induction, however co-administration of finasteride with DHT had no effect. George *et al* (1991) also showed that the steady state levels of 5αR-1 mRNA responded in much the same way as those of the 5αR-2 mRNA (Normington and Russell, 1992). Therefore in the regenerating prostate, 5αR-1 and 5αR-2 mRNAs are more efficiently induced by DHT than by testosterone.

In contrast to this pattern of regulation, liver 5αR-1 activity (Yates *et al.*, 1958) and mRNA (Lopez-Solache *et al.*, 1996) are induced by castration in male rats. Castrated-induced 5αR-1 activity was decreased by testosterone treatment (Yates *et al.*, 1958) and 5αR-1 mRNA was decreased by DHT administration (Lopez-Solache *et al.*, 1996). These findings suggest that liver 5αR-1 activity and mRNA are down-regulated by androgens. Andersson *et al* (1989) failed to show the inhibitory effect of androgens on 5αR-1 mRNA in the liver of castrated rats, probably because they used testosterone and not DHT in their experiments. This down-regulatory effect of androgens on 5αR-1 activity in liver is the opposite from that in the prostate, where 5αR-1 activity

declined in adult male prostate after castration and increased after treatment of castrated rats with androgens (Shimazaki *et al.*, 1969; Moore and Wilson, 1973).

The effect of DHT on 5αR (i.e. 5αR-1) mRNA and enzyme activity has also been examined in the adrenal cortex of 28 days male rats (Lephart *et al.*, 1991). Castration significantly increased adrenal 5αR-1 mRNA and activity (7-fold and 3-fold, respectively). Injecting castrated rats with DHT for 14 days reduced mRNA to undetectable levels, whereas activity was reduced to about one-third of controls. Thus, these results indicate that the mRNA encoding 5αR-1 in the adrenal cortex was negatively regulated by DHT presumably at the transcription level.

Several studies by Viger and Robaire (1991, 1992) have shown that the endocrine and developmental regulation of the 5αR-1 transcript in rat epididymis is regulated by many factors and is segment-specific. An intact connection between the testis and epididymis is critical for maintenance of 5αR activity, mRNA and protein expression, particularly in the initial segment of the epididymis (Robaire *et al.*, 1977; Viger and Robaire, 1991; Robaire and Viger, 1995). Both a nuclear and microsomal form of 5αR have been demonstrated in the epididymis, and it is now believed that the nuclear form represents 5αR-1 and the microsomal form represents 5αR-2 (Robaire and Viger, 1995).

Nuclear 5αR activity is highest in the caput epididymis and is abolished by castration, and testosterone administration has minimal restorative affects (Robaire *et al.*, 1977). It has been speculated that the regulation of nuclear 5αR activity (presumably 5αR-1) in the epididymis is controlled by a substance that is probably of Sertoli cell origin and whose synthesis is under the control of testosterone (Robaire and Viger, 1993). In contrast, microsomal 5αR activity is found throughout the epididymis, is expressed at a lower level, and is under the control of circulating androgens (Robaire *et al.*, 1991). Whereas unilateral efferent duct ligation caused a dramatic decrease in 5αR-1 mRNA levels in the initial segment, 5αR-2 mRNA levels increased by 2-fold (Viger and Robaire, 1996). These findings demonstrate that 5αR-1 and 5αR-2 mRNAs are

differentially expressed and regulated in the rat epididymis, and is the first demonstration of a tissue in which the mRNAs for the 5αR isoforms are regulated differently.

Pituitary 5αR-1 (i.e. activity assessed at pH 7.0) activity was increased in prepubertal rats after gonadectomy (Ghraf *et al.*, 1982), and DHT administration after gonadectomy suppressed 5αR-1 activity. Flutamide treatment inhibited the suppressive action of DHT on pituitary 5αR-1 activity, suggesting that androgenic regulation of pituitary 5αR-1 may be mediated via the AR.

Several studies have examined 5αR activity in human and rat genital skin fibroblasts, which exclusively express 5αR-2 activity (Horton *et al.*, 1993). DHT and insulin-like growth factor-I (IGF-I) significantly increased 5αR-2 activity, whereas testosterone, insulin-like growth factor-II (IGF-II) and insulin had no effect (Horton *et al.*, 1993). The effect of IGF-I was ~100 times that of androgen, suggesting that IGF-I might play an important role in regulating 5αR-2 activity. This hypothesis was confirmed by studies, which showed that addition of a monoclonal antibody against IGF-I reduced DHT-stimulation of 5αR-2, and simultaneous addition of a specific IGF-I receptor antibody blocked the induction of 5αR-2 activity. TGF-β1 and TGF-β2 also stimulated 5αR-2 activity in genital skin fibroblasts, and administration of both DHT and TGF-β1 or TGF-β2 caused a synergistic effect, potentiating the stimulation of 5αR-2 activity (Wahe *et al.*, 1993).

Activins and inhibins also affect 5αR-2 activity in human scrotal skin fibroblast cultures (Antonipillai *et al.*, 1995). Activin A significantly stimulated 5αR-2 activity in a dose dependent manner and was ~100 times more potent on a molar basis than androgen in inducing activity. Inhibin A alone did not alter basal activity but dose dependently inhibited DHT-induced activity. In conclusion, activin and inhibin potentially play opposite roles in DHT formation in sexual tissue, and the regulation of 5αR-2 may depend on the balance between androgen and activin/ inhibin interactions.

In summary, androgens have a positive regulatory effect on 5αR mRNA in the adult prostate (Andersson *et al.*, 1989; Normington and Russell, 1992), epididymis (Silver *et al.*, 1994a; Viger and Robaire, 1996), embryonic urogenital sinus (Berman *et al.*, 1995) and genital skin (Horton *et*

al., 1993). In contrast, testosterone has a negative regulatory effect on 5αR mRNA in liver (Moore and Wilson, 1973; Lopez-Solache *et al.*, 1996), adrenal cortex (Lephart *et al.*, 1991), and pituitary (Ghraf *et al.*, 1982).

1.6 5α-REDUCTASE IN THE TESTIS

1.6.1 Localization of Testicular 5α-Reductase Activity

Many studies in the early 1970s separated seminiferous tubules and interstitial tissue in an effort to determine the localization of testicular 5αR activity. The 5αR-2 gene had not yet been discovered, and given that all the 5αR enzyme activity assays were performed at neutral pH, information only on the 5αR-1 isoform was gained. To date, there is no data regarding 5αR-2 enzyme activity in rat testis.

Oshima and colleagues (1970) first showed that incubation of ^{14}C-androstenedione with immature (37 days) testicular rat preparations yielded testosterone and its 5α-reduced metabolites, DHT and 3α-Adiol. Furthermore, 5αR was localized to the microsomal fraction in whole testes of immature rats. Therefore it was clearly evident that the steroid 5αR was present in rat testes, at least during immaturity. Further studies incubated subcellular fractions from the interstitial tissue of day 30 rat testes (Yoshizaki *et al.*, 1978) to demonstrate that almost all of the 5αR activity was found in the microsomal fraction, and confirmed previous work (Oshima *et al.*, 1970) using subcellular fractions from whole testes.

For many years, the cellular localization of 5αR in the testis remained a contentious issue. Some laboratories reported the presence of 5αR-1 activity mainly in the seminiferous tubules and not in the interstitium (Rivarola and Podesta, 1972; Rivarola *et al.*, 1972; Dorrington and Fritz, 1975), whereas other laboratories showed 5αR-1 enzyme activity predominantly in the interstitium and not in the seminiferous tubules (Matsumoto and Yamada, 1972; Nayfey *et al.*, 1975; van der Molen, 1975). There are several differences in experimental designs amongst these studies, and

Table 4 outlines the similarities and differences (pH, temperature, 5αR source, rat strain, age) between these studies. A review of the localization of testicular 5αR-1 activity will be discussed, and is divided into those studies, which have found predominant 5αR-1 activity in the seminiferous tubules and/or interstitial tissue.

Some studies have shown that 5αR-1 activity is present in either the seminiferous tubules or in the interstitial tissue. Rivarola *et al* (1972a) showed 5αR activity in tubules but not in interstitial tissue of adult male rats. In another experiment, 5α-reduced products were studied in whole testis, isolated seminiferous tubules and interstitial tissue in rats of various ages (from days 1-365). This study demonstrated that 5α-reduced products (percentage of radioactivity converted) were significantly higher in seminiferous tubules compared to interstitial tissue (Rivarola *et al.*, 1972b). These studies (Rivarola *et al.*, 1972a, b) used male Wistar rats to measure 5αR activity at pH 7.4, without the addition of the cofactor NADPH (see *Table 4*) and suggest that 5αR-1 activity is predominately expressed in the seminiferous tubules.

5αR-1 activity in rat testis has also been investigated in the absence of the cofactor NADPH (Dorrington and Fritz, 1973, 1975). Isolated tubules from immature and mature rats metabolized testosterone to 5α-reduced products, and 5αR-1 activity was significantly higher in immature compared to adult testis (Dorrington and Fritz, 1973). Nayfey *et al* (1975a) also showed that 5αR-1 activity in immature rat testes was significantly higher in isolated tubules than in the interstitium.

In contrast to the studies that have shown 5αR-1 activity predominantly in the seminiferous tubules, other studies have shown that testicular 5αR-1 activity resides predominantly in the interstitial tissue. Van der Molen (1975) showed that compared to tubules, the interstitial tissue isolated from 10-90 day rat testis was responsible for almost all of the 5α-reduced products formed.

Yoshizaki *et al* (1978) demonstrated that 5αR-1 activity was 37 times greater in the interstitial tissue compared to tubules in day 29-35 rat testes, and 3β-HSD staining revealed that

Table 4: Outline of experiments conducted in the 1970s that investigated testicular 5α-reductase enzyme activity. All these studies were conducted at neutral pH, therefore 5αR type 1 activity was measured.

GROUP	Cofactor	pH	Temp (°C)	Preparation	Substrate	Rat Strain	Age (days)
Oshima *et al.*, 1970	NADPH	7.4	37	W.t, microsomes	^{14}C-testosterone	Wistar	37
Rivarola & Podesta, 1972	-	7.4	31 and 37	S.t, I.t	^{14}C-testosterone	Wistar	60-90
Rivarola *et al.*, 1972a	-	7.4	31	S.t, I.t	^{14}C-testosterone	Wistar	60-90
Rivarola *et al.*, 1972b	-	7.4	31 and 37	S.t, I.t	^{14}C-testosterone	Wistar	1-365
Matsmuto & Yamada, 1973	NADPH	7.4	34	S.t	^{3}H-testosterone	Wistar	9, 16, 20, 27, 41, 83
Sowell *et al.*, 1974	± NADPH	?	37	W.t, S.t, I.t	^{3}H-testosterone	Lewis	~42 & 142
Dorrington & Fritz, 1973, 1975	-	7.4	32	Cell fractions	^{14}C-testosterone	Wistar	27 & 42
Folman *et al.*, 1972	NADPH	7	32	3,500 x g pellet	^{3}H-testosterone	Lewis	45, 138-155
Folman *et al.*, 1973	NADPH	7	32	3,500 x g pellet	^{3}H-testosterone	Lewis	45, 148
Drosdowsky *et al.*, 1975	NADPH	7.2	31	S.t, germ cells	^{14}C-testosterone	Wistar	90
Van der Molen, 1975	NADPH	?	37	W.t, S.t, I.t	^{3}H-testosterone	Wistar	10 - 60
Yoshizaki *et al.*, 1978	± NADPH	7.4	34	600 x g pellet	^{14}C-testosterone	Sprague Dawley	29-35

W.t = Whole testis, S.t = Seminiferous tubules, and I.t = Interstitial tissue

5αR-1 was localized predominantly to the Leydig cells. This is the exact opposite to the results obtained by Rivarola *et al* (1972). A possible reason for this discrepancy is the addition of the cofactor, which would greatly increase the rate of testosterone conversion.

Other studies have shown that 5αR-1 activity is present in both the seminiferous tubules and interstitial tissue, but resides in one compartment predominantly. Folman *et al* (1973) separated interstitial tissue and tubules from immature and adult rats, and showed that 5αR activity at pH 7.0 (i.e. 5αR-1) was significantly higher in the tubules compared to the interstitium, suggesting that seminiferous tubules and interstitial tissue in immature and adult rats metabolize testosterone to DHT. They also showed that the type of 5α-reduced metabolite formed varying according to the preparation incubated: incubation of testicular homogenates yielded mainly 3α-Adiol, and incubation of microsomes (to eliminate activity due to 3α-HSD activity) yielded mainly DHT.

The enzyme activity studies discussed so far relate only to the 5αR-1 isoform, and have focused on 5αR-1 activity in the seminiferous tubules and/or interstitial tissue. More recently, Reyes *et al* (1997) used Northern and Southern blot analysis to show that adult rat testis expresses both 5αR genes. They also demonstrated a differential cytosine- and adenine-methylation pattern in 5αR-2 in reproductive and non-reproductive tissues that are sensitive to androgens, suggesting that the differential methylation pattern in the 5αR-2 isoform may contribute to the expression of this gene.

1.6.2 Evidence for 5α-Reductase in Sertoli Cells

Dorrington and Fritz (1973) showed that isolated Sertoli cells or cultured Sertoli cells from day 20 rats contained 5αR activity, and that these levels of activity were similar to those observed when tubules from immature rats were incubated under the same conditions. This data suggests that 5αR-1 (assessed at pH 7.0) activity was localized predominantly in Sertoli cells and that little, if any, 5αR-1 activity was present in the Leydig cells. Spermatocyte-enriched preparations (~90% spermatocytes and 6% immature spermatids) isolated from immature rats converted testosterone to

5α-reduced products, suggesting that in addition to Sertoli cells, spermatocytes also contain 5αR-1 activity (Dorrington and Fritz, 1975a, b). Sertoli cells produced 3α-Adiol as the predominant 5α-reduced metabolite whereas the major metabolite produced by spermatocytes was DHT. Furthermore, 5αR-1 activity in spermatid-enriched preparations was lower than that found in the spermatocyte preparations, suggesting that 5αR-1 was not present in spermatids.

Drosdowsky *et al* (1975) showed that 5αR-1 activity was similar between normal adult rat testis, and Sertoli-cell only tubules treated prenatally with busulphan to induce gonocyte degeneration, suggesting that 5αR-1 activity is absent or low in germ cells. This finding supports previous results (Folman *et al.*, 1972; Rivarola and Podesta, 1972; Rivarola *et al.*, 1972; Dorrington and Fritz., 1975a, b). Drodowsky *et al* (1975) also showed that isolated spermatids do not contain 5αR-1 activity, which supports the findings of Dorrington and Fritz (1973).

1.6.3 Evidence for 5α-Reductase in Leydig Cells

In contrast to the studies above which showed predominantly or solely 5αR-1 activity in the Sertoli cell, other studies have shown that 5αR was predominantly expressed in the Leydig cell. More than 90% of testicular 5αR activity in the adult rat was lost following Leydig cell destruction by treatment with EDS, an effect that was reversed during Leydig cell repopulation (O'Shaughnessy and Payne, 1982).

Vreeburg and colleagues (1988) demonstrated that 5αR activity at pH 7.0 (i.e. 5αR-1) in testes from normal immature and adult rats showed that 5αR-1 activity was 30-fold lower in the adult. However, thirty days after administration of EDS to adult rats, 5αR-1 activity increased 6-fold compared to untreated adult controls, but was still 5-fold lower than in immature rats. O'Shaughnessy and Murphy (1991) also investigated the effects of EDS treatment on 5αR-1 activity in adult Leydig cells. Three days after EDS treatment when all Leydig cells had disappeared, there was a 90% loss of 5αR-1 activity. This residual level of 5αR activity in the absence of Leydig cells suggests that 5αR-1 was predominantly, but not solely, present in Leydig

cells. 5αR-1 activity remained low for 14 days after EDS treatment and increased 3.5 fold 21 days, returning to control levels by 35 days. These EDS-treated studies suggest that 5αR-1 activity was present predominantly in Leydig cells.

Further evidence for the expression of 5αR-1 activity in Leydig cells comes from primary Leydig cell cultures. Purified immature Leydig cells contained considerable 5αR-1 activity (Murono and Washburn, 1990) and 5αR-1 mRNA was abundantly expressed in progenitor and immature Leydig cells, yet was almost undetectable in isolated adult Leydig cells (Ge and Hardy, 1998). In contrast, 5αR-2 mRNA was not detected at any stage of Leydig cell development (Ge and Hardy, 1998).

Viger and Robaire (1995) determined the steady state mRNA levels for both 5αR isoforms during postnatal (7 to 91 days) testicular development in the rat. Northern blot analysis showed that 5αR-1 mRNA levels increased by 3.5-fold between days 7 and 21, to reach peak expression at day 28. However, unlike enzyme activity which declines to reach undetectable levels in the adult (91 days) (Folman *et al.*, 1972; Sowell *et al.*, 1974), mRNA levels decreased and remained constant between days 42-91, at about one third of peak expression at day 28.

Viger and Robaire (1995) demonstrated a single hybridizing 5αR-1 transcript in the testis throughout development, however the size of the transcript varied with age; a 2.5 kb 5αR-1 transcript was expressed in the testis between days 21-28, which was identical in size to the 5αR-1 transcript previously reported in other tissues (Andersson *et al*, 1989; Viger and Robaire, 1994). However, a 2.7 kb 5αR-1 transcript was expressed at day 91, when 5αR activity was low (Coffey *et al*, 1971; Matsumoto and Yamada, 1973; van der Molen *et al*, 1975). Western blot analysis and immunocytochemical data support the low level of 5αR-1 protein in adult testis. Therefore, it was suggested that the 2.7 kb transcript may not be efficiently translated into a protein detectable by Western blot analysis or by immunocytochemistry (Viger and Robaire, 1995). Furthermore, whereas 5αR-1 mRNA was detected at all ages, 5αR-2 mRNA was barely detectable in the testis by Northern blot analysis (Viger and Robaire, 1995).

Viger and Robaire (1995) also used an antipeptide antiserum specific to rat 5αR-1 to immunolocalize 5αR-1 protein in the developing rat testis. 5αR-1 was localized predominantly to the interstitial tissue of the testis and the immunoreactive cells appeared to be Leydig cells at different stages of development. On postnatal day 7, clusters of interstitial cells resembling fetal Leydig cells were immunoreactive. The staining intensity increased steadily from day 7 onwards, so that by day 21 and 28, interstitial cells with the appearance of immature Leydig cells were intensely immunoreactive. This finding was consistent with the high levels of 5αR-1 enzyme activity that was reported in purified immature Leydig cells (Murono and Washburn, 1990). There was a progressive decrease in staining intensity after day 28, so that by day 91 Leydig cell immunoreactivity was barely detectable. Thus, the intensity of 5αR-1 protein was consistent with 5αR-1 mRNA levels and Western blot data (Viger and Robaire, 1995). Immunocytochemical staining revealed predominantly a cytoplasmic localization for 5αR-1, with no significant nuclear staining evident. The subcellular localization of 5αR-1 protein in the testis was consistent with the previously reported presence of 5αR-1 enzyme activity in testicular microsomes (Ichihara and Tanaka, 1989), and was comparable with what has been described for rat epididymis and prostate (Viger and Robaire, 1994; Berman and Russell, 1993; Thigpen *et al.*, 1993a).

In summary, these studies indicate that 5αR-1 protein is primarily expressed in the cytoplasm of Leydig cells, is dependent on age, and parallels enzyme activity. In contrast to this data, the RNA blots obtained from Normington and Russell (1992) suggest that adult rat testis contained both 5αR isoforms, and that 5αR-2 was the predominant isoform expressed. The reason for this discrepancy is not known. Therefore, the type of 5αR isoform(s) expressed and the level of 5αR isoform expression in rat testis still remain unclear.

1.6.4 Ontological Expression of 5α-Reductase Type 1 in the Testis

As well as examining the localization of 5αR-1 in the testis, the developmental regulation of 5αR-1 enzyme activity also attracted scientific interest in the early 1970s. It was initially

demonstrated that there was significantly higher 5αR activity at pH 7.0 (i.e. 5αR-1) in teased testicular tissue at days 30-45 than at days 140-160 (Folman *et al.*, 1972; Sowell *et al.*, 1974).

Rivarola and colleagues (1972b) demonstrated that 5αR activity at pH 7.0 (i.e. 5αR-1) in whole testes was low at birth, increased from day 20 to reach peak activity at days 30-45, and then declined by day 60 to reach very low levels after one year. Matsumoto and Yamada (1973) showed that 5αR activity at pH 7.0 (i.e. 5αR-1) in both isolated seminiferous tubules and whole testis, was low at day 9, increased to reach a peak of activity at day 27, and then decreased such that only low levels were seen by day 81. Furthermore, 5αR-1 activity was 20 times higher in the whole testis than in the isolated tubules in immature rats, suggesting by deduction that 5α-reduction was much higher in the interstitial tissue than in the tubules. This is in contrast to the findings of Rivarola *et al* (1972b) who showed no 5αR activity in the interstitial tissue. The discrepancy between these studies could be due to addition of NADPH cofactor or the loss of activity in the interstitial tissue during the isolation procedure.

A decline in 5αR-1 activity with age suggests that this enzyme may be regulated by a hormone that changes in concentration with increasing age. For example, the developmental profile for testosterone (Corpechot *et al.*, 1981) appears to be inversely related to the pattern of 5αR-1 expression. Thus, during puberty the concentration of testosterone is low whereas testicular 5αR-1 activity is high, and with increasing age testosterone levels rise and 5αR-1 activity declines. This suggests that 5αR-1 activity in the testis may be negatively regulated by testosterone.

The activity studies mentioned so far refer to 5αR-1, since these enzyme assays were performed at a neutral pH. To date, no studies have examined 5αR-2 activity in the testis, or the developmental profile for 5αR-2 enzyme activity or mRNA. Recently, Viger and Robaire (1992) used immunocytochemistry to investigate the developmental profile of 5αR-1. This study did not investigate 5αR-2 mRNA because it was not detected at any age by Northern blot analysis. 5αR-1 protein was immunolocalized to Leydig cells, and the intensity of staining was consistent with

enzyme activity previously reported for Leydig cells (Murono and Washburn, 1990), and was consistent with mRNA (Northern blots) and protein (Western blot) analysis (Viger and Robaire, 1995). Thus, according to these studies, 5αR-1 protein was primarily expressed in Leydig cells and was dependent on age.

In summary, the studies mentioned in this section have all demonstrated that 5αR-1 activity, assessed by the activity at a neutral pH or by using immunocytochemistry, increased to reach peak expression in young (day 30) animals, then declined rapidly to reach low levels by adulthood. However, there was no agreement as to which testicular compartment (seminiferous tubules or interstitium) or cell type was responsible for this activity, or what factor(s) regulated the developmental profile of 5αR-1 activity.

1.6.5 Regulation of 5α-Reductase Type 1 Activity in the Testis

The cellular localization and regulation of testicular 5αR remains controversial and poorly understood, despite the cloning of cDNAs for 5αR (Andersson *et al.*, 1989; Normington and Russell, 1992) and the development of 5αR antibodies (Viger and Robaire, 1991, 1994). The changes in testicular 5αR-1 at different stages of development appear to correlate well with maturational changes controlled by pituitary gonadotrophins and androgens in the testis. However, the mechanisms regulating testicular 5αR activity during various stages of development are unknown.

a) Regulation of Testicular 5α-Reductase Type 1 Activity by FSH and Testosterone

Several studies have examined the regulation of testicular 5αR-1 activity in the immature rat. Oshima *et al* (1970) examined the effect of androgen treatment on 5αR-1 (i.e. pH 7.0) activity in immature rat testis. Administration of human chorionic gonadotrophin and/or androgen for 7 or 14 days to normal immature rats significantly reduced 5αR-1 activity compared to control. Down-

regulation of 5αR-1 activity in immature rat testis by testosterone was similar to that observed in male liver (Yates *et al.*, 1958; Lopez-Solache *et al.*, 1996).

Murono and Payne (1979) showed that 7 days after hypophysectomy, 5αR-1 activity (i.e. pH 7.0) in immature rat testis was reduced to almost undetectable levels, LH treatment increased conversion of testosterone to DHT, and FSH had no effect on 5αR activity. The finding that FSH had no stimulatory effect on 5αR activity (Murono and Payne, 1979) is in contrast to Nayfey *et al* (1975a) who reported an increase in 5αR-1 activity in whole testicular tissue or in interstitial tissue of day 28 rats treated with FSH. The discrepancy between these studies may be due to the fact that Nayfey and colleagues used very high concentrations of FSH, which were likely to be contaminated with LH.

Nayfey *et al* (1975a) also measured 5αR-1 activity at pH 7.0 (i.e. 5αR-1) in immature rats after hypophysectomy and found that 5αR-1 activity decreased significantly in both isolated tubules and in the interstitium after one week of hypophysectomy. Treatment with LH or FSH for 4 days after hypophysectomy significantly increased 5αR-1 activity in whole testis. However, it was uncertain whether this effect was influenced by LH contamination (see above). Testosterone propionate did not increase 5αR-1 activity above untreated hypophysectomized control animals, suggesting that the stimulatory effect of gonadotrophins was not mediated by androgens.

Only a few studies examined the regulation of testicular 5αR-1 activity in the adult animal. Folman *et al* (1972) showed that administration of hCG to intact adult rats *in vivo* caused a 2-fold decrease in the amount of testicular DHT produced, and appeared to prevent formation of DHT *in vitro*. Dorrington and Fritz (1975b) showed that 5αR-1 activity at pH 7.0 (i.e. 5αR-1) in seminiferous tubules isolated from adult rats was quantitatively similar to that observed in tubules from intact rats. The authors suggested that 5αR-1 activity in tubules was not directly dependent on gonadotrophins. This study also showed that treatment of adult long-term hypophysectomized rats with FSH or LH for 3 days had no effect on 5αR-1 activity in tubules. Therefore, this study suggested that 5αR-1 in seminiferous tubules was not stimulated by gonadotrophins.

As well as examining the regulation of 5αR-1 activity in whole testis or seminiferous tubules, other studies looked at the regulation of 5αR-1 activity (i.e. pH 7.0) in isolated Sertoli cells. Administration of FSH and/or testosterone to Sertoli cells isolated from day 20 rats had no effect on 5αR-1 activity (Dorrington and Fritz, 1975b). Contrary to these results, an *in vitro* effect of FSH on 5αR-1 in Sertoli cells was reported by Welsh and Wiebe (1976), who demonstrated that FSH could stimulate 5αR-1 activity in Sertoli cells isolated from day 10 rats. Whether an FSH effect only occurs in Sertoli cells in very young rats is unknown.

The regulation of 5αR-1 activity has also been investigated in cultured Leydig cells. O'Shaughnessy and Payne (1982) showed that treatment of different populations of adult Leydig cells with hCG for 6 days did not effect 5αR-1 activity, except in population IA Leydig cells where hCG decreased 5αR-1 activity. Murono and Payne (1990) showed that percoll purified band 3 Leydig cells contained the highest amount of 5αR-1 activity among all of the cells comprising the interstitial tissue in immature rats. They also showed that LH/hCG directly stimulated 5αR-1 activity, whereas growth hormone, insulin, IGF-1, and epidermal growth factor (EGF) had no effect on 5αR-1 activity. The effects of hCG were mimicked by 8-Br-cAMP for the first 2 days, but after this period 8-Br-cAMP was no longer as effective as hCG, suggesting that long-term maintenance of the enzyme requires a second messenger other than cAMP. Whether the increase in 5αR-1 activity by hCG involves protein synthesis and/or stabilization of existing enzyme is unknown. Thus, these two studies suggest that the major factor stimulating 5αR-1 activity in Leydig cells during early maturation is LH, and this effect appears to be mediated, at least in part, by cAMP.

b) Regulation of Testicular 5α-Reductase Type 1 Activity by Other Factors

Several studies have investigated the regulation of testicular 5αR-1 activity (i.e. activity at pH 7.0) by factors other than testosterone and FSH. Chase and Payne (1985) demonstrated that prolactin suppression by bromoergocryptine treatment *in vivo* to intact immature rats decreased

5αR-1 activity by ~30%. Prolactin administration to intact rats did not effect 5αR-1 activity, however prolactin suppression (via bromoergocryptine-treatment) significantly increased 5αR-1 activity. Hypophysectomy almost abolished 5αR-1 activity and activity was increased 10-fold by LH administration. Following hypophysectomy, prolactin given alone had no effect whereas prolactin and LH together acted synergistically to maximally stimulate 5αR-1 activity. Thus, the action of prolactin on 5αR-1 required the presence of LH, and prolactin and LH may act together to maintain high 5αR-1 during immaturity.

Nicholson *et al* (1991) showed that administration of exogenous oxytocin via implants increased testicular DHT concentrations by ~50% whereas testosterone was suppressed after 1 and 2 weeks of treatment. After 3 and 4 weeks of treatment, testicular testosterone was even further suppressed yet DHT was similar to corresponding controls. Given that oxytocin treatment also suppressed serum FSH, it was possible that some of these effects could be due to changes in FSH. When the ratios of testicular DHT:testosterone were considered, the ratio was significantly increased in oxytocin-treated rats (1.1 after 1 week and 7.7 by 4 weeks) compared to control, suggesting that oxytocin effects the conversion of testosterone to DHT and that proportionally more was converted to DHT when low levels of testosterone were present.

In summary, pituitary hormones such as LH influence testicular 5αR-1 activity (Chase and Payne, 1985; Murono and Payne, 1979; Nayfey *et al.*, 1975b; Takeyama *et al.*, 1986). The stimulatory effect of LH on 5αR-1 activity occurs in both immature and adult rats (Murono and Payne, 1979; Takeyama *et al.*, 1986), and was reported to be potentiated by prolactin (Chase and Payne, 1985;Takeyama *et al.*, 1986). Furthermore, the LH-induced increase in prepubertal 5αR-1 activity occurred by stimulating Leydig cells with LH, an effect that was mimicked by 8-bromo-cAMP (Murono and Washburn, 1990). In contrast to LH, the role of FSH has been controversial (Nayfey *et al.*, 1975b; Murono and Washburn, 1990). It is clear, however, that FSH does not directly stimulate 5αR-1 activity in Leydig cells, at least in the immature rat (Murono and

Washburn, 1990). The regulation of 5αR-1 activity in various testicular compartments remains unclear, and warrants further investigation.

1.7 HUMAN SPERMATOGENESIS AND CONTRACEPTION

The next part of this review will discuss the relevance of 5αR to human male contraception. A brief overview of the hormonal regulation of non-human primate and human spermatogenesis, and the different hormonal male contraceptive methods will be outlined. While it is clear that gonadotrophins are necessary for spermatogenesis, the specific role and relative contribution of LH and FSH in the control of spermatogenesis are unclear in the human (see Matsumoto, 1989 for review).

A role for FSH in the initiation and maintenance of spermatogenesis was proposed in the non-human primate. FSH suppression by active or passive immunization (Wickings *et al.*, 1980; Moudgal *et al.*, 1992; Suresh *et al.*, 1995) resulted in the suppression of spermatogenesis and infertility (Moudgal *et al.*, 1992; Aravindan *et al.*, 1993). Treatment of pubertal monkeys with FSH induced germ cell proliferation, however quantitatively and qualitatively normal spermatogenesis was only achieved when co-administered with hCG (as an LH substitute) (Arslan *et al.*, 1993; Schlatt *et al.*, 1995). Long-term immunization against FSH induced oligozoospermia or azoospermia in monkeys (Srinath *et al*, 1983; Moudgal *et al*, 1992). The administration of purified FSH to GnRH-antagonist-treated cynomologous monkeys fully maintained spermatogonial numbers, but only partially maintained spermatocytes and spermatids (Weinbauer *et al.*, 1991). Overall these data suggest that FSH treatment alone was capable of partially maintaining spermatogenesis.

In humans, FSH appeared able to partially restore sperm counts in normal men after experimental gonadotrophin suppression, but that both FSH and testosterone were needed for quantitatively normal spermatogenesis (Matsumoto *et al.*, 1986). Sperm production was reinitiated by restoring testicular testosterone alone using hCG (Bremner *et al.*, 1981), or by FSH alone

(Matsumoto *et al.*, 1983), or by restoring LH despite markedly suppressed FSH levels (Matsumoto *et al.*, 1984), following suppression of gonadotrophin secretion by testosterone treatment. It can be concluded that testosterone and FSH, in the near absence of each other, are capable of partially stimulating sperm production.

Several recent studies support the role of FSH in the establishment of normal human spermatogenesis. Males homozygous for an inactivating FSH receptor mutation have variable degrees of spermatogenic failure, but are not azoospermic or necessarily infertile (Tapainen *et al.*, 1997). Gromoll *et al* (1996) showed that a man with complete gonadotrophin deficiency and an activating mutation of the FSH receptor had preservation of his testicular volumes, spermatogenesis and fertility.

In male hormonal contraceptive trials using either transdermal testosterone together with oral levonorgestrel (Büchter *et al.*, 1999), or intramuscular testosterone buciclate (a long-acting testosterone ester (Behre *et al.*, 1995), azoospermia was only achieved in those men with serum FSH levels below the lower limit of detection, supporting the role of FSH for the maintenance of spermatogenesis.

The role of testosterone in the regulation of spermatogenesis in the non-human primate has also been investigated. Testosterone alone initiated qualitatively normal spermatogenesis in immature monkeys (Marshall *et al.*, 1984) and was capable of maintaining qualitative spermatogenesis in GnRH antagonist-treated monkeys (Weinbauer *et al.*, 1988). High doses of testosterone alone qualitatively restored all germ cell types in pituitary stalk-sectioned monkeys (Marshall *et al.*, 1983), and was capable of inducing qualitatively complete spermatogenesis in immature cynomologous monkeys (Marshall *et al.*, 1984). It can be concluded that testosterone alone directly stimulated spermatogenesis through gonadotrophin-independent mechanisms in monkeys.

Many of these experimental paradigms are not possible in the human. However in addition to the studies of Matsumoto discussed above, other data identifies the need for both FSH and T and

gives insight in the sites in the spermatogenic process effected by gonadotrophin withdrawal in monkeys and man. In a recent monkey study, it was demonstrated that depletion of gonadotrophins via GnRH-antagonist treatment dramatically reduced the numbers of type B spermatogonia, and hence later germ cell types (Zhengwei *et al.*, 1998a).

In men receiving testosterone-based contraceptive treatment, the main lesion induced by gonadotrophin withdrawal was in the maturation of type A to type B spermatogonia (Zhengwei *et al.*, 1998b), however it was not clear whether this effect was due to FSH and/or testosterone suppression. Retention of mature spermatids in the epithelium of these men was also noted by Zhengwei, suggesting defective sperm release (i.e. spermiation). Studies in our lab showed that spermiation failure was also involved in the onset and maintenance of spermatogenic suppression in rats (Saito *et al.*, 2000).

1.7.1 A Testosterone-Based Hormonal Contraceptive for Men

The goal of male hormonal contraception is to abolish sperm production and eliminate sperm from the ejaculate, and thereby induce reversible infertility. The ideal contraceptive should leave sexual and general physical well being unaltered, be free of short and long term side-effects, have a rapid onset and be completely reversible (Comhaire, 1994). At present, the most common method of male contraception is vasectomy, however this is not an ideal method due to its surgical nature and problematic reversibility.

Testosterone-Treatment

Attempts to develop a hormonal male contraceptive have focused on testosterone treatment, which simultaneously inhibits gonadotrophin secretion (and thus spermatogenesis) while maintaining androgenic action. The contraceptive efficacy of a prototype testosterone-based, method has been demonstrated (WHO, 1990, 1993, 1996). Administration of weekly intramuscular injections of 200 mg testosterone enanthate rendered 70% of men azoospermic, while the remainder of men were suppressed to oligozoospermia (WHO, 1990, 1996). This method

of contraception had a relatively slow onset (~2- 3 months) and offset of action. The median time to recovery of normal sperm concentration (> 20 million/ ml) was 6.5 months in those men who became azoospermic. Discontinuations from the study were mainly because azoospermia was not achieved, side effects from the high testosterone dosage used, and because of dislike of the injection schedule.

The level to which sperm output must be reduced to ensure adequate contraceptive efficacy is uncertain. Amongst the 70% of men who became azoospermic, there was only one pregnancy during the 1486 months of the efficacy phase (WHO, 1990) giving a similar index of safety as the female oral contraceptive. In a further multicentre efficacy study, the fertility of azoospermic and oligozoospermic men following testosterone-induced spermatogenic suppression was established (WHO, 1996). This study showed that the risk of pregnancy was probably acceptable if severe oligozoospermia (< 3 million/ ml) was achieved (8.1 pregnancies per 100 person-years, however relatively small numbers of years of exposure were associated with a wide confidence interval [2.2 to 20.7 per 100 person-years]), while there were no pregnancies in the azoospermic men.

In conclusion, administration of exogenous testosterone in supraphysiological doses caused profound suppression of serum gonadotrophins and sperm production, presumably due to withdrawal of FSH action and the suppression of intratesticular testosterone levels. This regimen provided a safe, effective and reversible contraception for at least 12 months. However, the major limitations of this male hormonal contraceptive regimen were apparently the relatively slow onset and offset of action, the variability in the degree of spermatogenic suppression and sub-optimal patient acceptability (WHO, 1990, 1993, 1996).

Dose of Testosterone

The dose of intramuscular testosterone in early male contraceptive studies (~200 mg intramuscular weekly) resulted in supraphysiological testosterone serum levels and associated symptoms (WHO, 1990, 1996). The relatively short duration of action of testosterone enanthate and its pharmacokinetic characteristics generated wide swings in plasma testosterone levels which

peaked at two- to three-fold the upper limit of normal (Handelsman *et al.*, 1990, 1992). The fluctuating and intermittently supraphysiological plasma testosterone concentrations (Behre *et al.*, 1990; WHO, 1996; Anderson and Wu, 1996) make weekly intramuscular injections of testosterone enanthate impractical for general use, and an unlikely candidate as a future contraceptive for men. New long-acting injectable formulations (Behre *et al.*, 1995) or implants (Handelsman *et al.*, 1992) provide better testosterone delivery profiles. Recently, McLachlan *et al* (2000) demonstrated that testosterone implants (800-1200 mg every 3 months) provided adequate long-term spermatogenic suppression in ~70% of men, without significant androgenic side effects.

Racial Differences in Spermatogenic Suppression

Administration of exogenous testosterone induced azoospermia in 40-70% of Caucasian men, whereas (WHO, 1990, 1996; Handelsman *et al.*, 1995) consistent azoospermia was achieved with high frequency (91% to 100%) in contraceptive trials conducted in China (WHO, 1990, 1996; Handelsman *et al.*, 1995) and Indonesia (WHO, 1993). It was demonstrated that there were no differences between Chinese and non-Chinese men in mean testis size or sperm production prior to treatment (Handelsman *et al.*, 1995). The reason(s) for the heterogeneity in the spermatogenic response remains unclear.

Wang *et al* (1998) showed that there were no difference in pulsatile FSH secretion between Asian and non-Asian men in response to testosterone infusion, but at baseline Asian men had a significantly higher mean number of FSH pulses and mean incremental pulse heights than did white men. They postulated that the elevated basal serum FSH in Asian men suggested a small relative decrease is spermatogenic reserve and/or gonadal negative feedback. However, whether this difference contributed to the observed differences in variable suppression of spermatogenesis in Asian versus non-Asians in male contraceptive studies was unclear (Wang *et al.*, 1998).

Variable Testosterone-Induced Suppression of Spermatogenesis

The biggest problem with the testosterone-based male contraceptive regimes are their inability to uniformly suppress spermatogenesis in all men. A multicentre study was conducted to assess the contraceptive efficacy of testosterone-induced azoospermia, and showed that there were no differences between azoospermic and oligozoospermic responders with respect to age, height, weight, body mass index, mean testis volume, or pretreatment sperm concentration or motility (WHO, 1990). Handelsman *et al* (1995) examined potential factors that may account for oligozoospermia during testosterone-induced spermatogenic suppression, and found that the only differences between men who achieved azoospermia from those who did not were that azoospermic men had faster rates of suppression and recovery of sperm, and higher basal pretreatment levels of FSH. Furthermore, this study showed that the non-uniformity of testosterone-induced azoospermia was not attributed to incomplete gonadotrophin suppression, or on the timing and degree of gonadotrophin suppression.

Anderson and Wu (1996) showed that there were no significant differences in serum testosterone (bound or free), estradiol, LH, FSH, or in the rates of suppression between azoospermic and oligozoospermic men. Thus, the variability in spermatogenic suppression in response to testosterone treatment was unlikely to be due to differences in the pharmacokinetics or pharmacodynamics of testosterone enanthate, or in the sensitivity of the hypothalamic-pituitary-testicualr axis to sex steroid inhibition. These authors (Anderson and Wu, 1996) speculated that the partial maintenance of spermatogenesis in some men treated with exogenous testosterone may be related to individual differences in the metabolism of testosterone.

Testosterone-Treatment plus Progestins

Testosterone has been combined with other gonadotrophin-suppressing agents in contraceptive trials to achieve a more profound suppression of spermatogenesis with higher contraceptive efficacy. This addition of progestin has allowed the use of lower and more physiological doses of testosterone, minimizing the incidence of androgen-related side effects.

Various progestins have been used, including depot medroxyprogesterone acetate (DMPA) (Handelsman *et al.*, 1996), oral cyproterone acetate (CPA) (Bebb *et al.*, 1996; Meriggiola *et al.*, 1997, 1998; Meriggiola and Bremner, 1997; Büchter *et al.*, 1999), and oral desogestrel (Fotherby and Caldwell, 1994; Kuhl, 1996) and levonorgestrel (Anawalt *et al.*, 1999).

Testosterone-Treatment plus GnRH

Another approach to male contraception is to suppress gonadotrophins using GnRH antagonists, and then administering physiological levels of testosterone to maintain peripheral androgen-dependent functions (Bremner *et al.*, 1991). Studies in primates have shown that GnRH-antagonist treatment followed by testosterone substitution 6 weeks later resulted in consistent azoospermia (Weinbauer *et al.*, 1989). The combined use of GnRH-antagonist with delayed testosterone treatment has been tested in humans and this hormonal treatment induced reversible azoospermia while maintaining androgen-mediated effects such as libido (Pavlou *et al.*, 1991; Tom *et al.*, 1992). Availability, local side effects and high cost have limited research using these agents.

In summary, testosterone-induced azoospermia provides effective and reversible contraception with minimal side-effects (WHO, 1990, 1996), however azoospermia is only achieved in 40-70% of men. Addition of progestins increases the success rate of this contraceptive to ~90%, but complete spermatogenic suppression in all men has still not been achieved. A hormonal regimen that induces azoospermia in all men potentially would constitute an effective male contraception, therefore the factor(s) responsible for this incomplete spermatogenic suppression need to be elucidated.

1.7.2 A Role for 5α-Reduced Androgens in Contraception

Since 5αR determines the magnitude of the androgen stimulus, Anderson *et al* (1996) examined whether the maintenance of spermatogenesis in men remaining oligozoospermic while

receiving suppressive doses of testosterone was associated with increased 5αR activity. ^{3}H-testosterone was infused into azoospermic and oligozoospermic men, and the conversion of testosterone to DHT measured. There were no differences in the conversion rate of testosterone to DHT or in the levels of plasma 5α-reduced metabolites before treatment, however the conversion rate of testosterone to DHT was increased significantly after treatment in the oligozoospermic but not the azoospermic responders. While testosterone treatment increased plasma 5α-reduced metabolites in all men compared to baseline, the increase was significantly greater in the oligozoospermic men compared to azoospermia. This data suggested that there was a selective increase in 5αR activity in oligozoospermic compared to azoospermic men in response to supraphysiological doses of testosterone. Thus, increased 5αR activity may result in an amplification of androgen action in the testis of oligozoospermic men to allow the maintenance of low levels of sperm production (Anderson *et al.*, 1996).

Although oligozoospermic men appeared to have a greater increase in 5αR compared to azoospermia, the localization of this induced 5αR activity and the relative contributions of reproductive versus non-reproductive tissue to this overall effect were unknown (Anderson *et al.*, 1996). A further study by the same authors measured 5α-reduced androgens in seminal plasma (derived mainly from the epididymis which expresses predominantly 5αR-2) as a reflection 5αR activity in reproductive tissues, and in sebum (nongenital skin contains predominantly 5αR-1) as a reflection of 5αR activity in non-reproductive tissues, in oligozoospermic and azoospermic men (Anderson *et al.*, 1997). This study showed that there was a significant increase in the concentration of seminal DHT only in the oligozoospermic men after testosterone-treatment, suggesting that epididymal/ testicular 5αR activity may be greater in those men able to maintain oligozoospermia. This was consistent with the hypothesis that the persistence of spermatogenesis was associated with a relatively higher overall 5αR activity (Anderson *et al.*, 1996). These results suggested that the increase in 5αR activity during testosterone-treatment may be, at least in part,

be localized to the reproductive tract and may therefore reflect activity of 5αR-2 (Anderson *et al.*, 1997).

It has been postulated that increased 5αR activity during gonadotrophin suppression preserves some androgenic stimulus for the maintenance of lower levels of sperm production in the oligozoospermic but not the azoospermic responders (Anderson *et al.*, 1996, 1997). This hypothesis was further investigated by examining the extent of suppression of endogenous testicular and adrenal androgen secretion, before and after TE-suppression of spermatogenesis (Anderson *et al.*, 1997c). This study demonstrated that there were no differences between azoospermic and oligozoospermic responders, indicating that residual testicular steroidogenesis is unlikely to be a critical determinant in testosterone-induced spermatogenic suppression. Thus, these findings provided further support to the hypothesis (Anderson *et al.*, 1996) that differences in 5αR activity, rather than peripheral or testicular substrates, determine the degree of residual spermatogenesis during gonadotrophin suppression. This further highlights the importance of the postulated role of 5αR in maintaining a low level of spermatogenesis in the presence of suppressed gonadotrophins and depleted concentrations of testicular testosterone (Anderson *et al.*, 1997c).

Therefore, the testis may respond to the suppression of intratesticular testosterone (during contraception) by up-regulating 5αR to produce greater amounts of DHT to maintain androgen action, and this up-regulation may be the reason why oligozoospermic men continue to produce low levels of sperm. Studies in rodents (O'Donnell *et al.*, 1996b, 1999, see section 1.3.5) support this hypothesis by demonstrating that 5α-reduction of testosterone to DHT is involved in the restoration and maintenance of spermatogenesis in a setting of reduced testicular testosterone. Together these studies in humans and rodents suggest that inclusion of a 5αR inhibitor in existing testosterone-based contraceptive regimens may lead to a more effective and consistent suppression of sperm production.

Recently, the combination of a 5αR inhibitor with testosterone was investigated as a male hormonal contraceptive. McLachlan *et al* (2000) showed that inclusion of finasteride, a 5αR-2

inhibitor, did not enhance spermatogenic suppression in 16 men who remained oligozoospermic after 3 months of treatment with testosterone implants. However, this does not exclude the possibility that inclusion of a 5αR inhibitor may be beneficial for more reliably suppressing spermatogenesis in all men, given that the predominant 5αR isoform in humans. Aumüller *et al* (1996) used immunocytochemistry to show that 5αR-1 was localized to Leydig cells, Sertoli cells, peritubular cells and spermatocytes, whereas 5αR-2 was localized to spermatocytes and Leydig cells. Thus, as suggested by these authors (McLachlan *et al*., 2000), a dual inhibitor may prove to be a better option to suppress both 5αR isoforms in the testis.

Prostatic enlargement can occur following weekly intramuscular injections of testosterone enanthate to suppress spermatogenesis, and the pathological and clinical significance of short-term prostate changes is unclear (Wu *et al.*, 1996). Only the central zone of the prostate appears to be androgen sensitive, thus to monitor the prostate during contraceptive treatment, specialized exams and skill are required. Therefore, it would appear to be an advantage for contraceptive formulations that did not over-stimulate the prostate. The synthetic androgen 7-alpha-methyl-19-nortestosterone (MENT) produces a biologically inactive 5α-reduced androgen, therefore in contrast to testosterone, its action is not amplified in the prostate (Kumar *et al*, 1992). This favorable metabolic property could make MENT safer than testosterone in long term androgen administration.

1.8 CONCLUSIONS

Spermatogenesis is a complex process whereby spermatogonia undergo division and maturation to produce mature elongated spermatids. The regulation of spermatogenesis involves complex interactions between numerous cell types of the testis and between a number of autocrine, endocrine, and paracrine factors. Many different *in vivo* and *in vitro* models have been used to investigate the hormonal regulation of spermatogenesis, mainly using rodent models.

It is generally accepted that LH, acting via stimulation of testicular testosterone production, and FSH, both acting via receptors on the Sertoli cell, are the two major hormonal factors regulating spermatogenesis. Although many aspects of spermatogenic regulation are unknown or unclear, it is clearly evident that both FSH and testosterone are required for quantitative spermatogenesis.

The primary role for FSH in adult spermatogenesis is in promoting spermatogonial development and germ cell viability. Testosterone also exerts major effects on spermatogenesis, and is able to at least qualitatively maintain or restore complete germ cell development. A noticeable role of testosterone is in rat spermiogenesis, whereby round spermatids undergo morphological changes to produce elongated spermatids. In addition, FSH and testosterone appear to act co-operatively to maintain spermiation and overall normal sperm production in rats, primates and humans.

The 5αR enzyme, of which there are two cloned isoforms, 5αR-1 and 5αR-2, metabolize testosterone to the 5α-reduced metabolite DHT. Both androgens bind to the AR to promote androgen-dependent transcription, however DHT is more potent and thus serves to amplify androgen action. The potency of DHT is noticeable in androgen-dependent peripheral organs, such as the prostate, which are critically dependent on 5α-reduction of testosterone as a consequence of local testosterone concentrations being too low to promote sufficient androgen action.

The type of 5αR expressed and the regulation of the 5αR isoforms have been investigated in various tissues. However, testicular 5αR has remained relatively unexplored. There is no doubt

that 5αR-1 is expressed in the testis, however the regulation of this isoform has received little attention, and the available data, which has focused mainly on the immature animal, is inconsistent. There are no studies in the literature that have examined 5αR-2 activity, and of the two studies that have examined 5αR-2 mRNA, there is conflicting data on the expression of 5αR-2 in the testis.

A thorough understanding of the process of spermatogenesis and the factors that regulate this process are essential for the development of hormonal contraceptives for men. A recent approach to male contraception involves the administration of testosterone to men to suppress gonadotrophin secretion and spermatogenesis. However, this reversible and effective contraceptive does not induce azoospermia in all men. Clinical studies have speculated that a selective increase in 5αR activity in the reproductive tract may allow some men to continue to produce low levels of sperm despite gonadotrophin suppression. Therefore, it is critical for us to understand the physiology of testicular 5αR and its hormonal regulation, to elucidate its role in spermatogenesis in the contraceptive setting.

1.9 AIMS OF THESIS

There is now data to support a role for DHT in spermatogenesis when testicular testosterone concentrations are reduced to contraceptive levels, in both humans and rodents. Therefore, we have hypothesized that the 5αR isoform(s) are hormonally regulated, and the primary aim of this thesis was to explore the regulation of these isoforms to further understand their role in maintaining spermatogenesis at reduced testosterone levels.

The specific aims of this thesis are:

1) To develop and validate methods for the quantitation of 5αR-1 and 5αR-2 enzyme activity and mRNA expression in the testis,
2) To characterize the testicular 5αR isoforms, and
3) To explore the role of the 5αR isoforms in the hormonal regulation of spermatogenesis in rats, using *in vivo* models of hormonal suppression and replacement.

Chapter 2

General Methods

2.1 Animals

Sprague Dawley rats were obtained from the Monash University Animal House and maintained at 20°C in a fixed 12 h light/ 12 h day cycle with free access to food and water. All studies were approved by the Monash Medical Centre Animal Ethics committee (#1997/ 13 and 1997/ 17). The age of rats varied according to the experiment being conducted, and is stated in the relevant section. Rats were generally 30-35 days (90-130 g), 70-75 days (350-400 g), or 145-147 days (500-550 g).

2.2 Experimental Procedures

Unless otherwise stated, reagents were obtained from Sigma ❶ *[see Appendix 1]* and were of analytical grade.

2.2.1 Hormone Implants

Testosterone and 17β-oestradiol implants were prepared by packing either powdered testosterone or 17β-oestradiol into medical grade polydimethylsiloxane tubing (Dow Corning❶: id 19.8 mm; od; 3.18 mm). The tubing was cut into lengths 6 mm longer than required, so that the ends of the tubing (~3 mm on either side) could be sealed with medical adhesive silicone type A (Dow Corning❶). One side of the tubing was sealed and the adhesive allowed to dry overnight. The tubing was then filled with hormone powder and the open end sealed with adhesive silicone. Testosterone implants were prepared in lengths of 3 cm (i.e. 3 cm-*T.imp*) or 8 cm (3 x 8 cm = 24 cm-*T.imp*) implants. Oestradiol implants were 0.4 cm long (i.e. 0.4 cm-*E.imp*) in length. The hormone release rates from this silastic tubing have been described previously (Robaire *et al.*, 1979) and were 30 and 2.4 µg/ cm/ day for testosterone and oestradiol, respectively.

Animals were anaesthetized by ether❷ *[see Appendix 2]* (BDH Laboratory Supplies❶) inhalation and laid on their ventral side. To minimize the risk of infection during surgery, the

silastic implants and the skin were washed with 70% ethanol. Local hair on the dorsal aspect was trimmed and a small transverse incision was made in the skin midway between the head and the tail. Blunt dissection separated the underlying musculature from the skin and the implants were inserted in a caudal to cephalic direction. The surgical wound was closed with a steel surgical clip (Michel Clip, Lawton❶). Recovery to surgery was rapid and animals showed no obvious discomfort to the implants over the duration of the treatments.

2.2.2 Drug and Hormone Preparations

The following treatments were prepared in peanut oil (Biotech International❶):

(a) The androgen antagonist flutamide (Sigma❶) was administered at a dose of 20 mg/ kg (i.e. a 400 g adult rat receives 8 mg of flutamide in a 1 ml sc injection of peanut oil). Flutamide was first dissolved in methylene chloride and then added to peanut oil (1:2 methylene chloride:oil, v/v). The solvent was evaporated by bubbling with nitrogen and simultaneously warming the solution to 37°C to facilitate evaporation.

(b) Vehicle (oil) injections were prepared in the same manner as flutamide, except the drug was omitted, so that vehicle-treated control animals received the equivalent volume of peanut oil.

Vehicle and inhibitor were prepared fresh each day immediately prior to injection. Injections prepared in peanut oil were administered with a 5 ml syringe and an 18-gauge (G) needle.

The following treatments were prepared in 15% normal rat serum (NRS) in saline:

(a) Human chorionic gonadotrophin (hCG; 1500 IU/ ampoule, Pregnyl, Organon❶): is an LH-substitute, and was administered at 2.5 IU (high dose) or 0.5 IU hCG/ kg rat/ daily (low dose).

(b) FSH Antibody (FSH*Ab*, #1792): An Ig fraction of an anti-rat FSH antibody raised in sheep was used to selectively withdraw FSH (short term) from adult rats. The ability of this FSH antibody to neutralize serum rat FSH has been previously described (Meachem

et al., 1999). This study showed that administration of 2 mg/ kg of FSH.*Ab* by daily sc injection neutralized >90% of circulating FSH.

(c) Recombinant human FSH: (rhFSH, Gonal-F, Serono❶): Freeze-dried recombinant human FSH was administered at a dose of 10 IU/ kg rat/ day (i.e. a 400 g rat received 10 IU of rhFSH in 400 µl of 15% NRS diluted in saline).

(d) Vehicle (15% NRS in saline) for injections prepared in saline consisted of 15% NRS diluted in saline without the addition of any drugs.

The preparations diluted in 15% NRS diluted in saline were prepared aseptically and filtered through a 0.2 µ filter. These treatments were injected with a 1 ml syringe and 23 G needle.

2.2.3 Tissue Preparation

a) Blood

Blood was collected immediately post-mortem by cardiac puncture after CO_2 inhalation. An 18 G needle and 10 ml syringe was used to puncture the heart through the ribs to collect blood (5-10 ml) from the left ventricle. If insufficient blood was obtained with this method, then direct entry into the heart was gained by opening the thorax. The blood was allowed to clot overnight at 4°C, and then centrifuged at 1800 revolutions per minute (rpm) for 35 min at 4°C. The serum was aspirated, snap frozen and stored at –20°C for serum hormone assays.

b) Testicular Supernatants

Following CO_2 inhalation and cardiac puncture, a small incision was made in the scrotum. Each testis was excised, trimmed of fat, weighed, wrapped in aluminium foil, frozen in liquid nitrogen and stored at -70°C for later use.

Supernatants were used to measure 5αR activity in the testes because it has been shown that 5αR activity is predominantly, if not exclusively expressed in the microsomes (Yoshizaki *et al.*, 1978). The right testis was used to prepare a 9000 rpm supernatant for *in vitro* 5αR enzyme

activity assays. The frozen testis was decapsulated, placed into ice-cold 0.25 M sucrose and homogenized for 30 sec with a tissue disperser (Janke and Kunkel❶) at 20,500 rpm. The homogenate was then centrifuged (10,000 rpm for 20 min) and the supernatant collected, snap frozen in dry ice and stored at –70°C for testicular 5αR activity assays.

The left testis was used to prepare supernatants for measuring testicular steroid levels by High Performance Liquid Chromatography (HPLC) and radioimmunoassay (RIA, see section 2.3.3).

c) Epididymal and Liver Homogenates

Unlike testicular 5αR activity which is found in the microsomes, 5αR activity in the epididymis and liver is distributed between the nuclear and microsomal compartments (Frederiksen and Wilson, 1971; Moore and Wilson, 1972). Therefore, tissue homogenates were prepared to measure 5αR activity in the epididymis and liver. Once both testes were removed, the epididymes were excised, trimmed of fat, weighed, wrapped in aluminium foil, frozen in liquid nitrogen and stored at -70°C for later use. A section of the liver was also excised and stored frozen for later use. For measurement of epididymal or liver 5αR activity, the tissues were removed from the aluminium foil, cut into small pieces with a razor blade and homogenized in 0.25 M sucrose as above. The homogenates were stored at –70°C until required.

2.3 Hormone and Protein Assays

2.3.1 Serum FSH

The reagents for the serum rat FSH double antibody RIA was supplied by the Rat Pituitary Hormone Distribution Program (National Institute of Diabetes, Digestive and Kidney diseases, NIDDK❶).

Rat FSH (NIDDK rat FSH I-18) was iodinated by the lactoperoxidase method of Thorell and Johansson (1971) and purified by gel filtration (Robertson *et al.*, 1978). The rat FSH antiserum (NIDDK antiserum FSH S-11) was raised in rabbits against purified rat FSH and used at a dilution of 1:31,250 (diluted in 1:600 normal rabbit serum). The assay has a low cross-reactivity (<1%) with other pituitary hormones (rat thyroid stimulating hormone, LH, prolactin and hCG). The rat FSH standard (rat FSH RP-2) ranged from 0.39- 100 ng/ ml, and a goat-anti-rabbit IgG was used as precipitating second antibody (1:40). All samples were assayed at neat (100 µl) in duplicate.

All samples were assayed in two separate assays with a detection limit of 1.5 ng/ ml. Two quality control (QC) samples were included in each of the assays, and the values obtained for these QC's were 3.52 and 3.33, and 2.67 and 2.46.

The protocol for the FSH assays was as follows:

Day 1 - Add 100 µl sample/ standard/ QC (QC was a pool of normal and GnRH-immunized serum), 100 µl antiserum FSH S-11 (1:2500 in 1:500 NRS), and 100 µl buffer (0.1 M PBS❷ + 0.5% BSA❷, pH 7.4). Incubate for 24 h at 4°C *(final assay volume = 300µl)*.

Day 2 - Add 100 µl tracer at 10,000 cpm/ 100 µl and incubate for 48 h at room temperature *(final assay volume = 400 µl)*.

Day 4 - Add 100 µl second antibody Goat-anti-rabbit serum at 1:60 in PBS + 50 mM EDTA❷, 100 µl NRS to standard tubes and 100 µl buffer (0.1 mM PBS + 0.5% BSA, pH 7.4) to samples. Incubate overnight at 4°C *(final assay volume = 600 µl)*.

Day 5 - Add 2 ml cold saline (0.9%) to all tubes and centrifuge at 4000 rpm for 45 min at 4°C. Decant supernatant and drain tubes. Count for 2 min in an automatic gamma counter (Wallac 1470❶).

2.3.2 Serum LH

Serum LH was measured using an immunofluorometric (IFMA) assay based on the method of Haavisto *et al* (1993). The IFMA utilized two monoclonal antibodies, one to bovine (tracer

antibody, 5303, Medix❶) and the other to human LH (518B7, Dr. Rosen, University of California).

This assay required the following reagents which were obtained from Wallac❶: Wallac assay buffer, standard buffer (7.5% BSA in TSA❷ buffer), wash solution, Eu-labelled Streptavidin (0.1 mg/ ml), and enhancement solution. The rat LH standard (rLHRP-3, NIDDK) ranged from 0.00975- 2.5 ng/ ml and was diluted in standard buffer.

Maxisorp microtiter strips (NUNC❶) were coated by adding to each well 4 µg coating antibody (518B7) in 200 µl 0.1 M $NaH_2PO_4.2H_20$ (pH 4.4). The plate of strips was covered and placed into a plastic bag with a sponge soaked in 0.1% NaN_3, sealed, and incubated overnight at 37°C. After washing the strips once with Delfia wash buffer, 300 µl blocking solution containing 10 g/ L dialyzed BSA in 50 mM Tris-saline-azide (TSA) buffer, 0.9% NaCl and 0.1% NaN_3 (pH 7.5) was added to each well and incubated overnight at room temperature in a sealed plastic bag containing a sponge soaked in 0.1% NaN_3. After aspiration of the blocking solution, the coated plates were stored at 4°C in a sealed plastic bag containing a wet sponge.

Serum samples were assayed at neat (25 µl) in duplicate, and all samples were measured in two separate assays. The sensitivity of the IFMA assay was 0.02 ng/ ml, which corresponded to a concentration of 0.75 pg/ tube at a sample volume of 25 µl. The inter-assay coefficient of variation (CV) was 3.98% at a LH concentration of 0.5 ng/ ml, and 1.9% at a concentration of 0.23 ng/ ml.

The protocol for the serum LH assay was as follows:

a) Pre-wash 96-well streptavidin plate three times and dry.

b) Dilute the rat LH standard and normal rat serum in standard buffer.

c) To each well add:

- Standards: 100 µl standard, 75 µl Wallac assay buffer, and 25 µl standard buffer, and

- Samples: 100 µl standard buffer, 75 µl Wallac assay buffer, 25 µl serum sample.

d) Cover plate, shake at high speed for 5 min, and incubate for 85 min at room temperature while shaking. Wash plate six times and tap dry.

e) Add 200 µl 25 ng biotinylated antibody (5303) (diluted in Wallac assay buffer and filter sterilized). Incubate for 30 min and room temperature while shaking. Wash plate six times and tap dry.

f) Add 200 µl 50 ng European-Streptavidin (diluted in Wallac assay buffer and filter sterilized), and incubate for 30 min at room temperature while shaking. Wash plates six times and tap dry.

g) Add 200 µl enhancement solution, shake for 5 min and read using 1234 Delfia fluorometer for 1 sec/ well (Wallac❶).

2.3.3 Testicular Androgens

Testicular androgens (testosterone, DHT and 3α-Adiol) were separated and assayed using a previously published technique that was developed in our laboratory (O'Donnell *et al.*, 1996b). This method involves the separation of androgens by HPLC and subsequent measurement by RIA. The majority of these procedures were performed by Ms Fiona McLean at Prince Henry's Institute of Medical Research, and a brief overview of these methods will be given below.

Whole testes were stored at –70°C before androgen determination. Frozen testes were decapsulated and homogenized for 20 sec (Diax 600, Heidolph❶) in 2 ml homogenization buffer [0.1% (v/v) trifluoroacetic acid (TFA) and 60% (v/v) acetonitrile]. To follow steroid recoveries throughout processing, ~5000 cpm of radiolabeled [1α, 2 α -N-^{3}H]-Testosterone❷, [1,2-N-^{3}H]-DHT❷, [9,11-N-^{3}H]-3α-Adiol❷ (NEN Life Sciences❶; 40-60 Ci/ mmol) were added to each tube before homogenisation. The homogenate was centrifuged at 4°C for 20 min at 10,000 rpm, and the resulting supernatant was kept on ice. The pellet was resuspended in homogenisation buffer, vortexed, sonicated for 3 min, vortexed again, and centrifuged (10,000 rpm for 20 min at 4°C). The supernatants were combined and diluted 1:3 in deionized H_20 (MilliQ system, Millipore❶) and 0.1% TFA (v/v), and loaded onto a Sep-Pak C_{18} disposable column (Waters❶). The steroids

were eluted with 0.1% TFA and 60% acetonitrile (v/v) and lyophilized. Samples were resuspended in 200 µl HPLC buffer [0.1% TFA (v/v) and 40% acetonitrile (v/v)] and centrifuged (4000 rpm for 30 min at 4°C). The supernatant was transferred to a microfuge tube and applied to the HPLC.

A Waters µBondapak C_{18} column (30 x 0.39 cm; Waters❶) and guard column (4.3 mm ID x 1 cm, Activon❶) were equilibrated in HPLC buffer for 30 min at a flow rate of 1 ml/ min. Samples were loaded using a 250 µl glass syringe, and fractions (0.5 ml) were collected. The retention rates for each steroid were 14.5 min for testosterone, 23.5 min for DHT, and 21 min for 3α-Adiol. DHT and 3α-Adiol were completely separated using this system. After separation, 50 µl was taken from each fraction to determine the recovery of ^{3}H-labeled standards: scintillation fluid❷ (2 ml, Packard Emulsifier Safe, Packard Instrument Company, Inc. ❶) was added and counted for 10 min/ vial in a β-counter❷ (2500TR, Packard❶). The appropriate fractions were pooled (final volume, 1.5-2 ml) and dried down overnight. The recovery of radioactive standards were: 72 ± 7% for testosterone, 50 ± 6% for DHT, and 68 ± 9% for 3α-Adiol (mean ± SD; n = 5 HPLC runs).

Before quantitation of androgens by RIA, samples were resuspended in 1 ml assay buffer [0.1% (wt/v) gelatin in 0.1 M PBS (0.154 M NaCl), pH 7.4]. The primary antibody was Cox 0457 (Sirosera❶) diluted 1:400,000 in 1:800 normal sheep serum. Tracer was iodinated histamine-testosterone (10,000 cpm/ 100 µl, prepared at Prince Henry's Institute of Medical Research). The standards for the RIA (testosterone, DHT and 3α-Adiol) were diluted in 100% ethanol (1 mg/ ml) and then diluted to the top dose (250 pg for testosterone and DHT, and 1000 pg for 3α-Adiol). The assay was incubated overnight at 4°C. Second antibody (100 µl donkey anti-sheep IgG diluted 1:20 in assay buffer) was added and incubated at room temperature for 30 min, after which 1 ml 6% Polyethylene Glycol 6000 was added and incubated at 4°C for 30 min. Tubes were centrifuged at 4,000 rpm for 40 min at 4°C, drained and counted on a γ-counter (Wallac❶).

The between assay variations for testosterone, DHT, and 3α-Adiol were 8%, 3% and 8%, respectively, and the within-assay variations were 8%, 13%, and 12%, respectively (n = 6).

Standard curves for the assays ranged from 1.3-150 pg/ 100 µl for testosterone, 3.0-350 pg/ 100 µl for DHT, and 14-3,000 pg/ 100 µl for 3α-Adiol.

2.3.4 Protein Assay

The BCA Protein Assay Reagent (Pierce❶) was used for the quantitative calorimetric determination of total protein in dilute aqueous solutions. This reagent system uses bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment (Smith *et al.*, 1985). The purple-colored reaction product of this assay is formed by the interaction of two molecules of BCA with one cuprous (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562 nm, which is linear with increasing protein concentrations over a broad working range of 20 µg/ ml to 2 mg/ ml. The standard used was Bovine Serum Albumin❷ (No.23209, 2 mg/ ml, Pierce❶). The between assay variation for the BCA protein assay was 3.51% (n = 5 assays).

The protocol for the BCA protein assay was adjusted for microtiter plates and was as follows:

a) Prepare working reagent, dilute samples (dilute 30 µl protein in 270 µl 0.5 M sodium hydroxide; NaOH), and standards (5 – 50 µg) in 0.5 M NaOH. Allow samples to solubilize for 2 h.

b) Pipette 10 µl of each standard or unknown protein sample into a 96-well microtiter plate (Immuno Plate Maxisorp, NUNC❶). For background, replace protein sample with 0.5 M NaOH.

c) Add 200 µl working reagent to each well, and incubate at 37°C for 30 min. Read at 540/ 630 nm on the Microplate Reader MR7000 (Dynatech❶).

2.4 General Molecular Biology

2.4.1 Automated Sequencing

Sequencing was performed by the Wellcome Trust Sequencing Centre (a joint facility of Prince Henry's Institute of Medical Research and The Monash Institute of Reproduction and Development), using the ABI Prism™ 377 DNA Sequencer❷ (Perkin Elmer Biosystems❶).

2.4.2 Ethanol Precipitation of DNA

DNA was precipitated at -70°C overnight, following the addition of one-tenth the volume of 3 M sodium acetate❷ (pH 5.2) and equal volumes of 100% isopropanol. The solution was centrifuged (10,800 rpm for 10 min) to obtain a DNA pellet. The supernatant was removed and the pellet washed with 200 µl ethanol (70%) by centrifugation (10,800 rpm for 10 min). The supernatant was removed and the DNA pellet allowed to air-dry for 5-10 min. The DNA pellet was resuspended in diethyl pyrocarbonate-treated water (DEPC❷; ICN Biomedicals❶), allowed to dissolve for 5 min at room temperature and stored at –70°C.

2.4.3 Extraction of Total RNA from Testis

Total RNA was extracted using the RNeasy Mini extraction kit (Qiagen❶) according to the manufacturer's instructions. An additional step was included (isopropanol precipitation) to remove any contaminating substances that might interfere with subsequent steps. This RNA isolation system utilized guanidine-isothiocynate lysis and silica-gel-membrane purification to extract total RNA from tissues.

The protocol for the extraction of RNA was as follows:

a) Cut approximately ~30 - 50 mg frozen testis and place into a plastic tube kept on dry ice.

b) Add Lysis Buffer RLT (supplied; 350 µl for testis < 25 mg and 600 µl for tissue 25-50 mg) and homogenize lysate by passage through an 18 G and then a 23 G needle with a 1 ml syringe 9 - 12 times. Keep samples on ice after the addition of buffer RLT.

c) Centrifuge lysates (10,000 rpm for 3 min) and add 1 volume (either 350 or 600 µl) of ethanol (70%) to the cleared lysate. Pipette solution into an RNeasy mini spin column and centrifuge (10,000 rpm for 15 sec).

d) Wash the RNeasy column with 700 µl Wash Buffer RW1 (supplied) by centrifuging (10,000 rpm for 15 sec).

e) Transfer the RNeasy column to a new collection tube, add 500 µl RPE (supplied) and centrifuge (10,000 rpm for 15 sec).

f) The RNeasy membrane was washed with 500 µl Wash Buffer RPE by centrifuging (10,000 rpm for 2 min).

g) Transfer RNeasy column to a new 1.5 ml collection tube, add 40 µl RNase-free H_2O directly onto the RNeasy membrane and centrifuge (10,000 rpm for 1 min). Repeat this step (final volume of RNA in 80 µl Rnase-free H_2O).

Isopropanol Precipitation of RNA

a) Add one-tenth (8 µl) Na Acetate and equal volume (80 µl) of 100% isopropanol to the extracted RNA, vortex the mixture and store overnight at –20°C.

b) Centrifuge mixture (14,000 rpm for 5 min) and discard supernatant, taking care not to dislodge the pellet.

c) Add 200 µl ethanol (70%), vortex and briefly centrifuge (14,000 rpm for 10 min).

d) Discard the supernatant and air-dry for 5 min. Resuspend the RNA pellet in 40 µl DEPC H_20 and incubate for 3 min at room temperature. Resuspend RNA pellet by vortexing and store RNA at -70°C. Analyze RNA quality by running the extracted RNA on an agarose❷ gel (see section 2.4.4).

2.4.4 Quantitation of Total RNA Extracted

The Sub-Cell GT DNA Electrophoresis MiniGel Appartus❷ (BioRad❶) was washed once with 0.1 M NaOH for 15 min and then thoroughly washed with sterile H_2O. A 1.5% agarose gel was prepared as follows: microwave 2% agarose until properly melted, pour 30 ml into a polypropylene tube and add 10 ml of 10 X TBE❷ and ethidium bromide (10 mg/ ml). Mix well, allow to cool then pour into gel apparatus and allow gel to set.

Make a 1:60 dilution of the extracted RNA (prepared in section 2.3.3) and read the optical density (OD) of the RNA with a UV spectrophotometer❷ at a wavelength of 260 nm after blanking with sterile water (H_2O). Pure RNA samples had a 260/ 280 absorbance ratio >1.6. Lower ratios were caused by incomplete washing, DNA contamination, or protein contamination. The yield of RNA was calculated using the formula 1 OD unit (260 nm) = 40 µg/ µl of RNA, taking into consideration the dilution of the sample. Therefore, RNA was measured using the formula: $RNA\ (\mu g/\mu l) = (OD_{260} \times 60\ (dilution\ factor) \times 40) \div 1000$.

RNA samples were prepared for agarose gel analysis by adding 2 µg RNA and 2 µl DNA/ RNA loading buffer❷ with ethidium bromide to a microfuge tube, made up to a total volume of 20 µl with sterile H_2O. The gel was run at 75 V until the bromophenol blue❷ reached ~2 cm from the bottom of the gel. Ethidium bromide-stained RNA was visualized using short wavelength UV and a photo of the gel was taken using the Gel Doc 2000-gel documentation system❷. A ratio of 28S to 18S RNA components of 2:1 or greater, as visualized by ultraviolet light, was considered acceptable for further analysis. Strong bands at the top of the gel, near the well, suggested DNA or protein contamination, and smearing of the RNA indicated RNA degradation.

2.4.5 Purification of DNA Fragments from Agarose Gels

The QIAEX II Gel extraction kit (Qiagen❶) was used to purify DNA fragments from agarose gels. The principle of this procedure was based on the solubilization of agarose and selective adsorption of nucleic acids onto QIAEX II silica-gel particles in the presence of chaotropic salt.

The protocol for purifying DNA from agarose gels was as follows:

a) Excise and weigh the DNA band from the agarose gel, and add 3 x volumes of solubilization Buffer QX1 (supplied) and 2 x volume H_2O.

b) Add 30 µl QIAEX II (supplied) to the DNA sample and incubate mixture at 50°C for 10 min. Vortex mixture every 2 min to keep QIAEX II in suspension.

c) Centrifuge solution (9000 rpm for 30 sec) and dispose supernatant. Resuspend pellet in 500 µl Buffer QX1 and centrifuge (9000 rpm for 30 sec).

d) Resuspend pellet in 500 µl Elution Buffer (supplied) and centrifuge (9000 rpm for 30 sec).

e) Air-dry the pellet for 10 min and elute the DNA by adding 20 µl of 10 mM Tris-Cl (pH 8.5) and incubate for 5 min at 50°C.

f) Centrifuge the mixture (9000 rpm for 30 sec) and transfer the supernantant containing the purified DNA to a new sterile tube, and store at 4°C.

2.4.6 Purification of Polymerase Chain Reaction Products

Following polymerase chain reaction (PCR), unwanted products including primers, paraffin oil, unincorporated nucleotides, and polymerase enzymes that might inhibit subsequent enzymatic reactions (sequencing and cloning of PCR products) were removed using the High Pure PCR Product Purification Kit (Boehringer Mannheim❶).

This PCR purification kit was designed for the isolation of PCR products from amplification reactions, using salts to bind nucleic acids to pre-treated glass fibre. Binding was specific for nucleic acids and therefore separated the bound nucleic acids from impurities. The nucleic acids were removed with a washing step.

The protocol for purifying PCR products from agarose gels was as follows:

a) Add 500 µl binding buffer (supplied) to a 100 µl PCR reaction, and mix well. Pipette the sample into the High Pure filter tube connected to a collection tube and centrifuge (10,400 rpm for 30 sec).

b) Discard the flow-through and add 500 µl wash buffer (supplied) and centrifuge (10,400 rpm for 30 sec). Repeat this step with 200 µl wash buffer.

c) Place filter in a new collection tube and centrifuge (10,400 rpm for 30 sec). Elute DNA in 40 µl sterile H_2O. Repeat this step and store at 4°C.

2.4.7 Purification of Plasmid DNA from Bacterial Cultures

Two different procedures were used to purify plasmid DNA from bacterial cultures, depending on the volume of the bacterial culture:

The Quantum Prep Plasmid Miniprep Kit (BioRad❶) was used to purify the plasmid DNA from 5 ml bacterial cultures, according to the manufacturer's instructions. This procedure used the silicon dioxide exoskeleton of diatoms as the DNA binding matrix to provide a rapid purification of high-quality, high-yield plasmid DNA suitable for cell transfection.

The protocol for purifying plasmid DNA from bacterial cultures was as follows:

a) Remove 0.5 ml from bacterial culture (5 ml) and add equal volumes of sterile glycerol❷. Mix by inverting the tube and store stock at –70°C for later use (i.e. to grow 200 ml bacterial culture).

b) Centrifuge the remaining bacterial culture (10,800 rpm for 30 sec) and discard the supernatant.

c) Add 200 µl Cell Suspension Solution (supplied) and resuspend cell pellet by pipetting. Add 250 µl Cell Lysis Solution (supplied) and 250 µl Neutralization Solution (supplied), and mix by inverting the tube.

d) Pellet the cells by centrifuging (10,800 rpm for 5 min) and keep the supernatant that contains the plasmid DNA. Transfer the cleared lysate to a spin filter, add 200 µl Quantum Prep Matrix (supplied) and centrifuge (10,800 rpm for 30 sec).

e) Remove the spin Filter and discard the filtrate, add 500 µl Wash Buffer (supplied) and centrifuge (10,800 rpm for 30 sec). Repeat this wash step but centrifuge for 2 min.

f) Place spin filter in a new collection tube and centrifuge to remove all traces of ethanol. Add 100 µl of deionized TE (supplied) to elute the DNA by centrifugation for (10,800 rpm for 1 min).

g) Discard the spin filter and store the eluted DNA at –20°C.

The CONCERT High Purity Maxiprep Plasmid Purification System (Gibco❶) used a unique anion exchange resin to purify plasmid DNA from 200-500 ml bacterial cultures. This method yielded plasmid DNA that was suitable for cell transfections.

The protocol for purifying plasmid DNA from bacterial cultures was performed according to the manufacturers instructions with minor modifications:

a) Equilibrate column with 30 ml Equilibration Buffer (supplied) while harvesting cells by centrifuging (3000 rpm for 20 min at 4°C).

b) Resuspend pelleted cells in 10 ml Cell suspension Buffer containing RnaseA (supplied), lyse cells in 10 ml Cell Lysis Solution (supplied) and incubate at room temperature for 5 min.

c) Neutralize cells in 10 ml Neutralization Buffer (supplied) and centrifuge (3,000 rpm for 15 min at room temperature).

d) Load supernatant onto the equilibrated column and allow to drain by gravity flow. Wash column with 60 ml Wash Buffer (supplied) and elute DNA with 15 ml Elution Buffer (supplied).

e) Add isopropanol (10.5 ml) to the eluate and centrifuge the mixture (3,000 rpm for 40 min at 4°C). Wash plasmid DNA with 5 ml ethanol (70%) and centrifuge (3,000 rpm for 5 min at 4°C). Aspirate ethanol air-dry pellet for 10 min.

f) Dissolve the DNA pellet in 500 µl TE Buffer and centrifuge (3,000 rpm for 1 min at room temperature). Transfer the purified DNA to a new sterile tube and store at 4°C.

2.4.8 Culturing COS-7 Cells

Rat recombinant 5αR-1 and 5αR-2 cDNA were transfected into mammalian COS cells. COS cells are derived from African green monkey kidney cells by transformation with an origin-defective simian virus 40 (SV40) and are grown as a monolayer of fibroblasts. Several sublines of COS cells exist. The COS-7 subline expresses high levels of the SV40 large tumor antigen which is required to initiate viral DNA replication at the origin of SV40. SV40 large tumor antigen-mediated replication can amplify the copy number of plasmids containing the SV40 origin of replication to >100,000 per cell, which results in high expression levels from the transfected DNA. Therefore the COS-7 subline was used in these studies because it produced a higher plasmid copy number, and was obtained from ATCC❶.

Cell line stocks were stored in liquid nitrogen (N_2) by supplementation of 1 x 10^6 cells in medium with 20% dimethyl sulphoxide (DMSO). For regeneration of cells, cells were thawed quickly in a 37°C water-bath and transferred to a 175 cm^2 flask containing 50 ml warm DMEM. Cells were incubated in a humidified 37°C, 5% $C0_2$ incubator and passaged when ~90% confluency was reached. To passage cells, the media was removed and the cells were washed with PBS. To detach cells from the flask, 1% trypsin❷/EDTA was added for 2-5 min, and cells passaged 1:8 into 175 cm^2 flasks, with 50 ml media containing fetal calf serum❷ ($Media^{+FCS}$).

COS-7 cells were grown in DMEM-high glucose media supplemented with 10% Fetal Calf Serum (FCS) and 1% antibiotics (penicillin, streptomycin, and fungizone❷). Media was made by adding 4.5 g DMEM to ~700 ml MilliQ H_2O, 20 ml 7.5% Na Bicarbonate (1.5 g/ L final concentration), 25 ml 200 mM L-Glutamine, 10 ml 100 mM Sodium pyruvate (1 mM final concentration), at pH 7.3 - 7.4. This was made up to 1 L with H_2O and filter sterilized into 500 ml aliquots. Media was stored at -4°C, and 10% FCS (50 ml/ 500 ml) added before use.

Chapter 3

Validation of *In Vitro* Enzyme Assays for Measuring 5α-Reductase Type 1 and Type 2 Enzyme Activity

3.1 Introduction

Previous studies have examined both 5αR isoforms in various tissues by measuring enzyme activity, mRNA and protein (see section 1.5). Quantitation of enzyme activity is facilitated by the distinct pH optimas that the two 5αR isoforms display: 5αR-2 has a sharp peak at pH 5.0, whereas 5αR-1 has a broad neutral pH range (5.0 - 8.5). This distinct biochemical property is characteristic of many species, including rat and human. The studies described in this thesis will investigate 5αR-1 and 5αR-2 during *in vivo* hormonal manipulations as a means of understanding the role of 5α-reduced androgens in rat spermatogenesis. A method for the quantitation of specific 5αR isoform activity needed to be developed, so that the independent regulatory effects of the 5αR isoforms on spermatogenesis could be examined.

There are numerous early studies (prior to 1979) which have investigated 5αR activity in rat testis (Oshima *et al.*, 1970; Folman *et al.*, 1972; Nayfey *et al.*, 1975a; Murono and Payne., 1979). These studies however were performed at pH 7.0 and therefore only measure 5αR-1 activity. Furthermore, the majority of these studies focused on the localization and not the regulation of 5αR in the testis, and most of these studies were performed in immature rats. The reason for the lack of studies on testicular 5αR was probably two-fold: no one had ever speculated a role for 5α-reduced metabolites in adult rat testis, which normally express high levels of testosterone, and secondly 5αR activity declined dramatically after puberty to reach low levels in adulthood.

The 5αR enzyme has been more extensively studied in other tissues such as the prostate, epididymis, and human scalp, where the role of 5α-reduced androgens is critical for growth and development. Regardless of the tissue being examined, most investigators have utilized the distinct pH optimas of the 5αR isoforms to measure their activity *in vitro*. Thus, if the conversion of testosterone to 5α-reduced metabolites was measured as a function of pH, then a sample exhibiting a peak at pH 5.0 would be predicted to be 5αR-2, whereas a sample with a peak in the neutral pH range would be predicted to be due to 5αR-1 activity. Co-expression of both 5αR isoforms in a sample would be indicated by activity peaks at both acidic and neutral pH. Similarly, for enzyme

kinetic experiments, a micromolar K_m value for steroid substrates would indicate 5αR-1 activity, whereas a nanomolar K_m value would indicate 5αR-2 activity. Enzyme kinetic experiments are time-consuming and require large amounts of sample, and therefore are generally only used to determine which isoform of 5αR is expressed, and not the actual activity level of each isoform.

The use of pH 5.0 and 7.0 to measure 5αR-2 and 5αR-1 activity, respectively (Chase and Pahne, 1985; Liang *et al.*, 1985; Lephart *et al.*, 1990; George *et al.*, 1991; Harris *et al.*, 1992; Normington and Russell, 1992; Robaire *et al.*, 1995; Levy *et al.*, 1995; Haning *et al.*, 1996; Poletti *et al.*, 1997) is an accepted common approach, but becomes complicated when a tissue expresses both 5αR isoforms. In this case, the ratio of activities at pH 5.0 and 7.0 (pH 5.0/ 7.0) can be used to indicate the relative levels of 5αR-2 activity. However, this approach is a relative measure and does not provide quantitative information. Furthermore, use of the pH 5.0/ 7.0 ratio for assessing 5αR-2 activity can be difficult to interpret if the 5αR isoforms of a tissue are differentially regulated.

We have used an enzyme assay to measure 5αR activity by measuring the conversion of radioactive testosterone to the 5α-reduced metabolites, DHT and 3α-Adiol. The steroids were separated by thin layer chromatography (TLC) and quantified. During TLC, the solvent moves through the silica gel on the TLC plate. The component molecules are attracted to the stationary phase, retarding their movement, so that different component solutes will move at differing rates since each will have a slightly different affinity for the stationary phase. The resolution of separation in TLC is reflected by R_f values, which refer to the migration of component solutes 'relative to the solvent front'. The R_f values report the distance from the origin to the centre of the separated molecule, divided by the distance from the origin to the solvent front, and are calculated by the formula: R_f = Distance traveled by solute/ Distance traveled by solvent front. A suitable solvent should give R_f values ideally between 0.3 and 0.7 for the sample components of analytical interest.

In order to measure specific 5αR activity in a tissue that expresses both 5αR isoforms, a new quantitative method was required. To be specific for 5αR-2 activity, the overlapping effects of 5αR-1 at pH 5.0 had to be removed. This could theoretically be achieved by using inhibitors for 5αR-1, however it was difficult to be certain that 5αR-2 was not also being inhibited. Thus, we validated a method in which the activity at pH 5.0 attributed to 5αR-1 was calculated, allowing the measurement of 5αR-2. This was achieved using recombinant rat 5αR-1 (that is free of 5αR-1) measured at pH 5.0 and 7.0. This chapter describes the validation of this method for the quantitation of 5αR-2 activity in the presence of 5αR-1. Details of the *in vitro* enzyme activity assay, the steroid extraction process, steroid separation by TLC, and quantitation by β-counting, will be also be provided.

3.2 Material and Methods

3.2.1 *In vitro* 5α–Reductase Enzyme Activity Assay

An *in vitro* enzyme activity assay was used to measure 5αR activity in a range of tissues and cells. In brief, this assay measured the amount of ^{3}H-testosterone metabolized to the 5α-reduced metabolites, ^{3}H-DHT and ^{3}H-3α-Adiol. Thus, the amount of 5αR expressed in a sample was reflected by the amount of testosterone substrate that was converted under optimal conditions (i.e. the higher the conversion the more 5αR activity). Following the *in vitro* enzyme activity assay, steroids were extracted, separated by TLC and the radioactive substrate and products quantified in a β-counter.

The *in vitro* 5αR activity assay was carried out in a total volume of 550 µl, using 0.1 M Tris-Citrate❷ at either pH 5.0 or pH 7.0 (unless otherwise stated) as the assay buffer. The assay was performed in duplicate or triplicate, and each tube consisted of the following components:

a) 5 µl radioactive testosterone in 100% ethanol (0.105 µCi of ^{3}H-testosterone; ~230,000 dpm)

b) 15 µl non-radioactive testosterone in 100% ethanol (0.35 mM final concentration)

c) 430 µl 0.1 M Tris-citrate buffer containing 0.5 mM NADPH❷ (final concentration)

d) 100 µl sample (prepared as described in section 2.2.3).

All components of the 5αR assay were added to a 1.5 ml plastic tube in the order described above and kept on ice. The assay contents were mixed by vortexing and incubated at 37°C for 1 h, unless otherwise stated. Control incubations contained all assay components and were kept on ice during the 37°C incubation period.

3.2.2 Steroid Extractions

At the end of the incubation, sample tubes were placed on ice and the contents transferred to a glass vial containing 2 ml 0.1 M sodium hydroxide to terminate the reaction. The glass vial also contained: (a) 25 µg non-radioactive steroids (testosterone, DHT and 3α-Adiol) to aid visualization of steroids on the TLC plate, and (b) ~5000 dpm of ^{14}C-steroids (^{14}C-testosterone and ^{14}C-DHT, Du Pont-New England Nuclear❶, and ^{14}C-3α-Adiol was produced in our laboratory [see section 3.2.3]) to estimate the recovery of steroids during the extraction phase and chromatography.

The steroids were extracted by adding toluene:ether (1:5 ml, v:v), vortexing, and freezing the aqueous phase containing the protein in a solid CO_2/ ethanol bath. The organic phase containing the steroids was decanted into a glass tube and evaporated under N_2 to dryness. This glass tube was then washed with 450 µl ether to ensure maximal recovery of steroids, and the contents transferred to a 1.5 ml microfuge tube. The plastic tube was washed with 450 µl ethanol, and the extracted steroids following evaporation were reconstituted in 10 µl ethanol for spotting onto TLC plates.

3.2.3 Production of ^{14}C-3α-Androstanediol

^{14}C-3α-Adiol could not be commercially purchased and was therefore produced in the laboratory from ^{14}C-DHT. Undiluted immature (day 35) rat testis (10,000 g) supernatant was used

to convert ^{14}C-DHT to ^{14}C-3α-Adiol, using a similar assay protocol as that for the 5αR enzyme assay. The components of the assay for ^{14}C-3α-Adiol production included: ^{14}C-DHT (0.5 μCi), 430 μl 0.1 M Tris-citrate buffer (pH 7.0) containing 0.5 mM NADPH, and 100 μl undiluted immature rat testicular (10,000 g) supernatant. The assay was incubated at 37°C for 2 h.

The steroids were extracted as described above (section 3.2.2) and run on a TLC plate alongside non-radioactive DHT and 3α-Adiol. The area on the TLC plate corresponding to the ^{14}C-3α-Adiol region, as indicated by the sample lanes on the plate containing non-radioactive steroids, was cut and eluted with ethanol into a glass tube. This tube was sonicated for 5 min and vortexed. Finally, ^{14}C-3α-Adiol was reconstituted at ~5000 dpm/ 10 μl and its purity checked by TLC analysis.

3.2.4 Thin Layer Chromatography

TLC is a separation technique where component molecules in a sample are transported by a mobile phase (e.g. liquid solvent) over a stationary phase. The stationary phase is an active adsorbent (e.g. silica) spread as a layer on an inert support material Adhesion of a molecule to the adsorbent is dependent on two important properties, its particle size and its homogeneity.

Silica gel is the most commonly used adsorbent in TLC. It is prepared by the hydrolysis of sodium silicate to polysilicic acid, which on further condensation and polymerization yields silica gel material. The ability of an adsorbent to retain analyte species is determined by its chemical structure and properties. In silica gel, for example, there are several different types of active sites and the surface silanol groups are mainly responsible for its adsorptive properties. Substances are adsorbed onto the surface of the silica via hydrogen bonding, with the surface silanol groups serving as the hydrogen donors.

Instant TLC silica-impregnated glass fiber plates (ITLC-SA; 20 cm x 20 cm, Gelman❶) were used to separate testosterone, DHT, and 3α-Adiol, with a solvent system of chloroform❷:methanol❷, according to the procedure of Chase and Payne (1985). The solvent

system was allowed to equilibrate inside the TLC tank (Alltech❶) for 30 min by lining the inside of the tank with chromatography blotting paper (Advantec❶) soaked in solvent. The purpose of the equilibration time was to allow homogeneity of the atmosphere in the tank to minimize evaporation of the solvent from the TLC plate during development. During this equilibration step, the TLC plates were baked at 110°C for 30 min to activate the binding sites on the silica gel, to remove unbound water and improve separation.

Sample lanes with a width of 1.4 cm were marked on the chromatograms with a pencil, so that each chromatogram was subdivided into 14 sample lanes (*Figure 3-1*). The origin was marked 1.5 cm from the bottom of the plate. Samples were applied as spots at the origin using disposable glass pipettes (Drummond Microcaps, Drummond Scientific Company❶). The activated plate was placed at room temperature during spotting, and the samples spotted immediately to prevent exposure to a humid atmosphere. The samples were spotted onto the TLC plate by very lightly touching the plate at the origin under pressurized air to prevent the spots from spreading (the smaller the spot, the better the resolution and the better the separation). Once dried (~5 min) the chromatography plate was placed vertically into the tank with its lower edge immersed in the solvent to a depth of ~0.5 cm, and allowed to develop for 31 min. One plate was developed at a time, and at no time during chromatographic development was the lid of the tank opened.

Once molecules were separated, the components were detected by fluorescence or by chemical developing reagents, by exposing or spraying the vapors of reagents:

- Detection of testosterone: Organic compounds containing a double bond, such as testosterone, appear as dark spots if a TLC plate is irradiated with UV light. Therefore to detect testosterone, the plate was removed from the tank and allowed to air-dry for 5 min, and scanned by irradiating with UV (254 nm) light using a hand-held High Intensity-UV lamp (Model UVGL-58, Ultra-Violet Products, Inc❶). The areas corresponding to testosterone appeared as dark spots under UV light and were outlined with a pencil.
- Detection of DHT: Chemical methods of detection involve the application of a derivatising agent on the TLC plate to reveal coloured spots. For example, when iodine

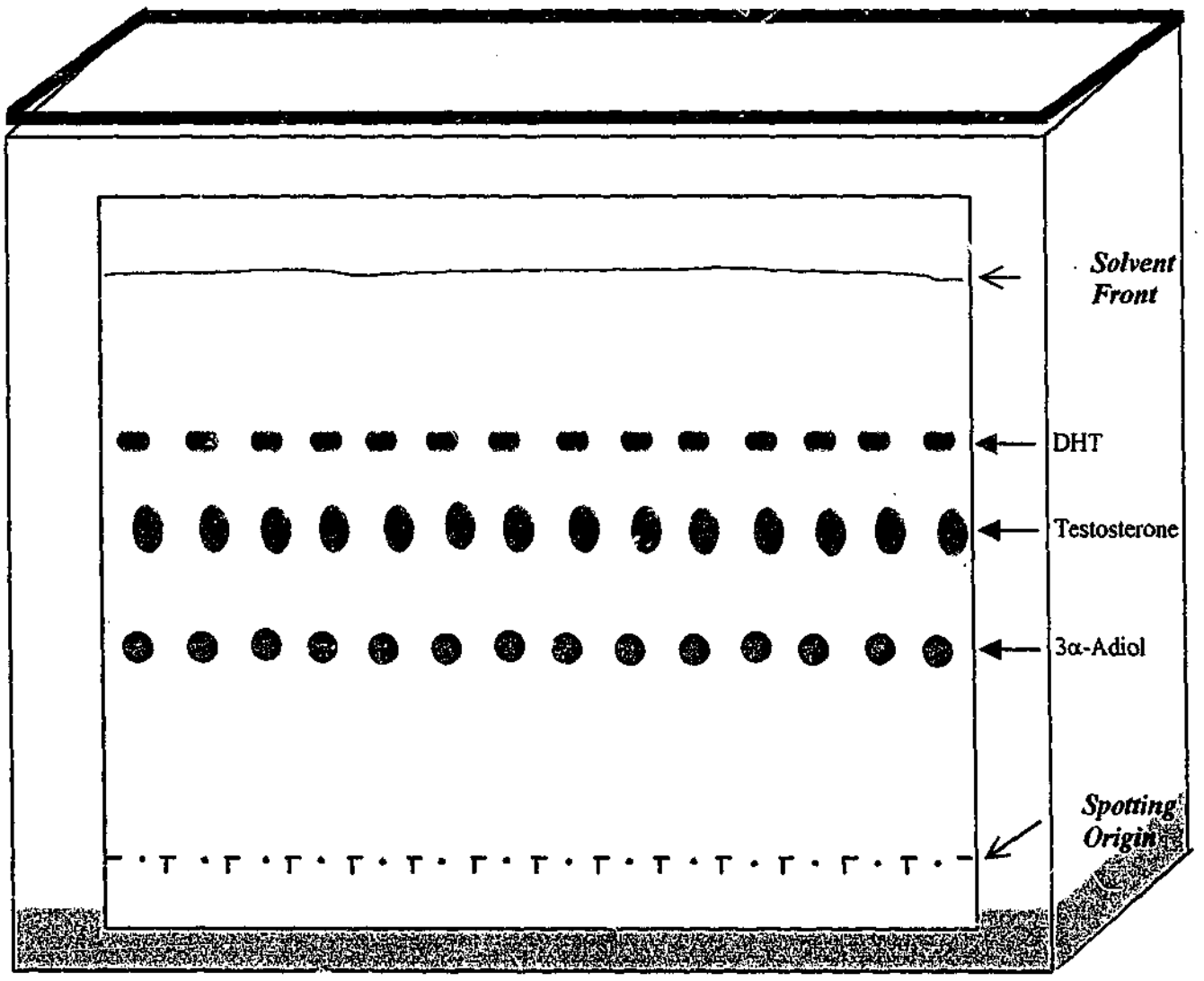


Figure 3-1: Steroid separation by silica-impregnated TLC plates. The spotting origin was marked 1.5 cm from the bottom of the chromatogram, and 14 sample lanes with a width of 1.4 cm were marked on the chromatogram with a pencil. Samples were applied at the spotting origin, and migrated upwards with the running solvent (chloroform:methanol, 98:2) during development for 31 min. The average R_f values for 3α-Adiol, testosterone, DHT were 0.45, 0.6, and 0.72, respectively (n = 15).

vapor is brought into contact with a TLC plate, it will readily dissolve in the solute spots that contain lipids, such as steroids. Therefore to detect DHT, the chromatogram was placed in another TLC tank containing iodine crystals (Sigma❶). The areas corresponding to DHT appeared as brown spots above the testosterone, and were outlined with a pencil. All lipids appeared as yellow spots on a white background, however this reaction was reversible, so the spots were outlined as soon as the plate was removed from the iodine tank.

- Detection of 3α-Adiol: Other derivatising reagents can also be applied to enhance the iodine vapor. For example, iodine spray stains general organic compounds and unsaturated compounds as white spots on TLC plates. To detect 3α-Adiol, the plate was sprayed with Iodine Vapor Spot Enhancer (Alltech❶) with a disposable spray unit (Alltech❶). The areas corresponding to 3α-Adiol, which appeared as a white spot below the elution spot of testosterone, were outlined with a pencil (see *Figure 3-1*).

After separation and detection of non-radioactive steroids, the component molecules were removed by cutting out each spot with a scalpel. The substrate in the 5αR assay (^{3}H-testosterone) and the recovery system (^{14}C-steroids) were assessed by liquid scintillation counting. The radioactivity profiles were determined by transferring each spot to a vial containing 2 ml Emulsifier Safe scintillation fluid (Packard❶), and measuring the radioactivity by dual isotope (i.e. ^{14}C and ^{3}H) liquid scintillation counting for 10 min/ vial using a β-counter (model 2500TR, Packard❶).

3.2.5 Recombinant Rat 5α-Reductase Type 1 and Type 2 Transcripts

The following section describes the development of a method for the measurement of 5αR-2 activity in the presence of 5αR-1. This method corrected for 5αR-1 activity at pH 5.0, to allow the quantitation of 5αR-2 activity at that pH 5.0. This method used recombinant 5αR-1 as a pure

source of 5αR-1 activity to measure the 5αR-1 overlap at pH 5.0. Once the activity at pH 5.0 due to recombinant 5αR-1 was known, specific 5αR-2 activity was calculated using the formula:

5αR-2 Activity = (pH 5.0/ 7.0 recombinant 5αR-1 – pH 5.0/ 7.0 sample) x Activity of sample at pH 7.0

To validate this approach, varying amounts of recombinant 5αR-2 were combined with a constant amount of recombinant 5αR-1, and this method applied to determine the accuracy and validity of this approach.

a) cDNA Template

The full-length rat cDNA encoding the 5αR-1 (GeneBank Accessory Number 206837, Rat S5 alpha) isoform (pB5α-RED1; the Eco R1- Not 1 insert corresponds to nucleotides 1-2470 of Figure 4 of Andersson *et al.*, 1989) and the partial length rat 5αR-2 (GeneBank Accessory Number M95058, Rat S2 5αR) isoform (pT801; the Eco R1 insert corresponds to nucleotides 1-1786 of Figure 1 of Normington and Russell, 1992), were generously provided by Dr. David Russell (Department of Molecular Genetics, Southwestern Medical Center, Dallas, TX). The cDNAs were contained within the pBluescript II SK, a vector that was not suitable for expression studies. It was not possible to use restriction enzymes❷ to cut the required segment of cDNA (i.e. mRNA coding region) out of the pBluescript vectors, and therefore we designed oligonucleotide primers and used the cDNA as a template for PCR.

b) Oligonucleotide Primers

Oligonucleotide primers for the mRNA coding region for 5αR-1 and 5αR-2 were designed using the MacVector 5.0 DNA analysis program (Oxford Molecular❶) and checked for primer dimers, secondary structures, and optimal PCR conditions using two programs, Amplify (University of Wisconsin, Madison, Wisconsin) and Primer Premier (Premier Biosoft❶). The oligonucleotide primers were purchased from Sigma Genosys❶ and were dissolved in H_2O at a concentration of 50 pM.

When designing primers the following aspects were considered:

1) Unique restriction enzyme sites were included in the primers to allow subcloning into the expression vector.
2) Additional base pairs flanking the recognition sites for restriction endonucleases were included to permit efficient cleavage by enzymes.
3) Recognition sites corresponding to the chosen restriction endonucleases were included. The oligonucleotide sequences for these enzymes were **g/aattc** for Eco R1, **c/tcgag** for Xho-1, and **t/ctaga** for Xba-1. These restriction sites were located between the additional base pairs at the 5'-end of the primer and the terminus of the fragment.

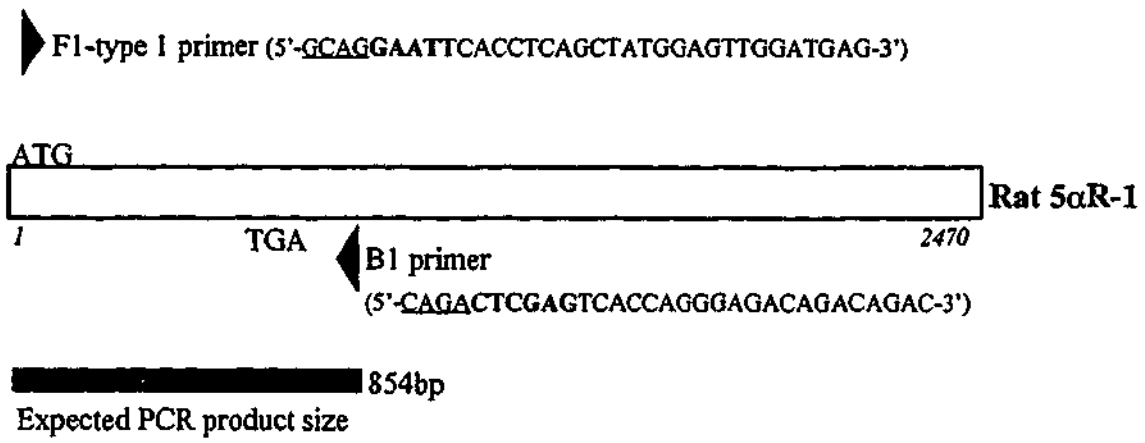
The forward and reverse primers designed for subcloning recombinant 5αR-1 and 5αR-2 are shown in *Figure 3-2*. The expected size of recombinant 5αR-1 and 5αR-2 was predicted to be 854 bp and 841 bp, respectively.

c) Vector Digestion

The 5.4 kb pcDNA3.1(+) vector was designed for high-level transient expression in mammalian cells with expression of the inserted gene controlled by the CMV promotor. In addition, the vector replicates episomally in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS-7).

The mRNA coding region for 5αR-1 and 5αR-2 was inserted into pcDNA3.1(+) expression vector, after the pcDNA3.1(+) vector was digested with the appropriate restriction enzymes (Eco R1 and Xho-1 for 5αR-1 and Eco R1 and Xba-1 for 5αR-2, Boehringer and Mannheim❶) to allow ligation of the corresponding cDNAs. The following components were added in order for vector digestion (5 µg pcDNA3.1(+) vector, 41 µl sterile H_2O, 1 x buffer H [5 µl; SuRE/ CUT Buffers, Boehringer and Mannheim❶], and 5 U restriction enzymes). This mixture was incubated overnight at 37°C. The digested vector was run on a 0.5% agarose gel with ethidium bromide and isolated using the QIAEX II gel extraction Kit (see section 2.4.5).

(A)



(B)

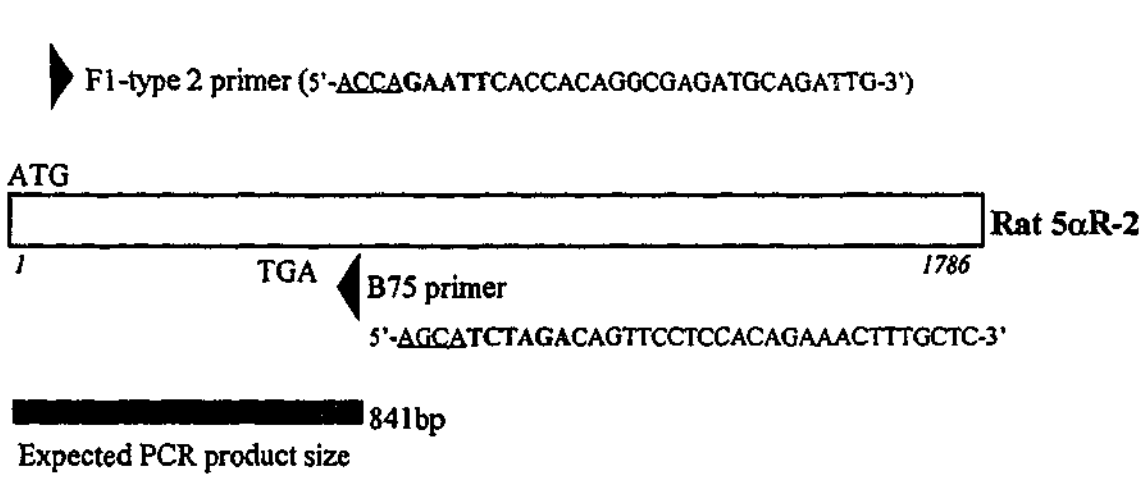


Figure 3-2: Oligonucleotide primers designed to recognize the mRNA coding region for rat 5α-reductase *(A)* Type 1 (5αR-1) and *(B)* Type 2 (5αR-2). The expected PCR product sizes were 854 bp and 841 bp, respectively. The sequence are shown in normal text, additional flanking bases are shown underlined, and recognition sites are shown in bold.

d) Polymerase Chain Reaction

For high-fidelity PCR amplification, the proofreading DNA polymerase *Pfu* (Stratagene❶) was used to amplify the rat recombinant 5αR isoforms. *Pfu*, derived from the hyperthermophilic archae *Pyrococcus furiosus*, was chosen as the DNA polymerase because: (a) it possessed 3' to 5' exonuclease proofreading activity that enabled it to correct nucleotide-misincorporation errors, (b) it is one of the most thermostable DNA polymerases, and (c) it has the lowest error rate of any polymerase (Cline *et al.*, 1996).

Plasmids containing 5αR cDNAs were diluted to a final concentration of 100 ng/ µl, and were used as templates for the following PCR reaction. The PCR reaction was performed in a sterile 0.6 ml PCR tube (Interpath Services Pty Ltd❶) using the RoboCycler Gradient 40 PCR Thermal Cycler (Stratagene❶), and contained: 10 x (10µl) *Pfu* polymerase buffer, 200 mM dNTP's, 75 pmol specific forward and reverse primer, 100 ng target DNA (template), made up to 99 µl with sterile H_2O. This mixture was centrifuged and heated to 95°C for 5 min, and then chilled to 45°C for 5 min. *Pfu* polymerase (2.5 U, 1 µl) was added, and the reaction heated at 72°C for 2 min. The mixture was briefly centrifuged and overlayed with 50 µl paraffin oil. To amplify the target DNA, 30 PCR cycles were performed [1 min at 95°C (denaturation), 1 min at 50°C (annealing), and 1 min at 72°C (extension)]. At the end of the cycles the reaction was chilled to 4°C.

The paraffin oil was removed by freezing the PCR reaction to -70°C on dry ice, and aspirating the oil. The PCR products were ethanol precipitated (see section 2.6.2) and then digested with restriction enzymes by adding the following components in order to the PCR pellet: 26 µl sterile H_2O, 1 x buffer H, 5 U relevant restriction enzymes. This was incubated overnight at 37°C. The digested PCR products were purified using the High Pure PCR Product Purification Kit (see section 2.4.6).

e) Ligation Reaction

Recombinant rat 5αR isoforms were ligated into the pcDNA3.1(+) vector using the Rapid DNA Ligation Kit (Boehringer and Mannheim❶). Briefly, plasmids (pcDNA3.1(+) with 5αR insert) were generated by the incubation of: PCR product (10 µl), digested pcDNA3.1(+) vector (0.5 µl), and 1 x DNA dilution buffer (2.1 µl). After thorough mixing, equal volumes T4 DNA ligation buffer (10.6 µl) were added, mixed, and T4 DNA ligase❷ (1 µl) was then added. The ligations were allowed to proceed at 30°C for 30 min, followed by an overnight incubation at 4°C. For control ligations, sterile H_2O replaced the recombinant plasmid.

f) Transformation

After 24 h at 4°C, the ligation reaction was brought to room temperature for 30 min prior to transformation into DH5α Competent Cells (Subcloning Efficiency, Gibco❶). For transformation, 5 µl ligation reaction was mixed into 50 µl DH5α cells and incubated on ice for 5 min. The remaining ligation reaction was stored at -20°C. Cells were incubated on ice for 30 min, heat-shocked for 20 sec at 37°C (not shaken), and placed on ice for 2 min. Cells were then mixed with 300 µl Luria-Bertaini (LB❷) broth and incubated at 225 rpm for 1 h at 37°C for expression. The transformation reaction was spread onto LB agar❷ plates supplemented with 100 µg/ ml ampicillin❷, and incubated overnight at 37°C.

g) Plasmid Extraction

Single colonies of transformed bacteria were seeded into 5 ml LB broth in the presence of ampicillin (100 µg/ ml) and incubated at 37°C overnight with constant agitation (225 rpm) in an orbital shaker. Bacterial clones were stored as glycerol stocks (50% LB broth/ 50% glycerol) at -70°C. The recombinant DNA from the bacterial cultures was purified using the Quantum Prep Plasmid Miniprep kit (see section 2.4.7). Plasmid concentrations were measured using a spectrophotometer.

To check for correct ligation, 1 μg DNA digested with the appropriate restriction enzymes [(1 μg purified plasmid DNA, 10 U/ μl relevant restriction enzymes, 2 μl 10 x Buffer, to a total volume of 20 μl with sterile H_2O, and incubated for 1 h at 37C°)]. The digest was run on a 1.5% agarose gel containing ethidium bromide (i.e. two bands on the gel were visualized, one band corresponding to the pcDNA3.1(+) digested vector and the other to the 5αR PCR insert (~854 bp for 5αR-1 and ~841 bp for 5αR-2).

To sequence the entire length of the recombinant 5αR isoforms, the T7 site on the pcDNA3.1(+) vector was used to prime from the 5'-end. To prime from the 3'-end, an oligonucleotide primer corresponding to the Bovine Growth Hormone (BGH) reverse priming site was constructed, and had the following sequence: 5'-TAG AAG GCA CAG TCG AGG C-3'.

After sequence confirmation, regrowth of stocks in 200 ml LB broth in the presence of 100 μg/μl ampicillin was achieved by scraping the frozen glycerol stock with a sterile inoculation loop, streaking onto LB ampicillin plates and incubating at 37°C overnight in an orbital shaker at 225 rpm. The Concert High Purity Maxiprep Plasmid Purification System was used to purify the plasmid DNA (see section 2.4.8). Some purified plasmid DNA was digested as previously and visualized on a 1.3% agarose gel to confirm the correct identity of the plasmids.

3.2.6 Transient Expression of Recombinant Rat 5α-Reductase Isoforms

Two different transfection procedures were used, one for the transient expression of 5αR-1 and the other for 5αR-2.

Transient Transfection with FUGENE (5α-Reductase Type 1)

FuGENE-6™ (Promega❶) was used to transiently express recombinant rat 5αR-1 into COS-7 cells. FuGENE is a blend of lipids (non-liposomal) and other components that produces high levels of transfection with high efficiency (50 - 70%) and minimal damage (yields >90% viable cells) in mammalian cell lines. The FuGENE and DNA form a simple complex when mixed in

media without fetal calf serum (media^{-FCS}), and can then be added directly to COS-7 cells that are cultured in either media^{-FCS} or media containing fetal calf serum (media^{+FCS}). According to the manufacturer's instructions, FuGENE was added to media^{-FCS}. Each DNA preparation was prepared in a 5 ml polystyrene tube on ice, and the protocol for transient transfection of recombinant 5αR-1 in COS-7 cells with FuGENE is described below:

a) On the day before transfection, cells were plated in 175 cm^2 flasks with 50 ml media^{+FCS} without antibiotics, to achieve 50 - 70% confluency for transfection the following day.
b) A FuGENE:recombinant 5αR-1 DNA ratio of 3:1 was used to transfect recombinant 5αR-1 into COS-7 cells. To transfect using 175 cm^2 flasks, 45 µg FuGENE, 15 µg recombinant 5αR-1, and media^{-FCS} (made up to 500 µl with media^{-FCS}) was mixed and incubated at room temperature for 5 min.
c) In separate tubes, 15 µg control plasmid [pcDNA3.1(+)] or recombinant plasmid [pcDNA3.1(+)/ r5αR1] or reporter gene [pCMV, to check for transfection efficiency] were added.
d) The FuGENE:DNA mix was added to the DNA, and incubated for 15 min at room temperature. Meanwhile, the media^{+FCS} was removed from the confluent COS-7, the cells rinsed with warm PBS and 20 ml media^{-FCS} added to the cells to be transfected.
e) The 500 µl mixture was added to the flask, gently shaken and incubated in a $C0_2$ incubator at 37°C. The media^{-FCS} was removed the next day and 50 ml media^{+FCS} added.
f) Cells were harvested 4 days after transfection with FuGENE. The media was aspirated, cells were washed twice with PBS and once with 0.25 M sucrose. Then, 8 ml 0.25 M sucrose was added and cells were scraped from the flask with a rubber policeman, resuspended and snap frozen. Harvested cells were thawed and homogenized before assaying for 5αR activity.

Measuring Efficiency of Transfection by β-Galactosidase Activity (5α-Reductase Type 1)

The expression vector pcDNA3.1(+) expresses E.coli β-Galactosidase (β-Gal) in mammalian cells, and can be used with a histochemical stain to assess the efficiency of transient transfections. Therefore, detection of β-Gal activity in situ in cultured cells was performed using the following protocol:

a) The media was aspirated and cells were washed twice with PBS. Cells were fixed with 2% gluteraldehyde/ 2% paraformaldehyde fixative. The fixative contained sodium phosphate [buffers the pH of the system and provides sodium ions], an activator of the β-Gal enzyme, the chromatographic indicator [5-bromo-4-chloro-3-indoyl-β-D-galactoside, or X-Gal], and potassium ferrocyanide and potassium ferricyanide [together these act as an oxidation catalyst to increase the rate of conversion of the soluble indoxyl molecules to the insoluble indigo form to enhance localization of the blue color]. Cells were fixed for 5 min at 4°C, and then the fixative was removed and the cells washed twice with PBS. The fixed cells were overlayed with the X-Gal substrate (substrate contained 385 µl 1 M Na_2HPO_4, 115 µl 1 M NaH_2PO_4, 6.5 µl 1 M $MgCl_2$, 100 µl X-Gal stock, 300 µl 50 mM potassium ferricyanide-$Ke_3Fe(CN)_6$, 300 µl 50 mM potassium ferricyanide-$Ke_4Fe(CN)_6$, and 3.79 ml H_2O).

b) The β-Gal was allowed to hydrolyze the substrate to generate galactose and soluble indoxyl molecules, which was converted to insoluble indigo during a 30 min incubation. The deep blue color generated by the hydrolysis of X-gal by β-Gal facilitated cellular localization of the β-Gal enzyme. The proportion of COS-7 cells that expressed the reporter gene protein was analyzed with a low power microscope to determine the efficiency of transfection.

Figure 3-3 shows the efficiency of transfection of recombinant 5αR-1 in COS-7 cells with FuGENE assessed by β-Gal activity. The abundance and intensity of β-Gal staining [*Figure 3-3(B)*] demonstrated that there was a high efficiency of transfection compared to non-transfected control cells [*(Figure 3-3(A)*].

(A)

(B

Figure 3-3: Transfected COS-7 cells stained for β-Galoctosidase activity. *(A)* Non-transfected COS-7 cells and *(B)* COS-7 cells after 4 days of transfection with fugene (x 4). Cells were fixed with 2% gluteralhyde/ 2% paraformaldehyde fixative for 5 min at 4°C, and then overlayed with X-Gal substrate for 30 min at 37°C.

Transient Transfection with Lipofectamine 2000 (5α-Reductase Type 2)

The transfection reagent Lipofectamine™ 2000 (Gibco❶) was used to transiently express 5αR-2 into COS-7 cells. Lipofectamine was used rather than FuGENE because it was a faster method (cells were harvested 2 days after transfection rather than 4 days). Lipofectamine is a cationic lipid reagent that produces rapid and high transfection efficiency in many cell types, including COS-7 cells. The efficiency of transfection was measured by fluorescence microscopy. The protocol for the transient expression of recombinant 5αR-2 with Lipofectamine 2000 is described below:

a) Cells were plated in 100 mm^2 dishes with 10 ml $media^{+FCS}$ without antibiotics, to achieve ~90% confluency the following day for transfection.

b) For each dish of cells to be transfected, 6 μg DNA (pcDNA3.1(+)/ r5αR2) was added with 4 μg pAdvantage (used to prevent inhibition of translation initiation; see below) and made up to 100 μl with $media^{-FCS}$. In separate tubes, 12 μg Lipofectamine was made up to 100 μl with $media^{-FCS}$ and incubated for 5 min at room temperature. The diluted Lipofectamine reagent was combined with the diluted DNA/ pAdvantage mixture and incubated for 20 min at room temperature to allow the DNA-Lipofectamine complexes to form.

c) The DNA-Lipofectamine complexes (200 μl) were added directly to each dish and incubated in a CO_2 incubator at 37°C for 24 - 48 h. The $media^{-FCS}$ was replaced the day after transfection with $media^{+FCS}$ if required. To harvest cells, the media was aspirated and cells were rinse twice with PBS and once with 0.25 M sucrose. The cells were resuspended in 1 ml 0.25 M sucrose and the cells scraped from the surface of the dish with a rubber policeman and snap frozen. Cells were thawed and homogenized before assaying for 5αR activity.

Measuring Efficiency of Transfection by pEGFP-2 Fluorescence (5α-Reductase Type 2)

The bacterial expression vector that encodes Green Fluorescent Protein (GFP) from the jellyfish, *Aequorea Victoria*, is a reporter molecule for monitoring the efficiency of gene

expression. These vectors are intended as a source of enhanced fluorescent protein coding sequences, so that GFP fluoresces bright green upon exposure to UV or blue light. The GFP variant, pEGFP-2 (Clontech❶) operates optimally for fluorescence microscopy (Cormack *et al.*, 1996). This GFP variant encodes a protein which has a single, red-shifted excitation peak that fluoresces ~ 35 times more intensely than wildtype GFP. The coding region of pEGFP-2 contains more than 190 silent base mutations, which correspond to human codon-usage preferences to ensure maximal mammalian expression. The increased expression of pEGFP-2 makes it ideal for fluorescence microscopy, and pEGFP-2 expression may be detected 24 to 72 h post-transfection. Fluorescing cells in culture dishes were directly observed with an inverted fluorescence microscope.

3.2.7 Calculating Enzyme Kinetics for Recombinant 5α-Reductase Isoforms

Human and rat 5αR isoforms display different affinities for several steroid substrates; 5αR-1 exhibits a substrate affinity in the micromolar range for testosterone (Andersson and Russell, 1990; Normington and Russell, 1992; Thigpen *et al.*, 1993a; Span *et al.*, 1995), whereas 5αR-2 exhibits a nanomolar affinity for testosterone (Faller *et al.*, 1993; Thigpen *et al.*, 1993a; Span *et al.*, 1995). Although 5αR-1 has a broad neutral pH optima and 5αR-2 has a sharp peak of activity at pH 5.0, there is now evidence that both 5αR isoforms operate at neutral pH (Thigpen *et al.*, 1993a). Therefore, by incubating tissue with a wide range of substrate concentrations, it is possible to measure the activity of both 5αR isoforms simultaneously.

The normal procedure for enzyme kinetic studies is to incubate a sample with a single substrate concentration. Velocity, however, depends strongly on substrate concentration. From the Michaelis-Menten equation: $v = (V_{max}[S]) / (K_m + [S])$ where [S] in the concentration, follows: when $S >> K_m$, a pH-profile would indicate V_{max} vs pH, and when $S << K_m$: $v = (V_{max})/ (K_m)[S]$. The velocity found is then proportional to V_{max}/K_m, and this ratio is also called the efficiency ratio. For the use of the V_{max}/K_m ratio as an index of potential enzyme activity one has to consider that

endogenous testosterone concentrations in most tissues are much lower than the K_m of either 5αR subtype (Krieg *et al.*, 1983). Therefore, applying K_m>>[S] in the Michealis-Menten equation gives $v = V_{max}/K_m[S]$. So at physiological testosterone concentrations, the enzyme reaction velocity is proportional to V_{max}/K_m. Unlike the classical pH optimum, the efficiency ratio V_{max}/K_m takes into account the substrate dependency of velocity, and reflects the potential *in vivo* velocity of DHT formation from testosterone (Span *et al.*, 1995, 1996a, b, c).

We performed enzyme kinetic experiments for the recombinant 5αR isoforms, to compare with previously published values and with the enzyme kinetic characteristics for the testis (see section 5.3). COS-7 cells transfected with either 5αR-1 and 5αR-2 expression plasmids were incubated with a wide range of substrate concentrations (1.9 μM to 9.5 μM testosterone) under standard enzyme kinetic conditions at pH 7.0 for 60 min. Velocities were plotted against testosterone concentration, and V_{max} and K_m values were calculated using a non-linear regression procedure based on the Michaelis-Menten equation for two isoform activities, with the computer program Graphpad Prism (Graphpad Software, Inc❷).

A Lineweaver-Burke plot is a double reciprocal plot of initial velocity against substrate concentration, and can be used to indicate multiple isoforms. However, the Eadie-Scatchard plot of velocity over substrate concentration (V/S) against velocity (V) gives a more equal weighting of points (Segel, 1975). Therefore, a non-linear Eadie-Scatchard plot indicates the presence of multiple isoforms. The abscissa intercepts on the Eadie-Scatchard plot give the respective V_{max} of the isoforms, while the slope indicates $-K_m^{-1}$. The Eadie Scatchard plots were used to graphically indicate the presence of multiple 5αR isoforms, in addition to the Michaelis-Menten equation for two isoforms, used by the computer package GraphPad Prism for the detection of multiple isoforms. The efficiency ratio V_{max}/K_m was calculated as an index of potential *in vivo* isoform activity.

3.3 Results

3.3.1 Separation of Androgens by Thin Layer Chromatography

Solvent System

A solvent system that was suited to separate the androgens of interest was investigated. The separation of testosterone, DHT and 3α-Adiol by TLC analysis, using a dual solvent system of chloroform:methanol at different ratios is shown in *Figures 3-4*. Initial experiments used chloroform:methanol (90:10) as outlined by the protocol of Murono and Payne (1979). However, all three steroids migrated too far up the plate with no clear separation of any of the three steroids [*Figure 3-4 (A)]*.

Methanol is a more polar solvent than chloroform, and therefore higher concentrations of methanol in a dual solvent system containing chloroform would carry component compounds further up the TLC chromatogram. Too much of a polar solvent like methanol would carry all components to the same area of the TLC plate, with poor separation. Therefore, the amount of methanol was decreased to chloroform:methanol (95:5) to slow migration, however there was insufficient separation between all steroids [*Figure 3-4 (B)]*.

A chlorofrom:methanol ratio of 98:2 [*Figure 3-4 (C)]* resulted in a good separation of all three steroids. This solvent system yielded average (n = 15) R_f ratios of 0.45, 0.6, and 0.72 for 3α-Adiol, testosterone, and DHT, respectively. Increasing the methanol concentration even further (99:1) [*Figure 3-4 (D)]* did not improve the separation between the steroids due to slow migration and poor separation of 3α-Adiol and testosterone. Therefore, a solvent system of chloroform:methanol (98:2) was used in all subsequent TLC procedures.

Once the ratio of solvents in the eluting mixture was established (i.e. chlorofom:methanol, 98:2, v:v), an experiment was conducted to confirm that the ^{14}C-, ^{3}H-, and non-radioactive steroids for testosterone, DHT and 3α-Adiol co-eluted in the same areas on the TLC plate. This was important because in all the experiments mentioned above, each TLC sample lane was cut into strips. Thus for each sample, nineteen 0.5 ml strips were cut and counted, which was a time-

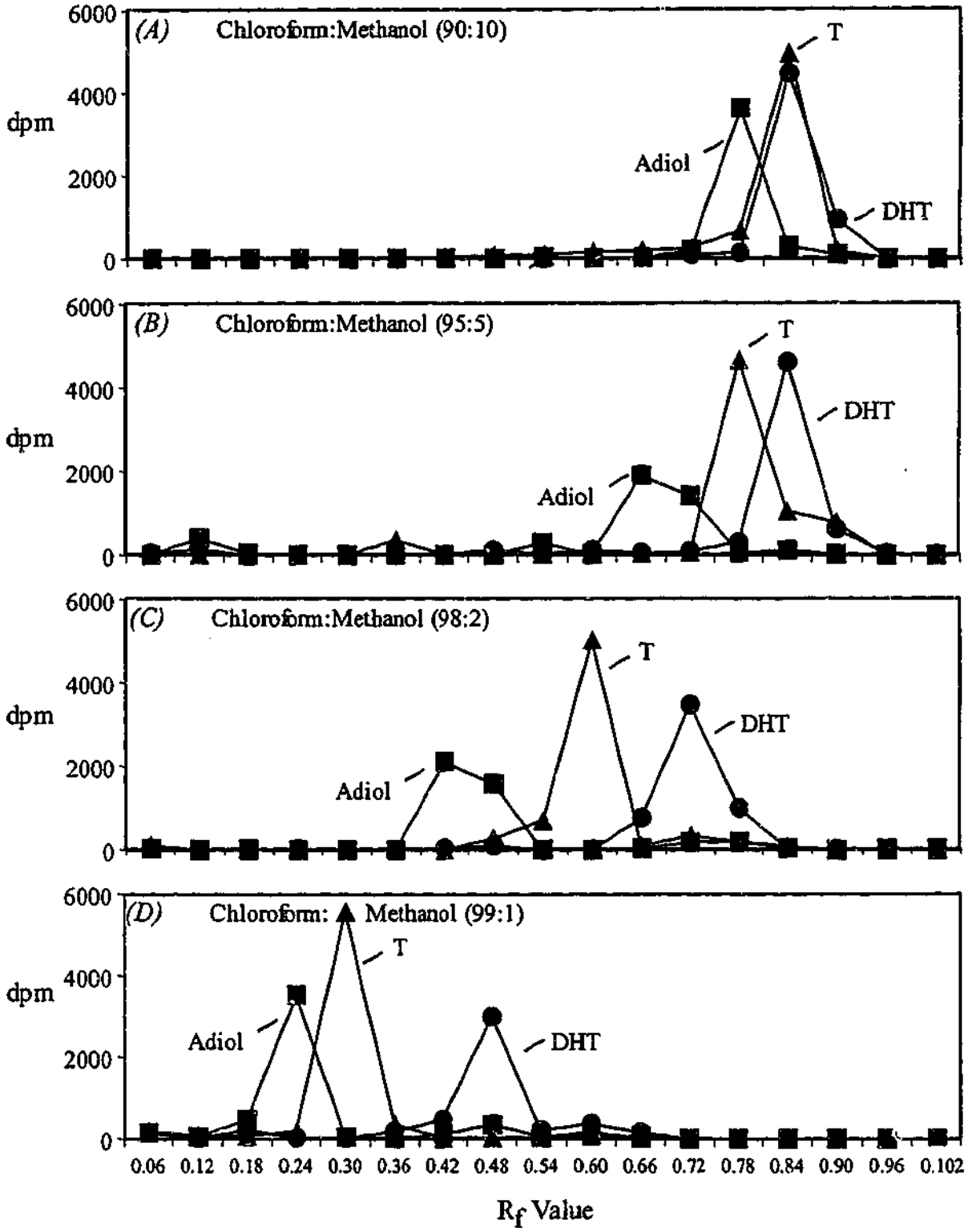


Figure 3-4: Typical elution profile of ^{3}H-testosterone, ^{3}H-DHT and ^{3}H-3α-Adiol by TLC, with different ratios of the component solvent system, chloroform:methanol at *(A)* 98:2, *(B)* 95:5, *(C)* 98:2, and *(D)* 99:1. The plate was cut into 0.5 cm strips and the R_f value for each strip is indicated on the x-axis. The radioactivity of each strip was determined by β-counting, and is presented on the y-axis as disintegrations per minute (dpm).

consuming procedure. Therefore, if non-radioactive steroids and iodine-identification techniques could be used to determine the specific location of each of the steroids of interest, then only three vials instead of seventeen would be required to be analyzed by β-counting.

To test this, samples containing a mixture of ^{14}C-, ^{3}H-, and non-radioactive 3α-Adiol [*(Figure 3-5 (A)*], testosterone [*(Figure 3-5 (B)*], or DHT [*(Figure 3-5 (C)*] were analyzed by TLC. *Figures 3-5* clearly demonstrate that all forms of each steroid co-eluted in the same area with identical R_f values. Therefore this steroid identification procedure was subsequently used instead of counting the entire lane for each sample. In addition, this experiment shows that there is no radioactivity on any part of the plate, other than the areas corresponding to the three steroids of interest.

Purification of ^{3}H-Testosterone

Commercially available ^{3}H-testosterone showed several impurities when analyzed by TLC, which potentially would increase background counts in the 5αR assays. Therefore, ^{3}H-testosterone was purified by TLC before use in the 5αR assays. Briefly, approximately 100 μCi ^{3}H-testosterone was spotted on a TLC plate. Non-radioactive and ^{14}C-testosterone were applied in adjacent sample lanes. The plate was developed for 31 min with chloroform:methanol (98:2), and the area of the plate corresponding to testosterone (as indicated by ^{14}C- and non-radioactive testosterone) cut out and the radioactivity eluted with 100% ethanol into a glass tube. The tube was sonicated, and diluted to ~5000 dpm ^{3}H-testosterone/ 10 μl ethanol. An aliquot of purified ^{3}H-testosterone, with ^{14}C-testosterone and non-radioactive testosterone, was run on a TLC plate to check its purity. Purified ^{3}H-testosterone contained minimal (<0.1%) contamination (see *Appendix A*) and thus provided low backgrounds for the 5αR assay.

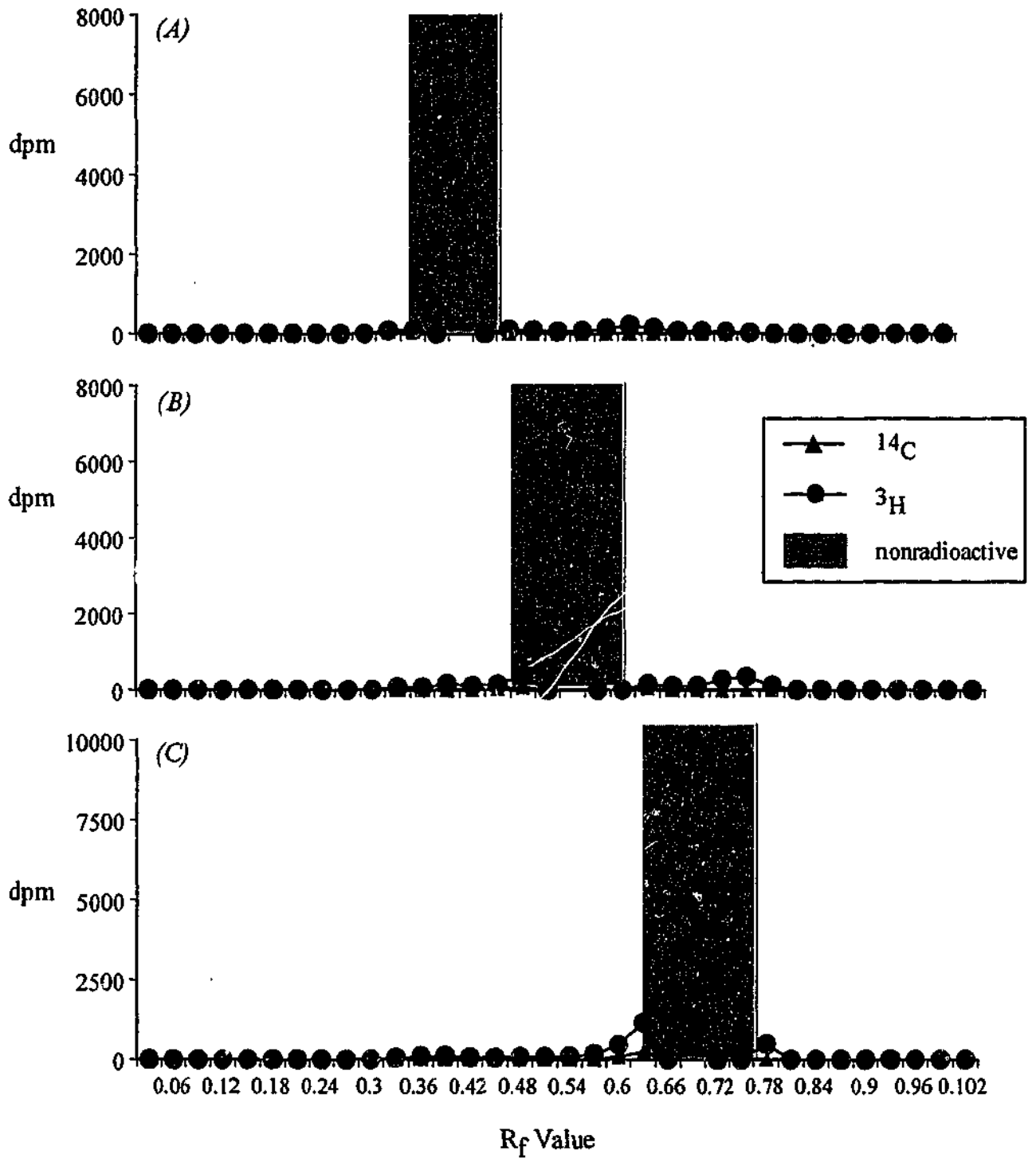


Figure 3-5: Co-elution of ^{3}H-, ^{14}C-, and nonradioactive (2.5 mg/ ml) *(A)* 3α-Adiol, *(B)* testosterone, and *(C)* DHT, following separation by TLC analysis with chloroform:methanol (98:2) as the running solvent.

Purity of ^{14}C-3α-Androstanediol

Figure 3-6 shows an aliquot of ^{14}C-3α-Adiol produced from ^{14}C-DHT, as well as ^{3}H- and non-radioactive 3α-Adiol. The co-elution of all three forms of 3α-Adiol shows the authenticity of the ^{14}C-3α-Adiol, and furthermore that there was no ^{14}C-DHT in the ^{14}C-3α-Adiol preparation. Therefore, pure ^{14}C-3α-Adiol was produced via conversion of ^{14}C-DHT relatively easily, and was required to determine the recoveries for 3α-Adiol losses during steroid extraction and TLC analysis. Greater than 85% of the ^{14}C-DHT incubated in this manner was converted to ^{14}C-3α-Adiol (n = 5).

3.3.2 Assay for Measuring 5α-Reductase Type 1 Activity

Background, Quality Control, and Detection Limits

The average (n = 15) background for the *in vitro* 5αR enzyme activity assay was 0.0102± 0.0036 pmoles (DHT+3α-Adiol)/ min (mean ± SD), which corresponded to 0.12% conversion of ^{3}H-testosterone. Thus, when 230,000 dpm ^{3}H-testosterone was added into the 5αR enzyme activity assay, approximately 276 dpm ^{3}H was recovered from the TLC plate in the areas where DHT and 3α-Adiol were eluted. Detection limits were set so that any value below two standard deviations above the average background (0.12 + [2*0.12] = 0.36%) would not be accepted as detectable (i.e. any value with a conversion of <0.36% was classified as non-detectable).

The within-assay variation was assessed for the average CV for sample replicates, and was 4% (n = 56). The between-assay variation was assessed from the reproducibility of measurement of a testicular extract which was included as a QC in every assay and was 3.6 ± 0.2% (mean ± SD, n = 14).

The average recovery of steroids from samples following the 5αR assay and during steroid extraction and TLC, as monitored by the ^{14}C-steroids, were 47.2 ± 5.1% for testosterone, 47.3 ± 5.0% for DHT, and 51.6 ± 4.7% for 3α-Adiol.

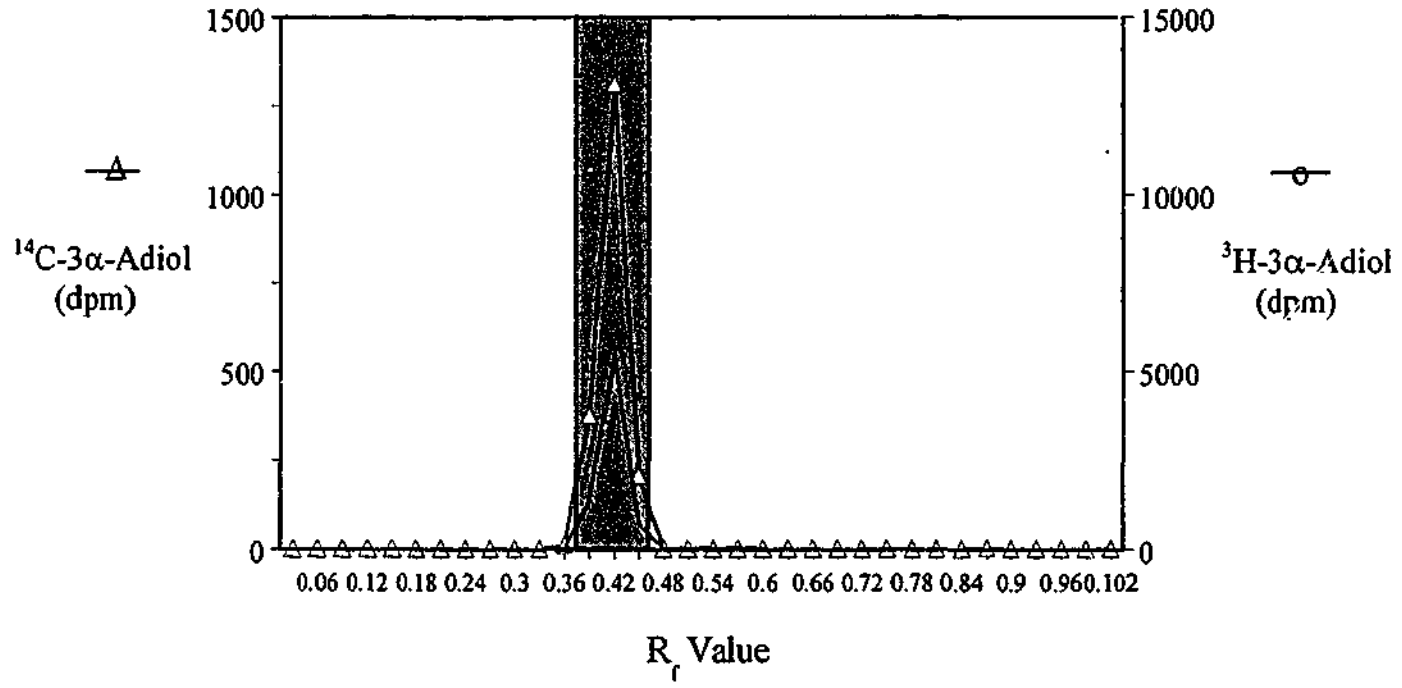


Figure 3-6: Elution profile of purified ^{14}C-3α-Adiol. ^{14}C-3α-Adiol was made by incubating 0.5 µCi ^{14}C-DHT with 100 µl immature (day 30) rat testicular (10,000 g) supernatant for 2 h at 37°C. After steroid extraction, the residue was spotted in TLC plates, and in separate lanes ^{3}H- and non-radioactive 3α-Adiol were also spotted. Non-radioactive 3α-Adiol (shown in the grey bar) and ^{3}H-3α-Adiol were eluted at the same location on the plate as ^{14}C-3α-Adiol. The corresponding area for ^{14}C-3α-Adiol was cut out and the radioactivity eluted.

3.3.3 Assay for Measuring 5α–Reductase Type 2 Activity

Expression of Rat Recombinant 5α–Reductase Type 1

Expression of the pcDNA3.1(+) vector alone (i.e. control) in COS-7 cells produced no 5αR activity at pH 5.0 or 7.0, whereas expression of recombinant rat 5αR-1 (pcDNA3.1(+)/ 5αR-1) yielded high activity at both pH 5.0 and 7.0 (*Table 1*). The activity for recombinant rat 5αR-1 was significantly higher at pH 7.0 compared to pH 5.0 (283 vs 35.3 fmoles DHT+3α-Adiol/ min/ mg protein, respectively). The pH 5.0/ 7.0 ratio for recombinant rat 5αR-1 reflected the degree of 5αR-1 overlap at pH 5.0, and was 0.124 ± 0.014 (mean ± SD, n = 14). Therefore, 12.4% of the activity at pH 5.0 was due to recombinant 5αR-1 activity.

Table 1: Recombinant rat 5α-reductase Type 1 (5αR-1) enzyme activity at pH 5.0 and 7.0.

Expression Plasmid	Recombinant 5αR-1 Activity (fmoles DHT+3α-Adiol/ min/ mg protein)		
	pH 5.0	pH 7.0	pH 5.0/ 7.0 ratio
pcDNA3.1(+)	0.2	0	
pcDNA3.1(+)/ 5αR-1	35.3 ± 10.2	283 ± 56.3	0.124 ± 0.014

Values are mean ± SD from five separate transfections and a total of 14 different measurements.

* Denotes at the detection limits of the assay (~0.36% conversion of substrate).

Expression of Rat Recombinant 5α–Reductase Type 2

COS-7 cells were initially transfected with 5αR-2 plasmid using the transfection procedure that was used for 5αR-1. However, 5αR-2 expression plasmid showed minimal activity at pH 5.0 and was similar to background levels (*Table* 2: 0.38 ± 0.15%, compared with >15% for recombinant 5αR-1 at pH 7.0). We decided to optimize expression of 5αR-2 activity by changing different transfection variables. Numerous experiments were required to optimize the transfection

protocol for recombinant 5αR-2 with Lipofectamine, and the results of these experiments are represented in *Table 2*.

Table 2: Optimization of transient expression of recombinant rat 5αR-2. 5αR-2 activity was determined at pH 5.0 by assaying for 1 h at 37C°. All transfections were performed in 100 mm^2 dishes, cells were resuspended in 1 ml 0.25 M sucrose and homogenized, and 100 µl taken for measuring 5αR-2 activity. Results are expressed as percent conversion of ^{3}H- testosterone to ^{3}H-DHT and ^{3}H-3α-Adiol.

Duration (hours)	Transfection Reagent (TR)	TR:DNA	Media (+/- FCS)	Cell Type	5αR-2 activity (%)
96	FuGENE	3:1	- FCS	COS-7	0.3±0.15
48	Lipofectamine	2:1	- FCS	COS-7	0.4±0.1
48	Lipofectamine	2:1	+ FCS	COS-7	1.25±0.22
48	Lipofectamine	2:1	+ FCS	COS-7	2.36
48	Lipofectamine	2:1	- FCS	COS-7	2.35*
48	Lipofectamine	2:1	- FCS	CHO	0.6±0.1
48	Lipofectamine + pAdvantage	2:1	- FCS	COS-7	10.2 (100 µl) 5 (50 µl) 2.1 (25 µl)

* denotes recombinant 5αR-2 sub-cloned from epididymal RNA.

Initial experiments demonstrated that the majority of cells did not survive after transfection with Lipofectamine if they were split to achieve ~70% confluency on the day of transfection, as was done previously with FuGENE. Therefore, to prevent cell death after transfection with Lipofectamine, cells were split to achieve >90% confluency on the day of transfection. This allowed cells to be harvested 2 days after transfection, as compared with 4 days for FuGENE.

The efficiency of transfection was critically dependent on the ratio of Lipofectamine to expression plasmid DNA. The ratio of Lipofectamine:recombinant 5αR-2 DNA was optimized for

the expression of recombinant 5αR-2 in COS-7 cells. Initial experiments investigated the effect of varying concentrations of Lipofectamine as recommended by the suppliers. Lipofectamine: recombinant 5αR-2 (3:1) caused major cell death after transfection, so that less than 30 - 50% of cells survived the transfection procedure (*Table 2*; 0.44 ± 0.11%). However, the majority of cells survived with Lipofectamine:recombinant 5αR-2 (2:1) to increase 5αR-2 activity by 3-fold (1.25 ± 0.22%, *Table 2*). A further 2-fold increase in 5αR-2 activity was obtained when cells were incubated with media^{-FCS} for the first day of transfection, compared to when cells were transfected with media^{+FCS} (2.66 vs 1.36%, respectively; *Table 2*).

We also used epididymal cDNA to subclone recombinant rat 5αR-2. This plasmid was constructed to contain a Kozak consensus region to increase translation efficiency. However, there was no improvement in the amount of 5αR-2 activity (2.35%, *Table 2*) using epididymal cDNA. Furthermore, there was no increase in 5αR-2 activity when other cell types were used to transfect recombinant 5αR-2 (2.42% with CHO cells and 0.34 ± 0.11% for 293 cells).

It has been reported (Farrell *et al.*, 1977) that mammalian cells with an expression vector can result in suboptimal expression of proteins, due to an increased amount of double-stranded RNA (dsRNA) in the cells after transfection (*Figure 3-7*). The dsRNA activates the dsRNA-activated inhibitor (DAI, a host cell's antiviral defense system), and activated DAI phosphorylates the translation initiation factor eIF-2, halting translation and therefore protein production (Farrell *et al.*, 1977). The ability of dsRNA to block gene expression is termed RNA interference (RNAi).

Inhibition of translation initiation can be prevented by co-transfecting with the pAdvantage™ Vector (Promega❶, which contains the VAI RNA genes from the adenovirus type 2 genome. Following co-transfection with the pAdvantage, the VAI RNA that is produced by RNA polymerase III binds to DAI, prevents its activation and allows for uninhibited translation initiation and increased protein expression (Kitajewski et al., 1986; O'Malley et al., 1986). Thus,

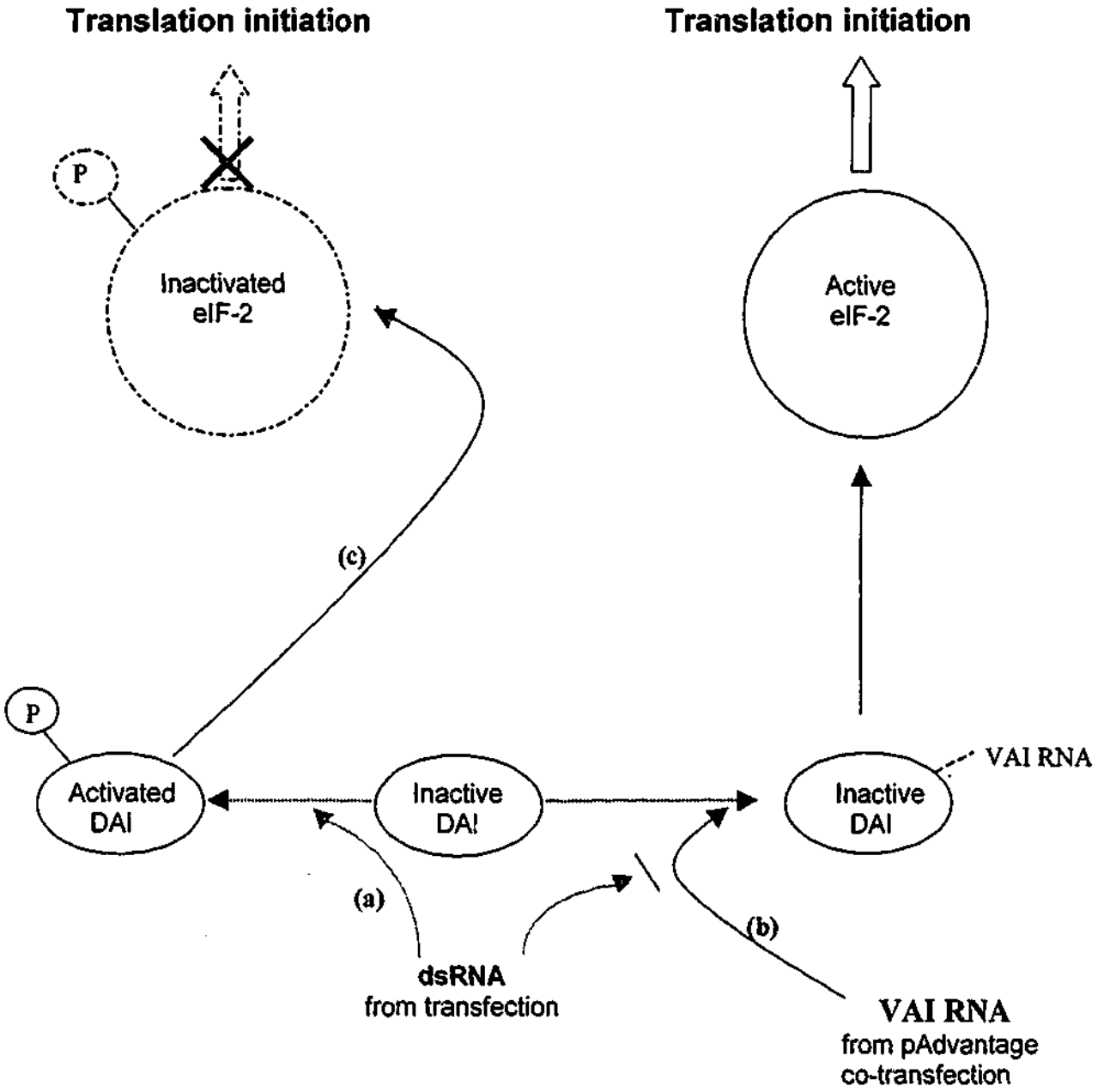


Figure 3-7: Preventing the inhibition of translation initiation with pAdvantage. Double-stranded RNA (dsRNA) generated during transfection activates the dsRNA- inhibitor (DAI) *(a)*. Activated DAI phosphorylates the translation initiation factor eIF-2, halting translation and therefore protein production *(b)*. Inhibition of translation by DAI can be overcome with the adenoviral VAI RNA that is produced by RNA polymerase III following co-transfection with the pAdvantage vector *(c)*. The VAI RNA binds to DAI, preventing its activation, allowing translation and protein expression.

the pAdvantage Vector can increase transient protein expression of transfected recombinant genes by increasing translation initiation.

Therefore, recombinant 5αR-2 was co-transfected with pAdvantage to try to increase the activity levels above 2.4%. Using pAdvantage:recombinant 5αR-2 (1:2.5), 5αR-2 activity increased by ~4-fold over previous experiments (10.2%, *Table 2*). Diluting the cells yielded proportionally lower activity as expected (5% for 50 µl and 2.1% for 12.5 µl sample, *Table 2*). Therefore, optimal conditions for transfecting 5αR-2 were obtained by co-transfection with the pAdvantage Vector to inhibit double-stranded RNA.

In summary, these experiments showed that the optimal conditions for transfecting recombinant 5αR-2 in COS-7 cells was to use Lipofectamine:recombinant 5αR-2 (2:1) for 2 days, incubating cells with media^{-FCS} for the first day of transfection, and using the pAdvantage Vector (pAdvantage:recombinant 5αR-2, 1:2.5) to inhibit formation of double stranded DNA and allow translation initiation.

Measuring 5α-Reductase Type 2 Activity in the Presence of Type 1 Activity

The proposed method for calculating 5αR-2 enzyme activity in the presence of confounding 5αR-1 effects at pH 5.0 was validated using the recombinant 5αR isoforms. *Figure 3-8* (one of three experiments performed in triplicate) shows that 10 µl recombinant 5αR-1 alone produced ~1.814 ± 0.147 fmoles DHT+3α-Adiol/ min at pH 5.0, and this represented the overlap of 5αR-1 activity at pH 5.0. The increasing amounts of recombinant 5αR-2 assayed alone (30 - 90 µl) produced proportionally increasing amounts of activity at pH 5.0. When a constant amount of recombinant 5αR-1 (10 µl) was assayed with increasing amounts of recombinant 5αR-2, the amount of activity detected at pH 5.0 for these samples, which contained both the 5αR-1 and 5αR-2 isoforms, did not reflect 5αR-2 activity alone, but rather the combined activities of 5αR-2 and the overlap of 5αR-1 at pH 5.0 (*Figure 3-8*). However, when our formula (**5αR-2 Activity = (0.124 – pH 5.0/ 7.0 sample) x Activity of sample at pH 7.0**) was used to eliminate overlapping 5αR-1

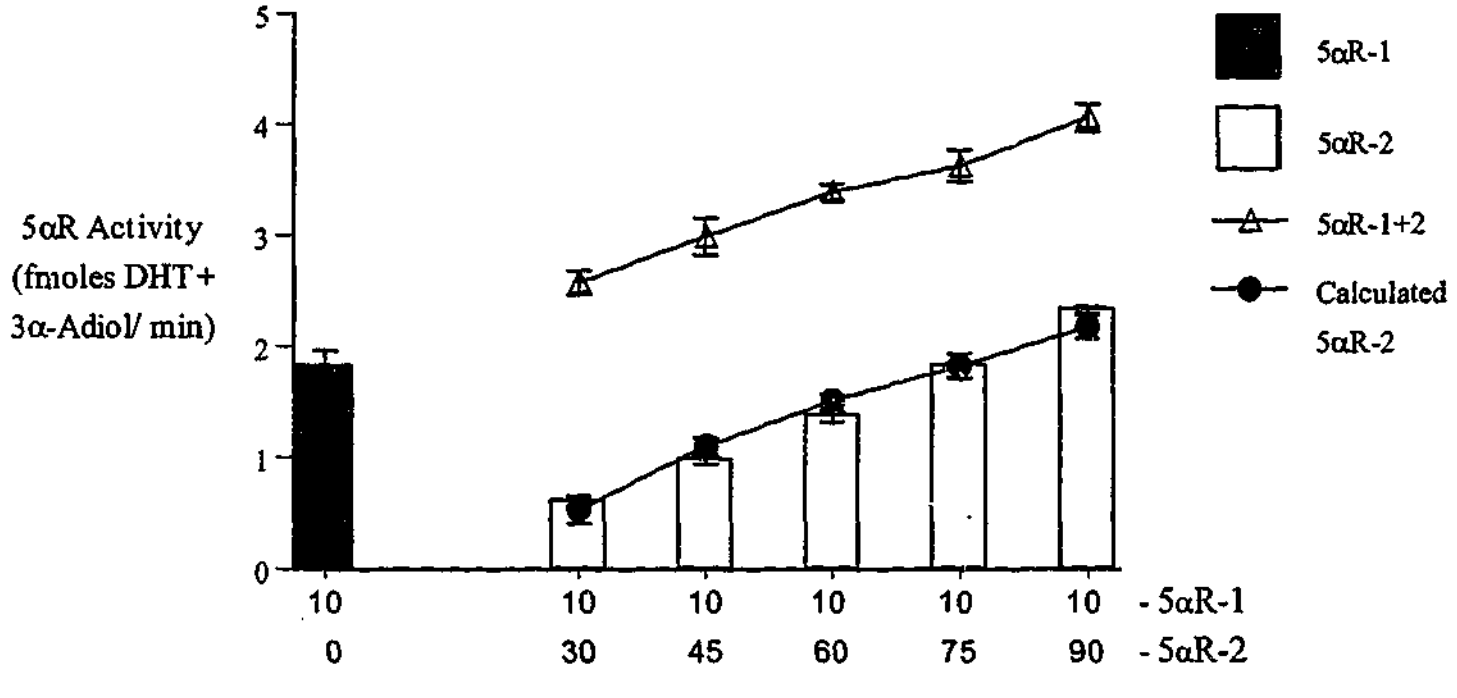


Figure 3-8: Validation of method for calculating 5αR-2 activity in the presence of 5αR-1 activity at pH 5.0. The open bars represent increasing amounts of 5αR-2 activity, and the solid bars represent the overlap of recombinant 5αR-1 (10 μl) activity at pH 5.0. The line graph with open triangles represents the activity measured when 10 μl 5αR-1 was combined with increasing amounts of 5αR-2 (30, 45, 60, 75, and 90 μl). The line graph with closed circles represents the 5αR-2 activity calculated using our formula. This experiment was performed 3 times in triplicate, and this is one representative experiment. Data is expressed as mean ± SD for triplicate measurements.

activity at pH 5.0, the amount of 5αR-2 activity was accurately determined, as shown by the similar values obtained for added and calculated 5αR-2 (*Figure 3-8*).

Figure 3-9(A) represents the summary of all three experiments in which the activity measured at pH 5.0 with increasing amounts of recombinant 5αR-2 (i.e. similar to the open bars in *Figure 3-8*) was plotted against the amount of calculated 5αR-2. The correlation of added recombinant 5αR-2 activity vs calculated 5αR-2 activity gave a linear response with a correlation coefficient of r = 0.98. Note, there appeared to be a tendency for deviation from linearity at the lower end of the graph, corresponding to low conversion rates.

It is important to note that this method for quantitating 5αR-2 activity depends on the activity of the sample measured at both pH 5.0 and 7.0, and requires the use of the pH 5.0/ 7.0 ratio. *Figure 3-9 (B)* shows the pH 5.0/ 7.0 ratio against the percentage accuracy of each sample. This graph shows that there was a high accuracy for calculating 5αR-2 activity when the pH 5.0/ 7.0 ratio was 0.15 or above, but was not accurate when the pH 5.0/ 7.0 ratio was less than 0.15. Therefore, the sensitivity of the assay for measuring 5αR-2 activity using our formula, was set at two standard deviations above the mean for the pH 5.0/ 7.0 ratio for recombinant 5αR-1 (i.e. 0.124 + [2 x 0.014]), and was 0.152. Thus, any sample with a pH 5.0/ 7.0 ratio of less than 0.152 was defined as non-detectable and assigned a pH 5.0/ 7.0 ratio equal to the sensitivity value of 0.152. When the samples with values less than 0.152 were excluded (*Figure 3-9(B)*, excluded data are shown in asterix), the average accuracy for calculating 5αR-2 was 99.3±14.7%.

3.3.4 Enzyme Kinetics

The enzyme kinetic data for the recombinant 5αR isoforms are represented graphically in *Figures 3-10* for 5αR-1, and *Figures 3-11* for 5αR-2. The Lineweaver-Burk plots were linear for 5αR-1 *(Figure 3-10[B])* and 5αR-2 *(Figure 3-11[B])*, as were the Eadie Scatchard plots for 5αR-1 *(Figure 3-10[C])* and 5αR-2 *(Figure 3-11[C])*. A single affinity constant for testosterone was

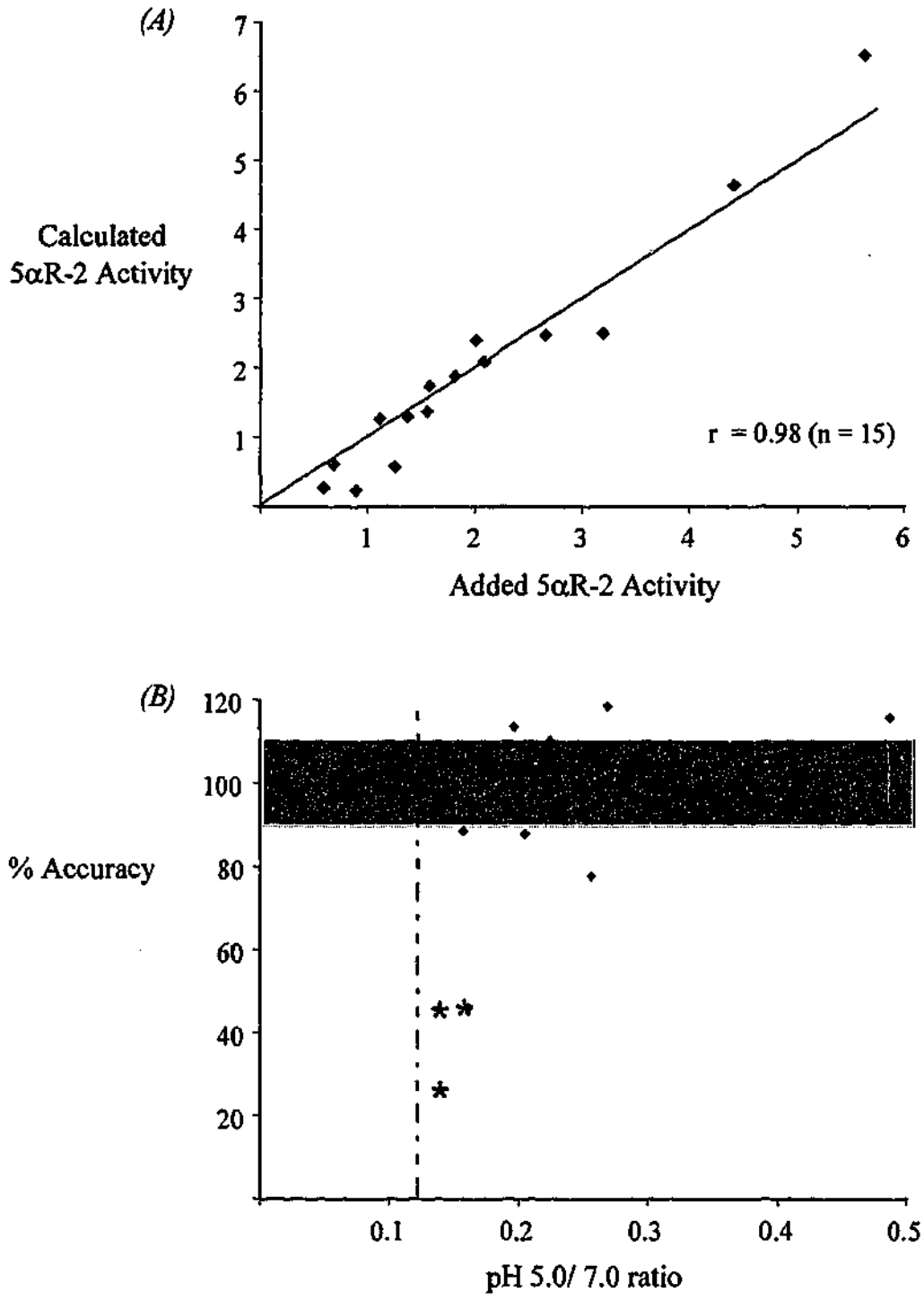


Figure 3-9: Validation of method for calculating 5αR-2 activity in the presence of 5αR-1 (data from 3 different experiments). *(A)* Correlation of added recombinant rat 5αR-2 activity, in increasing amounts, against calculated 5αR-2 activity. *(B)* Comparison of percentage accuracy of this method as a function of the pH 5.0/ 7.0 ratio. 5αR-2 activity was expressed as percent conversion of ^{3}H-testosterone to ^{3}H-DHT + ^{3}H-3α-Adiol. Each data point represents the average of triplicate measurements. The data in asterix indicate values that have a pH 5.0/ 7.0 ratio less than 0.152. The grey horizontal bar indicates a deviation of 5% above and below 100% accuracy.

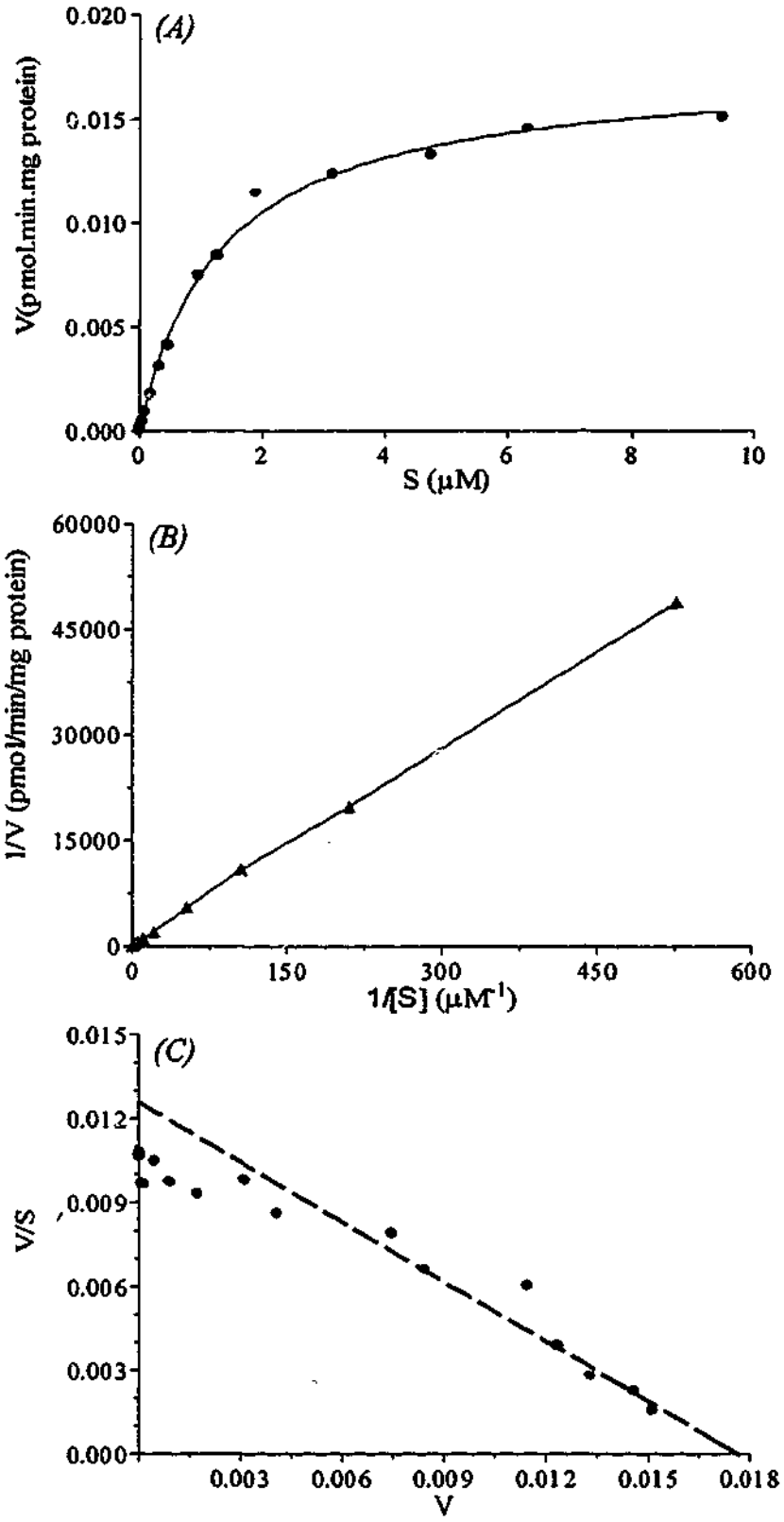


Figure 3-10: 5α-Reductase enzyme kinetics in recombinant rat 5αR-1. *(A)* Michaelis-Menten plot of estimated initial velocities (V) of 5αR-1 activity at pH 7.0 using substrate [S] concentrations from 1.9 nM to 9.5 mM. *(B)* Double reciprocal plot of the data reported in *A*. *(C)* Eadie-Scatchard plot of the data reported in *A* of estimated initial velocities over substrate concentration (V/S) against velocity (V), that can be described by a single enzyme activity, 5αR-1 (- - -). Values are the mean of duplicate measurements.

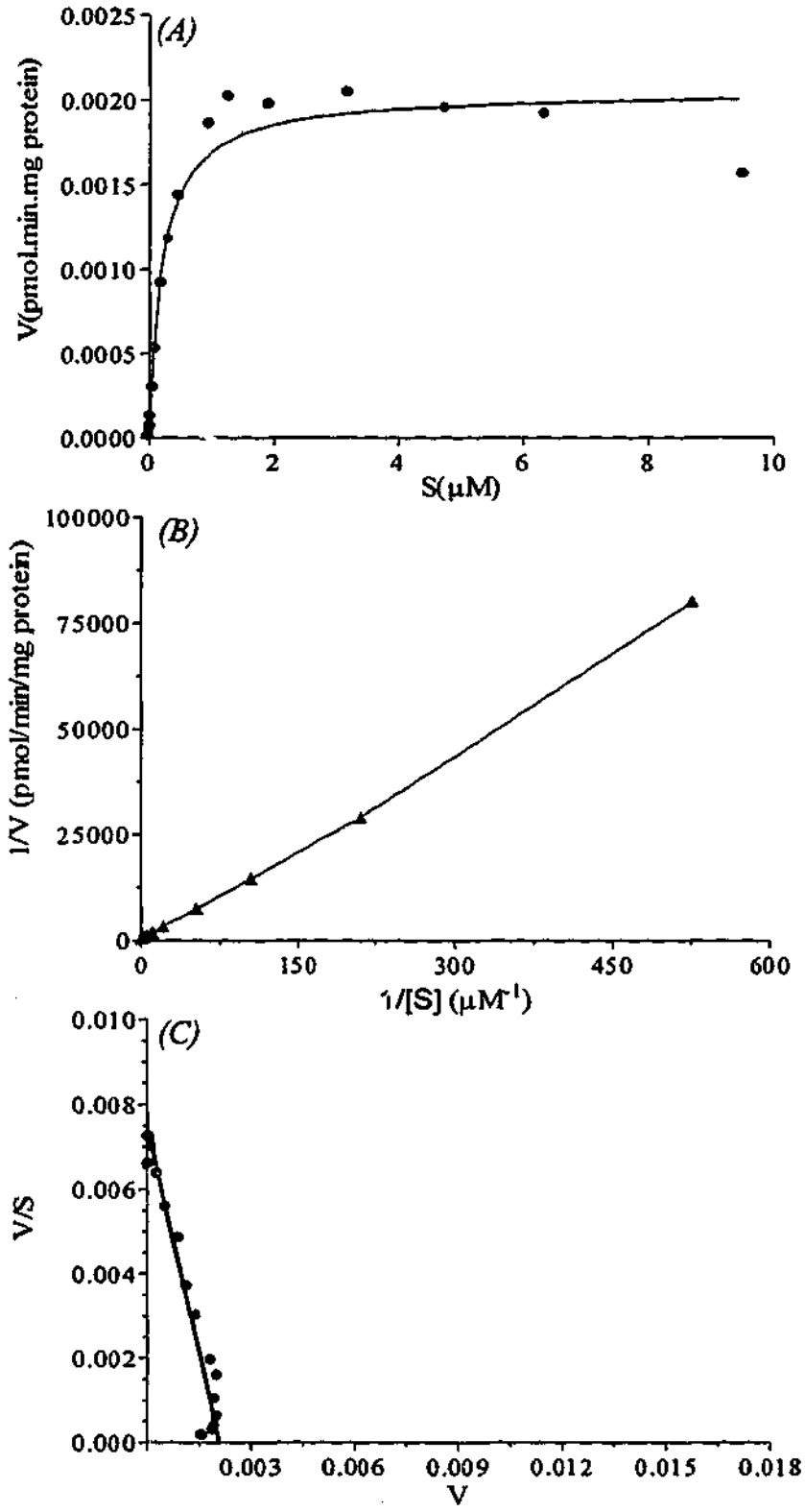


Figure 3-11: 5α-Reductase enzyme kinetics in recombinant rat 5αR-2. *(A)* Michaelis-Menten plot of estimated initial velocities (V) of 5αR-2 activity at pH 5.0 using substrate [S] concentrations from 1.9 nM to 9.5 mM. *(B)* Double reciprocal plot of the data reported in *A*. *(C)* Eadie-Scatchard plot of the data reported in *A* of estimated initial velocities over substrate concentration (V/S) against velocity (V), that can be described by a single enzyme activity, 5αR-1 (—). Values are the mean of duplicate measurements.

obtained for the recombinant isoforms, with K_m values of 1.64±0.40 for 5αR-1, and 0.27±0.02 for 5αR-2 *(Table 3)*.

Table 3: Enzyme characteristics (K_m, V_{max}, and V_{max}/K_m ratio) for recombinant rat 5α-reductase type 1 (5αR-1) and Type 2 (5αR-2). Details of enzyme assays and analysis are presented in the Methods section.

Recombinant rat 5αR	K_m (μM)	[illegible]	V_{max}/K_m ($\times 10^{-2}$)
[illegible]	1.64 ± 0.40	[illegible]	3.52
[illegible]	0.27 ± 0.02	[illegible]	11.9

Values are the mean ± SD from three separate experiments. * Denotes enzyme kinetics performed at pH 5.0.

3.4 Discussion

In order to understand the physiological role of 5αR in androgen-dependent tissues, it is important to know the relative expression of each 5αR isoform in a given sample and the regulation of each 5αR isoform. Therefore, the relative contributions of each enzyme to total enzyme activity is crucial in understanding the control of 5α-reduced androgen biosynthesis in a tissue. This chapter therefore validated a method that accurately measured 5αR-1 and 5αR-2 in a single sample, utilizing recombinant rat 5αR-1 activity at pH 5.0 and 7.0. A 5αR assay and TLC procedure for the separation of steroids were validated, and recombinant rat 5αR-1 and 5αR-2 were transiently transfected in COS-7 cells, and the method for the calculation of 5αR-2 activity in the presence of 5αR-1 was validated

This study showed that a solvent system of chloroform:methanol (98:2) separated testosterone, DHT, 3α-Adiol, with average R_f values of 0.45, 0.6 and 0.72, respectively. ^{3}H-Testosterone was used as the substrate for the 5αR activity assay, and purifying ^{3}H-testosterone by TLC reduced contamination to < 0.1%. This provided low backgrounds and high sensitivity for the

detection of ^{3}H-DHT and ^{3}H-3α-Adiol. ^{14}C-steroids were used to correct for losses during steroid extraction and separation. ^{14}C-3α-Adiol was not commercially available and therefore was produced via reduction of ^{14}C-DHT. The ^{14}C-3α-Adiol produced was specific and behaved in the same manner as ^{3}H- and non-radioactive 3α-Adiol.

Expression vectors for 5αR-1 and 5αR-2 were constructed from cDNAs kindly provided by Dr. D.W. Russell. Recombinant 5αR-1 and 5αR-2 was subcloned into the pcDNA3.1(+) vector and transfected into COS-7 cells. Expression of 5αR-1 with FuGENE was relatively straightforward, however transient expression of 5αR-2 was more complicated. Optimal conditions for expression of 5αR-2 were obtained with the transfection reagent Lipofectamine, and co-transfecting with the pAdvantage Vector to facilitate translation initiation. The pAdvantage Vector increased protein expression of transfected recombinant 5αR-2 by approximately four-fold.

The amount of overlapping 5αR-1 activity at pH 5.0 was 12.4 ± 1.4% of that at pH 7.0, and was incorporated into a formula to remove confounding effects of 5αR-1 at pH 5.0. This allowed 5αR-2 to be quantitated, even in a setting where 5αR-1 was predominantly expressed. This approach was validated by measuring differing amounts of recombinant 5αR-2 in the presence of a high amount of recombinant 5αR-1. A linear response was obtained when comparing added vs calculated 5αR-2 activity, and making the sensitivity of the assay to two times above the SD from the average pH 5.0/ 7.0 ratio for recombinant 5αR-1 (i.e. 0.152) yielded a 99.3 ± 14.7% accuracy.

Furthermore, enzyme kinetic studies showed that recombinant 5αR-1 and 5αR-2 produced K_m values in the micromolar and nanomolar range for testosterone, respectively, which are in accordance with previous published K_m values (Normington and Russell, 1992; Span *et al.*, 1995; Span *et al.*, 1996a; Faller *et al.*, 1993; Thigpen *et al.*, 1993a).

In summary, 5αR-1 and 5αR-2 was measured in a single sample using a method in which both isoforms were measured at their optimal pH (pH 7.0 and 5.0, respectively). The contribution of 5αR-1 activity at pH 5.0 was corrected for by assessing recombinant 5αR-1 activity, to

accurately determine the levels of 5αR-2. This method for measuring 5αR-1 and 5αR-2 was sensitive, highly reproducible within and between assays, and in terms of the method for determining 5αR-2, was >99% accuracy. This method was subsequently applied to the measurement of 5αR-1 and 5αR-2 in testicular samples.

Chapter 4

Validation and Optimization of Real Time Polymerase Chain Reaction for Measuring 5α-Reductase Type 1 and Type 2 mRNA Levels

4.1 Introduction

The regulation of the 5αR isoforms in rat testis have not been investigated at the mRNA level. Therefore, we aimed to establish a quantitative method that would allow measurement of testicular 5αR-1 and 5αR-2 mRNA levels. This chapter outlines the quantitative PCR approach that was used to measure mRNA levels, the equipment and reagents required for this procedure, and validation of this approach.

Steady state RNA levels can be quantified by different methods, including Northern blot analysis, RNAse protection assays and *in situ* hybridization. The main limitation of these methods is low sensitivity (Melton *et al.*, 1984). Alternatively, the method of reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive for detection of low-abundance mRNA and is the most flexible of the quantitation methods (Wang and Brown, 1999). RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA (Rappolee *et al.*, 1988), to allow the level of mRNAs in different sample populations to be compared. As RNA can not serve as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA template into complementary DNA (cDNA), followed by its exponential amplification in a PCR reaction. Thus;

(1) During reverse transcription, RNA-dependent DNA polymerases (i.e. reverse transcriptase enzymes) are used to generate cDNA tanscripts. The reverse transcription step can be primed using specific primers, random hexamers or oligo dT primers. The use of mRNA-specific primers decreases background priming, whereas the use of random and oligo-dT primers maximizes the number of mRNA molecules that can be analyzed from small amounts of RNA.

(2) Following reverse transcription, DNA-dependent DNA polymerases, such as Taq DNA polymerase, are used in the PCR reaction to amplify the cDNA produced during reverse transcription.

Quantitation of mRNA can be semi-quantitative or quantitative (Ferre, 1992). Quantitative procedures can use competitive RT-PCR techniques (Wang *et al.*, 1989; Vanden Heuvel *et al.*,

1993) and depend on spiking into the RNA samples, before reverse transcription, known amounts of internal standards. In competitive RT-PCR, a series of PCR tubes containing the target are spiked with serial dilutions of known copy numbers of the internal standard. After gel electrophoresis, a comparison of the intensities of ethidium-bromide-stained standard and target amplicons allows target quantification (Raemaekers, 1999). The disadvantages of competitive RT-PCR are high errors (~10%) and the procedure is time-consuming and requires the use of many costly reagents. Thus conventional RT-PCR-based procedures are not suitable for routine use.

More recently the application of fluorescence techniques to the RT-PCR, together with suitable instrumentation capable of combining amplification, detection and quantification, has led to the development of kinetic PCR methodologies that can quantitate nucleic acids in a more simple and efficient way (Orlando *et al.*, 1998; Bustin *et al.*, 2000). The LightCycler (Roche Molecular Biochemicals❶) instrument is a rapid and reliable PCR approach that uses a thermocycler for the rapid analysis and simultaneous evaluation of PCR experiments, using an airstream for heating and cooling (Wittwer *et al.*, 1989; Wittwer *et al.*, 1997b). Fluoremetric analysis of the PCR products formed are taken at every cycle of the PCR reaction, and are displayed in 'real time' (Wittwer *et al.*, 1990; Wittwer and Garling, 1991). An overview of the equipment and the optimization conditions will be discussed.

The LightCycler consists basically of a cycler component and a fluorescence-detection component. The PCR reaction takes place in a glass capillary that is made of composite plastic/glass and allows the rapid equilibration between the air and the reaction components because of its high surface to volume ratio. A capillary stopper is used to seal the capillary and provides a secure seal to reduce the risk of contamination.

The LightCycler uses the double stranded DNA binding dye, SYBR Green 1 (Roche Molecular Biochemicals❶), for fluorescence measurement. This is a specific dye that only fluoresces when bound to double stranded DNA (*Figure 4-1*). All of the DNA becomes single-stranded after denaturation, so the dye will not bind and the intensity of fluorescence signal is low. During annealing, PCR primers hybridize to the target sequence to result in small parts of double

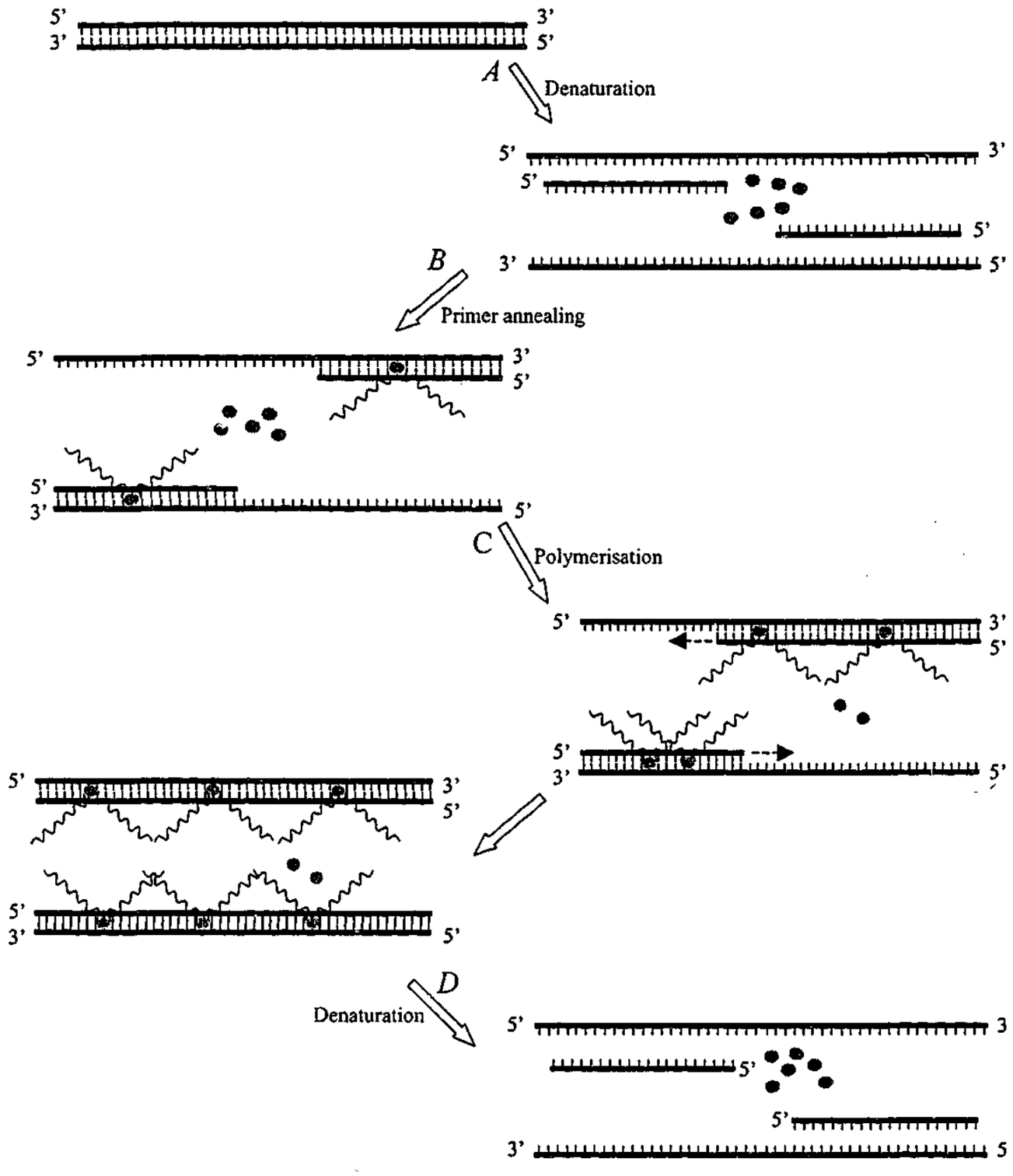


Figure 4-1: The LightCycler assay. *(A)* The DNA becomes single-stranded after denaturation, and SYBR Green 1 dye cannot bind. *(B)* During annealing, PCR primers hybridize to the target sequence to form double stranded DNA to which the dye binds, increasing fluorescence intensity. *(C)* During elongation, the PCR primers are extended and more dye binds. *(D)* During denaturation, the fluorescence can not bind to single stranded DNA and fluorescence is low.

stranded DNA to which the dye can bind, thereby increasing fluorescence intensity. In the elongation phase of the PCR, the PCR primers are extended, and more of the dye can bind. At the end of the elongation phase, the entire DNA has become double stranded, and a maximal amount of dye is bound. The fluorescence is recorded at the end of the elongation phase.

The SYBR Green 1 combined with Fastart Taq DNA polymerase minimizes non-specific amplification of products that contaminate the desired product. The Fastart Taq DNA polymerase is a modified form of thermostable recombinant Taq DNA polymerase, it is inactive at room temperature and therefore there is no elongation during the period when primers can bind non-specifically. The modified enzyme is "activated" by high temperatures (i.e. a pre-incubation step at 95°C for a maximum of 10 min is required). Thus, LightCycler-Fastart DNA Master SYBR Green 1 provides convenience, high performance, reproducibility, and minimizes the risk of contamination (Birch *et al.*, 1996).

Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction (*Figure 4-2*). The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (Gibson *et al.*, 1996). The point is defined as the threshold cycle (C_t), and will always occur during the exponential phase of amplification. The value of C_t is calculated based on the time (measured in PCR cycle number) at which the reporter fluorescent emission increases beyond a threshold level. A C_t value is reported for each sample, and this value can be translated into a quantitative result by constructing a standard curve. Relative quantification of mRNA transcription measures the changes in the steady-state abundance of a gene using a relative standard that is made by serial dilutions of a sample that has arbitrary units. The standard can be any nucleic acid, as long as its concentration and length are known.

The following criteria must be optimized when setting up real time PCR:

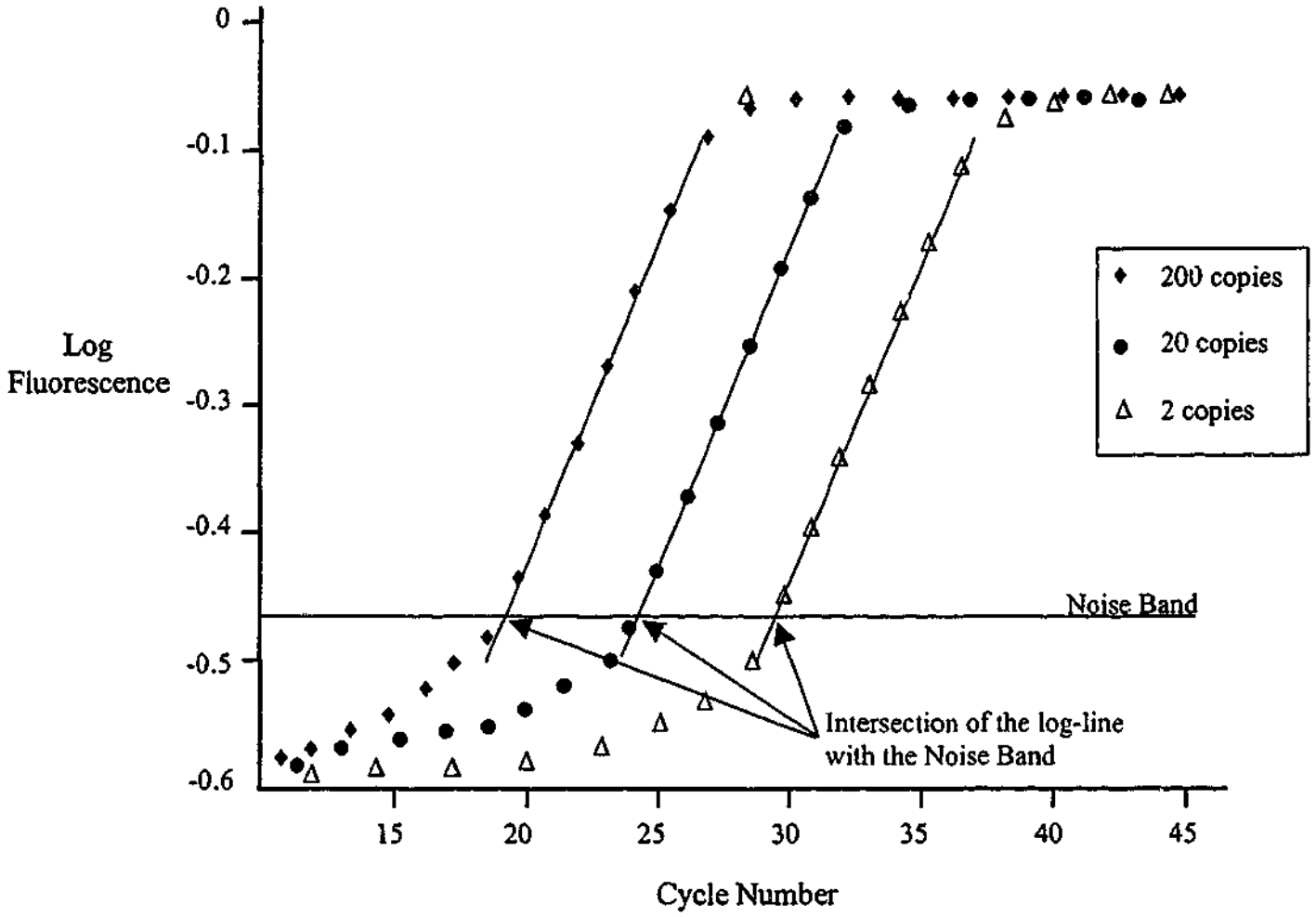


Figure 4-2: Quantification with the LightCycler. The graph shows the amplification of a target molecule at different concentrations, and the various points represent individual data points. The log-lines are extrapolated to the noise band line which defines the beginning of the log-linear phase. Significant differences in fluorescence can be seen in the log-linear phase of the PCR, whereas the fluorescence signal cannot be distinguished in the plateau phase.

A) Oligonucleotide primers must be designed to yield amplicon lengths of ~100 bp (Bustin et al., 2000). Shorter amplicons amplify more efficiently than longer ones and are more tolerant of reaction conditions because they are more likely to be denatured during the 92-95°C step of the PCR, allowing primers to compete more effectively for binding to their complementary targets. As the specificity of the PCR step is crucially dependent on the primers (Zhang and Bryne, 1999), reaction conditions must be optimized for different enzymes.

B) Magnesium chloride ($MgCl_2$) affects enzyme activity and increases the melting temperature of double-stranded DNA (Eckert and Kunkel, 1991). Therefore, since the PCR reaction in the LightCycler is critically dependent on the $MgCl_2$ concentration, the optimal $MgCl_2$ concentration for PCR was determined for both standards and samples. The Fastart DNA SYBR Green 1 contained a basic $MgCl_2$ concentration of 1 mM (final concentration) and optimal concentrations of $MgCl_2$ for PCR with the LightCycler vary between 1 to 5 mM.

C) Melting curve analysis is performed after amplification when a PCR product is formed. Real time PCR provides product verification through the melting curve analysis, a process whereby the melting curve of an amplicon is generated by plotting fluorescence as a function of temperature (Ririe et al., 1997). Starting from low temperatures, the temperature in the thermal chamber is slowly raised, and the fluorescence measured in each tube at frequent intervals (one measurement every 0.2 °C). The fluorescence of the dye that binds to the double-stranded amplicon drops sharply as the fragment is denatured. Each double-stranded DNA product has its own specific melting temperature (T_m), which is defined as the temperature at which 50% of the DNA becomes single-stranded, and 50% remains double-stranded. The T_m of a PCR fragment can be easily visualized by taking the first negative derivative (-dF/dT) of the melting curve.

Primer Dimers are non-specific target sequences that can also be formed during PCR. These non-specific PCR products usually take place as soon as PCR reagents are combined. Primer dimers are products of nonspecific annealing and primer elongation events, and are produced when there is no template or when template concentration is low. Formation of primer dimers compete with formation of specific PCR product, and thus leads to reduced amplification efficiency. Therefore, by knowing the T_m of the by-products that are formed during PCR, specificity of the PCR can be increased by increasing the temperature at which the LightCycler measures the fluorescence. Pure homogeneous PCR products produce a single, sharply defined melting curve with a narrow peak. In contrast, primer dimers melt at relatively low temperatures and have broader peaks.

D) Standard curves reflecting the range of the samples required validation. Both the amplification and melting curve analysis must be identical between standards and samples. To assess the variation between different LightCycler PCR experiments, quality control (QC) samples were included in every assay, and were measured at two different dilutions.

E) Unknown Samples to be analyzed were diluted accordingly to amplify at the same efficiency as the standards.

F) Negative Control Samples were included to assess the lower limits of detection. This was assessed by (A) *–ve control*: determined the contamination of genomic DNA in RNA samples and (B) *blank control*: assessed the sensitivity of each PCR reaction.

G) Method of Quantitation was selected from the LightCycler program to best describe the logarithmic part of the PCR reaction. Data was normalized using a housekeeping gene that was invariant throughout the biological treatments examined.

Therefore, the aim of this study was to set up and validate the LightCycler for the quantitation of mRNA concentrations. The levels of mRNA were compared to enzyme activity measurements in subsequent chapters during the withdrawal and replacement of testosterone and/or FSH using *in vivo* models of gonadotrophin suppression. The chapter will outline the methods for measuring 5αR isoform mRNA using the LightCycler instrument, the optimization and subsequent validation of this procedure.

4.2 Materials and Methods

4.2.1 Total RNA Preparation

Total RNA was isolated using the Qiagen RNeasy kit (Qiagen❶) with mini-columns as per manufacturer's instructions, and purified by isopropanol precipitation (see section 2.4.3). RNA was dissolved in RNase-free H_2O and quantified by its spectrophotometric absorption at A_{260}. Only RNA samples with a A_{260}/ A_{280} ratio of 1.9 - 2.1 were used for PCR. Confirmation of the quality of RNA was determined by electrophoresis at 80 V through a 1.5% agarose gel in 1 x TBE buffer❷. RNA integrity was confirmed by ethidium bromide staining and photographed using the Gel Doc 2000-gel documentation system (BioRad❶).

4.2.2 Reverse Transcription

Two-step RT-PCR was performed, whereby the reverse transcription of RNA into cDNA was performed outside the LightCycler in a PCR machine (RoboCycler Gradient 40 PCR Thermal Cycler, Stratagene❶), and the subsequent amplification of cDNA and on-line monitoring was performed using the LightCycler.

Synthesis of cDNA (reverse transcription) was performed using Expand Reverse Transcriptase (Roche Molecular Biochemicals❶) as described in the manufacturer's protocols. For

control incubations, the reverse transcriptase was omitted (i.e. these served as negative controls). The following components were used for reverse transcription:

a) *Primers*: 5αR-1: Random Primers (3 μg/ μl, Gibco❶) are oligonucleotides used to prime mRNA's with or without poly(A) for cDNA synthesis. These primers are suitable for cDNA synthesis using reverse transcriptase with mRNA templates. 5αR-2: Oligo $(dT)_{12-18}$ primer (0.5 μg/ μl, Gibco❶) hybridizes to the poly(A) tail of mRNA and is suitable for first strand cDNA synthesis with reverse transcriptase.

b) *RNase Inhibitor*: (40 U/ μl, Roche Molecular Biochemicals❶). Included during reverse transcription to protect the mRNA during cDNA synthesis. RNase inhibitor inactivates RNase by binding noncovalently to the enzyme molecule.

c) *Total RNA*: Gel electrophoresis was used to check the quality and concentration of RNA.

d) Expand Reverse Transcriptase: (50 U/ μl, Roche Molecular Biochemicals❶). An RNA directed DNA polymerase containing a point mutation within the RNase H sequence to eliminate RNA H activity to obtain higher amounts of full-length cDNA transcripts. The enzyme synthesizes with mRNA or single stranded DNA as substrate, in the presence of a primer, a complimentary DNA strand. Additional components provided were Expand Reverse Transcriptase Buffer (5 x concentration, [250 mM Tris-HCl, 200 mM KCl, 25 mM $MgCl_2$, 2.5% Tween 20 (v/v)], pH 8.3 at 25°C).

These solutions were stored at –20°C, thawed on ice prior to use, and immediately frozen after use.

Protocol for First Strand cDNA Synthesis of 5αR-1 and 5αR-2 using Expand Reverse Transcriptase:

The following components were added in order into a 0.6 ml nuclease-free PCR tube (Interpath Services Pty Ltd❶):

. 100 ng random primer for 5αR-1 or 0.125 μg oligo dT for 5αR-2

. 4 U RNase inhibitor

. 2 μg RNA

. Make up to a total volume of 12 μl with sterile DEPC-treated water

. Heat to 70°C for 10 min, quickly chill on ice.

. Add 8 μl of the following master mix added, containing:

. 4 μl 5 x First Strand Buffer,

. 2 μl 0.1 M DTT

. 1 μl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP)

. 1 μl Expand reverse transcriptase.

. Mix contents and incubate at 42°C for 50 min.

. Inactivate reaction by heating at 70°C for 15 min. The cDNA was now ready to be used as a template for amplification in PCR, and was kept frozen at –20°C until required.

4.2.3 Real Time PCR using the LightCycler

PCR primers were designed with the computer software MacVector 5.0 (Oxford Molecular❶) to recognize rat 5αR-1 and 5αR-2 mRNA sequences. The following criteria were used: minimal internal structure (i.e. hairpins, primer-primer, and primer dimer formations), compatible T_m's (i.e. each within 1°C of the other), optimal length (i.e. single stranded primers should be ~20 bases) and G/C content between 40-60%.

The sequences of these primers are listed below:

5αR-1 primers (128 bp PCR product)

Forward Primer (F3): 5'-TCC TGG TCA CCT TTG TCT TGG C-3'

Reverse Primer (B3): 5'-GTT TCC CCT GGT TTT CTC AGA TTC-3'

5αR-2 primers (207bp PCR product)

Forward Primer (F44): 5'-ACA TCC ACA GTG ACT ACA CCC TGC-3'

Reverse Primer (B38): 5'-TCC ATT CAA TAA TCT CGC CCA G-3'

To perform PCR using the LightCycler, capillaries were placed into the pre-cooled (4°C) cooling block and the PCR reaction mixture prepared, including forward and reverse primers, $MgCl_2$, PCR grade water, and SYBR Green 1 dye. Lastly, the cDNA was added to the pre-cooled capillary containing the PCR mixture, sealed with a stopper and centrifuged at 1000 rpm for 5sec. The capillaries were loaded into the LightCycler carousel, placed inside the thermal chamber, and the PCR reaction initiated by pressing 'RUN' on the LightCycler software program.

The real time PCR protocol consists of three programs:

1) Initial denaturation of template DNA to denature double-stranded cDNA template. This was set at 95°C for 10 min as recommended by the manufacturers.
2) Amplification of target DNA. Optimal conditions for specific PCR product formation (data not shown) were: 5αR-1: 95°C for 15 sec, 55°C for 5 sec, 72°C for 10 sec, and for 5αR-2: 95°C for 15 sec, 60°C for 5 sec, 72°C for 10 sec
3) Melting curve analysis will be discussed in the Results section.

Standards for PCR amplification should be as homologous as possible to the target to ensure similar amplification efficiency. Therefore, standards were prepared from PCR products amplified using 5αR isoform cDNAs as templates. Briefly, 15 µg of plasmids containing each 5αR isoform cDNA was digested (15 µg plasmid, 10 µl buffer H, 3 µl [EcoR1 and Xba1 for 5αR-1 and EcoR1 and Xho 1 for 5αR-2], made up to 100 µl with sterile H_20, and incubated overnight at 4°C). The digestion was run on a 1.5% agarose gel in 1 x TBE buffer and the insert extracted from the gel. The isolated cDNA inserts were then amplified using the primers designed for real time PCR (see above). The amplified products from this PCR reaction were run on a 1.5% gel, extracted from the gel, and the concentration determined by spectrophotometry. This was then used as a standard for PCR.

The QC sample ideally should be similar to the samples, therefore the QC was a pool of samples that were being analyzed (combined half of the reverse transcription reaction from each of the untreated control animals described in chapters 6 and 7). The QC was assayed at two different dilutions in duplicate.

An important step in the validation of real tme PCR assays is the demonstration that the signals observed are a result from the PCR amplification of RNA, and not from contaminating DNA. Therefore, two different negative controls were included to monitor for possible DNA contamination. The first negative control contained all the components included in standards and unknown samples, except the template was omitted, and was called the 'blank control'. This was equivalent to zero point on the standard curve.

The second negative control monitored for contaminating genomic DNA in the RNA samples that may be carried over from the reverse transcription reaction. This negative control contained all the components that were included in the standards and samples, except that the reverse transcription product was performed in the absence of the reverse transcription enzyme (Expand reverse transcriptase). This will be referred to as the '-ve control' sample.

PCR only has a few cycles in which the amount of product increase logarithmically before PCR efficiency drops to zero. Quantification is most accurate when only these few cycles are considered. Looking at later PCR cycles, which is normally done when the final amount of PCR product is considered, does not easily permit conclusions to be drawn about starting concentrations. Cycle-by-cycle monitoring identifies these cycles in which PCR is in its so-called log-liner phase (i.e. the PCR product doubles with each cycle).

After the PCR reaction has finished, the LightCycler software converts the raw data to copies of target molecules. The first step is performed by setting a noise band (threshold) on a fluorescence level, at which the fluorescence signal reflects that the PCR is in the log-linear phase. Then the logarithmic values for all the data points that are above this noise band are calculated. By interpolating a straight line through a user-defined number of data points above this threshold value, the software then determines these points of intersection with the noise band for all the

standards. These points of intersection, which are referred to as crossing points, are plotted against the logarithm of the concentration. The concentrations of target sequence in the standards and in the unknown samples are obtained by comparing the crossing points for the samples with the crossing points of the standards. This method of quantification is done automatically by the LightCycler software, and is termed 'second derivative maximum'.

The two underlying assumptions of quantification with external standards are that only a few log-linear cycles are used for quantification and that the reaction efficiency of standards and unknowns are the same. The slope of the log-linear region of the amplification can be used to verify that samples are amplified with the same efficiency as the standards. Since the noise band line defines the beginning of the log-linear phase, extrapolation errors can be minimized be setting the 'noise band' manually, if samples do not begin to amplify with the same efficiency. This method is referred to as 'fit points' analysis.

4.3 Results

A) *Optimal Primer Concentration*

To optimize the reaction conditions for real time PCR, first the optimal primer concentration was determined, then the optimal $MgCl_2$ concentration for both standards and samples, and lastly the dilution of sample yielding the best amplification was determined.

Different primer concentrations were tested as recommended by the manufacturers. We found that a primer concentration of 0.5 mM and 0.25 mM yielded the best PCR reaction for 5αR-1 and 5αR-2, respectively (data not shown). By decreasing the primer concentration to 0.25 mM for 5αR-2, mispriming and accumulation of non-specific product was reduced so that the primer dimer peaks on the melting curve analysis were reduced whilst the peaks corresponding to specific target DNA were increased. These findings were supported by agarose gel analysis, which showed a single band of correct product size.

B) Optimal Magnesium Chloride Concentration

Real time PCR reactions were optimized by examining the effects of $MgCl_2$ concentration on the resultant products, as analyzed by the amplification plots and melting curves from the LightCycler. The amplification curves *(Figure 4-3[A])* and melting curve analysis *(Figure 4-3[B, C])* for 5αR-2 are shown at different $MgCl_2$ concentrations. This data indicates that the amplification curves for standards and samples showed similar C_t's despite differences in $MgCl_2$ concentration *(Figure 4-3[A])*. Melting curve for standards showed a single peak at different $MgCl_2$ concentrations indicating that only one amplified product was present, irrespective of the amount of $MgCl_2$ *(Figure 4-3[B])*. However for samples, 5 mM $MgCl_2$ was optimal since this concentration minimized non-specific product formation *(Figure 4-3[C])*. Therefore, 5 mM $MgCl_2$ was used in all subsequent reactions.

These results were also representative for 5αR-1 (data not shown). A summary of the results indicating optimal primer concentration and $MgCl_2$ concentration are represented in *Table 1*, which outlines the PCR reaction mixture.

Table 1: The 'master mix' used for a 20 µl real time PCR reaction. Components supplied with the Fastart kit are indicated by a vial number.

Component	Volume	Final concentration
PCR grade H_2O (vial 1)	10.8 µl	-
$MgCl_2$ (vial 2)	3.2 µl	5 mM
Forward primer	1 µl	0.5 µM for 5αR-1 and 0.25 µM for 5αR-2
Reverse Primer	1 µl	0.5 µM for 5αR-1 and 0.25 µM for 5αR-2
SYBR Green master mix (vial 3)	2 µl	-
	Total Volume = 18 µl	

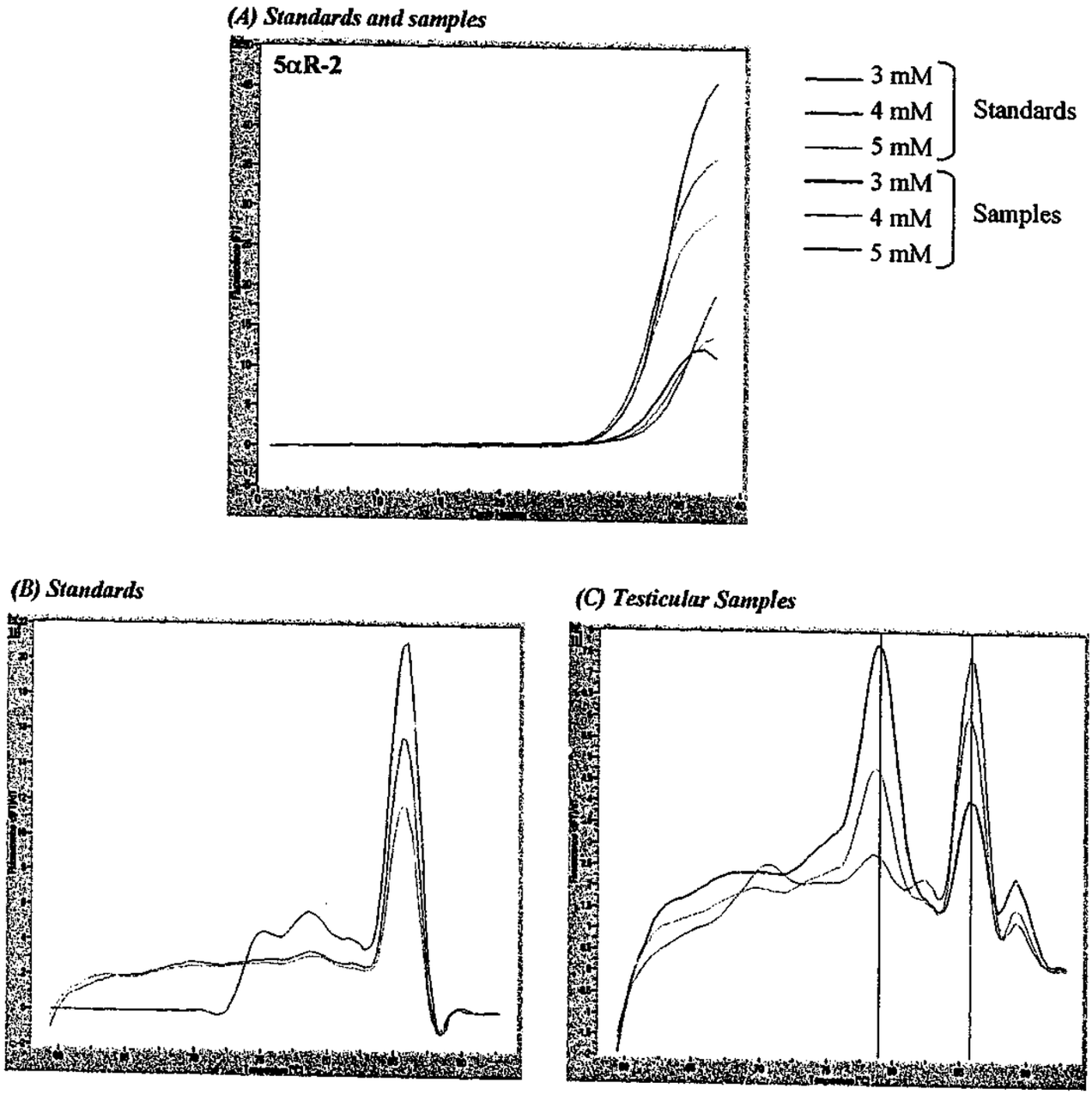


Figure 4-3: Effect of different magnesium concentrations (3, 4 and 5 mM) on PCR products using real time PCR. *(A)* Quantification curves of 5αR-2 mRNA standards and testicular samples, and melting curve analysis of the PCR products for *(B)* standards and *(C)* testicular samples.

C) Melting Curve Analysis

Figure 4-4 shows the melting curve analysis for 5αR-1 *(A)* and 5αR-2 *(B)*. These melting curves clearly indicate that at high concentrations of specific template alone, such as the standards, there was only a single T_m that corresponded to the specific product. The T_m of the PCR product was 87°C for 5αR-1 *(Figure 4-4[A])* and 85°C for 5αR-2 *(Figure 4-4[B])*. However, at lower concentrations of template or with no template (i.e. blank), there was a broader peak at a lower T_m (~80°C) which corresponded to non-specific products, probably primer dimers.

Thus, the LightCycler software was used to include an additional step in each PCR cycle, so that the temperature was increased to 84°C for 5αR-1 and 83°C for 5αR-2, before fluorescence measurement. Thus, by measuring at these elevated temperatures instead of at the elongation temperature (60°C for 5αR-2, 55°C for 5αR-1), sensitivity and specificity were increased because the contribution of signal derived from by-products was not included. *Table 2* describes the conditions for all three programs of the PCR reaction using the LightCycler.

Table 2: The protocol for the denaturation, amplification and melting curve analysis for real time PCR.

PCR Step	Conditions	
	5αR-1	5αR-2
Initial denaturation of template DNA	95°C for 10 min	95°C for 10 min
Amplification of target DNA	95°C for 15 sec 55°C for 5 sec 72°C for 10 sec	95°C for 15 sec 60°C for 5 sec 72°C for 10 sec
Melting curve analysis	84°C	83°C

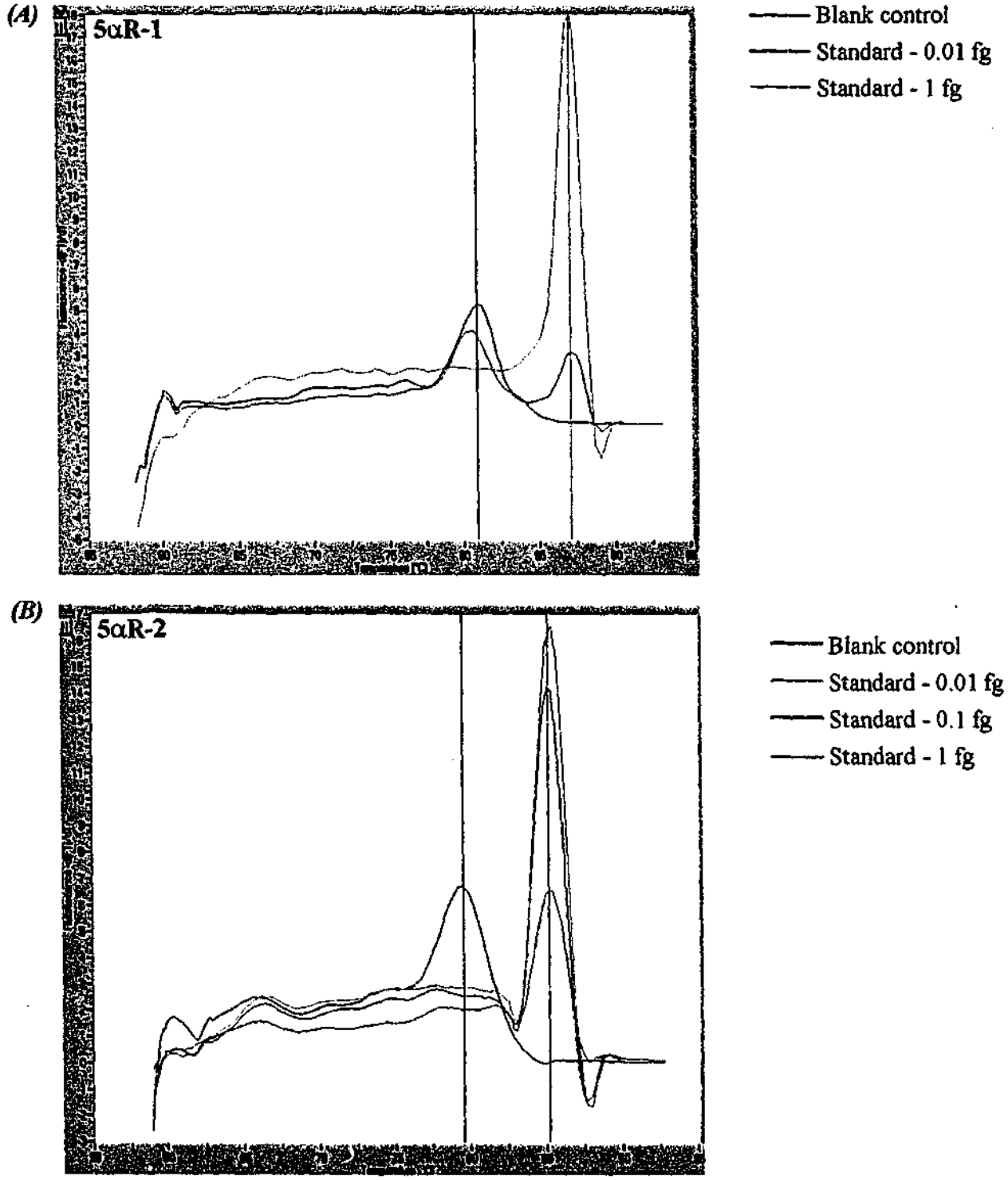


Figure 4-4: Melting curve analysis of a *(A)* 128 bp 5αR-1 fragment and a *(B)* 207 bp 5αR-2 fragment. The melting peaks clearly indicate a single peak at 87°C for 5αR-1 and 85°C for 5αR-2. However, at low template concentration or in the presence of no template (blank control) there is a second peak at a lower T_m (-80°C), which probably corresponds to primers dimers.

D) Standard Curves and Quality Control Samples

Figure 4-5(A) shows a standard curve for 5αR-1 from 100 pg to 10 fg in 10-fold dilutions, each reaction performed in triplicate. Testicular samples are also shown in this figure. The linear regression for the standard curve with the corresponding values for the slope of the regression, the intercept, error, and the coefficient regression values are shown below the amplification plots. *Figure 4-5(A)* demonstrates that the triplicate values are almost identical and that the standard is diluting out in parallel to yield a linear standard curve with minimal error. This was confirmed by a subsequent experiment *(Figure 4-5[(B])* showing reproducible values for the standards when performed in singlicate measurements.

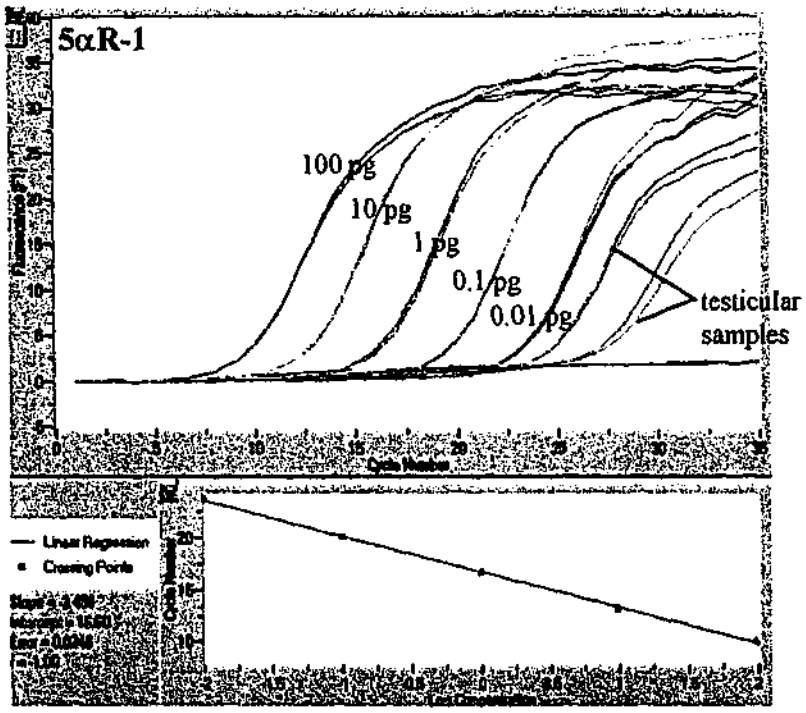
The two testicular samples in *Figure 4-5(A)* were outside the range of the standards. Therefore, the standards were diluted accordingly to be within the expected range of the samples. Furthermore, since we did not expect large differences in mRNA levels between the animals that were going to be examined in subsequent chapters (6 and 7), we decided to construct a standard curve in two-fold dilutions rather than ten-fold dilutions. The final standard curve consisted of five standard doses ranging from 1 fg to 0.0625 fg, in 2-fold dilutions *(Figure 4-6[A])*.

The LightCycler program has two different modes of quantitation, 'second derivative maximum' and 'fit points'. The standard curve quantified using the 'second derivative maximum' method is shown in *(Figure 4-6[A])*, and the standard curve quantified using the 'fit points' method is shown in *(Figure 4-6[B])*. These methods of quantification will be discussed later in section H.

Similar experiments were also performed for 5αR-2. *Figure 4-7* shows the five chosen doses for the 5αR-2 standard curve (0.2 - 0.0125 fg) in two-fold dilutions. Shown in duplicate measurements are the QC samples and 12 unknown samples, quantified using the 'second derivative maximum' *Figure 4-7 [A]* and 'fit points' *Figure 4-7[B]*. Samples were assayed (QC and unknown) in duplicate to ensure accurate quantification of unknown samples.

The QCs used to measure inter-assay variation for PCR amplification by the LightCycler are shown in *Table 3*.

(A)



(B)

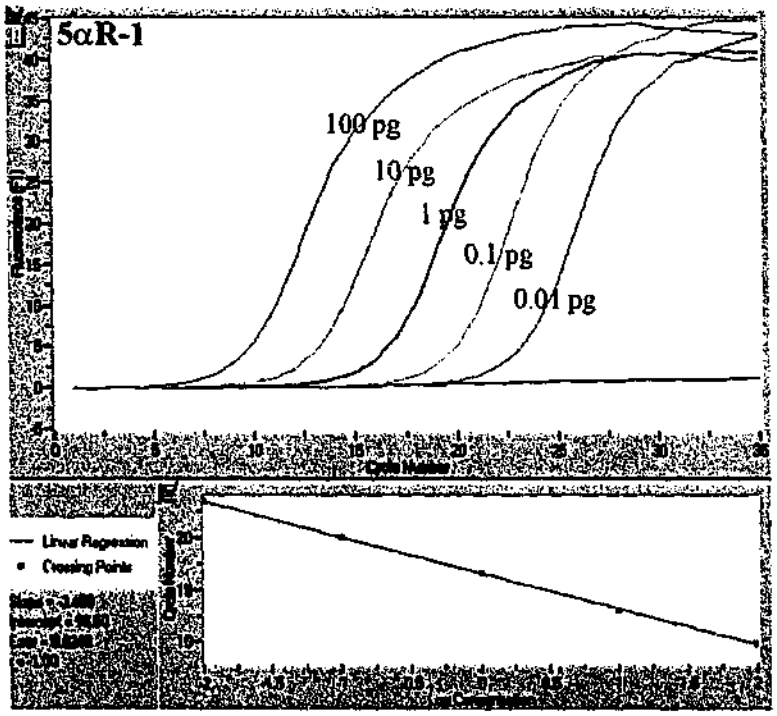


Figure 4-5: Standard curve (100 pg to 10 fg) for 5αR-1. Amplification plots and linear regression for 5αR-1 standard curve assayed *(A)* in triplicate or *(B)* in singlicates. Testicular samples are included in *(A)*.

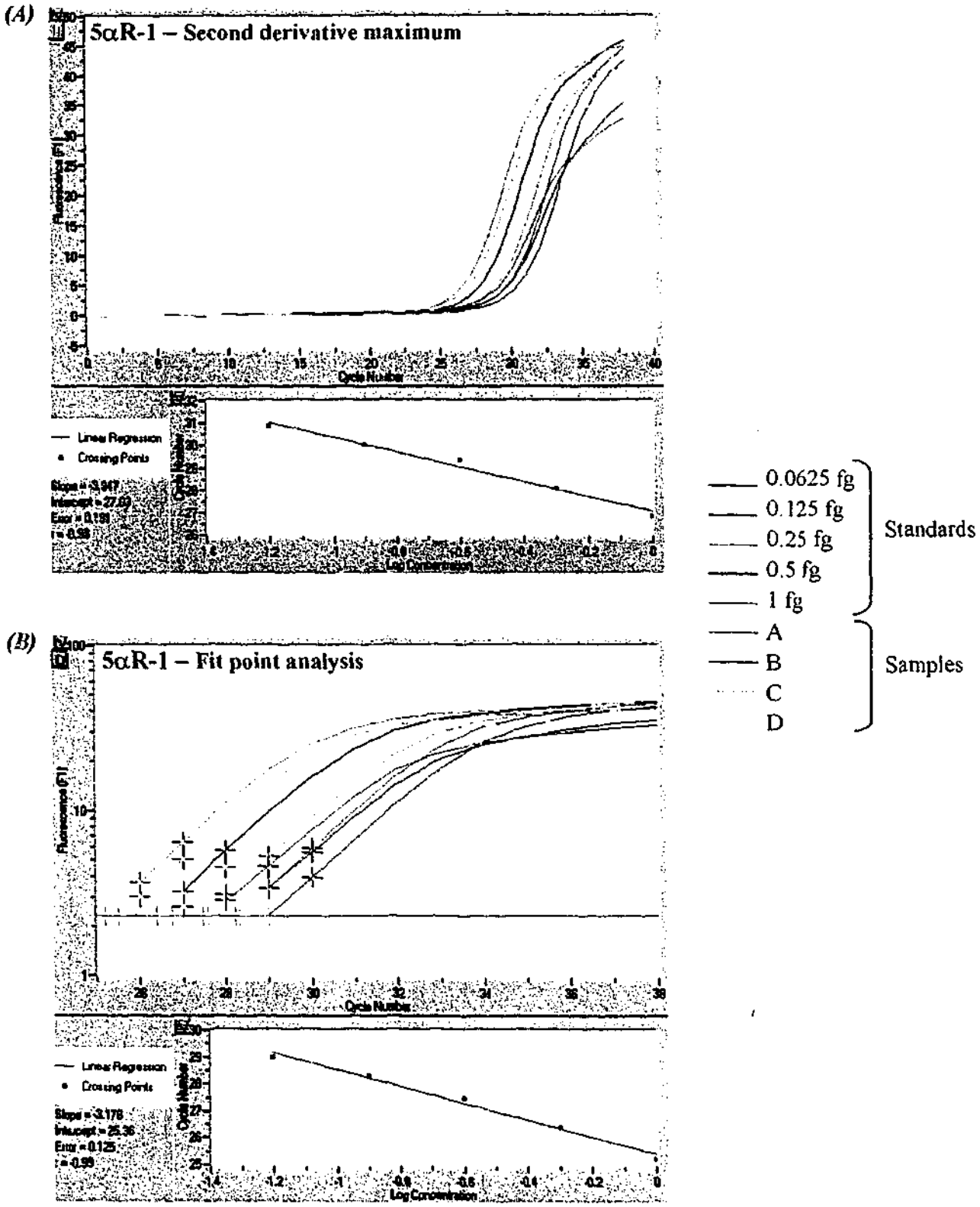
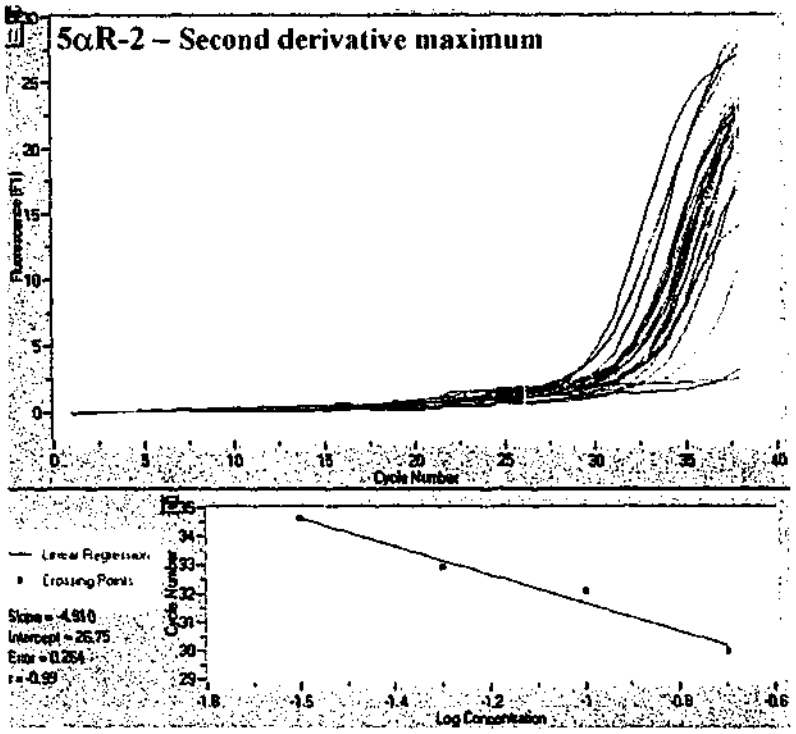


Figure 4-6: Standard curve for 5αR-1. Amplification plots and linear regression for 5αR-1 standard curve (1 fg to 0.0625 fg) and testicular samples assayed in singlicates, quantified using *(A)* 'second derivative maximum' and *(B)* 'fit points' analysis.

(A)



(B)

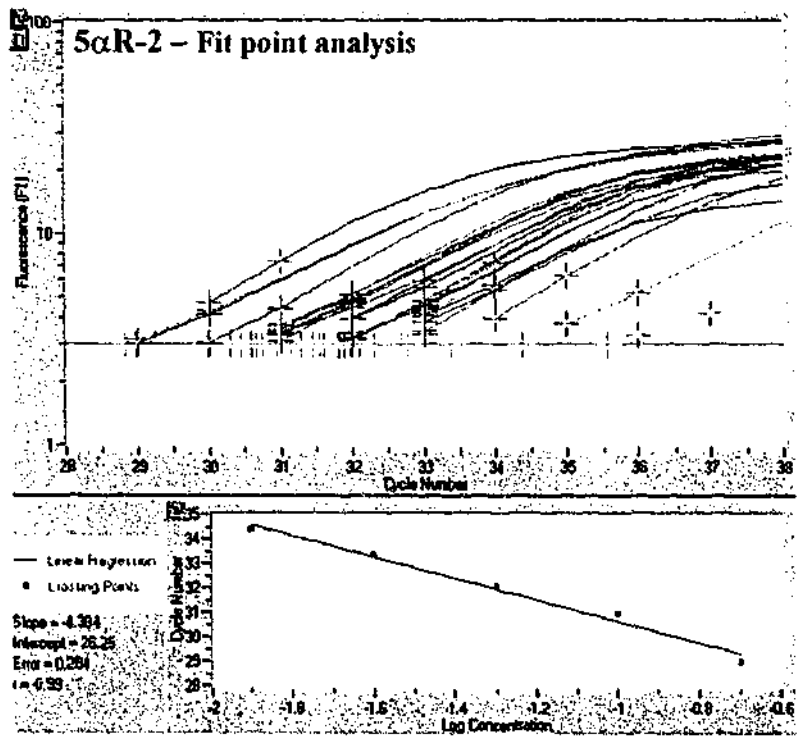


Figure 4-7: Standard curve for 5αR-2. Amplification plots and linear regression for 5αR-2 standard curve (0.2 fg to 0.0125 fg) assayed in singlicates, with blank control, QC samples and unknown samples, quantified using *(A)* 'second derivative maximum' and *(B)* 'fit points' analysis.

Table 3: Summary (mean, SD and coefficient of variation) of values for quality control (QC) samples, slope of the linear regression and intercept, for 5αR-1 and 5αR-2 mRNA measurement using the LightCycler (n = 8 assays). QC measurements are represented as fg 5αR mRNA, relative to the standards.

	QC (1:10)	QC (1:20)	Slope	Intercept
Mean	0.232	[illegible]	-3.44	[illegible]
SD	0.018	[illegible]	0.19	[illegible]
CV	7.72%	[illegible]	5.50%	[illegible]
Mean	0.065	[illegible]	-3.657	[illegible]
SD	0.001	[illegible]	0.474	[illegible]
CV	7.32%	[illegible]	12.96%	[illegible]

E) *Unknown Samples*

Once the appropriate standards and QCs were constructed, the amount of sample required to yield the optimal amplification and melting curve analysis was assessed. Theoretically, the amplification curves and the melting curve for all standards and samples should be identical, assuming the same product is formed. *Figure 4-8* shows the *(A)* amplification curves and *(B)* melting curve analysis for a 5αR-1 standard curve (2 fg and/or 1 fg standards), blank control (described in section E below), and various testicular samples at 1:10 dilution of the reverse transcription reaction. Whereas the melting curves for both standards and samples are identical with a single peak at 87°C *(A)*, the amplification curves for the samples did not show the same parallelism as those for the standards *(B)*, implying the samples were not amplified with the same efficiency as the standards.

The difference in amplification curves for standards and samples may have been due to inhibitory substances in the RNA that affects amplification efficiency. To avoid carry-over of any possible inhibitory substances that may be present in the reverse-transcription reactions, we used

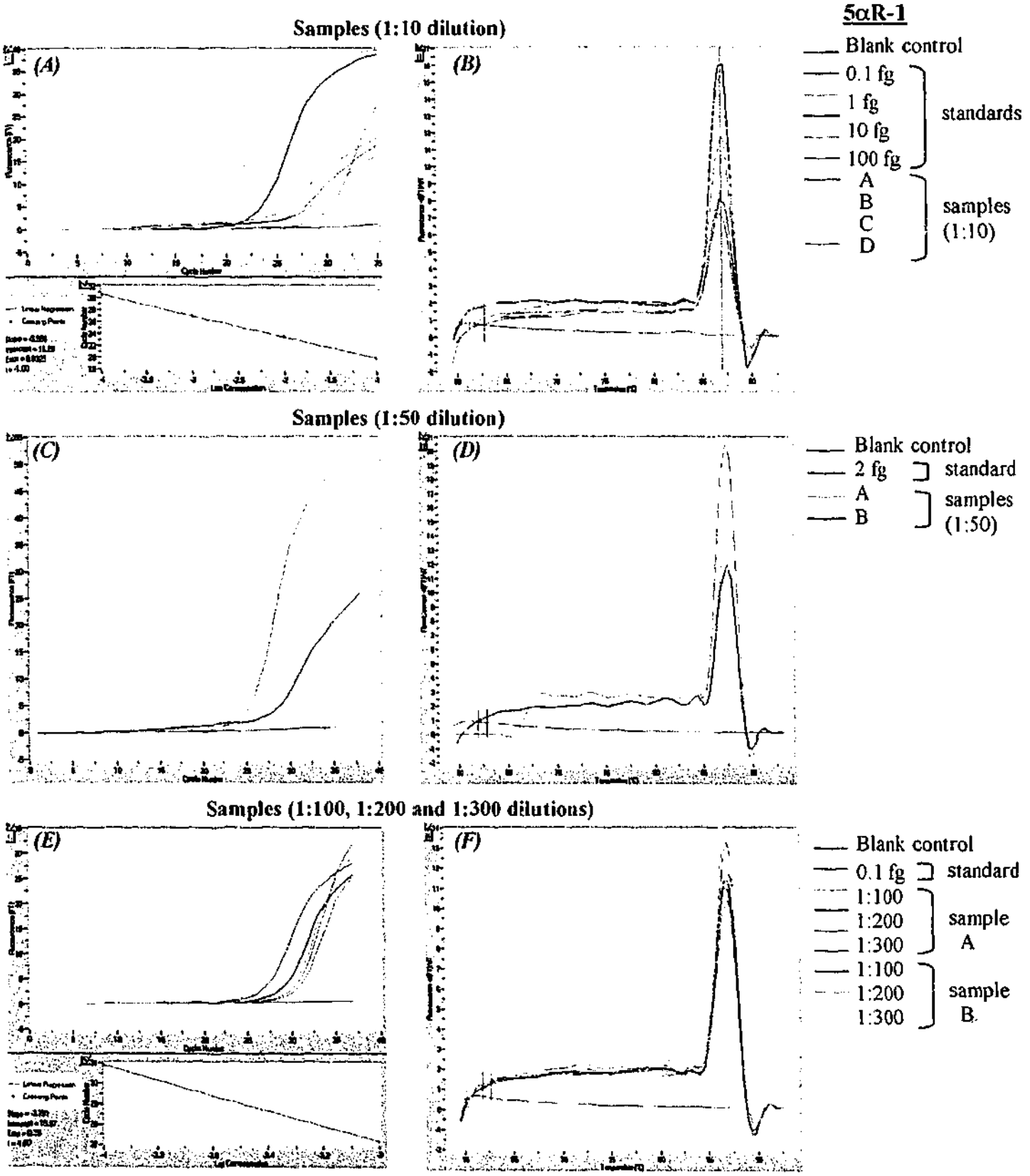


Figure 4-8: Comparison of amplification plots and melting curve analysis for 5αR-1 between standards/QC samples vs unknown samples, using 1:10 dilution *(A and B)* of the reverse transcription reaction or (1:50; *C and D*) and (1:100, 1:200 and 1:300; *E and F*) dilutions of the reverse transcription reaction.

less of the reverse-transcription reaction for PCR. *Figure 4-8* shows the amplification curves *[C]* and melting curve analysis *[D]* for a 5αR-1 standard, blank control, and two samples assayed at 1:50 dilution of reverse transcription reaction. By diluting the initial cDNA concentrations, parallelism of the amplification curves were improved, but were still different to those for the standard.

Figure 4-8 shows the amplification curves *(E)* and melting curves *(F)* for QCs a two dilutions, blank control, and two samples assayed at 1:100, 1:200 and 1:300 dilutions of the reverse transcription reactions. The amplification curves clearly show that by diluting the samples sufficiently, the amplification curves for testicular samples amplified in the same way as the standards. These experiments were repeated for 5αR-2, and it was demonstrated that using either a 1:50 or 1:100 dilution of the reverse transcription reaction yielded identical amplification curves for samples to those of 5αR-2 standards (data not shown). Thus, all samples were assayed at a 1:100 dilution of the reverse transcription reaction, in duplicate.

F) ***Negative Control Samples***

Blank control samples for three separate PCR amplifications are shown in *Figures 4-8 (in yellow)*. The amplification curves *(A, C, and E)* demonstrate that there is no amplification in the blank controls as indicated by the flat amplification plot, compared to samples and standards which were amplified exponentially. Furthermore, there was no T_m peaks on the melting curves for the blank controls *(B, D, and F)* indicating that there was no product formed.

Figure 4-9 shows the melting curve analysis for 5αR-1 standards and blank control, and samples reverse transcribed in the *(A)* presence or *(B)* absence (i.e. –ve control) of the reverse transcriptase enzyme (Expand). The melting curves show that samples reverse transcribed with Expand *(A)* showed a single T_m peak corresponding to 5αR-1, similar to the standards. In contrast, -ve control samples *(B)* showed a lower T_m peak, probably corresponding to primer dimers, similar

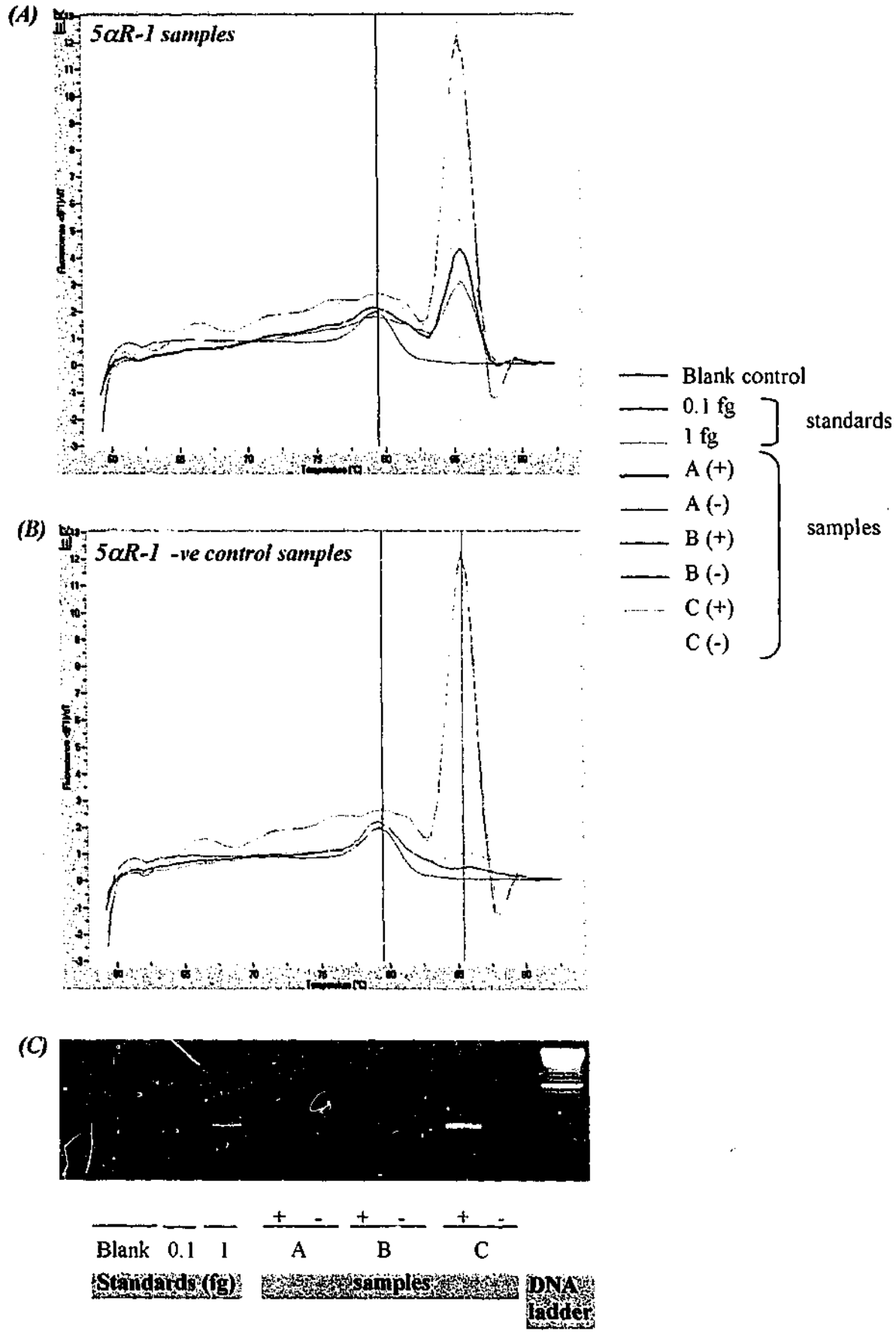


Figure 4-9: Melting curve analysis of standards, blank and unknown samples for 5αR-1. Unknown samples reverse transcribed with *(A)* 2 μg RNA *[indicated by +]* or *(B)* no RNA (i.e. -ve controls, *[indicated by -]*), and *(C)* an agarose gel of final PCR products.

to the blank control. Note, standards are not reverse transcribed and thus a melting curve is always seen for the standards, as in *Figure 4-9(B)*.

Melting curve analysis is not quantitative, therefore the height of the peak does not represent amount of product. The size of the product was assessed by agarose gel analysis. The agarose gel of the PCR products in *Figure 4-9 (A and B)* are shown in *Figure 4-9(C)*. The gel clearly shows that there is a 207 bp band for the standards and for the samples that were reverse transcribed with the enzyme Expand. In contrast, there was no band for the blank control or for the –ve control samples.

The melting curves for three samples and their corresponding –ve controls for 5αR-2 are shown in *Figure 4-10*. There is a predominant T_m peak corresponding to 5αR-2 for samples reverse transcribed with the Expand enzyme *(A)*. In contrast, the –ve controls displayed two broad T_ms, corresponding to primer dimers and 5αR-2 *(B)*. The agarose gel of these PCR products are shown in *Figure 4-10(C)*. A 128 bp 5αR-2 fragment from the standards and from samples reverse transcribed with the Expand enzyme is clearly visible on the gel, whereas there was no band for the blank and for –ve control samples.

The average percent backgrounds determined using these negative controls were 2.89 ± 0.59 for 5αR-1 (mean ± SEM, n = 17 samples) and 1.15 ± 0.76 for 5αR-2 (mean ± SEM, n = 10 samples). Thus, there was less than 3% contamination during the reverse transcription reaction, and was too low to interfere with quantification during PCR with the LightCycler.

G) *Method Of Quantitation and Normalization of Data*

The standard curve and unknown samples for 5αR-1 and 5αR-2 using the 'second derivative maximum' method are represented in *Figures 4-6 (A)* and *Figures 4-7 (A)*, respectively. The 5αR-1 standards and samples appear to be amplified with the same efficiency to provide a linear standard curve with minimal error. However, the standards and unknown samples for 5αR-2 do

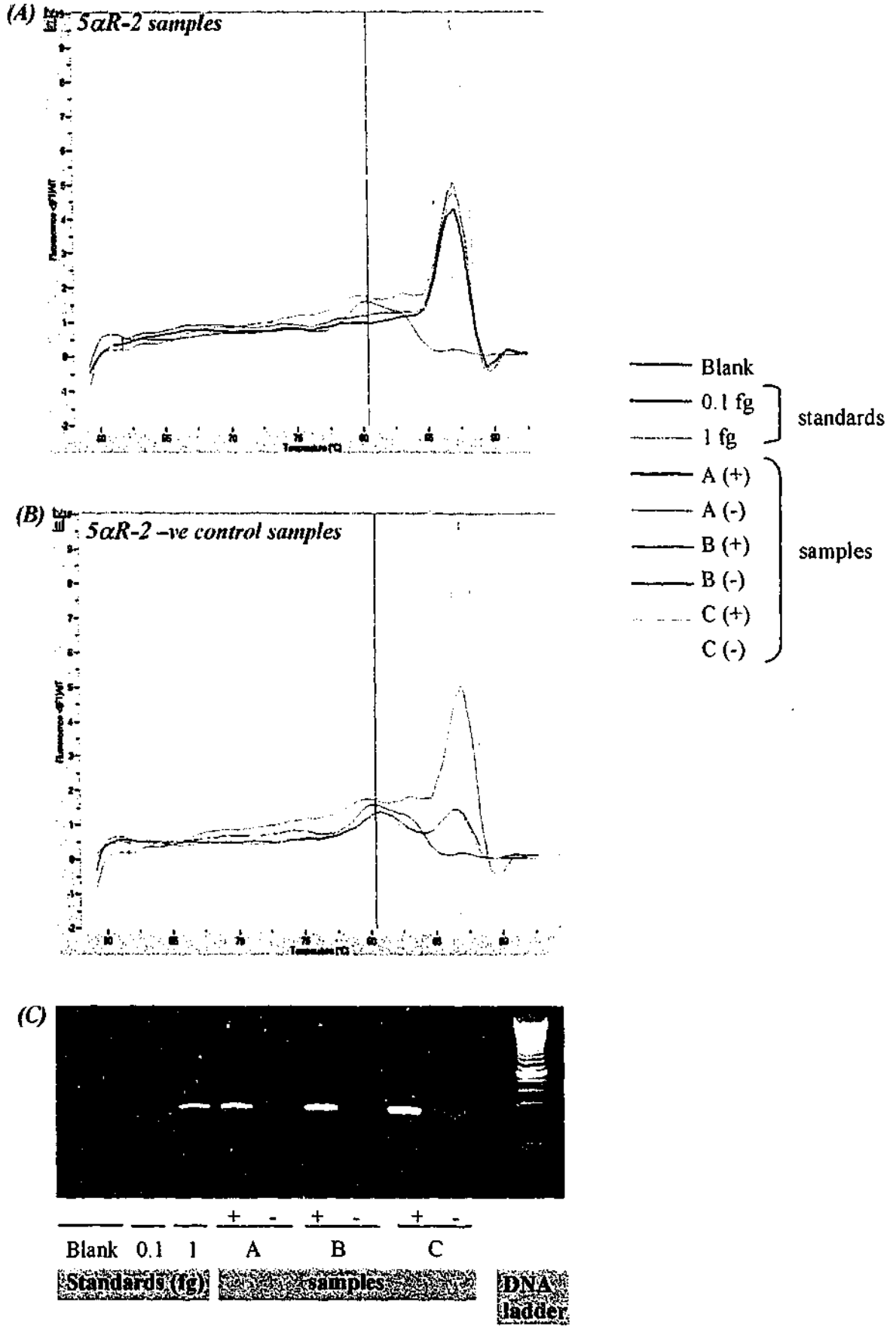


Figure 4-10: Melting curve analysis of standards, blank and unknown samples for 5αR-2. Unknown samples were reverse transcribed using either *(A)* 2 μg RNA *[indicated by +]* or *(B)* no RNA (i.e. negative controls, *[indicated by -]*) and *(C)* an agarose gel of final PCR products.

not appear to amplify with the same efficiency initially. Furthermore, one of the standards (0.0125 fg) did not even register a concentration reading, reducing the standard curve to 4 standards points.

The standard curve and unknown samples for 5αR-1 and 5αR-2 using the 'fit points' method are represented in *Figures 4-6 (B)* and *Figures 4-7 (B)*, respectively. These figures show that by setting the noise band manually, the amplification plots for all standards and samples are restricted to the log-linear phase, so that the amplification curves are similar between all samples and standards. In the case of the 5αR-2 standard curve all five standards are now included, whereas previously the lowest concentration of standard (0.0125 fg) could not be detected. Therefore, all samples were quantified using the 'fit points' procedure.

We normalized the LightCycler data by relating mRNA levels to total RNA concentration, that was measured accurately using a spectrophotometer (Bustin, 2000). The mRNA level was recorded as copy number per μg total RNA. Thus, the accuracy of the quantification was critically dependent on the accuracy of RNA measurement. The amount of poly(A) RNA recovered using the RNeasy extraction kit in our studies was sufficient to allow quantitation of RNA by spectrophotometry and fluorescent stains, which was used to determine that equal amounts of sample were analyzed, and thus was used as a housekeeping gene. Thus, mRNA data was corrected by normalizing 5αR-1 and 5αR-2 mRNA to 28S ribosomal RNA.

4.4 Discussion

This chapter outlines the validation and optimization of the real time PCR approach for measuring 5αR-1 and 5αR-2 gene expression levels in rat testis. These studies illustrate the advantages of the real time PCR approach to gene expression quantitation, and the ability to quantitate small amounts of mRNA in short periods of time.

PCR is a highly sensitive and specific method for detection of nucleic acids. However, quantification with conventional PCR is restricted by tedious protocols, which do not guarantee

measurement during the log-linear phase of a PCR. Advances in fluorescence technologies now enable a fast quantitative PCR approach which detects the amplification of nucleic acids in time, ensuring quantitation occurs during the log-linear phase. The LightCycler system consists of hardware components, the LightCycler instrument itself and a PC workstation, as well as software, consumables and reagents. Fluorescent technologies combined with rapid thermal cycling allow amplification and analysis in short periods of time by providing amplification and detection in the same tube while analyzing samples in real time while amplification is still in progress. Furthermore, this method allows you to study the target sequence of interest by looking at the melting behavior of the amplified product. The rapid microvolume amplification of the target sequence shortens the overall time to obtain results from hours to less than 40 min. The advantages of the LightCycler are: multiple reactions can be run simultaneously, the methodology is sensitive and therefore requires minimal sample, does not require postreaction manipulation, and is able to discriminate between two amplicons using the melting curve analysis.

In this study, RNA was extracted and reverse transcribed in a conventional thermocycler. Two different primers were used for reverse transcription: (A) 5αR-2 was primed with Oligo $(dT)_{12\text{-}18}$, which binds to endogenous poly$(A)^+$ tail at the 3'end of mammalian mRNA, and (B) 5αR-2 was reverse transcribed with random primers, which binds to the RNA templates at any complementary site. These primers were found to yield the best conditions for reverse transcription. Once the cDNA was formed, amplification of DNA by PCR was performed using the LightCycler.

An advantage of the LightCycler is that it permits differentiation of the signals coming from specific product from the signals coming from primer dimers, because the T_m of the by-products will be lower than that for the standards. This was performed via the melting curve analysis program. Melting curve analysis showed that 5αR-1 and 5αR-2 were denatured at 84°C and 83°C, respectively, whereas primer dimers were denatured at ~80°C. Thus, the LightCycler software was set so that fluorescence was acquired at a temperature above the melting point of the primer dimers, increasing the sensitivity and the accuracy of measurement.

PCR using the LightCycler instrument is critically dependent on the concentration of $MgCl_2$. A concentration of 5 mM $MgCl_2$ was shown to yield the best amplification of standards and samples, for both 5αR-1 and 5αR-2 primers. Optimal concentration of primers were found to be 0.5 mM and 0.25 mM for 5αR-1 and 5αR-2, respectively. These primers were designed to produce a fragment of 207 bp for 5αR-1 and 128 bp fragment for 5αR-2. Further experiments showed that samples were required to be diluted (1:100) to obtain similar amplification curves to those for the standards. This is critical because precise quantitation requires that the efficiency of amplification for all standards and unknown samples are identical.

Negative controls were used to monitor for genomic DNA contamination in the extracted RNA, which could potentially interfere with DNA amplification during PCR. Average percentage contaminations were 2.9 ± 0.69 and 1.2 ± 0.8 for 5αR-1 and 5αR-2, respectively. Thus, the negative controls (reactions performed in the absence of reverse transcriptase enzyme) revealed that RNA samples contained insignificant amounts of contaminating residual genomic DNA.

PCR products were used to construct standard curves for 5αR-1 and 5αR-2. These standards were performed in singlicates, in two-fold dilutions from 1 - 0.0625 fg for 5αR-1 and 0.2 - 0.0125 fg for 5αR-2. Each standard curve also included a blank, whereby the DNA template was omitted. This blank demonstrated that there was no non-specific amplification. Quality control samples were amplified simultaneously with the standard and unknown samples to measure inter-assay variation. The QC's were assayed at two dilutions, and were 0.232 ± 0.018 fg and 0.127 ± 0.013 fg for 5αR-1, and 0.065 ± 0.001 and 0.033 ± 0.004 fg for 5αR-2. The beginning of the log-linear portion of the amplification reaction, defined by the noise band, which was set manually by selecting the 'fit points' method for quantitation.

In summary, we have chosen to measure 5αR-1 and 5αR-2 mRNA by PCR in real time, using the LightCycler instrument. On-line monitoring of PCR allowed visual inspection of amplification and the efficiency of amplification in all samples. Subsequent analysis using the melting curve program allowed the specific products and the non-specific products, such as primer

dimers, to be visualized. Therefore, the PCR conditions were set so that quantitation did not begin until the specific product was formed, so that primer dimers were not included during quantitiation. This PCR approach was used to measure 5αR-1 and 5αR-2 mRNA levels in rats treated with testosterone and/or FSH in chapters 6 and 7.

Table 4: A summary of the PCR conditions used for measuring 5αR Type 1 (5αR-1) and Type 2 (5αR-2) in the LightCycler system.

PCR Step	Temperature (°C)	Time (sec)
Denaturation	95	600
Amplification (45 cycles)	95	0
	55 (5αR-1) and 60 (5αR-2)	
	72	60
Melting curve analysis	84 (5αR-1) and 83 (5αR-2)	
	95	0
	58 (5αR-1) and 60 (5αR-2)	0
	95	0

Chapter 5

Measurement of 5α-Reductase Type 1 and Type 2 Enzyme Activity in Rat Testis

5.1 Introduction

Despite numerous investigations, the type of 5αR isoform expressed in rat testis remains controversial (see section 1.6). This is in part due to the fact that the majority of the studies investigating testicular 5αR activity were performed before the second 5αR isoform, termed 5αR-2, was cloned. To date, testicular 5αR-2 activity in the rat has not been investigated.

The majority of early studies investigated 5αR activity in the testis at pH 7.0 with micromolar concentrations of substrate, and therefore this activity can be considered to reflect only 5αR-1 activity. These studies (see section 1.6.1) showed that whereas the location of 5αR-1 in the testis was controversial, its expression with age was consistent amongst all studies. In general, these studies showed that 5αR-1 enzyme activity was low at birth, increased to reach peak expression at ~day 30, then declined to reach almost undetectable levels by adulthood (>90 days). This developmental regulation of 5αR-1 activity was similar to the expression of mRNA encoding 5αR-1. Viger and Robaire (1995) showed that 5αR-1 mRNA increased by ~3.5-fold from day 7 to days 21-28 d, then declined ~3-fold by day 91. However, unlike enzyme activity, which decreased to almost undetectable levels during adulthood, mRNA levels remained at 30 - 40% of peak expression.

As mentioned previously, 5αR-2 enzyme activity in the testis has not yet been investigated. Recently however, two studies used Northern blot analysis to examine the level of 5αR-2 mRNA and its localization. Normington and Russell (1992) isolated RNA from 7-week old rats and showed that 5αR-2 was the predominant isoform in the testis. In contrast, Viger and Robaire (1995) showed that 5αR-2 mRNA was barely detectable in rat testis between days 7 to 91, whereas 5αR-1 mRNA was detectable at all ages examined. Thus, the relative expression of 5αR-2 mRNA in rat testis remains unresolved.

As described in chapter 3, different pHs can be used to measure the 5αR isoforms. The activity at pH 7.0 reflects the level of 5αR-1 isoform. However, 5αR-2 can not be measured with the activity at pH 5.0 due to the overlap of 5αR-1 at pH 5.0. Therefore, we have developed an

approach that will allow the quantitation of 5αR-2 activity in the presence of 5αR-1, so that the activities of the 5αR isoforms can be distinguished based on pH optima (see chapter 3). Similarly, the 5αR isoforms can be distinguished by their affinities for steroid substrates since 5αR-1 has a micromolar affinity (i.e. K_m value) and 5αR-2 has a nanomolar affinity for steroid substrates. Thus, these enzyme kinetic methods are useful for determining which 5αR isoforms are expressed in tissues.

The current study was undertaken to establish which testicular 5αR isoform(s) are expressed in the rat. Therefore, to investigate 5αR isoforms in rat testis at days 35 and 75, the activity of 5αR-1 and 5αR-2 were examined using enzyme kinetics, pH profiles, and our formula (using recombinant rat 5αR) used to quantitate 5αR-2 activity.

5.2 Materials and Methods

5.2.1 Animals

All animals used in the subsequent experiments were male Sprague Dawley rats. They were obtained from the Monash University Animal House and maintained at 20°C in a fixed 12h light/ 12 h day cycle with free access to food and water. All studies were approved by the Monash Medical Centre Animal Ethics committee. The body weights of rats at days 30, 75, and 147 were 110, 370, and 500 g, respectively, with testicular weights of ~0.4, ~1.7, and ~2 g, respectively.

5.2.2 Experimental Procedures

Animals were euthanised by CO_2 inhalation, the testes were removed and testicular supernatants prepared, and homogenates for epididymal and liver were prepared (see section 2.1.3 b and c). Protein levels were determined using the BCA method (Smith *et al.*, 1985) against a standard of bovine serum albumin (Pierce❶, 2mg/ ml, No. 23209). The assay was modified for microtitre plates and had a sensitivity of 5μg per well (see section 3.3.4).

5.2.3 pH profiles for 5α-Reductase in Testis

To ensure that the *in vitro* activity assay was functioning under steady state conditions, experiments were conducted to determine the effect of enzyme concentration and time on 5αR activity. Once these conditions were set (i.e. linear reactions), 5αR activity was measured under these optimized incubation conditions for all tissues.

The activity of the 5αR isoforms were investigated at different pHs, using Tris-Citrate buffer from pH 5.0 to 9.5, at 0.5 increments. For comparisons and representative examples of predominantly 5αR-1 and 5αR-2 expression, pH profiles for liver and epididymis, respectively, were also investigated. The pH profile for rat testis was analyzed at days 30 and 75, and in adult (day 75) epididymis and liver. All components of the *in vitro* 5αR enzyme activity assay were identical for all tissues and as described in chapter 3, and 5αR activity was expressed as μmoles DHT + 3α-Adiol/ min/ mg protein.

5.2.4 Enzyme Kinetics for the Detection of Multiple 5α-Reductase Isoforms

We investigated the kinetic characteristics of the 5αR isoforms in the testis, and also in liver, epididymis, and recombinant 5αR isoforms. As the substrate concentration should ideally be in the range of the K_m (Segel, 1975), samples were incubated with a wide range of substrate concentrations (1.9 nM to 9.5 μM testosterone) under standard enzyme kinetic conditions at pH 7.0 at 37°C (20 min for liver and epididymal homogenates, and 60 min for testicular supernatants and recombinant isoforms), as described in section 3.2.7. This technique has been used to detect both 5αR isoforms in rat prostate (Span *et al.*, 1995), rat epididymis (Span *et al.*, 1996a), and benign prostatic hyperplastic tissue (Span *et al.*, 1996c). Thus, enzyme kinetics was used to determine which 5αR isoforms were expressed in the testis.

5.2.5 Measurement of 5α-Reductase Isoform Activity in Testis

The distinct pH optimas for the 5αR isoforms allows the activity of each enzyme to be assessed independently of the other. Therefore, measuring the activity at pH 7.0 indicates the level of 5αR-1 activity. However, due to the overlap of 5αR-1 activity at pH 5.0, the activity at pH 5.0 alone can not be used to indicate 5αR-2 enzyme activity. Thus, 5αR isoform activity was measured using the method that was described and validated in chapter 3.

5.3 Results

pH Profiles

Figure 5-1[A] showed a linear enzyme reaction up to 60 min at pH 7.0 using immature (day 30) testicular (10,000 g) supernatant, and a linear reaction up to 80 min at pH 7.0 using adult (day 128) testicular (10,000 g) supernatant (*Figure 5-1[B]*). In both these preparations, testes were homogenized in 1 ml 0.25 M sucrose, centrifuged at 9,1000 rpm, and a 100 µl aliquot of the supernatant used for 5αR activity assays. *Figure 5-1[C]* shows 5αR enzyme activity using immature testicular supernatant at different dilutions and at different time periods. This graph shows that with a 10 µl (0.3 mg protein) sample, a linear reaction was obtained regardless of incubation time. However at higher concentrations (50 and 100 µl, 0.6 and 1.2 mg protein) the time and enzyme concentration-dependent reaction was only linear up to a 30 min incubation time (i.e. >18% conversion), after which 5αR activity did not increase linearly. Thus, all subsequent experiments used a 60 min reaction time with a sample concentration yielding less than 18% conversion of substrate. Similar experiments were also performed for epididymis and liver *(see Appendix B and C)*.

The activity of 5αR across a range of pHs should give an indication of which 5αR isoforms are expressed and their level of expression. 5αR activity as a function of pH was first examined in the liver and epididymis, which are known to express predominantly 5αR-1 and 5αR-2 enzyme

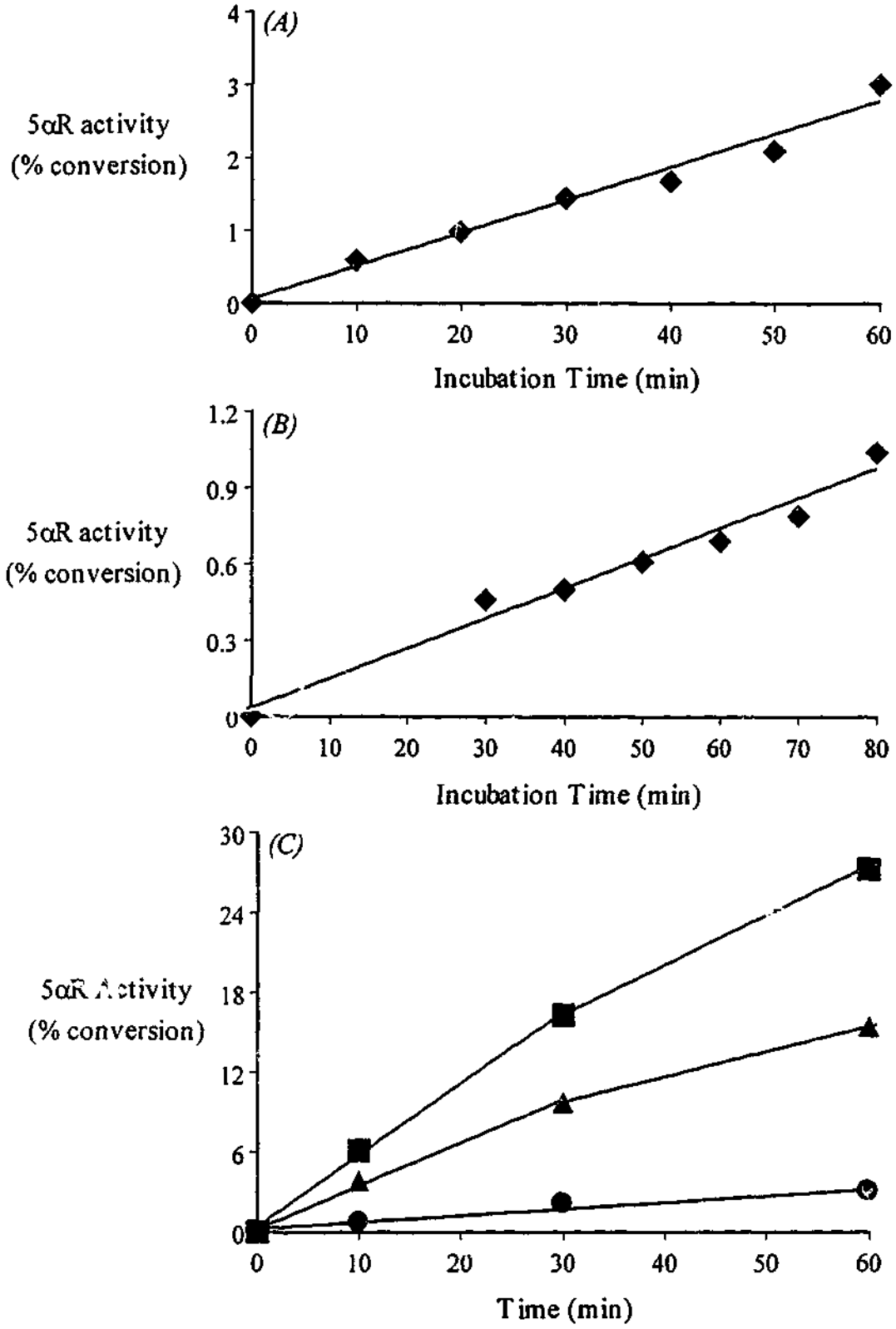


Figure 5-1: 5α-Reductase activity at pH 7.0 in *(A)* immature (day 35) and (B) adult (day 75) testicular (10,000 g) supernatants for different time periods. *(C)* Immature (day 30) rat testicular supernatant assayed using different amounts of sample preparation for different time periods. Enzyme activity is expressed as percent conversion of ^{3}H-testosterone after subtracting backgrounds. Values are the average of duplicate measurements.

activity and mRNA, respectively. There was a peak of 5αR activity at pH 5.0 for the epididymis *(Figure 5-2[A])* which is indicative of 5αR-2, with lower levels of activity in the neutral pH range which corresponds to 5αR-1. In contrast, the liver *(Figure 5-2[A])* showed 5αR activity predominantly in the neutral range (5.5 - 8.0).

The dependency of 5αR was examined in the testis at two ages to determine testicular 5αR isoform expression. *Figure 5-2[B]* represents 5αR activity as a function of pH for immature (30 d) and adult (day 75) rat testis. Regardless of age, testicular 5αR activity predominated across a broad neutral pH range (5.5 - 8.0), similar to that seen in the liver. However, the activity at pH 5.0 when compared to the corresponding activity at pH 7.0 was different for the two ages, such that the level of pH 5.0 activity was approximately 2-fold higher at day 30 compared to day 75. Note the different y-axes used to represent the activity in the testis, such that the level of pH 7.0 activity at day 30 was approximately 40 times greater than at day 75.

Enzyme Kinetics

Enzyme activity for 5αR was established at pH 7.0 with 15 different substrate concentrations, ranging from 0.19 nM to 9.5 μM. The Michaelis-Menten plot for adult male rat liver is shown in *Figure 5-3[A]*, and the double reciprocal plot (i.e. Lineweaver-Burk) of these data appeared to be linear *(Figure 5-3[B])*. However, the Eadie-Scatchard plot of V/S against, which gives a more even weighting of points, was clearly non-linear *(Figure 5-3[C])*. These graphs are examples of representative experiments with duplicate measurements. The average enzyme characteristics from three separate experiments in duplicate are shown in *Table 1*. The affinity constants of the subtype activities calculated were characteristic for the already established 5αR isoforms: the average K_m was 2.41 ± 0.66 μM for 5αR-1, and 0.11 ± 0.03 μM for 5αR-2. The average V_{max} value of 5αR-1 was ~22-fold higher than that of 5αR-2 (104 ± 21.6 vs 4.74 ± 0.86 fmol/ min/ mg protein).

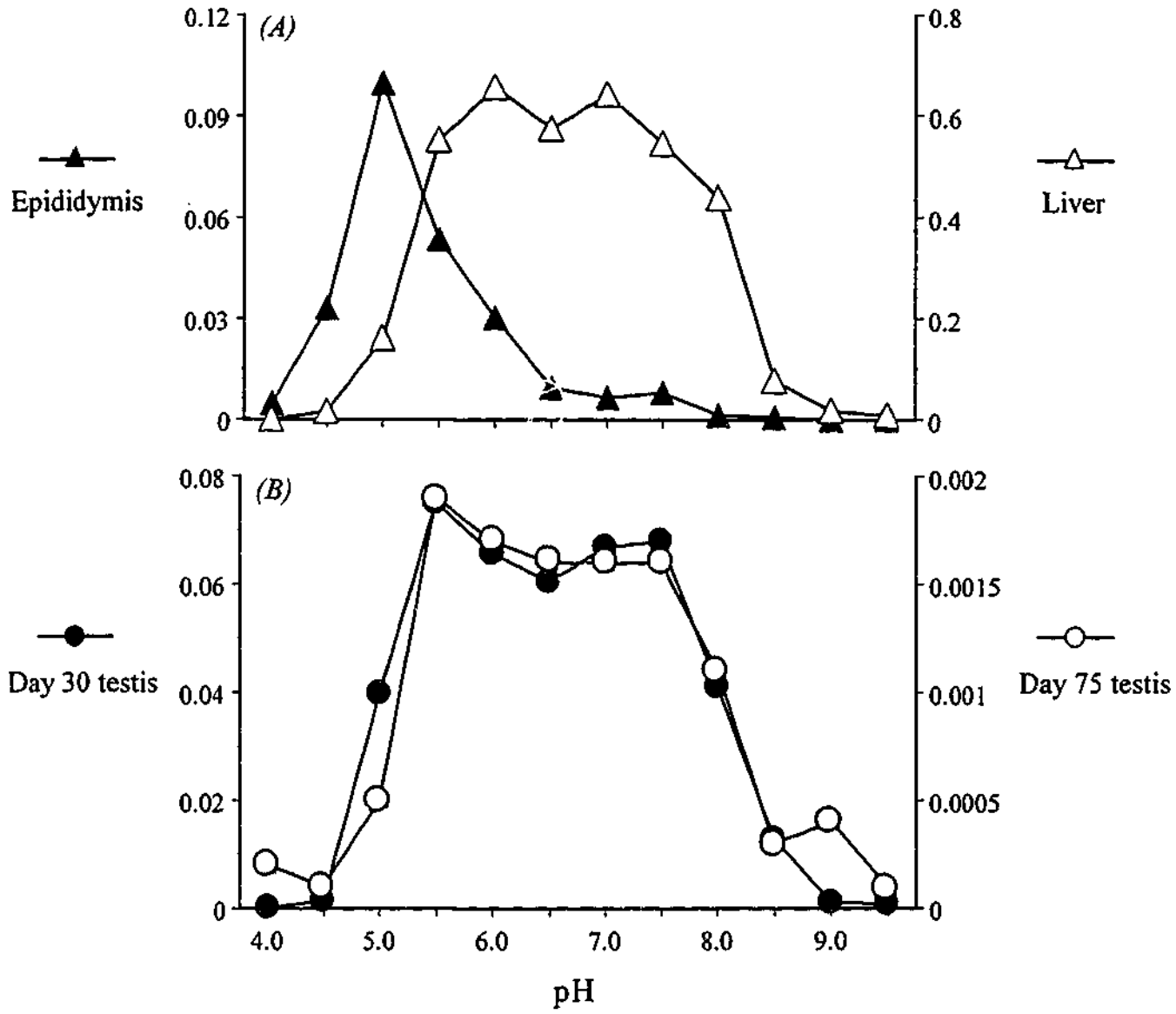


Figure 5-2: pH profiles for 5αR activity for *(A)* adult (day 75) male rat epididymis and liver, and *(B)* immature (day 30) and adult (day 75) rat testis. The optimum pH for 5αR activity in epididymis is pH 5.0, and in liver pH 7.0. The testis shows a peak of activity in the neutral range, and differing amounts of activity at pH 5.0. Values are the average of duplicate measurements and 5αR activity is expressed as μmoles DHT+3α-Adiol/ min/ mg protein. Note the different y- axis for each graph.

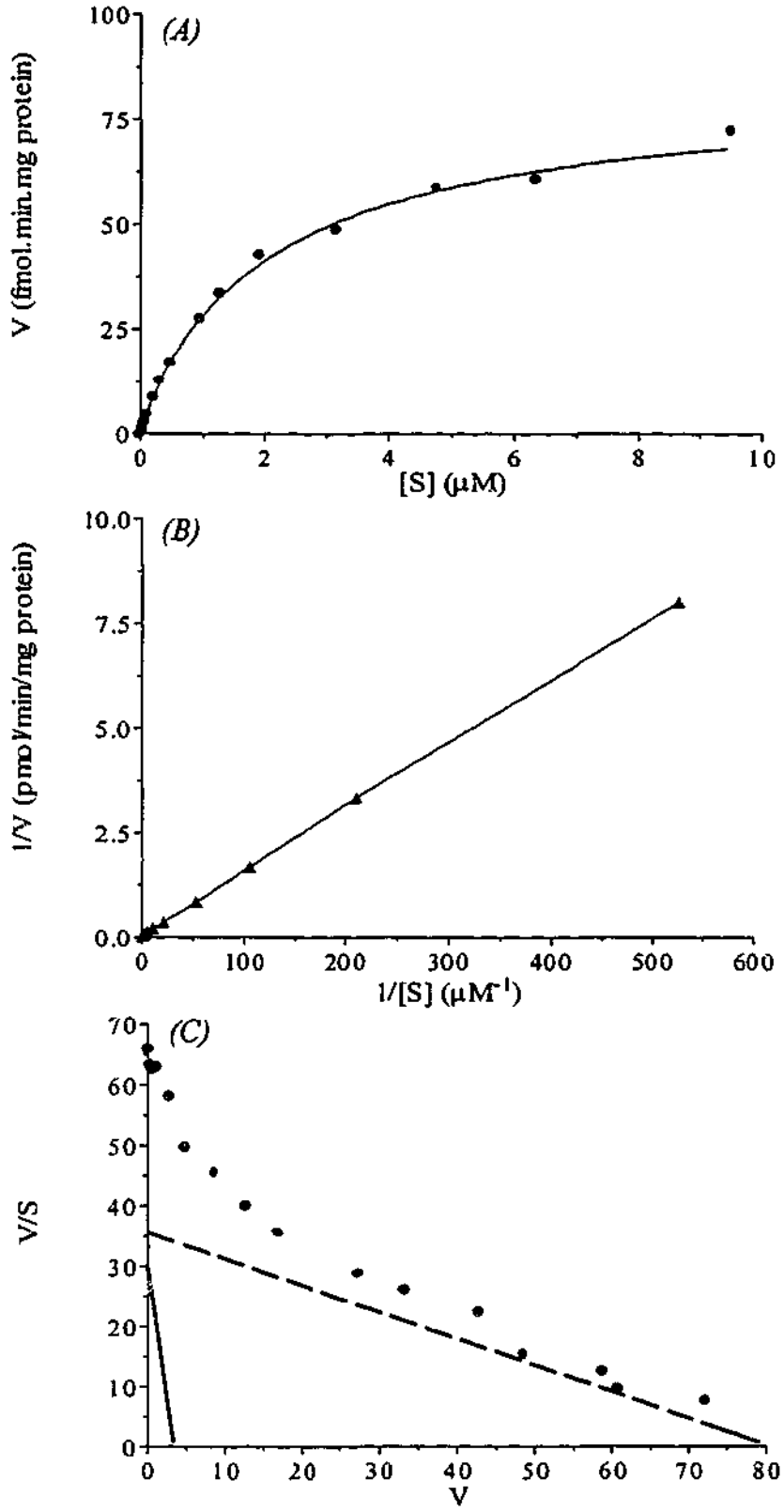


Figure 5-3: 5α-Reductase enzyme kinetics in adult (day 75) male rat liver at pH 7.0. *(A)* Michaelis-Menten plot of estimated initial velocities (V) of 5αR activity using substrate [S] concentrations ranging from 1.9 nM to 9.5 mM. *(B)* Double reciprocal Lineweaver-Burke plot of the data reported in *A*. *(C)* Eadie-Scatchard plot of the data reported in *A* of estimated initial velocities over substrate concentration (V/S) against velocity (V). This plot gives a more even weighting of points and is clearly non-linear. It could be described by two enzyme activities, 5αR-1 (—) and 5αR-2 (----). The values are the mean of duplicate values.

Table 1: Enzyme characteristics (K_m, V_{max}, and V_{max}/K_m ratio) for 5α-reductase (5αR) activity in day 30 and day 75 rat testis, epididymis and liver at day 75. Pure recombinant rat 5αR Type 1 (5αR-1) and Type 2 (5αR-2) are also shown. Details of enzyme assays and analysis are presented in the Methods section.

	5αR-1			5αR-2		
Rat tissue / Cell	K_m (μM)	V_{max} (fmol/min/mg protein)	V_{max}/K_m (x10^{-12})	K_m (μM)	V_{max} (fmol/min/mg protein)	V_{max}/K_m (x10^{-12})
day 30 testis	2.25 ± 0.96	[illegible]	1.66	0.13 ± 0.09	0.26 ± 0.2	1.93
day 75 testis	3.06 ± 0.97	0.36 ± 0.01	0.12	ND	ND	
Epididymis	2.82 ± 1.39	[illegible]	0.48	0.03 ± 0.03	0.35 ± 0.16	11.8
Liver	2.41 ± 0.66	[illegible]	43.2	0.71 ± 0.03	4.74 ± 0.86	6.6
	Recombinant 5αR-1			Recombinant 5αR-2*		
	1.64 ± 0.40	[illegible]	8.32	0.27 ± 0.02	3.16 ± 2.17	11.9

Values are the mean ± SD from three separate experiments. ND denotes non-detectable and * denotes enzyme kinetics performed at pH 5.0.

The Michaelis-Menten plot for rat epididymis is shown in *Figure 5-4[A]*, and the corresponding Lineweaver-Burk plot for epididymis is clearly non-linear (*Figure 5-4[B]*). The non-linear Eadie-Scatchard plot *Figure 5-4[C]* indicates that the epididymis expresses two 5αR enzymes. The dotted line corresponds to 5αR-1 and the solid line to 5αR-2, with average K_m values of 2.82 ± 1.39 μM and 0.03 ± 0.03 μM, respectively (*Table 1*). The average V_{max} values for 5αR-1 in the epididymis was ~4-fold higher than the V_{max} for 5αR-2 (1.35 ± 0.11 and 0.35 ± 0.16, respectively; *Table 1*).

The Michaelis-Menten plot for day 30 testis is shown in *Figure 5-5[A]*, and there appears to be a deviation from linearity at the higher substrate concentrations for the Lineweaver-Burk plot *(Figure 5-5[B])*. This is clearly evident from the non-linear Eadie-Scatchard plot *(Figure 5-5[C])*, which represents two 5αR isoforms. The average K_m values for immature testis for 5αR-1 and 5αR-2 were 2.25 ± 0.96 μM and 0.13 ± 0.09 μM, respectively (*Table 1*). A linear Lineweaver-Burk plot *(Figure 5-6[A])* and a linear Eadie-Scatchard plot *(Figure 5-6[C])* were obtained for day 75 testis, with a average K_m value of 3.06 ± 0.97 μM which corresponds to 5αR-1 *(Table 1)*.

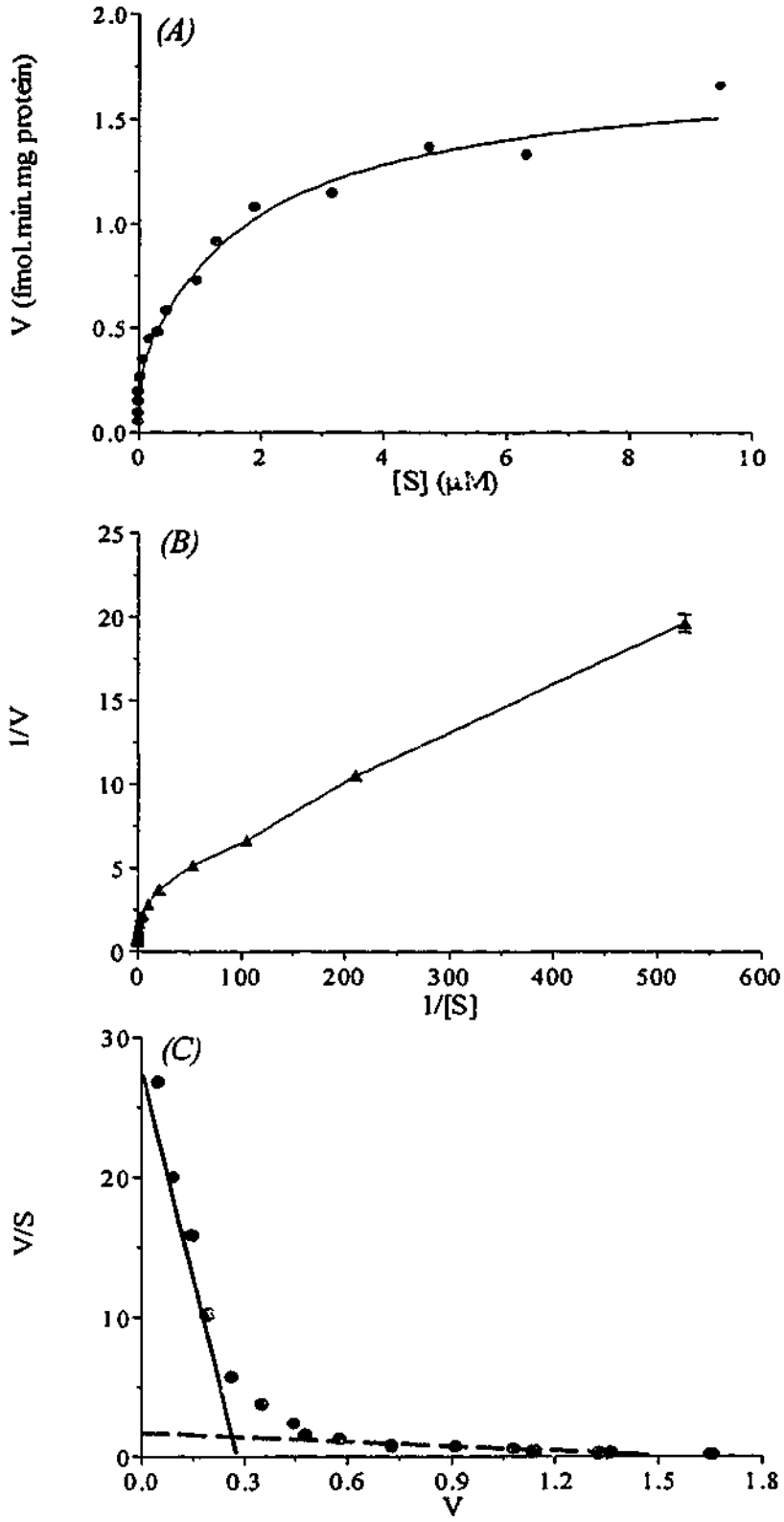


Figure 5-4: 5α-Reductase enzyme kinetics in adult (day 75) epididymis at pH 7.0. *(A)* Michaelis-Menten plot of estimated initial velocities (V) for 5αR activity. *(B)* Double reciprocal Lineweaver-Burke plot of the data reported in *A*. *(C)* Eadie-Scatchard plot of the data reported in *A* of estimated initial velocities over substrate concentration (V/S) against velocity (V), that can be described by two enzyme activities, 5αR-1 (- - -) and 5αR-2 (——). The values are the mean of duplicate values.

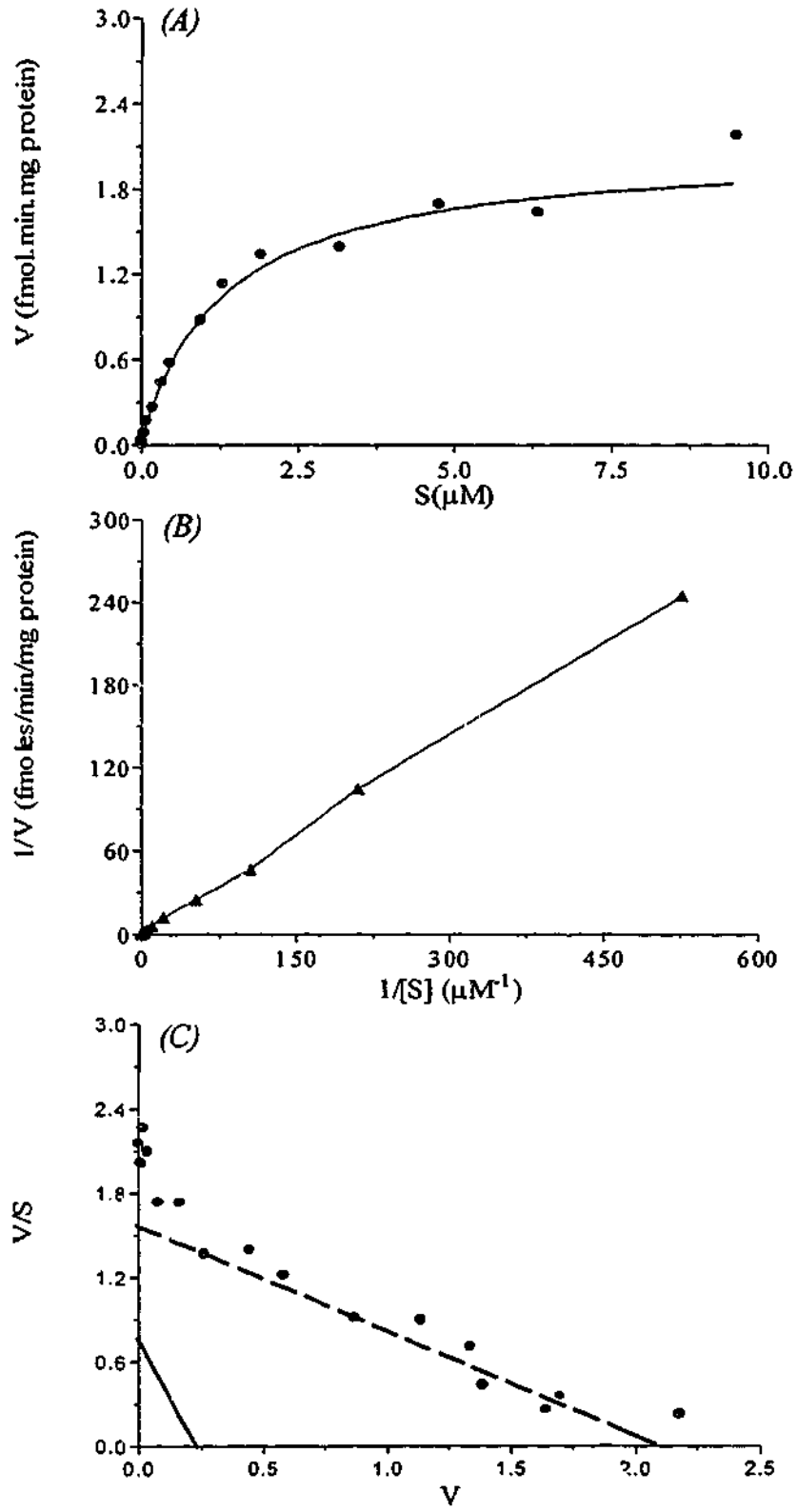


Figure 5-5: 5α-Reductase enzyme kinetics in immature (day 35) rat testis at pH 7.0. *(A)* Michaelis-Menten plot of estimated initial velocities (V) of 5αR activity using substrate [S] concentrations ranging from 1.9 nM to 9.5 mM. *(B)* Double reciprocal plot of the data reported in *A*. *(C)* Eadie-Scatchard plot of the data reported in *A* of estimated initial velocities over substrate concentration (V/S) against velocity (V), that can be described by two enzyme activities, 5αR-1 (——) and 5αR-2 (---). The values are the mean of duplicate values.

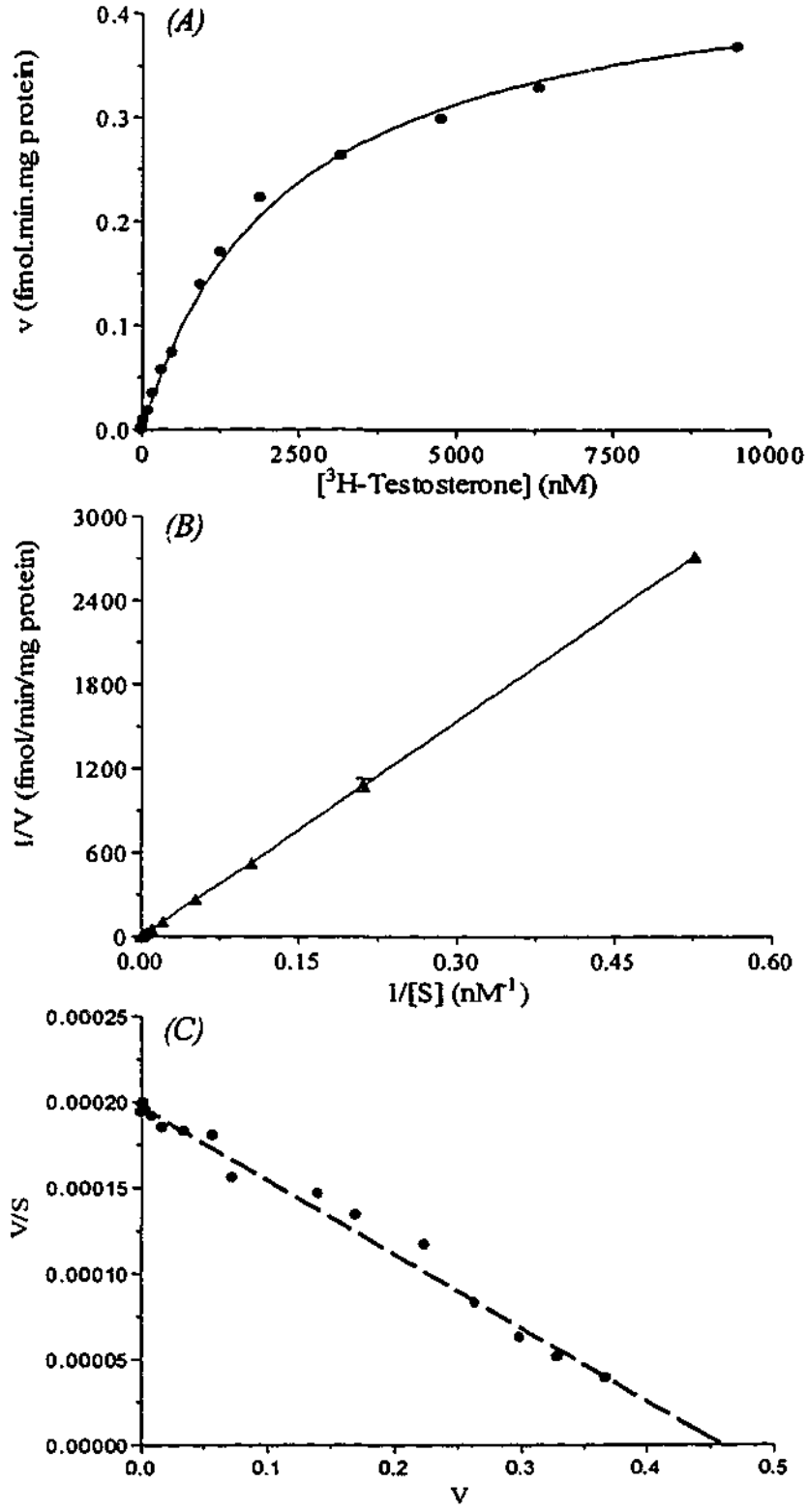


Figure 5-6: 5α-Reductase enzyme activity in adult (day 75) rat testis at pH 7.0. *(A)* Michaelis-Menten plot of estimated initial velocities (V) of 5αR activity using substrate [S] concentrations ranging from 1.9 nM to 9.5 mM. *(B)* Double reciprocal Lineweaver-Burke plot of the data reported in *A*. *(C)* Eadie-Scatchard plot of the data reported in *A* of estimated initial velocities over substrate concentration (V/S) against velocity (V), that can be described by a single enzyme activity, 5αR-1 (- - -). The values are the mean of duplicate values.

The V_{max} values and the efficiency ratio V_{max}/K_m for epididymis and immature testis are represented in *Figures 5-7*. The values obtained for the maximal velocity (V_{max}) are an index of the total amount of active enzyme present in the preparation. The average V_{max} for 5αR-1 in the epididymis is ~4-fold higher than the value for 5αR-2 *(Figure 5-7[A])*. When the V_{max} value is taken as a measure of enzyme concentration, these results suggest that there is four times the amount of 5αR-1 compared to 5αR-2 in the epididymis. The efficiency ratio (V_{max}/K_m) of 5αR-1 isoform in the epididymis was 0.48, whereas 5αR-2 had a 25-fold higher V_{max}/K_m ratio of 11.8 *(Table 1)*.

The V_{max} values for the 5αR isoforms in day 30 testis *(Figure 5-7[B])* suggest that 5αR-1 is the predominant 5αR isoform expressed. Although the 14-fold higher V_{max} for 5αR-1 suggests that it is quantitatively the predominant isoform in day 30 testis, the potential *in vivo* activity at pH 7.0 would be attributed to both isoforms equally, as suggested by the almost equal efficiency ratios for 5αR-1 and 5αR-2 (1.66 and 1.98, respectively; *Figure 5-7[B]*).

These enzyme kinetic experiments were conducted at neutral pH (7.0). When the substrate affinity constants for testis were determined at pH 5.0, the pH optimum for 5αR-2, a nanomolar K_m value was obtained for day 30 but not in day 75 testis (data not shown). This provides further support for the enzyme kinetic experiments performed at neutral pH.

5α-Reductase Type 1 and Type 2 Enzyme Activity in Rat Testis

Table 2 shows the activity at pH 7.0 (i.e. 5αR-1) in rat testis at varying ages. The activity detected at pH 7.0 represents 5αR-1, and was highest at day 30, 17-fold lower by day 75, and even lower by day 147 (46.1, 2.70 - 1.90, and 0.25 pmoles DHT+3α-Adiol/ min/ mg protein, respectively).

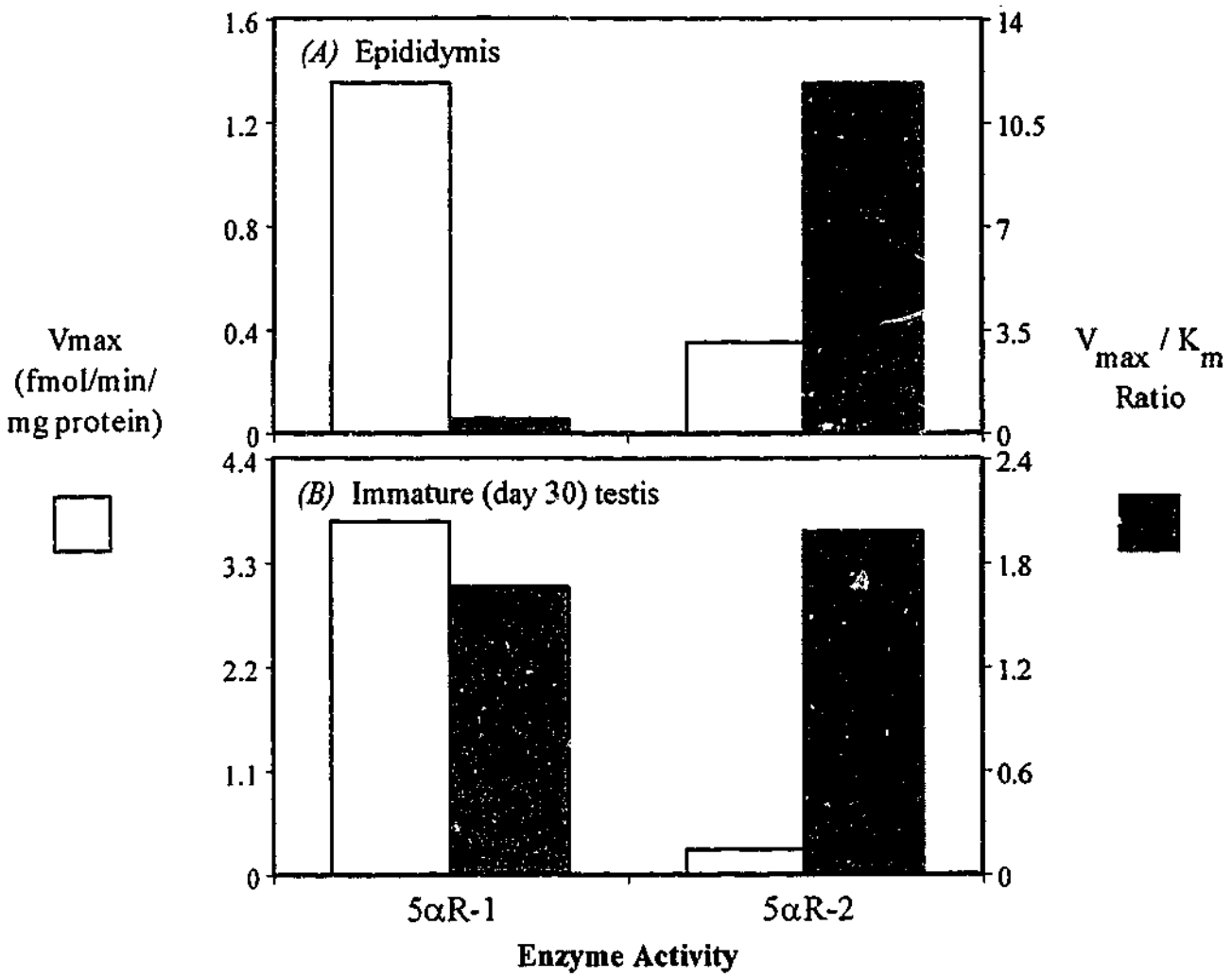


Figure 5-7: 5α-Reductase Type 1 (5αR-1) and Type 2 (5αR-2) isoform activity in *(A)* epididymal homogenates and *(B)* immature (day 30) testicular (10,000 g) supernatants as measured by the V_{max} (open bars) or V_{max}/K_m ratio (solid bars) at pH 7.0. The V_{max} values are used to measure the amount of enzyme, whereas the V_{max}/K_m can be used to assess the efficiency of the enzyme.

Table 2: Measurement of 5α-Reductase Type 1 (5αR-1) and Type 2 (5αR-2) activities in day 30, 75, and 147 rat testis. Some animals at day 75 had a pH 5.0/ 7.0 ratio less than 0.152 and are shown separately. 5αR activity is expressed as pmoles DHT+3α-Adiol/ min/ mg protein.

		5αR-1	5αR-2		
Rat Testis	n	pH 7.0	pH 5.0	pH 5.0/ 7.0 ratio	Calculated 5αR-2
day 30	11	[illegible]	15.4 ± 3.23	[illegible]	3.44 ± 1.49
day 75	3	[illegible]	0.49 ± 0.24	[illegible]	0.16 ± 0.04
	3	[illegible]	0.25 ± 0.03	[illegible]	0.05 ± 0.01
day 147	7	[illegible]	0.08 ± 0.03	[illegible]	0.05 ± 0.02

Mean ± SD. Calculated 5αR-2 activity was determined as validated in chapter 3.

The various measures that have been used previously to assess 5αR-2 activity (pH 5.0 and pH 5.0/ 7.0 ratio) and the calculated values for quantitation of 5αR-2 using our approach with recombinant rat 5αR isoforms that was validated in chapter 3, are also represented in *Table 2*. The pH 5.0 measurements indicate that there is a 31-fold decrease in 5αR-2 activity from days 30 to 75 (15.4 to 0.49 pmoles DHT+3α-Adiol/ min/ mg protein, respectively), and a further decline in activity from day 75 to 147 (0.49 to 0.08 pmoles DHT+3α-Adiol/ min/ mg protein, respectively). However, these values can not be used to estimate 5αR-2 levels because of the overlap of 5αR-1 at pH 5.0. Therefore, many investigators have resorted to using the pH 5.0/ 7.0 ratio to indicate the relative level of 5αR-2 activity. The pH 5.0/ 7.0 ratio shows that the activity at pH 5.0 accounts for 34% of 5αR-1 activity at pH 7.0 at day 30 and 147, and 19% at day 75.

Using our method to quantitate specific activity, 5αR-2 at day 30 was 3.44 pmoles DHT+3α-Adiol/ min/ mg protein, which is 7.5% of the 5αR-1 activity at pH 7.0 *(Table 2)*. The pH 5.0/ 7.0 ratio for three out of the six animals at day 75 was below 0.152, which is the sensitivity of the assay for 5αR-2. Therefore, these three animals were assigned a pH 5.0/ 7.0 ratio of 0.152 (see Chapter 3 for validation of assay), and are represented separately from the other day 75 animals. The amount of 5αR-2 activity at day 75 was lower than that at day 30, and was 0.16 and 0.05

pmoles DHT+3α-Adiol/ min/ mg protein, 6% and 3% of the activity at pH 7.0. There was even a lower level of 5αR-2 activity at day 147, where 5αR-2 activity was 0.06 pmoles DHT+3α-Adiol/ min/ mg protein, which is 24% of 5αR-1 activity at pH 7.0.

5.4 Discussion

The ability of the testis to synthesize 5α-reduced androgens during sexual maturation is well established, however the 5αR isoform that is responsible for this conversion is controversial. This study has used enzyme kinetics and *in vitro* enzyme activity assays to show that day 30 rat testis expresses both 5αR isoforms. Enzyme kinetic experiments were able to detect a micromolar substrate affinity constant corresponding to 5αR-1 at day 75, as well as enzyme activity at pH 7.0. However, 5αR-2 activity was not detected at day 75 when using enzyme kinetic studies, but can be detected and quantitated using our method validated in chapter 3, which eliminates the overlap of 5αR-1 activity at pH 5.0. This is the first study which has examined rat testicular 5αR isoforms using: enzyme kinetics to characterise 5αR isoforms in the testis, pH profiles to determine which 5αR isoforms are expressed, and measured enzyme activity levels for both 5αR-1 and 5αR-2.

Enzyme kinetic studies were performed to investigate the 5αR isoforms in rat testis. These studies showed that a micromolar and a nanomolar K_m value, corresponding to 5αR-1 and 5αR-2, respectively, could be detected in day 30 testis. These values were in accordance with previous published K_m values (Normington and Russell, 1992; Span *et al.*, 1995; Span *et al.*, 1996a; Faller *et al.*, 1993; Thigpen *et al.*, 1993a), and were similar to those values obtained for the recombinant rat 5αR isoforms, and epididymis and liver. Thus, these two distinct K_m values indicate that both 5αR isoforms are expressed in the testis during immaturity.

The Eadie-Scatchard plot is particularly suitable for identifying two isoforms as it leads to a more even weighting of points as compared to the Lineweaver-Burk plot (Segel, 1975). The Eadie-Scatchard plots for immature testis were clearly non-linear over essentially the whole substrate

range tested and could be described by two enzyme activities, as shown by two lines, which correspond to 5αR-1 and 5αR-2.

The V_{max} values are an index of the total amount of active enzyme, and the 14-fold higher V_{max} for 5αR-1 suggests that it is the predominant isoform expressed in the testis at day 30. Unlike the classical pH optimum, which only determines velocities at a single substrate concentration, the efficiency optimum (V_{max}/K_m) has the advantage of taking into account the substrate dependency of enzyme reaction velocity (Thigpen *et al.*, 1993a). Thus, the V_{max}/K_m ratio is considered to be a valid estimation of the potential *in vivo* isoform activity, and was calculated as this ratio reflects the enzymatic activity at substrate concentrations much lower than K_m (Thigpen *et al.*, 1993a; Krieg *et al.*, 1983). Even though the V_{max} values indicate that 5αR-1 is the predominant 5αR isoform in the testis, the potential *in vivo* activity at pH 7.0, as indicated by the V_{max}/K_m ratio, would indicate that both isoforms would contribute equally to total 5αR activity.

Other studies have used this approach to dissect the relative physiological roles of the 5αR isoforms when co-expressed in the same tissue. For example, it is well known that the epididymis expresses both 5αR-1 and 5αR-2 mRNA and enzyme activity (Normington and Russell, 1992; Viger and Robaire, 1994). More recent studies have applied this enzyme kinetic methodology to show that even though both 5αR isoforms are expressed in equal amounts in the epididymis, as assessed by their V_{max} values, 5αR-2 has a higher potential *in vivo* activity ratio (i.e. V_{max}/K_m), suggesting that 5αR-2 would play a major role in the 5α-reduction of testosterone at physiological concentrations and at neutral pH.

A micromolar K_m value corresponding to 5αR-1 was detected in day 75 testis, and was in accordance with the value for 5αR-1 obtained in all the other tissues examined and the value obtained for recombinant 5αR-1. The V_{max} values indicate the amount of 5αR-1 activity at day 75 was about one-tenth of that expressed at day 30. A nanomolar K_m corresponding to 5αR-2 was not detected at 75 d, suggesting that only 5αR-1 was present at this age. An alternative explanation

was that the level of 5αR-2 expression at day 75 was extremely low, and thus could not be detected using enzyme kinetic studies.

The low K_m value measured *in vitro* for 5αR-2 suggests that it would be well suited to act under conditions of limiting substrate. For example, production of testosterone by the fetal testis is low, and thus conversion to DHT is crucial for sexual development (Griffin and Wilson, 1989; Wilson *et al.*, 1993). However, even under conditions of saturating substrate concentrations, such as is the case in the sexually mature adult, a small amount of 5αR-2 might be expected to synthesize considerable amounts of DHT (Thigpen *et al.*, 1993). We can therefore speculate that the expression of high levels of 5αR-1 and 5αR-2 activity during immaturity might be required to amplify the androgen milieu of the testis by converting the low levels of testosterone to DHT, at a time when androgens are crucial for the sexual maturation and the onset of spermatogenesis. In support of this concept, there is a higher concentration of testicular 5α-reduced androgens than that of testosterone at day 30 (Corpechot *et al.*, 1981). There does not appear to be a role for 5α-reduced androgens in adulthood normally, given the high local concentrations of testosterone (Corpechot *et al.*, 1981; O'Donnell *et al.*, 1996b, 1999). If this is the case, then why 5αR is expressed during adulthood is unknown. Perhaps some regulatory functions are dependent on DHT rather than testosterone; DHT produced locally within the testis is required to act as an endocrine hormone (Wilson, 1996) to be utilized by androgen-dependent peripheral organs; or the level of 5αR isoforms could be amplified when testosterone levels are too low and do not allow testosterone to act directly on the AR.

Enzyme kinetics is a suitable approach to detect the expression of multiple isoforms. The disadvantages of this technique are that it is time-consuming and requires large quantities of sample. Therefore, it was not an appropriate method for analyzing many samples or when sample quantities were limited. An alternative means of detecting 5αR isoform activity was using the distinct pH optimas of these isoforms. The activities at pH 7.0 and pH 5.0 are generally considered indicative of 5αR-1 and 5αR-2 isoform activity, respectively (Thigpen *et al.*, 1993b). Our method

validated in chapter 3 allows a valid estimation of 5αR-1 and 5αR-2 using this pH optima approach.

The pH profile for epididymis, which expresses predominantly 5αR-2 (Russell and Wilson, 1994; Normington and Russell, 1992), has a sharp peak of activity at pH 5.0. The pH profile for liver, which expresses high amounts of 5αR-1 (Normington and Russell, 1992) covers a broad neutral pH range. This shows that the classical pH optima for 5αR-1 and 5αR-2 (pH 7 and 5.0, respectively; Normington and Russell, 1992) hold true under our experimental conditions. This is important because there is a wide variation in the literature for these pH optima. For example, 5αR-2 has been reported to operate maximally at pH 5.0 (Jenkins *et al.*, 1992; Normington and Russell, 1992) or at pH 5.5 (Fisher *et al.*, 1978; Voigt *et al.*, 1970; Harris *et al.*, 1992)

The pH profiles for day 30 and day 75 testis showed optimal activity over a broad neutral pH range, similar to that seen for the liver. The neutral pH range for 5αR is characteristic of 5αR-1, both in its native form and when expressed in mammalian or yeast cells (Russell and Wilson, 1994; Normington and Russell, 1992; Poletti *et al.*, 1996). This suggests that 5αR-1 is expressed in the testis, and is in agreement with the enzyme kinetics studies which demonstrated a micromolar K_m value. There was no clearly identifiable peak of activity at pH 5.0 for either age in the testis. Even though there was no peak activity at pH 5.0 for the testis, there were different amounts of activity at pH 5.0 between testis at days 30 and day 75 when compared to their respective pH 7.0 activities. The pH profiles show that the activity at pH 5.0 was two-fold higher at day 30 compared to day 75. This variable activity at pH 5.0 could not be due to an overlap of 5αR-1 activity at pH 5.0 alone, simply because of the variation of activity detected at pH 5.0. This suggests that there might be different levels of 5αR-2 activity in the testis at these two ages, and that the activity at pH 5.0 is due to the combination of 5αR-1 overlap and 'true' 5αR-2 activity.

To gain better insight into which 5αR isoforms were expressed in rat testis, and to quantitate the levels of each 5αR isoform, the activities of both 5αR-1 and 5αR-2 were evaluated in testicular supernatants. As mentioned previously, the activity at pH 7.0 was used to assess 5αR-1,

and our method incorporating the pH 5.0/ 7.0 ratio for recombinant 5αR-1 used to quantitate 5αR-2 activity. This study showed that the level of 5αR-1 activity declined with increasing age. This data is consistent with previous findings which have shown that 5αR-1 activity increased from birth to reach maximum levels by day 30, then declined to almost undetectable levels by adulthood (Rivarola *et al.*, 1972; Matsumoto and Yamada, 1973; Podesta and Rivarola, 1974). These findings are also in agreement with Viger and Robaire (1995) who showed that testicular 5αR-1 mRNA and protein were developmentally regulated. They showed that 5αR-1 mRNA was most abundant at day 30, but then declined by ~3.5-fold by day 90.

Using our formula to quantitate 5αR-2 in the testis, we were able to detect 5αR-2 activity at day 30, and the level of this activity was 13-fold less than 5αR-1. This is very similar to the V_{max} values obtained for the 5αR isoforms in immature testis, where the V_{max} value for 5αR-2 was 14-fold less than that for 5αR-1. Therefore, we can conclude that 5αR-1 is the predominant 5αR isoform in the testis at day 30. Using this method to quantitate 5αR-2 activity, we were also able to measure low levels of 5αR-2 activity at day 75, which were previously undetectable using enzyme kinetic studies. Therefore, this method appeared to be more sensitive than detecting multiple K_m values, especially, such as is the case in the testis, when 5αR-1 activity is relatively low compared to other tissues, and 5αR-2 activity is expressed at much lower levels than 5αR-1.

Our finding that 5αR-2 was expressed in the testis is supported by the studies of Normington and Russell (1992), who used Northern blot analysis to show that 5αR-2 was expressed in the testis at day 49. However, this study showed that 5αR-2 mRNA was the predominant 5αR isoform expressed in the testis. In contrast, Viger and Robaire (1995) could not detect 5αR-2 mRNA by Northern blot analysis, between days 7 to 91. These investigators suggest that the weak expression of the 5αR-2 transcript in the testis was not a result of low specific activity of the 5αR-2 probe because it recognised the positive controls. However, one must keep in mind that the level of 5αR-2 in the epididymis is relatively high, thus it is not surprising that the 5αR-2 probe would work sufficiently well in the epididymis and not in the testis.

In summary, kinetic analysis of 5αR enzyme activity at pH 7.0 for rat testis revealed both 5αR isoforms at day 30, with affinity constants that compared favorably to the human and rat 5αR-1 and 5αR-2, respectively. On the other hand, only a single affinity constant, corresponding to 5αR-1, was detected at day 75 using enzyme kinetic studies. However, using our method validated in Chapter 3, we were able to detect testicular 5αR-2 activity at day 75, and this was significantly less than that at day 30. Furthermore, we were able to detect 5αR-2 activity at day 147 as well, which was even lower than that at day 75. Thus, these results suggest that both 5αR-1 and 5αR-2 are expressed in rat testis, and that the level of these 5αR isoforms decrease with age.

Chapter 6

The Role of Testosterone and Follicle Stimulating Hormone in the Regulation of Testicular 5α-Reductase Type 1 and Type 2 Enzyme Activity and mRNA in Testosterone-Treated Rats

6.1 Introduction

The role of 5α-reduced androgens in spermatogenesis has not received much attention from scientists and clinicians over the years, and the regulation of testicular 5αR isoforms is relatively unexplored. However, there is growing evidence to suggest that 5αR may be important for maintaining spermatogenesis when testicular testosterone is suppressed, in both rats (O'Donnell *et al.*, 1996b, 1999) and in humans (Anderson *et al.*, 1996, 1997a).

The primary evidence for a role for 5α-reduced androgens in spermatogenesis comes from studies in rats administered a 5αR inhibitor (O'Donnell *et al.*, 1996b, 1999). In these studies, spermatogenesis was suppressed by low doses of testosterone (i.e. TE implants) which suppress serum LH levels and consequently intratesticular testosterone. Higher doses of testosterone can then be administered to restore spermatogenesis in a dose-dependent manner (McLachlan *et al.*, 1994a; Zirkin *et al.*, 1989). In this setting, co-administration of a 5αR inhibitor with testosterone implants impaired the dose-responsive restoration of spermatogenesis, suggesting that 5α-reduced androgens do play a role in spermatogenesis when testosterone levels are low, by amplifying androgen action (O'Donnell *et al.*, 1996b, 1999). Therefore, consideration of the testicular level of 5αR is of interest when studying the hormonal regulation of spermatogenesis. Previous studies (O'Donnell *et al.*, 1999) also showed that the androgen receptor antagonist, flutamide, when administered to TE-treated rats significantly increased testicular DHT and 3α-Adiol levels, in the absence of a rise in serum LH or an increase in intratesticular testosterone. This rise in 5α-reduced steroids suggests that 5αR activity in the testis may be up-regulated in the absence of androgen action. In men, there is also evidence to suggest that 5αR may be up-regulated in response to a testosterone-based contraceptive, and that it may play an important role in maintaining low levels of sperm production in a setting of reduced intratesticular testosterone levels (Anderson *et al.*, 1996, 1997a).

The studies mentioned above in the rat (O'Donnell *et al.*, 1996b, 1999) only measured the concentrations of 5α-reduced androgens, and thus do not indicate which 5αR isoform was up-

regulated by testosterone. The regulation of 5αR enzyme activity has been previously investigated, however only 5αR-1 activity was assessed, and these studies concentrated mainly on the immature animal (Oshima *et al.*, 1970; Nayfey *et al.*, 1975a; Murono and Payne, 1979). Furthermore, the regulation of testicular 5αR isoform mRNA expression has not been investigated.

Having already established the presence of both 5αR-1 and 5αR-2 enzyme activities in adult rat testis (Chapter 5), this chapter aimed to investigate the regulation of 5αR isoforms in a setting of reduced testicular testosterone. The methods for quantitation of enzyme activity and mRNA levels (Chapters 3 and 4, respectively) were used to investigate the hormonal regulation of testicular 5αR isoforms in the adult rat. In this study, the TE-treatment model with subsequent flutamide administration or high dose testosterone treatment were used to manipulate the levels of LH and testosterone, and androgen action via its receptor was inhibited by flutamide treatment. Administration of an FSH*Ab* allowed investigation of the effects of acute suppression of FSH.

6.2 Materials and Methods

6.2.1 Animals

Adult male Sprague-Dawley rats (day 80) were obtained from the Monash Central Animal House (Clayton, Australia) and housed under 12 h light/ dark cycle with free access to food and water, in groups of 3 - 4 animals per box. This study was approved by the Monash Medical Centre Animal Ethics Committee.

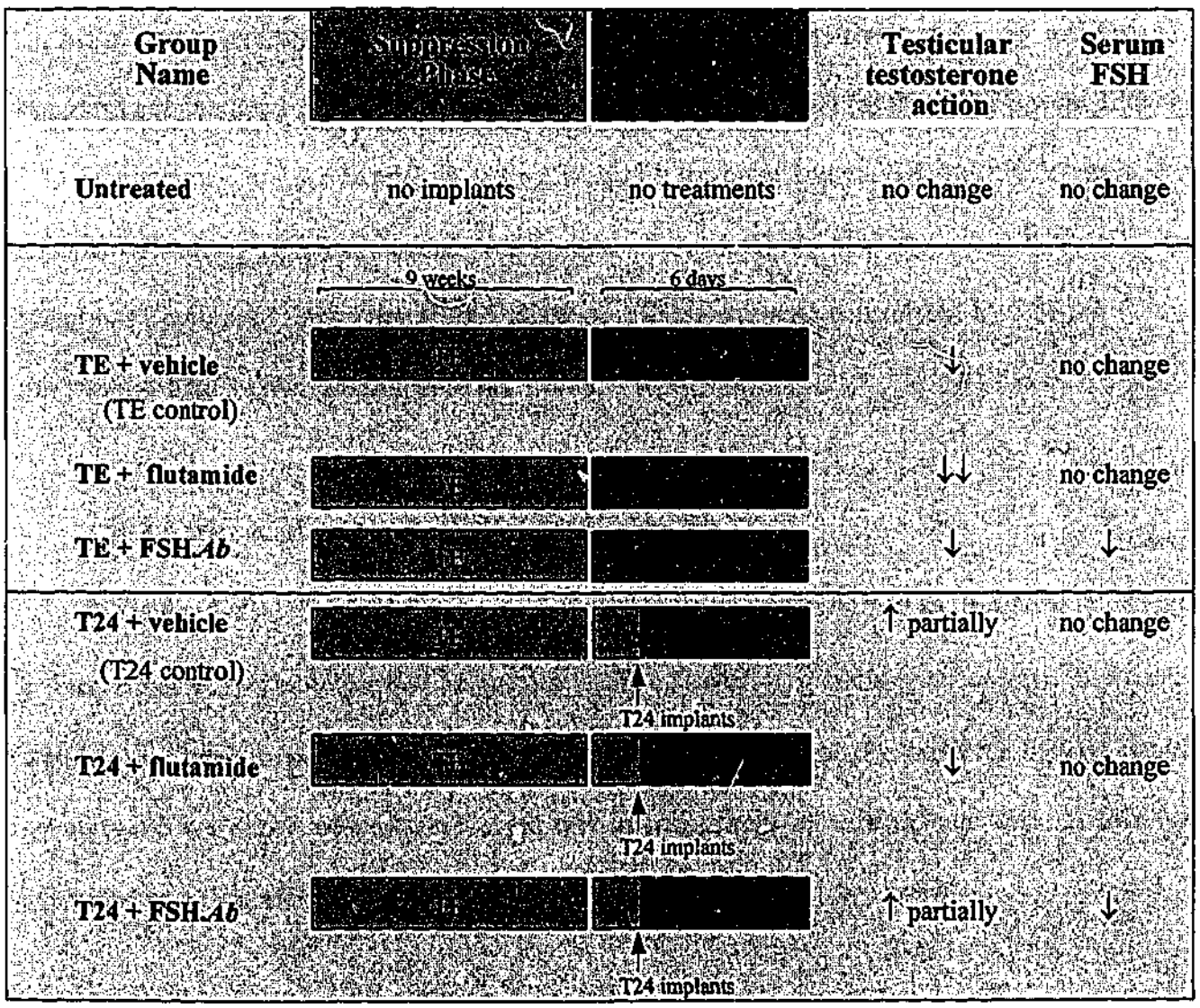
6.2.2 Experimental Design

Table 1 describes the suppression and treatment phases in the TE-treatment model. All animals except untreated rats (i.e. 6 groups of animals, n = 8 per group) received TE implants for 9 weeks to suppress serum LH and testicular testosterone levels to ~3% of untreated levels (O'Donnell *et al.*, 1999). After TE-treatment, the TE implants were retained in 3 groups of animals

during the treatment phase (TE-treatment). The other 3 groups of animals (n = 8 per group) had their TE implants removed on the second day of the treatment phase and replaced with 24 cm-*T.imp* (i.e. T24-treatment).

Animals received either vehicle (oil and/ or NRS injections, see section 2.2.2), FSH*.Ab* (2 mg/ kg) to acutely inhibit circulating FSH, or the AR antagonist flutamide (20 mg/ kg) to block residual androgen action. These treatments were administered for a total of 6 days to ensure that they were effective from the beginning of the 4-day implant period (O'Donnell *et al.*, 1996b, 1999). TE- and T24-treated animals receiving vehicle injections are referred to as TE and T24 control animals.

Table 1: Description of the suppression and treatment phases in the various groups of animals, and the anticipated effects on testicular testosterone and serum FSH action compared to untreated animals (n = 8). The TE implants were replaced with T24 implants on day 2 of the treatment phase in T24-treated animals.



6.2.3 Tissue Collection

Animals were sacrificed by CO_2 inhalation, blood was collected by cardiac puncture and the testes removed, weighed and frozen in liquid nitrogen. Plasma was snap frozen and stored at –20°C for hormone assays and testes were stored at -70°C for measurement of 5αR levels and analysis of testicular steroids (section 2.3.3).

6.2.4 Serum LH and FSH

Serum LH was measured using an IFMA (see section 2.3.2) based on the protocol of Haavisto *et al* (1993), using a rat LH standard (rLHRP-3, NIDDK●). All samples were measured in two assays, with a sensitivity of 0.02 ng/ ml, and the inter-assay variation (assessed by QC samples) was 4% and 1.9% at a LH concentration of 0.5 and 0.23 ng/ ml, respectively.

Serum FSH was measured by RIA (see section 2.3.1) using a rat FSH standard (rat FSH RP-2). All samples were measured in two assays, with an average sensitivity of 1.5 ng/ ml. Two QC samples were included in each of the assays, and the values obtained for these QC's were 3.52 and 3.33 for one assay, and 2.67 and 2.46, respectively, for the other assay. FSH was not determined in animals treated with FSH.*Ab* due to interference of the antibody with the assay. However, our group has previously shown that >90% of serum FSH is immuno-neutralized by FSH.*Ab* (2 mg/ ml) treatment for 7 days (Meachem *et al.*, 1998).

6.2.5 5α-Reductase Activity Measurements

Testicular 5αR-1 and 5αR-2 enzyme activity levels were determined as described in Chapter 3. Enzyme activity was expressed as pmoles DHT + 3α-Adiol/ g testis (wet weight). The results were similar when expressed per mg protein (see Appendix E and F), however limited sample amounts did not allow measurement of protein in two groups of animals, and thus all data was expressed as 5αR activity per g testis (wet weight).

6.2.6 5α-Reductase mRNA Measurements

Testicular 5αR-1 and 5αR-2 mRNA concentrations were determined using real time PCR, and mRNA levels were normalized to 28S ribosomal RNA, as outlined in Chapter 4. The mRNA levels were measured in selected treatment groups.

6.2.7 Testicular Steroids

Testicular testosterone, DHT and 3α-Adiol were extracted with acetonitrile, separated by HPLC and measured by a double antibody RIA, as described previously (O'Donnell *et al.*, 1996b) and outlined in section 2.3.3. Testicular steroids were only measured in those treatment groups where mRNA levels were measured.

6.2.8 Statistics

The Bartlett's test was used to test for equality of variance. If the variances were homogenous, a One-Way ANOVA was used to test for differences between all groups, followed by independent t-tests to compare selected treatments groups. All of the statistics mentioned above were performed by Microsoft Excel 97®. If variances were not homogenous, the Kruskal-Wallis test was used to test for differences amongst all groups, followed by the Dunn's Multiple Comparison Test to compare selected treatments groups. These data analyses was performed by Graphpad Prism®.

The Dunnett's test was used to compare untreated to treated animals. All data was expressed as mean ± SEM, unless otherwise stated (n = 8 per group).

6.3 Results

Testis Weights

TE-treatment significantly suppressed testis weights compared to untreated animals, and there were no significant differences in testis weights among treatment groups *(see Appendix D)*.

Serum LH and FSH Concentrations

LH levels were significantly ($p < 0.05$) suppressed in TE and T24 control and FSH.*Ab* treated groups compared to untreated animals *(Figure 6-1[A])*. However, administration of flutamide to TE- and T24-treated animals significantly elevated LH compared to TE and T24 controls.

Serum FSH was not affected in TE or T24 control animals compared to controls *(Figure 6-1[B])*. Flutamide administration to TE-treated rats increased ($p < 0.05$) serum FSH above TE-treated control levels (6.47 vs 3.06 ng/ ml, respectively).

5α-Reductase Type 1 and Type 2 Enzyme Activity

TE-treatment increased ($p < 0.05$) testicular 5αR-1 enzyme activity above untreated levels. Flutamide administration to TE- and T24- treated animals increased ($p < 0.05$) 5αR-1 activity above TE and T24 control levels, respectively *(Figure 6-2)*. There was no difference between TE and T24 control animals (i.e. T24-treatment had no effect), and acute withdrawal of FSH (FSH.*Ab* treatment) did not alter 5αR-1 activity.

There were no significant differences (one-way ANOVA, $p = 0.125$) among untreated and TE-treated groups in testicular 5αR-2 enzyme activity *(Figure 6-3)*. When T24-treated rats were administered FSH.*Ab*, there was a ~50% reduction in 5αR-2 activity compared to T24 control rats. However, due to the large data variability between these two groups this difference failed to reach significance ($p = 0.059$).

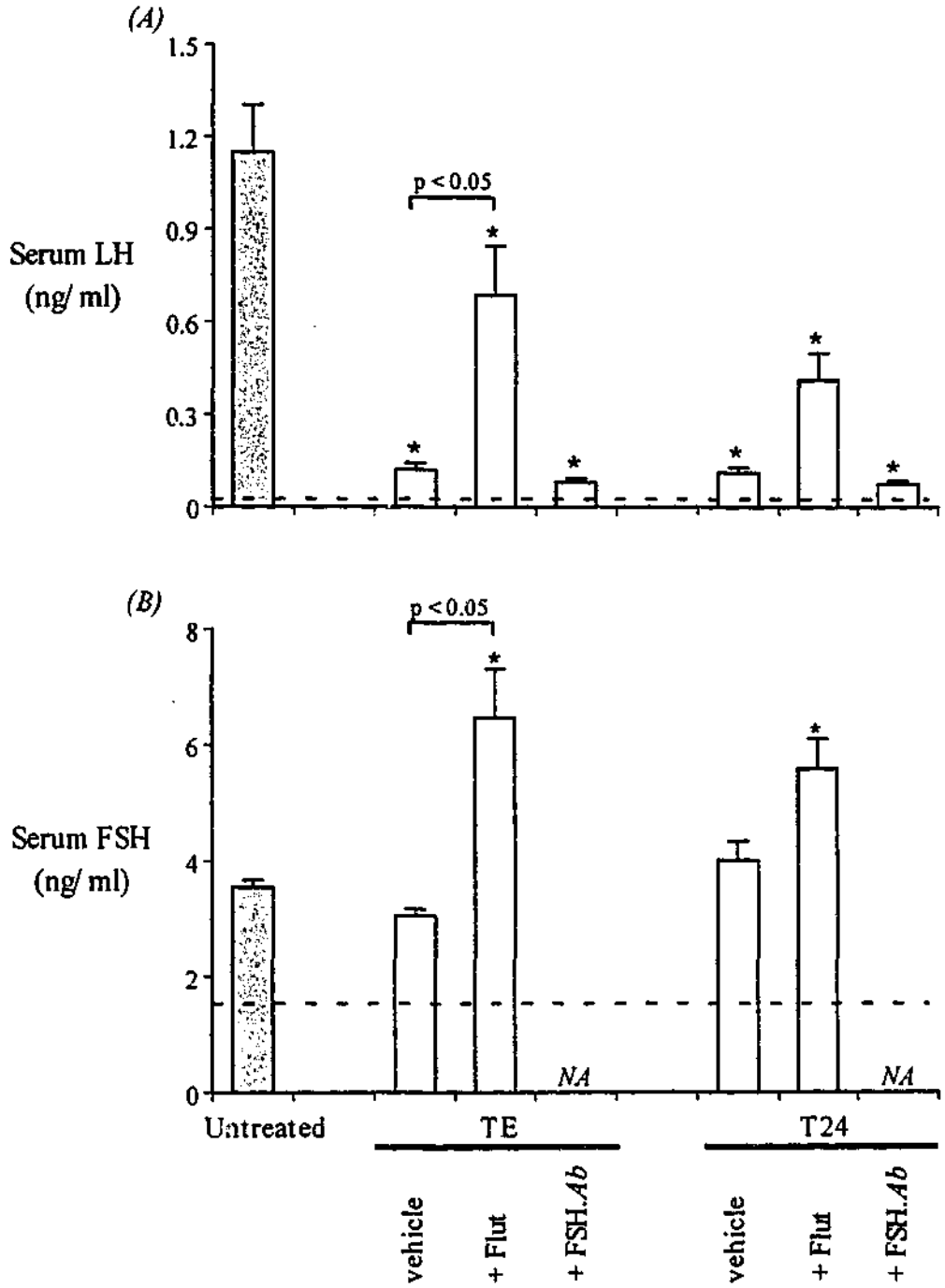


Figure 6-1: Concentration of *(A)* serum LH (ng/ ml) and *(B)* serum FSH (ng/ ml) in untreated rats in the shaded bar, and in the open bars TE- and T24-treated rats administered either vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 8). *NA* denotes serum FSH not assayed because of interference of FSH.*Ab* with the assay. * Indicates a significant difference ($p < 0.01$) between untreated and treated animals. Differences between treatment groups are indicated at the $p < 0.05$ level. Dotted lines represent sensitivities of assays.

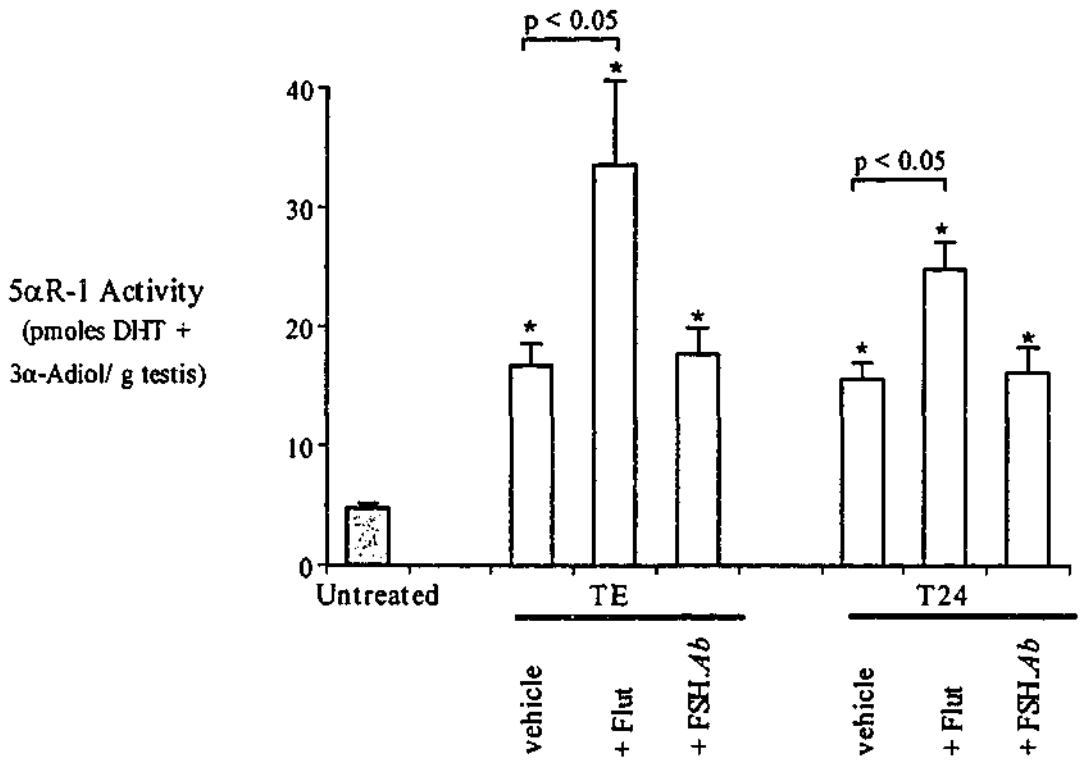


Figure 6-2: 5α-Reductase Type 1 (5αR-1) enzyme activity (pmoles DHT + 3α-Adiol/ min/ g testis) in untreated animals, and TE- and T24-treated animals administered vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 8). * Indicates a significant difference ($p < 0.05$) compared to untreated animals. Differences between treatment groups are represented at the $p < 0.05$ level.

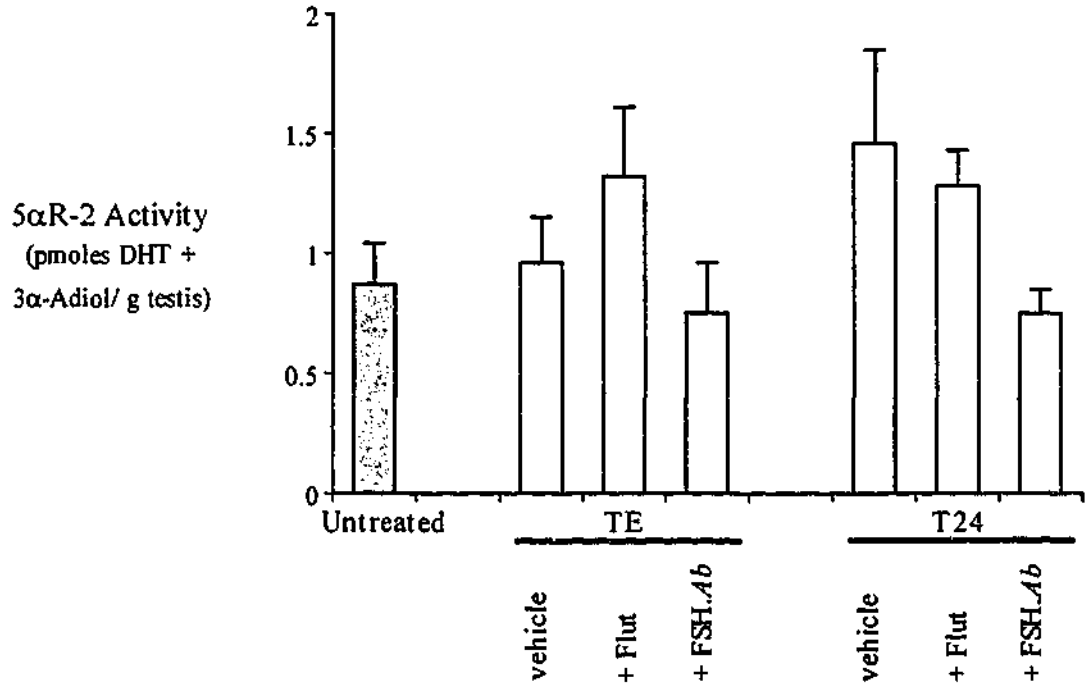


Figure 6-3: 5α-Reductase Type 2 (5αR-2) enzyme activity (pmoles DHT + 3α-Adiol / min/ g testis) in untreated animals, and TE- and T24-treated animals administered vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 8). There were no significant differences between any of the treatment groups.

As mentioned previously, 5αR-1 and 5αR-2 activity were similar when expressed as DHT + 3α-Adiol/ g testis or DHT + 3α-Adiol / mg protein (see Figure 6.2 vs Appendix E and Figure 6.3 vs Appendix F).

5α-Reductase Type 1 and Type 2 mRNA Levels

The housekeeping gene 28S ribosomal RNA was used to normalize mRNA levels. There were no significant differences in 28S ribosomal RNA expression among untreated and TE-treated animals *(Figure 6-4)*.

TE-treatment increased ($p < 0.05$) 5αR-1 mRNA above untreated levels, and flutamide administration caused a further increase in 5αR-1 mRNA, such that it was significantly ($p < 0.05$) above TE control animals *(Figure 6-5)*. Acute withdrawal of FSH by FSH*Ab* administration after TE-treatment decreased ($p < 0.05$) 5αR-1 mRNA compared to TE control animals.

TE-treatment suppressed ($p < 0.05$) 5αR-2 mRNA by ~3-fold compared to untreated levels *(Figure 6-6)*. Acute withdrawal of FSH by FSH*Ab* administration further suppressed ($p < 0.05$) 5αR-2 mRNA by ~ 4.5-fold below TE control levels. The administration of flutamide to TE-treated animals did not alter 5αR-2 mRNA levels.

Testicular Steroids

TE-treatment suppressed ($p < 0.01$) testicular testosterone levels to 2.7% of untreated levels *(Figure 6-7[A])*. Testosterone levels were not altered when TE-treated animals were administered flutamide or FSH*Ab*. Although TE-treatment suppressed testosterone by >97% compared to untreated animals, DHT and 3α-Adiol levels were suppressed by 26% and 54%, respectively *(Figure 6-7[B and C])*. Flutamide administration significantly ($p < 0.05$) increased testicular DHT *(Figure 6-7[B])* and 3α-Adiol *(Figure 6-7[C])* above TE control levels. Acute withdrawal of FSH did not alter the production of 5α-reduced metabolites.

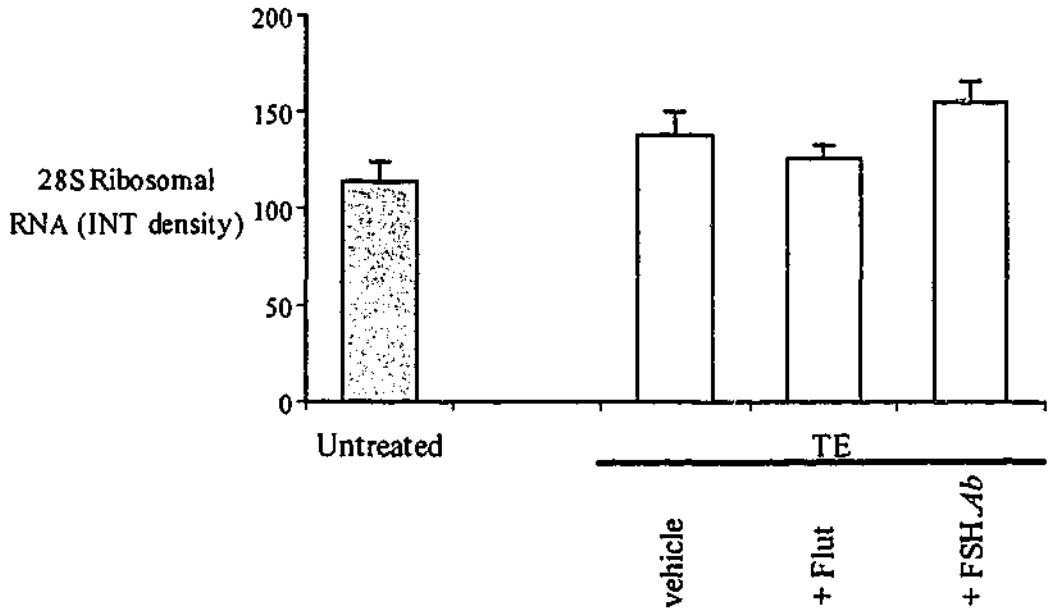


Figure 6-4: There were no significant differences between treatment groups at the $p < 0.05$ level for the housekeeping gene 28S ribosomal RNA (intensity represented as INT units, mean ± SEM).

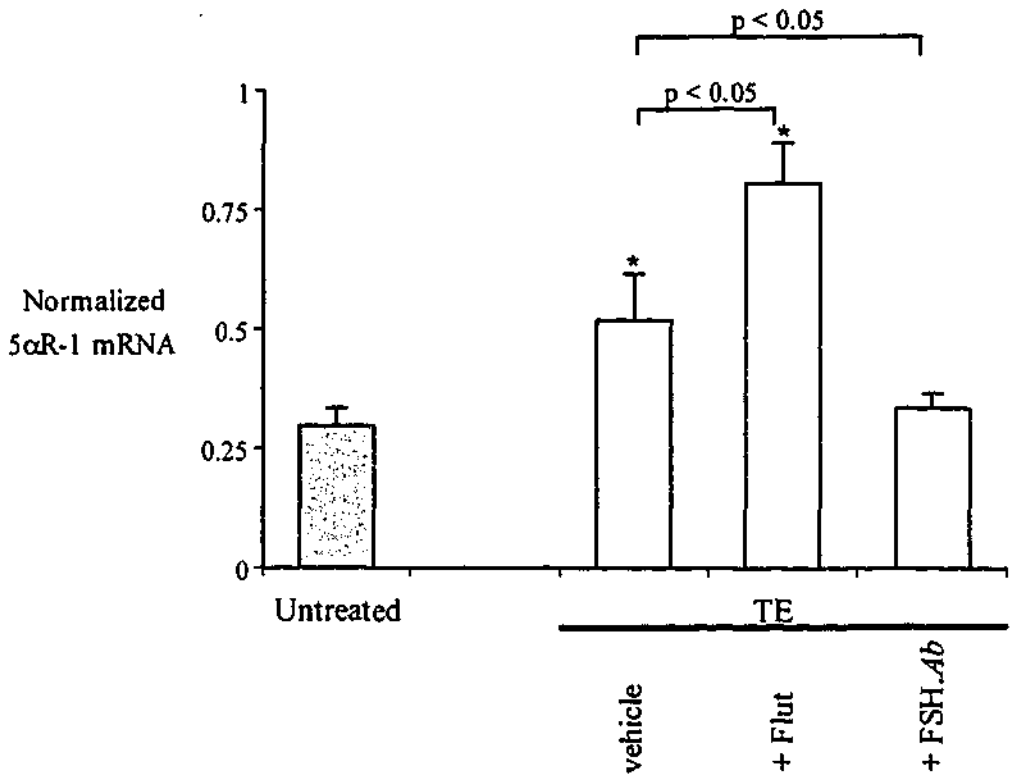


Figure 6-5: 5α-Reductase Type 1 (5αR-1) mRNA levels corrected for RNA loading (mRNA/ 28S ribosomal RNA) in untreated and TE-treated rats administered vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 8). * Indicates a significant difference ($p < 0.05$) compared to untreated animals. Differences between treatment groups are represented at $p < 0.05$. One of the TE + FSH.*Ab* treated animals was removed from this analysis as an outlier since it was more than two standard deviations away from the mean (before removal of the outlier FSH.*Ab* treatment reduced [not significant] 5αR-1 mRNA to 84% of TE control, and after removal of the outlier 5αR-1 mRNA was reduced significantly to 64% of TE control levels).

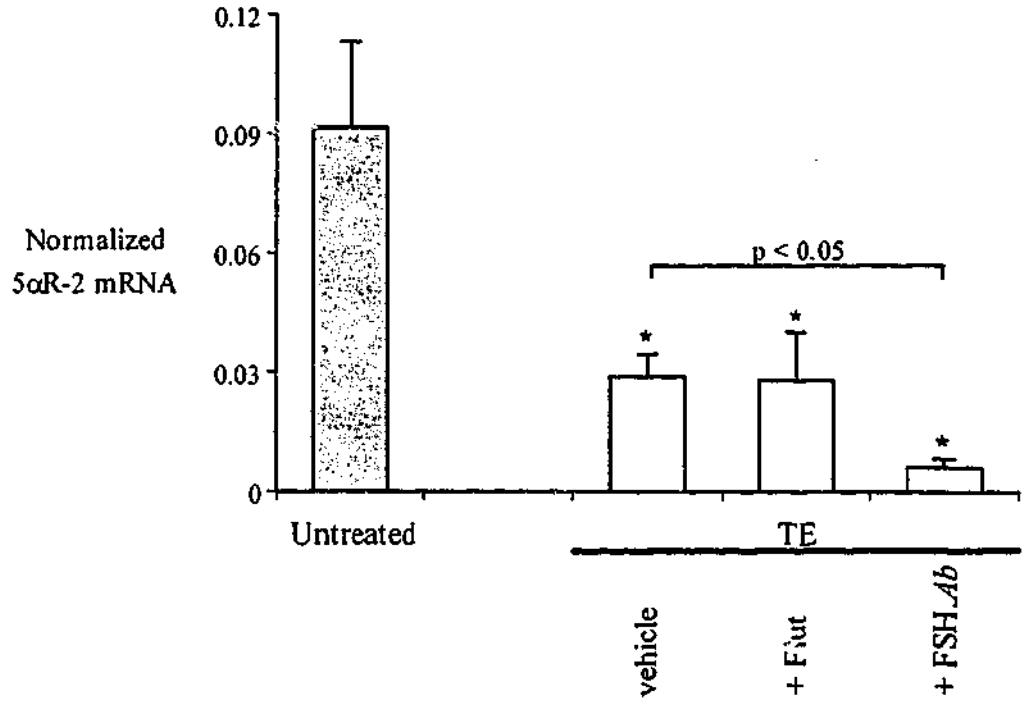


Figure 6-6: 5α-Reductase 2 (5αR-2) mRNA levels corrected for RNA loading (mRNA/ 28S ribosomal RNA) in untreated and TE-treated rats administered vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 8). * Indicates a significant difference ($p < 0.05$) compared to untreated animals. Differences between treatment groups are represented at $p < 0.01$.

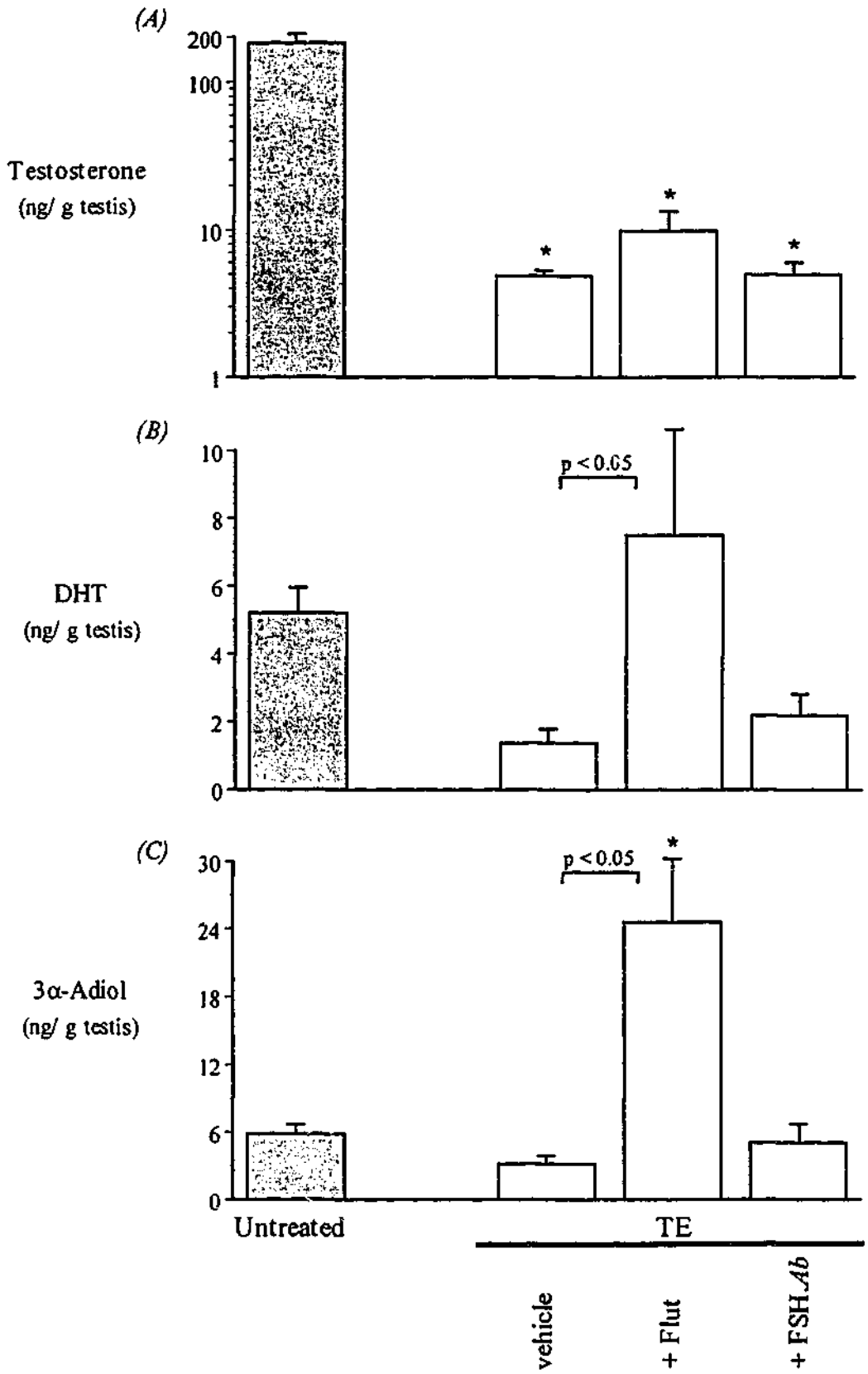


Figure 6-7: Testicular concentration (ng/ g testis) of *(A)* testosterone, *(B)* DHT, and *(C)* 3α-Adiol in untreated and TE-treated rats administered vehicle, flutamide [Flut] or FSH antibody [FSH*Ab*] treatment. Each bar represents mean ± SEM (n = 8). * Indicates a significant difference ($p < 0.01$) compared to untreated animals. Differences between treatment groups are represented at $p < 0.05$. Note the log scale on the y-axis for testosterone.

Figure 6-8 represents the percentage of DHT + 3α-Adiol in terms of testosterone concentration for each group. TE-treatment increased ($p < 0.05$) 5α-reduced metabolites (~ 16-fold) compared to untreated levels. Flutamide administration increased ($p < 0.05$) 5α-reduced metabolites (~ 7-fold) above TE control levels. FSH*Ab* treatment had no effect on the 5α-reduction of testosterone compared to TE control animals.

6.4 Discussion

This study used the TE-treatment model of spermatogenic suppression to selectively suppress testicular testosterone in order to investigate the regulation of 5αR-1 and 5αR-2 enzyme activity and mRNA levels in adult rat testis. This data shows that 5αR-1 (mRNA and activity) in the testis was negatively regulated by androgens, whereas testosterone did not alter the testicular 5αR-2 isoform. It appeared that FSH positively regulated 5αR-1 and 5αR-2 mRNA levels, but did not affect the activity of these isoforms.

(A) Evidence for the Regulation of 5αR-1 Isoform by Testosterone and FSH

Effect of Testosterone Suppression on 5αR-1 Enzyme Activity and mRNA

Blocking the action of androgens by administering the AR antagonist, flutamide, following TE-treatment, increased 5αR-1 enzyme activity and mRNA levels. Thus, inhibition of androgen action up-regulates the 5αR-1 isoform, suggesting that the 5αR-1 isoform in the testis is negatively regulated by androgens. Up-regulation of the 5αR-1 isoform by testosterone suppression was also apparent in TE-treated rats when compared to untreated animals.

The finding that flutamide caused an increase in DHT and 3α-Adiol production is consistent with our previous data (O'Donnell *et al.,* 1999), and suggests that the negative regulation of 5αR-1 by testosterone occurs via the AR rather than by conversion to oestradiol. The graph (*Figure 6-8*) representing the production of 5α-reduced metabolites as a percentage of testosterone directly

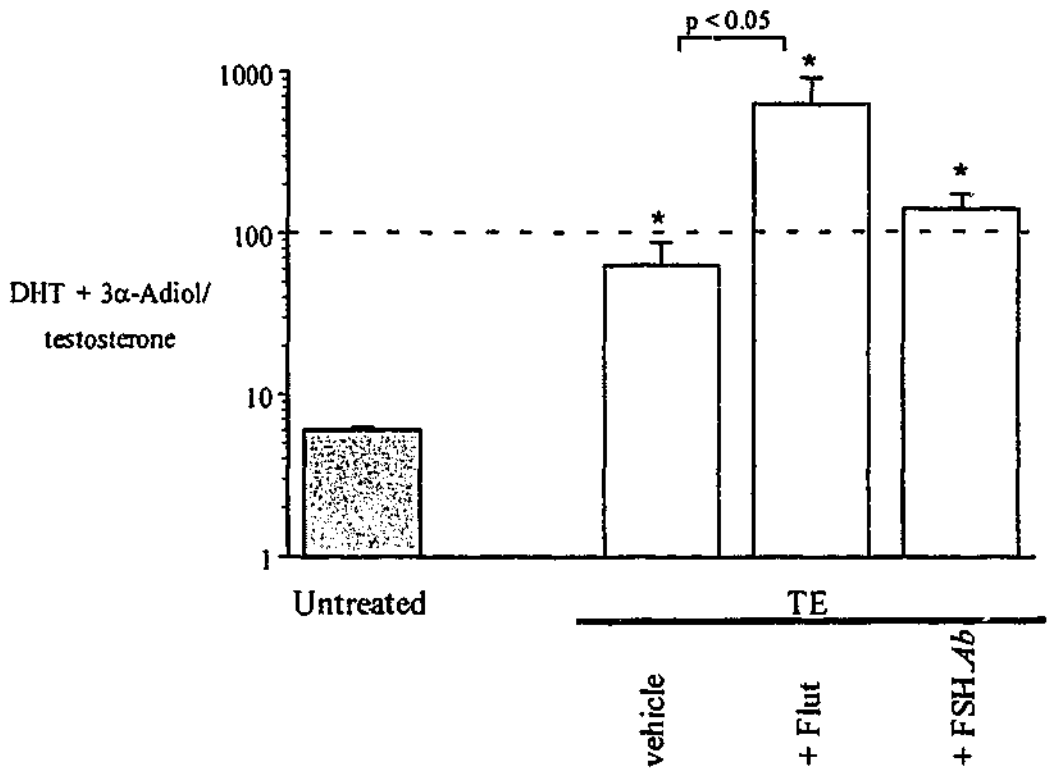


Figure 6-8: 5α-Reduced androgens as a percentage of testosterone (DHT + 3α-Adiol/ testosterone) in untreated and TE-treated rats administered vehicle, flutamide [Flut] or FSH antibody [FSH*Ab*] treatment. Each bar represents mean ± SEM (n = 8). * Indicates a significant difference ($p < 0.01$) compared to untreated animals. Differences between treatment groups are shown at $p < 0.05$.

parallels 5αR-1 mRNA and activity, suggesting that 5αR-1 is primarily responsible for testicular 5α-reduced metabolite biosynthesis.

The current study confirms the hypothesis we proposed earlier (O'Donnell *et al.*, 1999) that testosterone negatively regulates 5αR in the testis, and extends this hypothesis by specifying that it is the 5αR-1 isoform that is negatively regulated by androgens. This finding poses the question for the reason why testicular 5αR-1 is up-regulated in response to androgen suppression. A possible explanation is that this may be a mechanism by which the testis can continue to provide androgenic support to androgen-dependent testicular cells when testosterone levels are reduced, such as during puberty. Therefore, in response to testosterone deficiency, 5αR-1 is up-regulated in the testis to increase the amount of DHT, which given its high androgenic potency, would provide an amplified androgenic stimulus to the seminiferous epithelium. The exact mechanism by which the androgen-induced up-regulation of 5αR-1 occurs is unknown, but is likely to be via the AR.

Effect of Partial Testosterone Restoration on 5αR-1 Enzyme Activity

If testosterone withdrawal increases the 5αR-1 isoform, then restoring testosterone should suppress 5αR-1. However, there was no change in 5αR-1 between TE- and T24-treated animals *(Figure 6-2)*. Although the concentration of testicular steroids in T24-treated animals was not measured, other studies in our laboratory have demonstrated that T24-treatment restores testicular testosterone to ~10% of control levels (O'Donnell *et al.*, 1994, 1996b, 1999), causing a ~5-fold increase in testicular testosterone compared to TE control animals. It is possible that higher doses of testicular testosterone are required to suppress 5αR-1 activity levels (see hCG treatment in Chapter 7). No comment can be made regarding mRNA levels in these animals because they were not analyzed.

These data support previous findings, which suggested that testicular 5αR-1 activity was down-regulated by androgens in immature animals. Oshima *et al* (1970) showed that 5αR-1 activity was reduced following hCG or testosterone propionate administration to normal immature

rats. In adult animals, androgen treatment (hCG administration) appeared to decrease testicular 5αR-1 activity (Folman *et al.*, 1972). Negative regulation of the 5αR-1 isoform by androgens has also been demonstrated in other tissues. For example, in the liver it has been shown that 5αR-1 activity and mRNA are induced by castration in male rats (Lopez-Solache *et al.*, 1996), and the castrated-induced 5αR-1 activity (Yates *et al.*, 1958) and mRNA (Lopez-Solache *et al.*, 1996) decreases following androgen treatment. These findings suggest that similar to the testis, 5αR-1 activity and mRNA are down-regulated by androgens in the liver.

Effect of FSH Suppression on 5αR-1 Enzyme Activity and mRNA

The role of FSH on the 5αR isoform was assessed by administering an FSH*Ab* during TE-treatment (over the 6-day treatment phase) to withdraw FSH. Acute withdrawal of FSH reduced 5αR-1 mRNA *(Figure 6-5)* but had no affect on enzyme activity *(Figure 6-2)*. The reason for the lack of correlation between enzyme activity and mRNA for 5αR-1 in response to the withdrawal of FSH is unclear, and will be discussed later in this chapter. *Table 2* summarizes the regulation of the 5αR-1 isoform in the testis by testosterone and FSH.

Table 2: Summary of changes in mRNA and enzyme activity levels for 5αR-1 after TE-treatment, comparing TE control animals to other treatment groups (no comparisons to untreated animals).

Treatment	5αR-1 mRNA	5αR-1 Activity
Androgen blockade	increases (↑55%, *)	increases (↑ [illegible])
FSH withdrawal	decreases (↓54%, *)	no change [illegible] (ns)

* denotes significance at $p < 0.05$ level and *ns* denotes not significant ($p > 0.05$)

(B) Evidence for the Regulation of 5αR-2 by Testosterone and FSH

Effect of Testosterone Suppression of 5αR-2 Enzyme Activity and mRNA

5αR-2 enzyme activity and mRNA levels were not altered by blocking residual testosterone levels following TE-treatment *(Figures 6-3 and 6-6)*. Thus, unlike the 5αR-1 isoform it appears that 5αR-2 is not regulated by testosterone. Differential regulation of the 5αR isoforms has been previously reported for other tissues, such as the epididymis (Viger and Robaire, 1991, 1996). For example, unilateral efferent duct ligation increased 5αR-2 and decreased 5αR-1 mRNA in the initial segment of the epididymis in the rat. Furthermore, 5αR-2 mRNA does not show any developmental changes whereas 5αR-1 mRNA and activity vary with increasing postnatal age (Viger and Robaire, 1996). Taken together, these results indicate that the 5αR mRNAs are differentially regulated in the rat epididymis. It is not surprising that these two isoforms may be differentially regulated considering that they are two separate genes, which are likely to have distinct promoters.

However, it is worthy to note that whilst suppression of testicular testosterone by TE-treatment did not alter 5αR-2 activity, which is in agreement with the results mentioned above with flutamide treated animals, TE-treatment reduced 5αR-2 mRNA to 31% of untreated levels. Although we have referred to changes between TE-treated and untreated animals throughout the results, we have restricted ourselves from drawing conclusions between TE-treated and untreated animals in the discussion of this chapter. The reasons for this are: (1) the size of the testis varies between untreated and treated animals, therefore if the data was expressed as 5αR activity/ testis rather then 5αR activity/ g testis, the interpretations and conclusions would vary dependent on the mode of data expression, (2) the cellular composition of the testis varies dramatically between treated and untreated animals (i.e. there are qualitative and quantitative differences in germ cell types), so that if 5αR was expressed in germ cells then comparisons could not be made due to the decrease or total loss of some cell types following TE-treatment.

Effect of FSH Suppression on 5αR-2 Enzyme Activity and mRNA

The withdrawal of FSH after TE-treatment did not significantly alter 5αR-2 enzyme activity, but decreased 5αR-2 mRNA. Thus, the inhibition of 5αR-2 mRNA after acute FSH withdrawal suggests that FSH positively regulates the testicular 5αR-2 isoform at the mRNA level. This is similar to the 5αR-1 isoform, where FSH suppression increased 5αR-1 mRNA but had no affect on enzyme activity. From this study alone we can not ascertain why acute FSH suppression affected 5αR mRNA but not activity.

There are several studies in the literature that have reported a lack of correlation between enzyme activity and mRNA levels. For example, androgen withdrawal by castration increased steroid sensitive gene-1 (SSG1) mRNA levels whereas protein levels decreased (Marcantonio *et al.*, 1999a). SSG1 mRNA and protein expression were also discordantly regulated in rat mammary glands following estrogen treatment (Marcantonio *et al.*, 1999b). Yeap *et al* (1999) have demonstrated a discordant pattern of AR mRNA and protein regulation in response to androgens in LnCap prostate cancer cells and in MDA 453 breast cancer cells. Therefore, there does seem to be some precedence for the apparent discordant regulation of mRNA and activity in response to hormonal treatment.

The reason for the discordant regulation of 5αR mRNA and activity in our study is unknown. It is possible that the 6-day time point may be too early to observe a decrease in 5αR activity with FSH treatment. It is worthwhile noting that recombinant human 5αR-1 and 5αR-2 proteins have long half lives (~30 h) (Thigpen *et al.*, 1993). Based on the data on the turnover of recombinant 5αR enzymes (Thigpen *et al.*, 1993) it is reasonable to suggest that a decrease in 5αR-2 activity would not be apparent until at least 2 days after mRNA expression had been affected. *In vitro* and *in vivo* time course studies on the relationship between mRNA expression and 5αR activity are required to confirm this hypothesis.

Above we have mentioned that it appears that FSH is positively regulating 5αR-2 mRNA but not activity. Thus, it is feasible to predict that higher concentrations of FSH might stimulate 5αR-2

mRNA. Elevated FSH levels as a result of flutamide treatment however, did not increase 5αR-2 mRNA expression compared to TE controls. This could be due to the fact that FSH in TE control animals is nearly at untreated levels, and thus increasing the concentration of FSH even further by flutamide would not necessarily induce an increase in 5αR-2 mRNA, or that it has reached a maximum level of expression. *Table 3* summarizes the regulation of the 5αR-2 isoform in the testis by testosterone and FSH.

Table 3: Summary of changes in mRNA and enzyme activity levels for 5αR-2 after TE-treatment, comparing TE control animals to other treatment groups (no comparisons to untreated animals).

Treatment	5αR-2 mRNA	5αR-2 Activity
Androgen blockade	no change (3%↑, *ns*)	no change (20%↓, *ns*)
FSH withdrawal	decreases (36%↓, **)	no change (20%↓, *ns*)

** denotes significance at $p < 0.01$ level and *ns* denotes not significant ($p > 0.05$)

In conclusion, this study has investigated the regulation of testicular 5αR-1 and 5αR-2 enzyme activity and mRNA in the TE-treated adult rats. Testosterone withdrawal significantly increased 5αR-1, and this testosterone-induced increase in 5αR-1 was enhanced by blocking residual levels of testosterone with flutamide. Therefore, these findings indicate that testosterone negatively regulates testicular 5αR-1 mRNA and activity in the rat. In contrast, the 5αR-2 isoform was not regulated by testosterone. FSH did not regulate 5αR-1 or 5αR-2 enzyme activity, however FSH positively regulated 5αR-1 and 5αR-2 mRNA.

Chapter 7

The Role of Testosterone and Follicle Stimulating Hormone in the Regulation of Testicular 5α-Reductase Type 1 and Type 2 Enzyme Activity and mRNA in GnRH- Immunized Rats

7.1 Introduction

It is well known that the two main regulators of spermatogenesis are testosterone and FSH (Sharpe *et al.*, 1994; Zirkin *et al.*, 1989; McLachlan *et al.*, 1996), and thus these are the two main targets for suppression by hormonal contraceptives for men. The previous Chapter, using the TE-treatment model to selectively suppress testosterone, showed that androgen suppression up-regulated the 5αR-1 isoform and that withdrawal of FSH suppressed 5αR-1 and 5αR-2 at the mRNA level.

In order to extend these studies a different animal model was used in which both LH/testicular testosterone and FSH were suppressed by GnRH-immunization for 12 weeks (Awoniyi *et al.*, 1989b, 1992a; McLachlan *et al.*, 1994a, b). Thus, this model is one of severe gonadotrophin suppression that can be used to study the effect of chronic withdrawal of FSH and androgens on the 5αR isoforms. Since this model suppresses both LH and FSH, this model is more analogous to the effects produced by testosterone-based contraceptives in human males. Therefore studying the 5αR isoforms in a setting of long term FSH and LH suppression in rats may provide preliminary information that could be used to predict the effects of contraceptive treatment on the testicular 5αR isoforms in humans.

After chronic gonadotrophin suppression, the acute effects of FSH and testosterone on the testicular 5αR isoforms were studied in order to further elucidate the hormonal regulation of these enzymes. FSH was increased by administration of rhFSH or hCG (FSH concomitantly increases with during hCG treatment, McLachlan *et al.*, 1994b) over a 6-day treatment period. To examine the role of testosterone in the regulation of the 5αR isoforms, hCG was administered to increase testosterone levels in the testis and the concomitant rise in FSH inhibited by FSH.*Ab* treatment.

7.2 Materials and Methods

7.2.1 Animals

Adult male Sprague-Dawley rats (day 65) were obtained from the Monash Central Animal House (Clayton, Australia) and housed under 12 h light/dark cycle with free access to food and water, in groups of 3 - 4 animals per enclosure. This study was approved by the Monash Medical Centre Animal Ethics Committee.

7.2.2 Experimental Design

To withdraw FSH and LH long term adult rats were actively immunized against GnRH with a proprietary GnRH immunogen preparation (BA-1666-4, Biotech Australia❶) incorporating an adjuvant free of myobacterial components (Stewart *et al.*, 1992). Briefly, animals received 100 µl GnRH immunogen every 4 weeks at a single sc site over a 12 week period (McLachlan *et al.*, 1994b, 1995; Meachem *et al.*, 1998). The response to immunization was assessed after 12 weeks by measurement of testicular weights. Based on previous data (McLachlan *et al.*, 1994b), testicular regression to less than 0.55 g was considered to be an indication of successful immunization. The success rate of the GnRH immunogen was 98%.

Table 1 describes the suppression and treatment phases in the GnRH-immunization model. Untreated animals did not receive implants during the suppression phase nor any treatments during the treatment phase. After GnRH-immunization two groups of animals received daily sc hCG (2.5 IU/ kg/ day) to restore testicular testosterone action. Treatments began on the same day as hCG administration and continued for 6 days. The other three groups of animals received either vehicle, FSH*Ab* (2 mg/ kg), or rhFSH (25 IU/ kg/ day) treatment. GnRH-immunized and GnRH-immunized + hCG treated animals receiving vehicle injections were referred to as GnRH-immunized controls and GnRH-immunized + hCG treated controls, respectively.

7.2.3 Experimental Procedures

The collection of tissues, serum LH and FSH assays, measurement of 5αR isoform activity, mRNA, and testicular steroids, and statistical analyses were undertaken as outlined in Chapter 6 for the TE-treatment model.

Table 1: Summary of experimental design for the GnRH study. All groups of animals except untreated animals received 12 weeks of GnRH-immunization prior to the treatment phase. The various treatments given to each group are summarized in the 'treatment phase' column. The anticipated effects on the concentrations of testicular testosterone and serum FSH compared to untreated animals are shown in the last two columns (n = 7 - 8 per group).

Group Name	Suppression Phase	Treatment Phase	Testicular testosterone action	Serum FSH
Untreated	no implants	no treatments	no change	no change
GnRH-Immunization + vehicle (GnRH control)	12 weeks GnRH	6 days	↓	↓
GnRH-Immunization + FSH*Ab*	12 weeks GnRH	6 days	↓	↓↓
GnRH-Immunization + rhFSH	12 weeks GnRH	6 days	↓	↑
GnRH-Immunization + hCG (GnRH + hCG control)	12 weeks GnRH	6 days	↑	↑
GnRH-Immunization + hCG +FSH*Ab*	12 weeks GnRH	6 days	↑	↓

7.3 Results

Testis Weights

GnRH-immunization significantly ($p < 0.01$) suppressed testis weights compared to untreated animals (*see Appendix G*). GnRH-immunization + hCG treatment (±FSH*Ab* treatment) partially restored ($p < 0.01$) testis weight compared to GnRH-immunized controls.

Serum LH and FSH Concentrations

GnRH-immunization markedly suppressed ($p < 0.05$) serum LH to 12.5 % of untreated levels *(Figure 7-1[A])*. There were no significant differences in LH between any of the treatment groups.

Serum FSH was suppressed ($p < 0.05$) to 53% of untreated levels and approached the sensitivity of the FSH assay *(Figure 7-1[B])*. GnRH-immunization + hCG treatment increased FSH above GnRH control levels, but below untreated levels.

Serum FSH was not measured in treatment groups receiving FSH*Ab* because of interference of the antibody with the assay, however, a previous study (Meachem *et al.*, 1998) showed that FSH*Ab* treatment suppressed circulating FSH levels to less than 10% of normal. Serum FSH levels could not be measured in the GnRH-immunized group receiving rhFSH, however, past studies have shown that a lower dose of rhFSH (10 IU/ kg/ day) for 7 days restored all germ cell types except elongated spermatids (McLachlan *et al.*, 1995; Meachem *et al.*, 1998).

5α-Reductase Type 1 and Type 2 Enzyme Activity

GnRH-immunization increased ($p < 0.05$) testicular 5αR-1 enzyme activity compared to untreated levels *(Figure 7-2)*. In comparison to GnRH-immunized controls, 5αR-1 activity was not altered by suppressing residual FSH levels (FSH*Ab* treatment) nor by rhFSH treatment after GnRH-immunization. Restoration of testosterone action by hCG treatment (± FSH*Ab* treatment) suppressed ($p < 0.05$) 5αR-1 activity compared to GnRH-immunized controls.

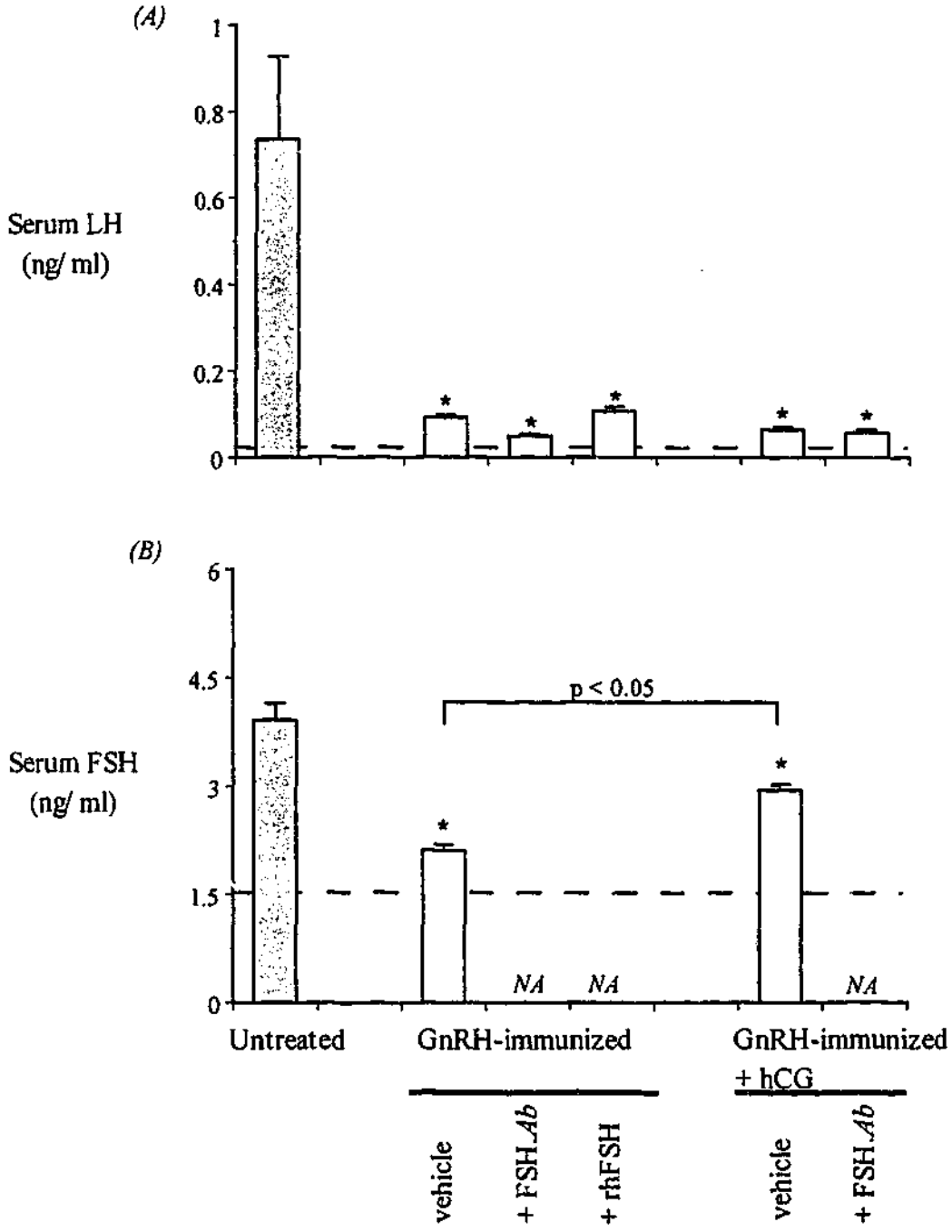


Figure 7-1: Concentration of *(A)* serum LH (ng / ml) and *(B)* serum FSH (ng / ml) in untreated, GnRH-immunized and GnRH-immunized animals treated with human chorionic gonadotrophin (hCG; 2.5 IU / kg / daily). Animals were treated with either vehicle, FSH antibody [FSH.*Ab*], or recombinant human FSH [rhFSH]. Each bar represents mean ± SEM (n = 7 - 8). * Indicates a significant difference compared to untreated animals ($p < 0.05$). Differences between treatment groups are represented at $p < 0.05$. *NA* denotes not assayed because of interferences of FSH.*Ab* or rhFSH with the assay. The dotted lines represent the sensitivities of the assays.

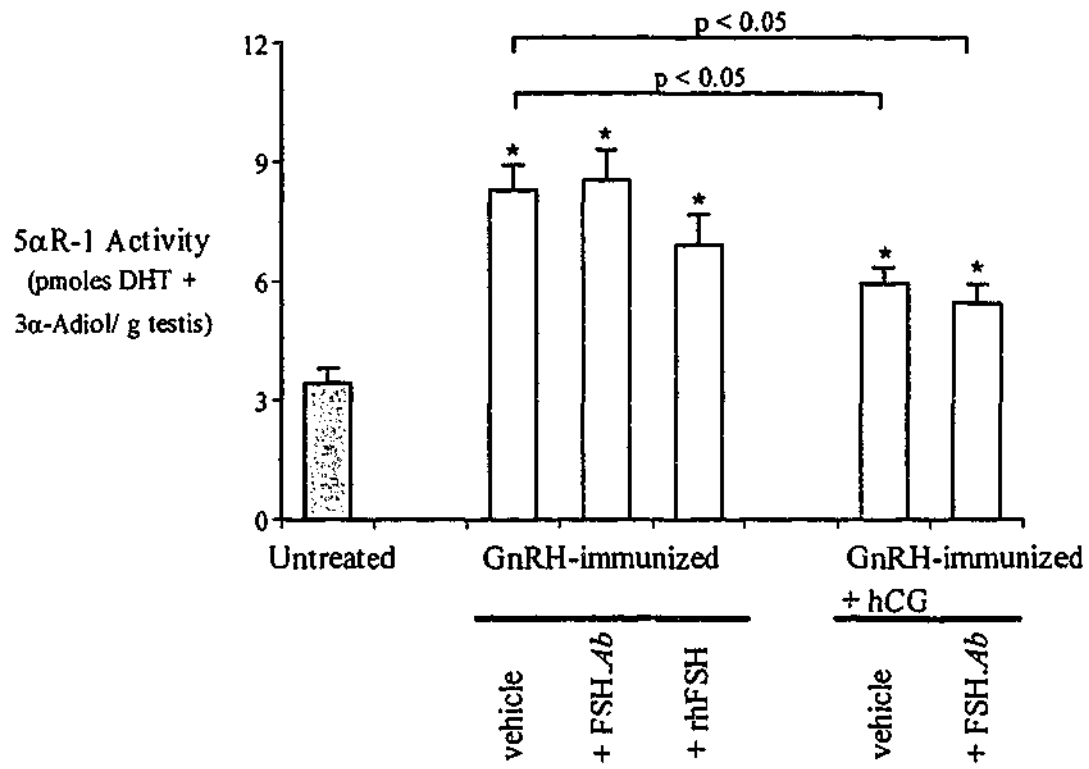


Figure 7-2: 5α-Reductase Type 1 (5αR-1) enzyme activity (pmoles DHT + 3α-Adiol/ min/ g testis) in untreated, GnRH-immunized and GnRH-immunized rats administered human chorionic gonadotrophin (hCG; 2.5 IU/ kg /daily). Animals were treated with vehicle, FSH antibody [FSH.*Ab*], or recombinant human FSH [rhFSH]. Each bar represents mean ± SEM (n = 7-8). * Indicates a significant difference compared to untreated levels ($p < 0.05$). Differences compared to GnRH control are shown at $p < 0.05$.

GnRH-immunization suppressed ($p < 0.05$) 5αR-2 enzyme activity below untreated levels *(Figure 7-3)*. Blocking residual FSH levels following GnRH-immunization did not significantly alter 5αR-2 activity, whereas increasing FSH by rhFSH treatment significantly increased ($p < 0.05$) 5αR-2 activity compared to GnRH-immunized controls, such that it was not different to untreated levels. Increasing both testosterone and FSH levels by hCG treatment following GnRH-immunization significantly increased ($p < 0.05$) 5αR-2 activity compared to GnRH-immunized controls, such that it was similar to untreated levels. Suppression of the hCG-induced rise in FSH caused a significant decrease ($p < 0.05$) of 5αR-2 activity, compared to GnRH-immunized + hCG treated controls.

5α-Reductase Type 1 and Type 2 mRNA Levels

The housekeeping gene 28S ribosomal RNA was used to normalize mRNA levels. There were no significant differences in 28S ribosomal RNA expression between untreated and treated animals *(Figure 7-4)*.

GnRH-immunization suppressed ($p < 0.05$) 5αR-1 mRNA compared to untreated levels *(Figure 7-5)*. Following GnRH-immunization, blocking residual FSH levels by FSH*.Ab* treatment did not alter 5αR-1 mRNA, whereas increasing FSH by rhFSH treatment significantly increased ($p < 0.05$) 5αR-1 mRNA compared to GnRH-immunized controls *(Figure 7-5)*. Restoration of testosterone and FSH by hCG treatment also increased ($p < 0.05$) 5αR-1 mRNA compared to GnRH-immunized controls. Thus, restoration of FSH by rhFSH or hCG treatment restored 5αR-1 mRNA to near untreated levels. Suppression of the hCG-induced rise in FSH with FSH Ab caused a significant decrease ($p < 0.05$) of 5αR-2 activity to 32% of GnRH-immunized + hCG treated controls and ~one-half that of ($p < 0.05$) GnRH-immunized control levels.

After chronic gonadotrophin suppression by GnRH-immunization, 5αR-2 mRNA levels were lower but not significantly different to untreated levels *(Figure 7-6)*. Following GnRH-immunization, restoration of FSH by rhFSH or hCG treatment suppressed ($p < 0.05$) 5αR-2

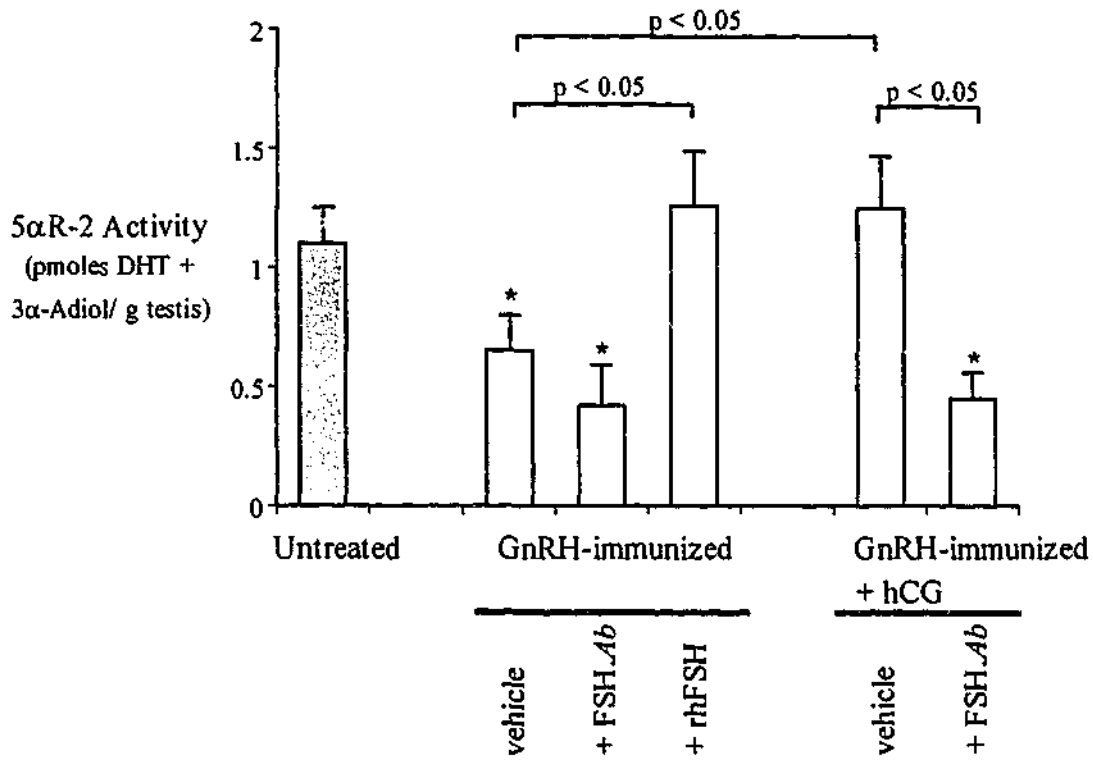


Figure 7-3: 5α-Reductase Type 2 (5αR-2) enzyme activity (pmoles DHT + 3α-Adiol/ min/ g testis) in untreated, GnRH-immunized and GnRH-immunized rats administered human chorionic gonadotrophin (hCG; 2.5 IU/ kg/ daily). Animals were treated with vehicle, FSH antibody [FSH*Ab*], or recombinant human FSH [rhFSH]. Each bar represents mean ± SEM (n = 7-8). * Indicates a significant difference compared to untreated levels ($p < 0.05$). Differences between treatment groups are represented at $p < 0.05$.

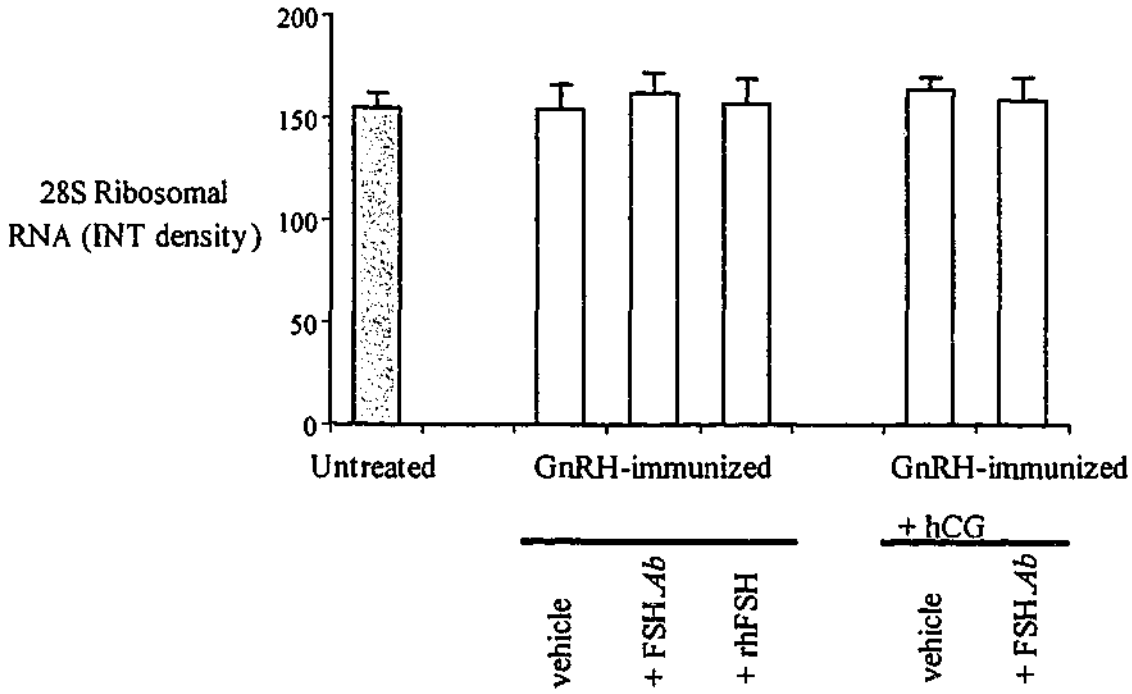


Figure 7-4: There were no significant differences between treatment groups at the $p < 0.05$ level for the housekeeping gene 28S ribosomal RNA (intensity represented as INT units, mean ± SEM).

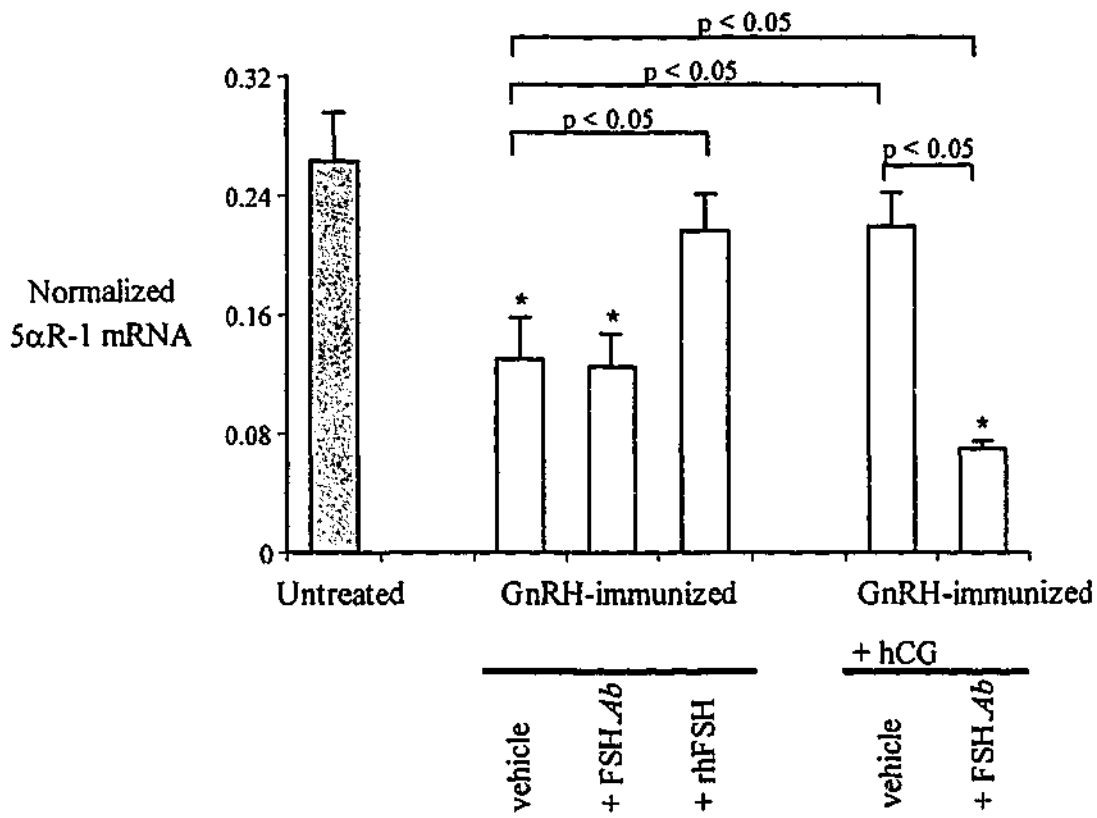


Figure 7-5: 5α-Reductase Type 1 (5αR-1) mRNA levels corrected for RNA loading (mRNA/ 28S ribosomal RNA) in untreated, GnRH- mmunized and GnRH-immunized rats administered human chorionic gonadotrophin (hCG; 2.5 IU/ kg/ daily). Animals were treated with either vehicle, FSH antibody [FSH *Ab*], or recombinant human FSH [rhFSH]. Each bar represents mean ± SEM (n = 7- 8). * Indicates a significant difference ($p < 0.05$) compared to untreated levels. Differences between treatment groups are represented at $p < 0.05$.

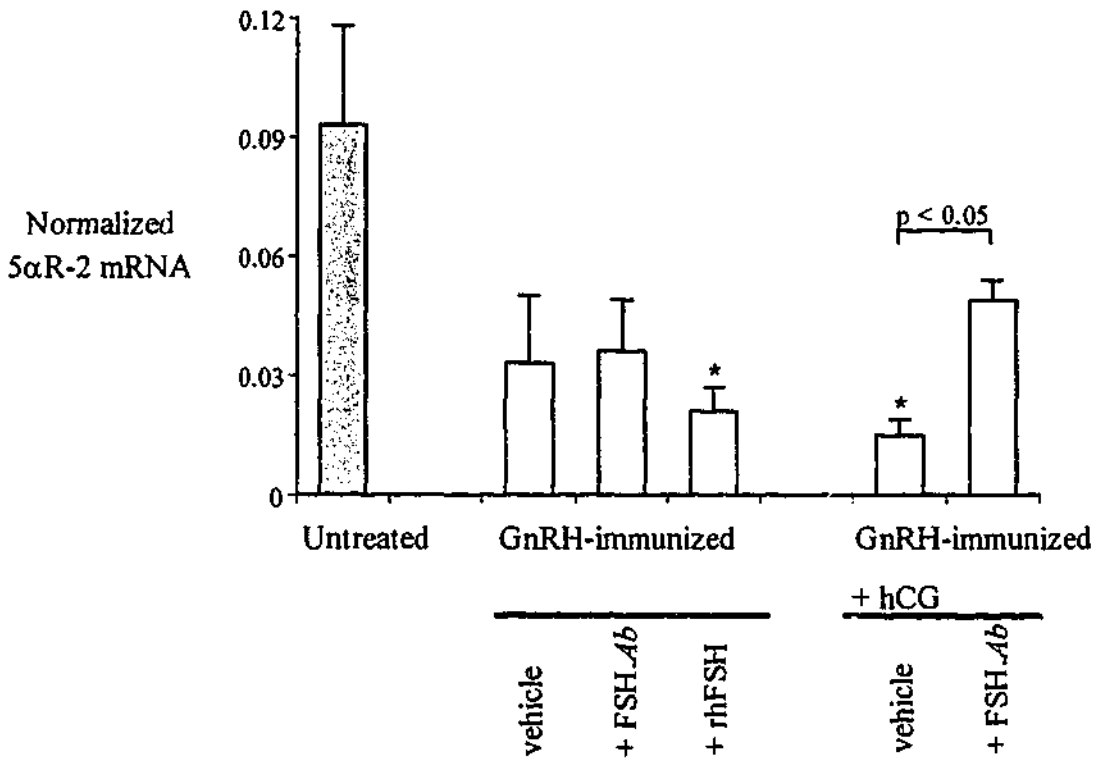


Figure 7-6: 5α-Reductase Type 2 (5αR-2) mRNA levels corrected for RNA loading (mRNA/ 28S ribosomal RNA) in untreated, GnRH-immunized, and GnRH-immunized rats given human chorionic gonadotrophin (hCG; 2.5 IU/ kg/ daily). Animals were treated with either vehicle, FSH antibody [FSH.*Ab*], or recombinant human FSH [rhFSH]. Each bar represents mean ± SEM (n = 7-8). * Indicates a significant difference ($p < 0.05$) compared to untreated levels. Differences between treatment groups are represented at $p < 0.05$.

mRNA, such that it was lower than untreated levels but not different to GnRH-immunized control animals. Suppression of the hCG-induced rise in FSH by FSH Ab caused a significant increase ($p < 0.05$) in 5αR-2 mRNA compared to GnRH-immunized + hCG treated controls.

Testicular Steroids

GnRH-immunization suppressed ($p < 0.01$) testicular testosterone to < 2% of untreated levels *(Figure 7-7[A])*. Suppressing or restoring FSH did not alter testosterone, DHT or 3α-Adiol levels compared to GnRH-immunized control animals. Administration of hCG to GnRH-immunized rats (± FSH*Ab*) increased ($p < 0.01$) testicular testosterone, DHT *(Figure 7-7[B])* and 3α-Adiol *(Figure 7-7[C])* compared to GnRH-immunized controls.

Figure 7-8 represents the percentage of DHT + 3α-Adiol in terms of the testosterone concentration. GnRH-immunization increased ($p < 0.01$) the amount of 5α-reduced metabolites formed compared to untreated animals, and this was not altered by suppressing residual FSH levels or restoring FSH. Restoration of testosterone following GnRH-immunization (± FSH*Ab* treatment) suppressed ($p < 0.01$) the 5α-reduced metabolites compared to GnRH-immunized control levels.

7.4 Discussion

Spermatogenesis was suppressed using the GnRH-immunization model of gonadotrophin withdrawal to chronically suppress testosterone and FSH in order to investigate the regulation of testicular 5αR-1 and 5αR-2 enzyme activity and mRNA levels in adult rats in this setting. It has been shown that severe regression of spermatogenesis via GnRH-immunization occurs within 12 weeks (McLachlan *et al.*, 1995). Following this suppression phase, a 6-day treatment phase was used to examine the effects of FSH (rhFSH treatment) or testicular testosterone production (hCG treatment) on the 5αR isoforms in the testis.

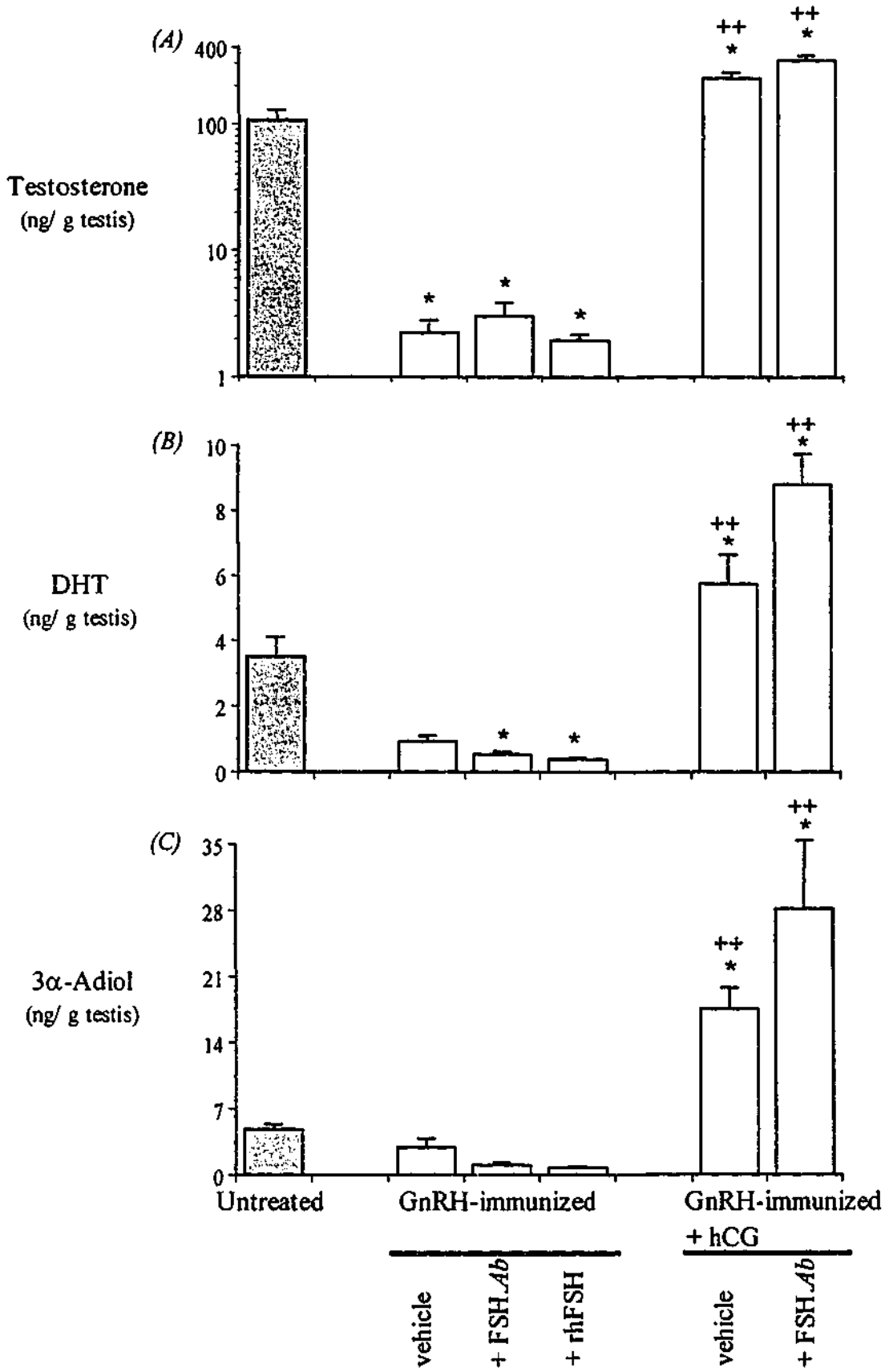


Figure 7-7: Testicular concentration (ng/ g testis) of *(A)* testosterone, *(B)* DHT, and *(C)* 3α-Adiol in untreated, GnRH-immunized and GnRH-immunized rats administered human chorionic gonadotrophin (hCG; 2.5 IU/ kg/ daily), treated with either vehicle, FSH antibody [FSH.*Ab*], or recombinant human FSH [rhFSH]. Each bar represents mean ± SEM (n = 7- 8). * Indicates a significant difference ($p < 0.01$) compared to untreated levels. ++ Indicates a significant difference compared to GnRH controls.

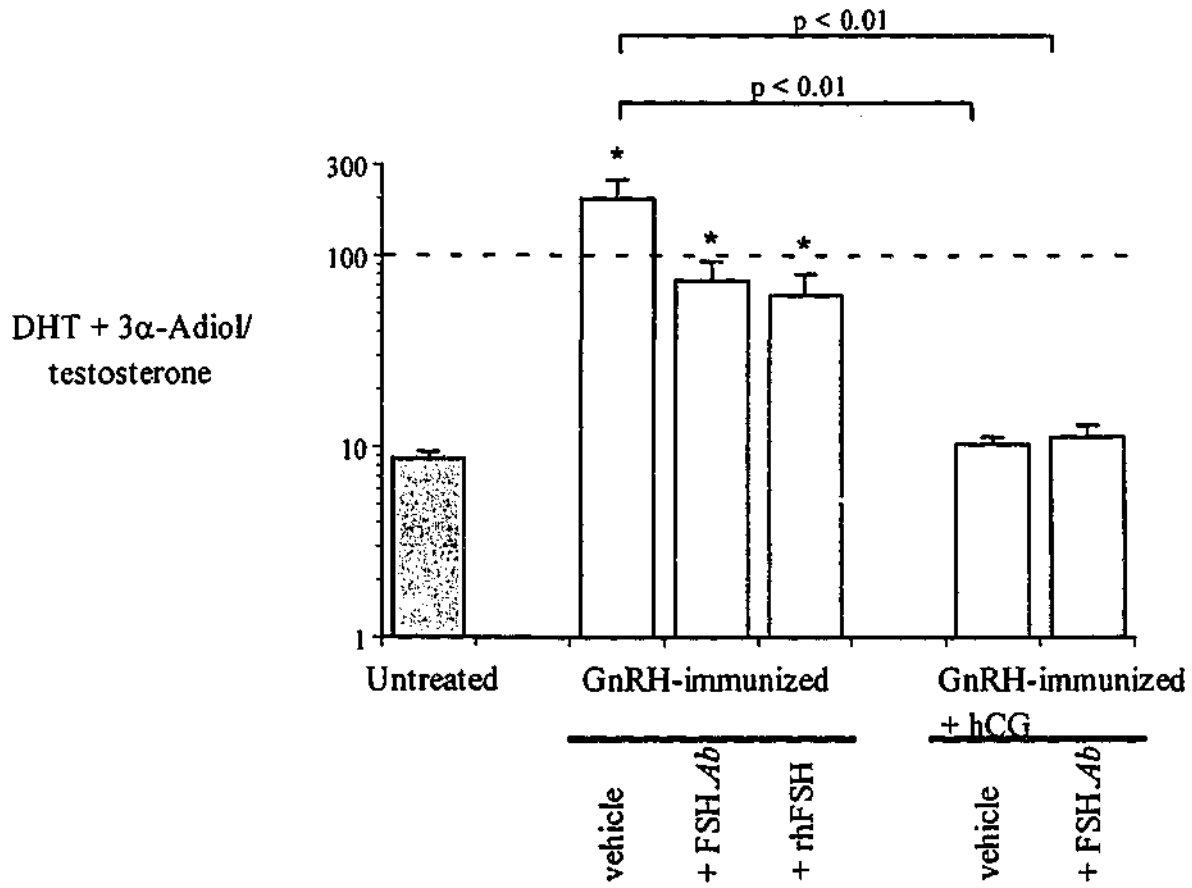


Figure 7-8: 5α-Reduced androgens as a percentage of testosterone (DHT + 3α-Adiol/ testosterone) in untreated, GnRH-immunized and GnRH-immunized rats administered human chorionic gonadotrophin (hCG; 2.5 IU/ kg/ daily), treated with either vehicle, FSH antibody [FSH.*Ab*], or recombinant human FSH [rhFSH]. Each bar represents mean ± SEM (n = 7- 8). * Indicates a significant difference compared to untreated levels ($p < 0.01$). Differences between treatment groups are represented at $p < 0.01$.

GnRH-immunization suppressed serum LH in all treatment groups below untreated levels, and no treatment altered the levels of LH compared to corresponding vehicle-treated controls. FSH levels were suppressed to approach the sensitivity of the FSH RIA by GnRH-immunization, although the FSH levels only decreased to 53% of untreated animals. Administration of hCG to restore testosterone action also caused a significant increase in serum FSH levels. These results are similar to our previously published data (McLachlan *et al.*, 1994b; Meachem *et al.*, 1998).

(A) Evidence for the Regulation of the 5αR-1 Isoform by Testosterone

Testicular testosterone can be acutely restored in GnRH-immunized animals by hCG treatment (Meachem *et al.*, 1997). However, hCG treatment also increases FSH levels in rodents via a GnRH-independent mechanism (Rea *et al.*, 1986; Bhasin *et al.*, 1987; Arslan *et al.*, 1989; Sharma *et al.*, 1990). To investigate the effects of testosterone restoration alone, FSH*Ab* was co-administered with hCG treatment to block the concomitant rise in FSH. Restoration of testosterone alone after GnRH-immunization significantly decreased the level of 5αR-1 enzyme activity compared to GnRH-immunized controls. Similar to the 5αR-1 enzyme activity, restoration of testicular testosterone also decreased 5αR-1 mRNA. This supports the finding from the TE-treatment model which showed that testosterone suppression increased the 5αR-1 isoform, and again suggests that testosterone negatively regulates the testicular 5αR-1 isoform.

Enzyme activity for 5αR-1 directly paralleled the amount of 5α-reduced steroids produced, providing further evidence to the data presented in Chapter 6 that 5αR-1 is primarily responsible for 5α-reduced androgen biosynthesis. This is an important point when considering the affinity of each 5αR isoform. Whereas 5αR-1 has a micromolar affinity for steroid substrates, 5αR-2 has a nanomolar affinity for substrates and thus would be better suited at low substrate concentrations (Normington and Russell, 1992; Thigpen *et al.*, 1993a). Therefore, it is important to note that in our model of gonadotrophin suppression, the low affinity 5αR-1 isoform is still able to convert the

reduced levels of testosterone to 5α-reduced metabolites, emphasizing the importance of this enzyme in the contraceptive setting.

As stated in Chapter 6, the reason for the negative regulation of the 5αR-1 isoform by testosterone is unknown, but we believe that it may be a mechanism by which DHT production can be increased in the testis when testosterone is reduced to levels that are unable to support spermatogenesis. Normally, in the adult animal the level of testosterone in the testis is sufficiently high to allow the weak androgen, testosterone, to directly interact with the AR, and thus conversion to DHT is presumed not to be essential. A decrease in testicular testosterone would stimulate the 5αR-1 isoform, leading to increased DHT production to compensate for the decline in androgenic function. Suppression of testicular testosterone would inevitably also reduce the amount of testosterone available for androgen-dependent peripheral organs, and thus up-regulation of testicular DHT production could possibly also supply these peripheral tissues with the more potent androgen DHT to counteract the decline in testosterone locally within the tissues. Conversely, this mechanism could be thought of as a normal function of the testis rather than a survival mechanism. That is, 5αR-1 is suppressed in the normal testis by the high local concentrations of testicular testosterone in order to prevent an overload of androgen production, which could lead to hypertrophy of tissues such as the prostate.

In summary, taken together the results from the TE-treatment and GnRH-immunization models suggest that 5αR-1 activity and mRNA are up-regulated during testosterone suppression and down-regulated during testosterone restoration, indicating that testosterone negatively regulates the testicular 5αR-1 isoform.

It is interesting to note that whereas GnRH-immunization increased 5αR-1 activity, the mRNA for 5αR-1 declined. We have already discussed in Chapter 6 that comparisons between treated animals are more meaningful than comparisons between untreated and treated animals because of the way we have expressed our data and because of the difference in cell composition between untreated and GnRH-immunized animals. For example, in the normal testis there are

~790 million germ cells (McLachlan *et al.*, 1995; Meachem *et al.*, 1998), 30 million Leydig cells (Duckett *et al.*, 1997a, b), and 35 million Sertoli cells (McLachlan *et al.*, 1998). Thus, Leydig cells account for ~3.8% of these cell types. However, the percentage of Leydig cells after GnRH-immunization increases to ~20% (Duckett *et al.*, 1997a). The fact that 5αR-1 activity per gram of testis is increased could be due to the fact that Leydig cells, which are probably the primary source of 5αR-1 (Viger and Robaire, 1995, and Chapter 5) will be "enriched" in the testis after GnRH-immunization (increase from ~3.8% to ~20% of total cells). It is clear however that 5αR-1 mRNA is low in GnRH-immunized animals, perhaps due to Leydig cell atrophy (Duckett *et al.*, 1997a). Alternatively, post-translational regulation of the 5αR isoforms may also play an important role under these circumstances. These suggestions are purely speculative, and clearly further studies are required as well as determining which cell types express the 5αR isoforms to further our understanding on the mechanism(s) of hormonal regulation of testicular 5αR isoforms.

(B) Evidence for the Regulation of the 5αR-1 Isoform by FSH

The regulation of testicular 5αR-1 enzyme activity by FSH in previous studies has been investigated only at the enzyme activity level and has focused mainly on the immature rat. Furthermore, the data is inconsistent, showing that FSH treatment after hypophysectomy either does (Nayfey *et al.*, 1975a; Weish and Wiebe, 1976) or does not (Dorrington and Fritz, 1975b; Murono and Payne, 1979) affect 5αR-1 activity. There is no data available in the literature on the role of FSH in regulating the 5αR-2 isoform or on the 5αR-1 isoform at the mRNA level. Therefore, FSH was administered following chronic suppression by GnRH-immunization to explore the role of FSH on the regulation of the testicular 5αR isoforms.

Suppression of residual FSH in GnRH-immunized animals did not alter 5αR-1 activity or mRNA, suggesting that the low levels of FSH that remain after GnRH-immunization probably do not play a role in regulating the testicular 5αR-1 isoform. More importantly, 5αR-1 activity was not altered when FSH levels were restored by rhFSH treatment, nor when FSH levels were

restored by hCG treatment. Therefore, this data suggests that 5αR-1 activity is not regulated by FSH.

The restoration of FSH after GnRH-immunization by either rhFSH or hCG treatment increased 5αR-1 mRNA. Thus, although FSH did not alter 5αR-1 activity there were FSH-induced changes in 5αR-1 mRNA, suggesting that FSH stimulates 5αR-1 mRNA but does not affect the activity of the 5αR-1 isoform. The fact that FSH affects 5αR-1 mRNA is also supported by the fact that blocking the FSH rise in hCG treated animals suppressed 5αR-1 mRNA.

The level of 5αR-1 mRNA in GnRH-immunized animals treated with hCG + FSH*Ab* were significantly lower than any other group of GnRH-immunized animals *(Figure 7-5)*. The reason for this suppression of 5αR-1 mRNA may be two-fold: (a) we showed that testosterone negatively regulates 5αR-1 mRNA, therefore hCG treatment would suppress 5αR-1 mRNA, and (b) we showed that FSH positively regulates 5αR-1 mRNA, therefore the prevention of the concomitant rise in FSH with hCG treatment by FSH*Ab* administration would not stimulate 5αR-1 mRNA. Thus, it is possible that suppression of 5αR-1 mRNA may be due to the combined effects of androgen inhibition and FSH deprivation.

In summary, FSH suppression decreased 5αR-1 mRNA and FSH restoration increased 5αR-1 mRNA levels, yet no changes were seen in 5αR-1 activity in response to FSH restoration. This finding supports the data obtained in the TE-treatment model where acute FSH withdrawal suppressed 5αR-1 mRNA but did not alter activity levels.

A difference in the half-lives of the protein and mRNA for 5αR could explain this discordant pattern of regulation for 5αR-1 mRNA and activity with FSH treatment. Clearly, time course studies are required to test whether the profile for 5αR-1 mRNA expression in response to FSH restoration varies to that for the activity. Nayfey *et al* (1975a) showed that FSH treatment after hypophysectomy increased 5αR-1 activity. In this study, animals were hypophysectomized at day 28, and FSH treatment started 3 days later for 4 days. In contrast, Dorrington and Fritz (1975b) showed that FSH did not alter 5αR-1 activity levels. In this study, animals were

hypophysectomized at day 21, and FSH treatment started 6 days later for 5 or 10 days. Thus, it is possible that enzyme activity for 5αR-1 in response to FSH restoration is stimulated by 4 days, similar to the mRNA, however with time 5αR-1 activity declines and thus does not remain elevated by 6 days. The reason for this is unclear, but could possibly be related to post-translational modifications of the 5αR isoform. The regulation of the 5αR-1 isoform in the testis by testosterone and FSH is summarized in *Table 2*.

Table 2: Summary of changes in mRNA and enzyme activity levels for 5αR-1 in response to acute hormone treatment after GnRH-immunization.

Treatment	5αR-1 mRNA	5αR-1 Activity
Testosterone restoration	decreases* (↓47%*)	decreases* (↓35%*)
FSH restoration	increases* (↑66%*)	no change (↓15%ns)

* denotes significance at $p < 0.05$ level and *ns* denotes not significant ($p > 0.05$)

(C) Evidence for the Regulation of the 5αR-2 Isoform by FSH and Testosterone

FSH restoration by rhFSH or hCG treatment stimulated 5αR-2 activity *(Figure 7-3)*, suggesting that FSH positively regulates enzyme activity for the 5αR-2 isoform. When the rise in FSH with hCG treatment was blocked by administration of FSH*Ab*, the increase in 5αR-2 activity was suppressed, suggesting that the increase in 5αR-2 activity in hCG treated animals is due to FSH effects rather than testosterone. However, restoration of FSH did not alter 5αR-2 mRNA levels *(Figure 7-6)*. This data collectively suggests that FSH dos not appear to regulate 5αR-2 mRNA, but positively regulates the 5αR-2 isoform at the enzyme activity level.

Restoration of testosterone alone following GnRH-immunization (hCG + FSH*Ab* treatment) did not alter 5αR-2 activity or mRNA levels compared to GnRH-immunized control animals. This supports the findings from the TE-treatment model which showed that blocking residual levels of

testosterone with flutamide after TE-treatment did not alter 5αR-2 activity or mRNA levels. The regulation of the 5αR-2 isoform in the testis by testosterone and FSH is summarized in *Table 3*.

Table 3: Summary of changes in mRNA and enzyme activity levels for 5αR-2 in response to hormone treatment after GnRH-immunization.

Treatment	5αR-2 mRNA	5αR-2 Activity
Testosterone Stimulation	no change (↑48%, *ns*)	no change (↑53%, *ns*)
FSH Stimulation	no change (↓36%, *ns*)	increased (↑92%, *)

* denotes significance at $p < 0.05$ level and *ns* denotes not significant ($p > 0.05$)

Chapter 8

General Discussion

8.1 Introduction

The 5αR enzyme, by virtue of its ability to metabolize testosterone to the more potent androgen, DHT, has been suggested to be involved in maintaining some aspects of androgen action in the testis in a setting of low testicular testosterone (Anderson *et al.*, 1996b, 1997a, c; O'Donnell *et al.*, 1996b, 1999). These observations are important for understanding why some men respond adequately to a male contraceptive whereas others do not (Anderson *et al.*, 1996b, 1997a, section 1.7.2). Accordingly, it is of interest to understand the characteristics of testicular 5αR isoforms, as well as to understand what factors regulate the 5αRs in the testis. The studies presented in this thesis, including the development of methodologies for measuring testicular 5αR mRNA and enzyme activity, characterization of 5αR-1 and 5αR-2 in the testis, and analysis of the hormonal regulation of the testicular 5αR isoforms, provide us with a better understanding of these enzymes and their role in the testis.

A large portion of this thesis was concerned with the development of appropriate methods to measure 5αR isoforms in the testis. An *in vitro* assay was used to measure 5αR-1 and 5αR-2 enzyme activity, taking advantage of the distinct pH optimas for each of the 5αR isoforms. However, we were required to validate a new approach to measure 5αR-2 activity in the testis due to the overlapping 5αR-1 activity at the optimal pH for 5αR-2. These assays were validated and then used to measure 5αR isoform activity in rat testis at various ages and during hormonal treatment.

Secondly, a quantitative PCR approach with the LightCycler instrument was validated and used to measure 5αR-1 and 5αR-2 mRNA levels in hormone-treated adult animals. This approach allowed rapid analysis of PCR amplification, confirmation of specific product amplification and sensitive quantitative analysis. Together, these techniques allowed us to investigate the 5αR isoforms at both the transcriptional and translational level.

To investigate the *in vivo* hormonal regulation of the 5αR isoforms in adult rat testis, two models of gonadotrophin deficiency, TE-treatment and GnRH-immunization, were used. In the TE model,

LH/ testicular testosterone was suppressed to levels unable to maintain spermatogenesis, whereas in the GnRH immunization model both FSH and LH were suppressed. Following chronic/ long-term gonadotrophin suppression, various treatments were used to either block residual hormone levels or to increase testosterone or FSH. For example, the androgen receptor antagonist, flutamide, and FSH.*Ab* were administered to block the action of residual levels of testosterone and FSH, respectively. Testosterone action was restored by hCG administration and FSH action was restored with rhFSH.

8.2 Methods for the Measurement of Testicular 5α-Reductase Isoform Activity and mRNA Expression

An *in vitro* activity assay (Chapter 3) was used to measure testicular 5αR-1 and 5αR-2 enzyme activity at pH 7.0 and pH 5.0, respectively, by measuring the conversion of ^{3}H-testosterone to the 5α-reduced metabolites, ^{3}H-DHT and ^{3}H-3α-Adiol. The steroids were extracted with toluene:ether (1:5) and separated on ITLC-chromatography plates for 31 min, using chloroform:methanol (98:2) as the running solvent. After separation, each of the androgens were quantitated by β-counting, and 5αR activity expressed as the sum of the 5α-reduced metabolites formed. Standard procedures such as correcting for losses during experimental procedures (using ^{14}C-steroids) and subtraction of background levels were included.

5αR-1 and 5αR-2 enzyme activity has been assessed in many different tissues by examining the activity at the optimal pH for each isoform. Thus, pH 7.0 measurements have been used to assess 5αR-1 and pH 5.0 measurements have been used to assess 5αR-2 activity (Andersson *et al.*, 1990; Normington and Russell, 1992). Similar to previous studies investigating testicular 5αR-1 activity (e.g. Folman *et al*, 1972; Rivarola *et al.*, 1972; Dorrington and Fritz, 1975), we used the activity detected at pH 7.0 to measure 5αR-1 activity.

However, due to the overlap of 5αR-1 activity at pH 5.0 (the pH optima for 5αR-2), pH 5.0 measurements could not be used to measure 5αR-2 activity where 5αR-1 activity was in abundance. It was thus proposed to develop a specific 5αR-2 activity assay which was specific for 5αR-2 activity even in the presence of large amounts of 5αR-1. To this end we determined the overlap of 5αR-1 activity at pH 5.0 using recombinant rat 5αR-1 which was devoid of 5αR-2. These studies showed that 12.4 % of 5αR-1 activity was detected at pH 5.0 (based on data from 14 different measurements and 5 different transfections). Therefore, by correcting for this overlap of 5αR-1 activity at pH 5.0, we showed that 5αR-2 activity could be measured at pH 5.0. This conclusion was substantiated by additivity experiments whereby recombinant rat 5αR-2 activity was determined in the presence of recombinant rat 5αR-1. We have published the validation of the *in vitro* 5αR-1 and 5αR-2 assays and their application for measuring the 5αR isoforms in the testis (Pratis *et al.*, 2000; see *Appendix H*).

The advantages of the *in vitro* 5αR isoform assays were accuracy, sensitivity and reliability. The TLC procedure for separating testosterone from its 5α-reduced derivatives was practical since a large number of samples could be analyzed simultaneously in a short period of time. For example, in our laboratory we use HPLC to separate testicular androgens before measuring the concentration of each androgen by RIA. HPLC requires testes to be homogenized, centrifuged, passed through a column, separated by HPLC, and the relevant fractions determined by β-counting, before RIA analysis (see section 2.3.3). Using HPLC, 20 days would be required to analyze 150 samples, compared to just 2 days using TLC. Considering the amount of work that was required to validate procedures and analyze samples, we found that it was worthy, financially and time-wise, to use the TLC procedure instead of HPLC.

The second method used in this thesis was a quantitative PCR procedure using the LightCycler instrument (Roche®) to measure 5αR-1 and 5αR-2 mRNA (see Bustin *et al.*, 2000 for review and Chapter 4). This technique was used to monitor PCR reactions in 'real time' by

visualizing the PCR amplification as it occurred, followed by subsequent quantification and verification of specific product amplification via melting curve analysis. This procedure is commonly referred to as real time PCR, and is fast becoming the preferred choice for quantitative PCR.

The advantages of real time PCR are: (a) unlike other PCR methods, the visualization of PCR amplification with the LightCycler allows adjustments to cycle number to be made during the experiment, (b) it allows the simultaneous verification of PCR product using the melting curve program. Thus, the PCR products of interest obtained at a specific melting temperature could be differentiated from non-specific products which have lower melting temperatures. In addition, the quantification procedure allows the experimenter to choose the temperature at which quantification begins, and this is usually set above the non-specific product temperature to eliminate non-specific products from contributing to quantitation, (c) the LightCycler has short PCR cycles because of airstream cooling/heating rather than the conventional block methods, (d) it is more sensitive than other methods used to measure steady state RNA levels, including Northern blot analysis, RNAse protection assays and *in situ* hybridization (Bustin *et al.*, 2000), and (e) real time PCR is the best routine technique available for quantitative PCR analysis, and is fast replacing competitive RT-PCR which has high errors and is time-consuming (Raemaekers, 1999).

Based on the advantages listed above and particularly because of its sensitivity and accuracy, the LightCycler PCR method was applied in the determination of testicular 5αR-1 and 5αR-2 mRNA levels. Whereas testicular 5αR-2 mRNA has not been investigated, 5αR-1 mRNA has been examined recently in the testis using Northern blot analysis, however its level of expression is contentious (Normington and Russell, 1992; Viger and Robaire, 1995; Reyes *et al.*, 1997).

8.3 5α-Reductase Isoforms in the Testis

In Chapter 5 it was shown that the pH profiles for 5αR activity in immature (day 30) and adult (day 75) animals displayed high levels of activity in the neutral pH range. These pH profiles were similar to the liver which expresses high levels of 5αR-1 (Andersson *et al,* 1989; Lopez-Solache *et al.*, 1996; Normington and Russell, 1992), and to recombinant rat 5αR-1 (Anderson *et al.*, 1991; Normington and Russell, 1992). Enzyme kinetic studies confirmed that pH 7.0 measurements in the testis corresponded to 5αR-1 activity. The micromolar substrate affinity constants for 5αR-1 in rat testis (at days 30, 75 and 147) were similar to those obtained for the liver and recombinant 5αR-1 in our studies, and were in accordance with previously published data for recombinant and native rat 5αR-1 (Andersson and Russell, 1990; Normington and Russell, 1993; Span *et al.*, 1996, 1996a; Thigpen *et al.*, 1993a). Using the activity measured at pH 7.0 to assess 5αR-1 activity in rat testis, we showed that 5αR-1 enzyme activity was high at day 30 post birth, decreased at day 75, and then declined further by day 147. These data are in agreement with previously published data (Dorrington and Fritz, 1973, 1975, Rivarola *et al.*, 1972a; van der Molen, 1975).

To the best of our knowledge, there are no descriptions of 5αR-2 enzyme activity in rat testis. The pH profiles for day 30 and day 75 rat testis revealed low but dissimilar amounts of activity at pH 5.0, such that pH 5.0 activity was higher at day 30 compared to day 75. This suggested higher levels of 5αR-2 activity during immaturity. However, it was not apparent whether the activity at pH 5.0 was due to 5αR-2 activity or an overlap of 5αR-1 activity. Applying the procedure for measuring 5αR-2 activity in the presence of high overlapping 5αR-1 activity (using recombinant 5αR-1), this thesis showed that 5αR-2 activity in rat testis was high at day 30, was decreased at day 75, and was reduced even lower by day 147. Thus, the pattern of 5αR-2 enzyme activity in rat testis was similar to that of 5αR-1 activity, but 5αR-2 activity levels were lower than 5αR-1. A comparison of the two 5αR isoforms showed that 5αR-1 activity was approximately 14-, 17-, and 4-fold higher than 5αR-2 activity at days 30, 75, and 147, respectively.

Despite the decrease in both 5αR isoforms with age, expression of 5αR-2 activity relative to 5αR-1 was highest at day 147. This suggests that the 5αR-2 isoform might be important for supporting the spermatogenic process as testosterone levels decrease with age (Corpechot *et al.*, 1981). In agreement with this, the enzyme kinetic studies for day 30 testis in this thesis showed that whereas 5αR-1 was quantitatively the most abundant 5αR isoform, the efficiency of the 5αR-2 isoform (i.e. its potential *in vivo* activity) was equivalent to 5αR-1. This type of analysis could not be applied to day 147 testes because 5αR-2 was not detectable at this age by enzyme kinetics.

8.4 Regulation of 5αR-1 and 5αR-2 mRNA and Enzyme Activity in the Rat Testis

In the rat, the number of round spermatids (O'Donnell *et al*, 1996b) and subsequently elongated spermatids (O'Donnell *et al*, 1999) produced in the testis under the influence of low testicular testosterone can be suppressed by inhibiting 5αR (O'Donnell *et al*, 1996b, 1999), indicating that at low testosterone doses the 5α-reduction of testosterone is required for androgen action on spermatogenesis. We have also previously shown that the level of testicular 5α-reduced androgens were elevated by blocking androgens with flutamide after TE-treatment, despite no change in serum LH or testicular testosterone levels (O'Donnell *et al.*, 1996b, 1999). These results suggested that 5αR activity in the testis might be up-regulated in the absence of androgens, perhaps due to the up-regulation of the 5αR enzyme. The type(s) of 5αR isoform affected by androgen suppression were not determined in that earlier study.

During testosterone-induced spermatogenic suppression in humans, persistence of low levels of spermatogenesis in some men may depend on their ability to form the potent androgen DHT (Anderson *et al.*, 1996b, 1997a). This highlights the importance for understanding the role of 5αR during contraception, and warrants the investigation of the testicular 5αR isoforms, particularly in a setting of reduced testicular testosterone. Therefore, we were interested in investigating the

regulation of mRNA and activity for the testicular 5αR isoforms in the adult rat, in a setting where spermatogenesis was suppressed by inhibiting pituitary gonadotrophins.

(A) Regulation of the Testicular 5α-Reductase Isoforms by Testosterone

A summary of the regulation of the 5αR isoforms by testosterone is represented in *Table 1*. In the section below, the regulation of the 5αR-1 mRNA and enzyme activity by testosterone will be discussed, followed by the regulation of the 5αR-2 isoform, and finally the relevance to the regulation of spermatogenesis during contraception.

Table 1: The effect of increasing and decreasing testosterone on 5αR-1 and 5αR-2 mRNA and enzyme activity.

Treatment	mRNA	Enzyme activity	Explanation
5αR-1 (Liver)			
↓ androgens [a] ↑ androgens [b]	Increases *(Figure 6-5)* Decreases *(Figure 7-5)*	Increases *(Figure 6-2)* Decreases *(Figure 7-2)*	Testosterone negatively regulates 5αR-1
5αR-2 (Liver)			
↓ androgens [a] ↑ androgens [b]	- *(Figure 6-6)* - *(Figure 7-6)*	- *(Figure 6-3)* - *(Figure 7-3)*	Testosterone does not regulate 5αR-2

[a] refers to the TE-treatment and [b] refers to the GnRH-immunization model, - denotes no significant change

Testicular 5αR-1 Isoform

The hormonal regulation of testicular 5αR-1 activity is a contentious issue (Dorrington and Fritz, 1975b; Folman *et al.*, 1972). This thesis used the TE-treatment model and flutamide administration (Chapter 6) to investigate the effect of androgen blockade on the 5αR-1 isoform in adult rat testis. Blocking androgens with flutamide increased 5αR-1 enzyme activity and mRNA levels, to result in a parallel increase in the production of testicular 5α-reduced metabolites without a significant increase in testicular testosterone, which is consistent with our previous study (O'Donnell *et al.*, 1999). The GnRH-immunization model and hCG treatment (Chapter 7) was used to investigate the effect of testosterone restoration on the 5αR-1 isoform. Increasing testicular testosterone levels after GnRH-immunization by hCG treatment decreased 5αR-1 mRNA and enzyme activity levels, and also decreased the amount of 5α-reduced metabolites formed in the testis. Thus, this thesis has shown that 5α-reduced enzyme activity and mRNA levels are up-regulated by decreasing testicular testosterone levels and 5αR-1 is down-regulated by increasing testosterone levels *(Table 1)*.

This data suggests that testosterone negatively regulates the testicular 5αR-1 isoform, confirming and extending our previous hypothesis that testosterone suppression up-regulates testicular 5α-reduction to increase the levels of DHT and 3α-Adiol, by increasing the 5αR-1 isoform. In Chapter 6 we discussed that up-regulation of 5αR-1 during testosterone-induced spermatogenic suppression may help amplify androgen action within the testis, to maintain spermatogenesis at low levels. The fact that flutamide treatment resulted in an increase in 5αR-1 suggests that the regulation of 5αR-1 by testosterone may occur via the AR.

In support of our findings, testicular 5αR-1 activity in immature rats was inhibited by testosterone treatment and enhanced by gonadotrophin administration (Oshima *et al.*, 1970) and LH increased 5αR-1 activity after its abolishment by hypophysectomy (Murono and Payne, 1979). Similar to the testis, 5αR-1 has also been shown to be negatively regulated by androgen at both the mRNA and activity level in other tissues. For example, liver 5αR-1 activity (Yates *et al.*, 1958)

and mRNA (Lopez-Solache *et al.*, 1996) levels increased in response to castration in male rats, and androgen treatment decreased 5αR-1 activity (Yates *et al.*, 1958) and mRNA (Lopez-Solache *et al.*, 1996). In the adrenal cortex of male rats, castration increased 5αR-1 activity by 3-fold and mRNA by 7-fold compared to intact animals (Lephart *et al.*, 1991). After castration, DHT treatment for 14 days decreased 5αR-1 activity and mRNA to one-third and one-half that of intact animals, respectively (Lephart *et al.*, 1991). In contrast, 5αR-1 enzyme activity (George *et al.*, 1991; Moore and Wilson, 1973) and mRNA (Andersson *et al.*, 1989; Normington and Russell, 1992) in rat ventral prostate increased after castrated animals were treated with testosterone, suggesting that androgens positively regulate 5αR-1 in the prostate. The reason for the differences between different tissues is not clear, but indicates that the factors regulating 5αR-1 may be tissue-specific.

Testicular 5αR-2 Isoform

As outlined in the literature review (section 1.5.5) the regulation of 5αR-2 has been shown to vary between tissues and between species (Andersson *et al.*, 1989; George *et al.*, 1991; Horton *et al.*, 1993; Antonipillai *et al.*, 1995; Cornwall and Hann, 1995). The regulation of the 5αR-2 isoform in the testis has not been previously investigated.

This thesis has shown that the 5αR-2 isoform (mRNA and enzyme activity) were not altered by blocking testosterone action with flutamide (Chapter 6) or by increasing testosterone levels with hCG treatment after GnRH-immunization (Chapter 7), suggesting that testosterone does not regulate the 5αR-2 isoform in the testis *(see Table 1)*. These results are in contrast to those findings for the 5αR-1 isoform, and suggests that the testicular 5αR isoforms are differentially regulated in rat testis. It is not surprising that these two isoforms may be differentially regulated considering that 5αR-1 and 5αR-2 are products of two separate genes. Differential regulation of 5αR isoforms has been reported for rat epididymis (Viger and Robaire, 1991, 1996). For example,

5αR-1 mRNA, enzyme activity and protein increase dramatically during postnatal development (Scheer and Robaire, 1980, 1983; Viger and Robaire, 1992) whereas 5αR-2 mRNA expression does not change during development (Viger and Robaire, 1996).

The regulation of the 5αR-2 isoform by testosterone has been investigated in a number of non-gonadal tissues in the rat, and it appears that androgenic regulation of 5αR-2 is tissue-specific. Similar to our observations in the testis, androgens have been demonstrated not to influence 5αR-2 mRNA in the genital tubercle of the fetal rat (Tian and Russell, 1997). In contrast, 5αR-2 in rat prostate has been demonstrated to be positively regulated by androgens (Normington and Russell, 1992), and this is mediated by DHT rather than testosterone (George *et al.*, 1991; Normington and Russell, 1992). Similarly in human and rat genital skin fibroblasts, DHT and not testosterone increases 5αR-2 activity (Horton *et al.*, 1993). Further studies are required to determine if DHT, rather than testosterone specifically, affects the testicular 5αR-2 isoform.

Relevance of Findings

In the normal testis, testosterone is thought to be the predominant androgen involved in the regulation of spermatogenesis (Wright and Frankel, 1979) since the level of testosterone far exceeds that of any other androgen. However, during experimentally-induced spermatogenic suppression in rats, 5αR has been shown to be important for maintaining spermatogenesis (O'Donnell *et al.*, 1996b, 1999). This highlights the importance of the 5αR enzyme and DHT in spermatogenesis at reduced levels of testosterone.

The 5α-reduction of testosterone to DHT is also thought to be of major significance in the human testis during contraception. Testosterone-based male contraceptives (weekly testosterone enanthate injections) provide adequate and consistent spermatogenic suppression (azoospermia) in two thirds of Caucasian men, while the other one third of men remain oligozoospermic (WHO, 1990, 1995, 1996; Anderson and Wu, 1996).

There are several lines of evidence to suggest that there is selective up-regulation of 5αR activity in oligozoospermic men after testosterone treatment: (a) 5αR activity (conversion of testosterone to DHT) in oligozoospermic men was significantly increased compared to their baseline, suggesting that the contraceptive increases 5αR activity, (b) the conversion ratio of testosterone to DHT is higher in oligozoospermic compared to azoospermic men (Anderson *et al*, 1996), (c) there are higher levels of 5α-reduced metabolites in the reproductive tract (epididymis and/or testis) of oligozoospermic men (Anderson *et al.*, 1997a), and (d) residual testicular steroidogenesis does not appear to account for the incomplete suppression of spermatogenesis (Anderson *et al.*, 1997c). These data suggest that production of 5α-reduced metabolites during testosterone-induced spermatogenic suppression may allow low levels of spermatogenesis despite gonadotrophin and testosterone suppression. It is also interesting to note that, despite marked decreases in testicular testosterone levels during contraceptive treatment, testicular DHT levels are maintained in both primates (Narula, Gu, O'Donnell, Stanton, Robertson, McLachlan, Bremner, *submitted*) and humans (Robert McLachlan, *manuscript in preparation*). This also suggests that testicular 5αR is up-regulated during contraception.

The work from this thesis together with the fact that 5αR appears to play an important role in spermatogenesis when intratesticular testosterone levels are low, in both rats (O'Donnell *et al.*, 1996b, 1999) and humans (Anderson *et al.*, 1996, 1997a, c) suggests that to achieve consistent and uniform spermatogenic suppression in testosterone-based contraceptives, the 5αR enzyme must also be inhibited to prevent the conversion of testosterone to the more potent androgen DHT. This could be achieved by the co-administration of a 5αR inhibitor in existing testosterone-based contraceptive regimens, or by using non-5α-reducible androgens such as MENT.

McLachlan *et al* (2000) used testosterone implants in a contraceptive efficacy study to suppress spermatogenesis in men, and obtained similar results (70% of men became azoospermic) to the WHO (1990, 1996) trials. However, administering finasteride, which is a specific 5αR-2 inhibitor (Normington and Russell, 1992) to oligozoospermic men did not enhance spermatogenic

suppression. However, the type of 5αR isoform expressed in the human testis is unclear. Aumüller *et al* (1996) showed immunohistochemically that in human testis, 5αR-1 is present in Leydig cells and Sertoli cells, whereas 5αR-2 is present in the Leydig cells and spermatogonia. In the cynomologous monkey, 5αR-1 mRNA was abundantly expressed whereas 5αR-2 mRNA was not detectable (Mahony *et al.*, 1997). Thus, further clinical trials are required to test the significance of adding a 5αR inhibitor with testosterone as a male contraceptive, using either a specific 5αR-1 inhibitor or a dual or non-specific inhibitor to suppress both 5αR isoforms. Alternatively, non-5α-reducible androgens such as MENT could replace the use of testosterone (Sundaram and Kumar, 2000).

Furthermore, this thesis has shown that 5αR-1 appears to be the enzyme responsible for the 5α-reduced metabolites formed in the testis, since 5αR-1 activity directly paralleled the concentration of testicular DHT and 3α-Adiol. The 5αR-1 enzyme may also be important in humans since McLachlan *et al* (2000) did not see an improvement in spermatogenic suppression when co-administering a specific 5αR-2 inhibitor with testosterone implants.

The dose of testosterone used in male contraceptive studies produces high serum testosterone levels, which may have adverse effects on blood cholesterol metabolism, potentially leading to increase heart disease. Furthermore, the long-term effects of prolonged administration of testosterone on the prostate is unknown. Thus, the benefit of co-administering a 5αR inhibitor with testosterone is that it may prevent adverse effects on the prostate as a result of excessive androgenic stimulation.

(B) Regulation of the Testicular 5α-Reductase Isoforms by FSH

A summary of the regulation of the 5αR isoforms by FSH is represented in *Table 2*. In the section below, the regulation of 5αR-1 mRNA and enzyme activity by FSH will be discussed, followed by the regulation of the 5αR-2 isoform, and the relevance of these findings to contraceptive studies will be discussed.

Table 2: The effect of increasing and decreasing FSH on 5αR-1 and 5αR-2 mRNA and enzyme activity.

Treatment	mRNA	Enzyme activity	Explanation
5αR-1 isoform			
↓ FSH [a]	Decreases *(Figure 6-5)*	- *(Figure 6-2)*	(no change in 5αR-1 activity could be due to protein half-life or transcript size) FSH positively regulates 5αR-1 mRNA
↑ FSH [b]	Increases *(Figure 7-5)*	- *(Figure 7-2)*	(no change in 5αR-1 activity could be due to time course)
5αR-2 isoform			
↓ FSH [a]	Decreases *(Figure 6-6)*	- *(Figure 6-3)*	(no change in 5αR-2 activity could be due to protein half-life) FSH positively regulates 5αR-2
↑ FSH [b]	- *(Figure 7-6)*	Increases *(Figure 7-3)*	(no change in 5αR-2 mRNA could be due to mRNA half-life and time course)

[a] refers to the TE-treatment and [b] refers to the GnRH-immunization model, - denotes no significant change

Testicular 5αR-1 Isoform

The role of FSH in the regulation of 5αR-1 activity is unclear (Nayfey *et al.*, 1975a; Dorrington and Fritz, 1975b; Welsh and Wiebe, 1976; Murono and Payne, 1979). This thesis showed that withdrawal of FSH (TE-treatment + FSH*Ab*, Chapter 6) decreased 5αR-1 mRNA and increasing FSH (GnRH-immunization + rhFSH or GnRH-immunization + hCG, Chapter 7) levels increased 5αR-1 mRNA, suggesting that FSH positively regulates 5αR-1 mRNA. In contrast to

5αR-1 mRNA, 5αR-1 enzyme activity was not changed by FSH *(Table 2)*. We are not the first to report differences between enzyme activity and mRNA levels for the 5αR isoforms. In humans there are several tissues that express 5αR-1 mRNA but do not appear to have detectable protein (Jenkins *et al.*, 1992; Thigpen *et al.*, 1993b). Viger and Robaire (1995) showed that in rat testis, 5αR-1 mRNA expression at day 90 was approximately one-third of that expressed at day 30, whereas 5αR-1 enzyme activity at day 90 was approximately 20- to 40-fold less than that expressed during day 30 (Rivarola *et al.*, 1972; Matsumoto and Yamada, 1973; Pratis *et al.*, 2000). This difference between 5αR-1 mRNA and activity during normal testicular development suggests post-transcriptional or translational regulation, and could explain the difference in mRNA and enzyme activity levels we observed in our studies.

Recently, there have been several reports in the literature demonstrating discordant patterns of mRNA and protein expression. For example, a discordant pattern of AR mRNA and protein regulation in response to androgens was observed in prostate and breast cancer cell lines (Yeap et al., 1999). This study showed that DHT treatment decreased total AR mRNA but increased AR protein expression in LnCap and MDA 453 cells. Marcantonio *et al* (1999b) showed that in prostatic smooth muscle cells, androgen withdrawal by castration increased SSG1 mRNA levels whereas SSG1 protein levels decreased after castration. SSG1 mRNA and protein expression are also discordantly regulated in the rat mammary gland following estrogen treatment (Marcantonio et al., 1999a).

We showed that testosterone negatively regulated the 5αR-1 isoform at the mRNA and activity level, whereas FSH only affected 5αR-1 mRNA and not activity. There are several reasons that could explain the differential effects of FSH on 5αR-1 mRNA vs activity. We believe the following two points are important and deserve further consideration.

a) Time Course Studies: The time course response to hormone treatment may differ between mRNA and protein expression. Studies in our laboratory (Stanton *et al.*, 2000 *Abstract*) have investigated the mRNA of several cell adhesion molecules in adult rat testis involved in the

binding of germ cells to Sertoli cells induced by testosterone treatment. These studies showed that N-Cadherin, β1-integrin and β-catenin mRNAs increased between 0 and 24 h after TE-treatment and testosterone replacement, and these mRNA levels decreased to 0 h control levels by 36 h. Whereas the mRNA for these molecules increased as early as 12 to 24 h after testosterone replacement, the conversion of step 7 to step 8 round spermatids (is critically dependent on the expression of these molecules) was restored at 96 h. These results suggest that the protein changes for these molecules occurs several days after the peak of mRNA expression, and occurred at a time when mRNA was low. Chung *et al* (1999) has also shown a time-dependent transient increase in N-cadherin expression, which peaked by 3- 5 days and returned to control levels by 6 days.

Observations in our laboratory have recently shown that, when considering the onset of 5αR mRNA and activity during testicular development, mRNA levels peak 5 days earlier than activity (Killian *et al.*, unpublished observations), thus providing an explanation for why we saw increases in 5αR-2 mRNA but not activity when rhFSH was administered to GnRH-immunization animals. Also, these studies showed that while testicular expression of 5αR-1 mRNA was switched off at 45 days of age, activity was still high, providing a possible explanation for the lack of decrease in 5αR-1 activity, despite decreases in mRNA, after FSH*Ab* was given to TE-treated rats *(Table 2)*. This is also supported by the fact that both 5αR isoforms have a long half-life (Thigpen *et al.*, 1993).

b) <u>Transcript size:</u> It has been demonstrated that there are tissue-specific differences in the sizes of rat 5αR-1 mRNA transcripts (Normington and Russell, 1992) and multiple mRNAs have been detected for both human and rat 5αR isoforms (Normington and Russell, 1992; Thigpen *et al.*, 1993b). In rat testis, Viger and Robaire (1995) demonstrated a change in the size of the 5αR-1 transcript with increasing age. They showed a characteristic 2.5 kb 5αR-1 transcript (Andersson *et al.*, 1989; Viger and Robaire, 1991) in immature (day 21- 28) rat testis coinciding with the time when 5αR-1 mRNA and enzyme activity were maximally suppressed. In contrast, a 2.7 kb 5αR-1 mRNA was observed in the adult (day 91), which coincided with low levels of 5αR-1 enzyme

activity and mRNA expression. The authors concluded that the 2.7 kb transcript did not appear to be translated into protein. Thus, it is possible that the methods we have used to measure enzyme activity and mRNA are actually detecting these different size mRNAs. The primers we designed to use in the LightCycler are not likely to discriminate between the two possible transcript sizes, whereas the *in vitro* assay would only be measuring transcripts that are translated into protein. This could explain the differences we observed between 5αR-1 mRNA vs activity after FSH treatment *(Table 2)*.

Testicular 5αR-2 Isoform

The regulation of the testicular 5αR-2 isoform has not been previously investigated. This thesis showed that acute withdrawal of FSH (TE-treatment + FSH.*Ab*) decreased 5αR-2 mRNA by ~3.5 fold whereas 5αR-2 enzyme activity was not significantly altered (~26% decline, $p > 0.05$), suggesting that FSH positively regulates 5αR-2 mRNA which, in turn may lead to a change in activity *(Table 2)*. Interestingly, FSH suppression by FSH.*Ab* treatment caused a more marked suppression in 5αR-2 mRNA compared to 5αR-1, suggesting that the 5αR-2 isoform is particularly sensitive to FSH. Previously, we discussed how the discordant regulation of 5αR-1 mRNA and activity after FSH treatment could be due to different half lives and thus time-dependent effects, and how a discordant regulation of mRNA and activity has been demonstrated for a number of proteins (Marcantonio *et al.*, 1999a, 1999b; Yeap *et al.*, 1999). Clearly, similar to the 5αR-1 isoform, time-course studies are also required to investigate the regulation of 5αR-2 mRNA and activity after FSH treatment.

To study the effect of FSH administration after chronic testosterone and/FSH suppression, GnRH-immunized animals were treated with rhFSH to restore FSH action. This treatment increased 5αR-2 enzyme activity by nearly 2-fold, but did not significantly alter 5αR-2 mRNA levels *(see Table 2)*. The reason for this difference is unclear and requires further investigation, but

again this could be related to time-dependent events. That is, 5αR-2 mRNA in response to FSH treatment after chronic FSH suppression by GnRH-immunization may rapidly turn on transcription of 5αR-2 mRNA, which would then increase 5αR-2 protein levels. However, at the time point we have chosen to analyze our samples (i.e. 6 days), mRNA levels may have already decreased whereas protein levels are still increasing as a result of the initial peak of mRNA several days previously. This proposition is supported by observations on mRNA and protein time-course studies in testicular cells (Stanton *et al.*, 2000 *Abstract*).

Alternatively, several factors may synergize or act in concert with one another to regulate the 5αR-2 isoform. For example, in genital skin fibroblasts 5αR-2 enzyme activity has been shown to be stimulated by DHT and IGF-I (Horton *et al.*, 1993), TGF-β1 and TGF-β2 (Wahe *et al.*, 1993), and activin A (Antonipillai *et al.*, 1995). 5αR-2 activity in genital skin fibroblasts was not altered by testosterone, IGF-II, or insulin (Horton *et al.*, 1993), and inhibin A dose dependently inhibited DHT-induced activity (Antonipillai *et al.*, 1995). Furthermore, the stimulatory effect of IGF-I (Horton *et al.*, 1993) and activin A (Antonipillai *et al.*, 1995) on 5αR-2 activity was ~100 times greater than the DHT effect, and there was synergism when both DHT and TGF-β1 or TGF-β2 were co-administered to potentiate the stimulation of 5αR-2 activity (Wahe *et al.*, 1993). These data suggest factors other than androgens might play an important role in regulating 5αR-2 activity. We can not be certain that the changes observed in this thesis are attributed directly and/or solely to testosterone and FSH. *In vitro* studies are required to assess these direct effects. Thus, it is possible that the changes we observed *in vivo* are the result of a number of factors that act in a coordinated fashion to regulate testicular 5αR-2 expression.

Relevance of Findings

This thesis has shown that FSH positively regulates both the 5αR-1 and 5αR-2 isoforms in rat testis. Thus, suppressing serum FSH levels by GnRH-immunization or by active immunization

with FSH.*Ab* treatment would lead to suppression of both 5αR isoforms, and thus would inevitably decrease the concentration of testicular DHT and 3α-Adiol in the testis. In the rat however, changes in the 5αR-2 activity did not parallel changes in the testicular levels of 5α-reduced products. One must keep in mind however that the level of 5αR-1 activity is greater than that for 5αR-2 in rat testis, and thus any change in testicular 5α-reduced products by 5αR-2 may be masked by the 5αR-1 effects.

These findings suggest that in humans, residual FSH levels after gonadotrophin suppression may continue to stimulate the 5αR isoforms to produce the more potent androgen DHT, despite suppression of testicular testosterone. Furthermore, since the 5αR-2 isoform is also positively regulated by FSH, up-regulation of this high affinity enzyme could metabolize low levels of residual testosterone, whereas the 5αR-1 isoform has reduced activity at testosterone concentrations lower than ~ 1μM (Andersson *et al.*, 1990; Normington and Russell, 1992). It is interesting to note that recent unpublished observations in our laboratory indicate that testicular testosterone levels in men undergoing 12 weeks of testosterone enanthate treatment (200 mg im weekly) were approximately 0.05 μM (R. McLachlan, *unpublished observations*), and thus it could be expected that 5αR-2 is more active enzyme at this substrate concentration, although the relative contribution of 5αR-1 and 5αR-2 to androgen biosynthesis in human contraceptive-treated testis remains to be determined.

Recent studies in our laboratory have shown that following testosterone-induced spermatogenic suppression in cynomologous monkeys, azoospermic animals had lower levels of FSH than oligozoospermic animals, suggesting a crucial role of residual FSH in maintaining primate spermatogenesis (Narula G.U., O'Donnell L., Stanton P.G., Robertson D.M., McLachlan R.I., and Bremner B, *submitted*). Testicular DHT levels were not increased in oligozoospermic compared to azoospermic animals, however testicular 5αR isoform expression has yet to be assessed.

Whether or not the persistence of FSH levels are related to the persistence of oligozoospermia in contraceptive-treated men is a contentious issue, and thus it is difficult to determine whether FSH-induced up-regulation of testicular 5αR in humans has clinical relevance. Some studies have shown that serum FSH was suppressed more profoundly in azoospermic men compared to those men whose sperm counts were not as severely suppressed (Bochter *et al.*, 1999; McLachlan *et al.*, 2000). More sensitive FSH assay methods may in fact detect differences between azoospermic and oligozoospermic men in terms of serum FSH. Current studies in our laboratory are focusing on supplying more sensitive assays for measuring serum FSH levels in humans.

8.5 Future Directions

This thesis has highlighted the importance of the 5αR isoforms in producing more potent androgens such as DHT during suppression of testosterone and FSH levels. Further studies are required to clarify these effects and determine the mechanism(s) by which these hormones regulate 5αR. Below is an outline of some experiments that are in progress or are required to further our understanding of the regulation of the 5α-reduced metabolites and their role in maintaining spermatogenesis during contraception.

Localization of 5αR

Several studies examined the localization of 5αR-1 in rats (section 1.6.1) by isolating seminiferous tubules and interstitial tissue. However, these crude preparations do not describe which cell types within these testicular compartments are expressing 5αR activity. For example, the 5αR-1 activity measured in seminiferous tubules could be due to Sertoli cells or germ cell(s), or a combination of these. Clearly, the localization of the 5αR isoforms in rat testis needs further investigation. Knowing the cellular localization of the 5αR isoforms is crucial for assigning a physiological role for the testicular 5αR isoforms and for understanding how these enzymes are

affected in response to hormonal changes. For example, manipulating testicular testosterone and/or serum FSH levels may change the localization of the 5αR isoform(s) from one cell type to another.

The localization of 5αR can be investigated by culturing isolated Sertoli cells and Leydig cells. Studies in our laboratory have developed and used *in vitro* primary cell cultures for both immature (Perryman *et al.*, 1996) and adult (Lampa *et al.*, 1999) Sertoli cells, as well enzymatic digestion and elutriation separation and percoll purification for isolating various germ cell populations. With advancing laser technology, it is now possible to use 'laser capture micro-dissection' to isolate particular germ cell types from their environment for further analysis. Thus, an analyses of 5αR isoform expression and activity in various testicular cell populations, from both normal and hormone-treated testis, would clarify the issue of 5αR localization

In Vitro Studies

This thesis has investigated the *in vivo* regulation of the 5αRs, providing valuable information about the physiological relevance of these enzymes in spermatogenesis. However, one disadvantage of *in vivo* studies is that they do not allow the investigation of direct effects. For example, GnRH-immunization suppresses testosterone and FSH but also suppresses inhibin, and rhFSH administration after GnRH-immunization stimulates inhibin production (McLachlan *et al.*, 1994). Thus, a change in 5αR cannot be directly attributed to FSH alone since other factors are also affected. An advantage of *in vitro* methods is that they allow direct effects to be investigated, however the disadvantage is that these experiments may not reflect true biological events. Thus, there are advantages and disadvantages of both methods, but both methods provide valuable information that will help us further understand the regulation of the 5αR isoforms and their physiological significance.

In addition to primary cell cultures, one could use cell lines that have been derived from Leydig and Sertoli cells to investigate factors that regulate 5αR isoform expression and activity. For example, the TM3 and TM4 cell lines are derived from murine Leydig cells and Sertoli cells,

respectively (Mather *et al.*, 1980). These cell lines are easy to grow and maintain, and could be used to study the type of 5αR(s) expressed in Sertoli and Leydig cells, as well as studying the *in vitro* regulation of the 5αR isoform(s) in these cells by testosterone and FSH, as well as other factors that have been demonstrated to alter 5αR activity in androgen-dependent tissues (see Sharpe, 1994).

Further *In Vivo* Studies

This thesis has investigated the role of testosterone and FSH on the regulation of the 5αR isoforms in the testis. We chose to study the role of testosterone and FSH because these are the two main hormonal regulators of spermatogenesis (see Sharpe, 1994 for review). However, there are a number of different hormones and factors that have been demonstrated to affect expression of 5αR activity and/or mRNA in various tissues (see Russell and Wilson, 1994 for review). Thus, further *in vivo* studies are required to determine the role of these factors on the expression of 5αR.

For example, inhibin A dose dependently inhibited DHT-induced 5αR-2 activity in genital skin fibroblasts (Antonipillai *et al.*, 1995). Further studies are required to determine the individual and synergistic effects of different hormones, such as inhibin, on the regulation of the 5αR isoforms in the testis. Furthermore, it has been demonstrated that the androgenic regulation of 5αR activity in rat prostate and genital skin is mediated by DHT rather than testosterone (George *et al.*, 1991; Horton *et al.*, 1993). Thus, studies are required in the testis to investigate the role of testosterone vs DHT in the regulation of testicular 5αR isoforms.

Ontological Studies

This thesis has shown that both 5αR isoforms are expressed in the testis and that the enzyme activity of both isoforms declined from day 35 to day 75, and then declined even further by day 147. Other studies have shown that Leydig cell 5αR-1 mRNA expression varied as a function of age (Viger and Robaire, 1995). Thus, it appears that both 5αRs are developmentally regulated. It is

interesting to note that the pattern of 5αR-1 expression across development appears to have an inverse relationship to the concentration testicular testosterone (Corpechot *et al*, 1981). That is, during puberty testosterone levels are low and 5αR-1 activity is high, and during adulthood testosterone levels are high and 5αR-1 activity is low. This thesis has shown that testosterone negatively regulates testicular 5αR-1, and thus the negative regulation of the 5αR-1 isoform may explain why 5αR-1 levels change with age.

This thesis has also shown that FSH positively regulates 5αR-1 and 5αR-2 mRNA. Previous investigations have shown that serum FSH levels rise during postnatal life and reach a maximum usually between 30 and 40 days of age, and then gradually falls (Negro-Villar *et al.*, 1973; Döhler and Wuttke, 1975). Thus, it is possible that the high levels of 5αR during puberty are partly due to the high FSH levels at this age.

Current studies in our laboratory are focusing on the expression of both 5αR isoforms during development at the enzyme activity and mRNA level, and correlating these with testicular testosterone and serum FSH levels. To date, some of the findings from these studies have shown that both 5αR-1 and 5αR-2 are maximally expressed at puberty when FSH levels are at their highest and testosterone levels are at their lowest, and that the changes in mRNA expression are correlated with activity changes, but are displaced by several days, suggesting post-transcriptional or translational regulation.

Size of mRNA Transcript

Viger and Robaire (1995) showed that a 2.5 kb 5αR-1 mRNA was present in immature rat testis, whereas a 2.7 kb 5αR-1 mRNA was observed in the adult animal. These authors concluded that the 2.7 kb mRNA present in the adult might not be translated into a protein since the appearance of the 2.7 kb transcript occurred when enzyme activity was dramatically reduced, whereas mRNA was still abundantly expressed. The reason for this is unclear but suggests post-transcriptional or translational regulation. A 2.5 kb 5αR-1 transcript has also been previously

reported for rat epididymis and liver, which express high levels of 5αR-1 activity (Andersson *et al.*, 1989; Viger and Robaire, 1991).

In this thesis we have not investigated the size of either 5αR mRNA transcript. Thus, it is possible that an induction in 5αR-1 activity is due to production of a 2.5 kb transcript whereas the suppression of 5αR-1 is due to production of a 2.7 kb 5αR-1 mRNA. In addition, the 5αR-2 mRNA needs to be investigated to determine whether different size mRNAs exist for the 5αR-2 isoform. To investigate this, Northern blot analysis could be used to determine the size of the 5αR-1 and 5αR-2 mRNA transcripts during testosterone and/or FSH manipulation.

Time Course Studies

This thesis has shown a discordant pattern of mRNA vs enzyme activity regulation with FSH treatment on both 5αR-1 and 5αR-2. As discussed previously, we believe that this difference may be due to time effects as a consequence of different half-lives for the protein and activity, which would lead to different time-dependent profiles for the mRNA and activity for 5αR. Studies in our laboratory (Stanton *et al.*, 2000 *Abstract*) as well as others (Chung *et al.*, 1999) have demonstrated that, due to the time-dependent induction of a peak in mRNA transcript, followed by a peak in protein production, analysis of mRNA and protein at a single time point may produce discordant results.

This time-dependent phenomenon we believe is the reason why we did not see an increase in 5αR-1 mRNA after FSH treatment whereas 5αR-1 activity was increased after our 6-day treatment period (i.e. that a peak in mRNA occurred prior to the 6-day time point and was then down-regulated). This proposition is currently been investigated by measuring mRNA and activity at various times after increasing serum FSH levels following GnRH-immunization. In order to examine this, we GnRH-immunized adult male rats and supplemented them with rhFSH. Animals were sacrificed 1, 3 or 6 days after rhFSH treatment, to measure 5αR-1 activity and mRNA levels.

We expect to see an increase in mRNA levels at 1 to 3 days after FSH supplementation but not at 6 days, whereas the activity would be elevated at 6 days.

.....

The existence and the need for two 5αR isoforms has puzzled researchers. The presence of two 5αR isoforms in the testis, that essentially carry out the same biological function but with different efficiencies, may be to safeguard reproductive ability and success. Thus, multiple 5αR isoforms together with isoform-specific hormonal regulation, may serve to preserve the androgen-dependent spermatogenic process under a variety of onslaughts to the testis.

Appendices

Appendix 1 - List of Suppliers

Activon	N.S.W., Australia
Advantec	Pleasanton, CA, USA
Alltech	Sydney, Australia
ATCC	Rockville, MD, USA
BDH Laboratory Supplies	Poole, England, UK
BioRad	N.S.W, Australia
Biotech Australia	Rocklea, Queensland, Australia
Boehringer Mannheim	Boehringer, Germany
Clonetech	Sydney, Australia
Dow Corning	Midland, MI, USA
Drummond Scientific Company	Broomall, PA, USA
Du Pont-New England Nuclear	Boston, MA, USA
Dynatech	Melbourne, Australia
Gelman	Lane Cove, NSW, Australia
Gibco	NY, USA
Heidolph	Germany
Integrated Sciences	UK
Janke and Kunkel	Staufen, West Germany
Lawton	Turlington, West Germany
Merck, Sharp and Dohme	Rahway, New Jersey
Millipore	Milford, MA, USA
NIDDK	Bethesda, MD, USA
NUNC	Roskilde, Denmark
Organon	Sydney, Australia
Oxford Molecular	Madison, Wisconsin, USA

Packard	Downers Grove, IL, USA
Packard Instrument Company, Inc	Meridan, CA, USA
Perkin Elmer Biosystems	Scoresby, VIC, Australia
Pierce	Rockford, IL, USA
Premier Biosoft	Palo Alto, California, USA
Promega	Madison, WI, USA
QIAGEN	Clifton Hill, Victoria, Australia
Roche Molecular Biochemicals	Mannheim, Germany
Serono	Melbourne, Australia
Sigma	St Louis, MO, USA
Sigma Genosys	St Louis, MO, USA
Sirosera	Sydney, Australia
Strategene	La Jolla, CA, USA
Ultra-Violet Products, Inc	San Gabriel, CA, USA
Wallac	Turku, Finland
Waters	Milford, MA, Australia

Appendix 2 - Buffers

0.1% DEPC-treated H_2O:

Add 1 ml DEPC per L H_2O, stir overnight and autoclave before use.

0.1 M PBS:

Add 0.9% NaCl, 42 ml Solution A (13.12% $NaH_2PO_4.H_2O$ = 31.2g/ L), 208 ml Solution B (12.84% Na_2HPO_4 = 28.4g/ L), and 0.01% NaN_3, pH 7.4.

0.1 M Tris-Citrate Buffer:

Add 12.11 g of Trizma base [Tris(hydroxymethyl)aminomethane, $C_4H_{11}NO_3$, MW = 121.1] to 1 L H_20. Adjust the pH of the buffer with citric acid monohydrate powder (MW = 210), to end up with 0.1 M Tris-citrate buffer at the required pH (5.0 – 9.5).

0.5% BSA:

Add 5 g BSA / L H_2O.

1 x Phosphate buffered saline (PBS) (pH 7.4):

Add 8 ml phosphate buffer A, 41.6 ml phosphate buffer B, 9 g NaCl, 0.1 g NaN_3, per L H_2O at pH 7.4.

1 x TBE

Add 121.14 g Tris Base (500 mM), 52 g Boric Acid (420 mM), 7.44 g ADTA (10 mM) and make up to 2 L with sterile H_2O, pH 8.4. This makes 10 x TBE, so dilute 1:10 to get 1 x TBE.

3 M Sodium acetate

Add 40.81 g Sodium Acetate.$3H_2O$ to 80 ml H_2O and adjust pH to 5.3 with glacial acetic acid. Make up to 100 ml with H_2O and autoclave before use.

7.5% BSA in TSA Buffer

Add 10 ml 10 x TSA (500 mM Tris/ HCl, 9% NaCl, 1% azide, pH 7.5) to 90 ml H_2O. Add to this 7.5g BSA / L H_2O. Allow to dissolve and aliquot into 20 ml and sap freeze. Store at –20C°.

50 mM EDTA

Add 9.25 g EDTA/ L H_2O.

Blue Juice (DNA/ RNA loading buffer):

Add 0.125 g bromophenol blue, 0.125 g xylene cyanol, 20 g sucrose, and make up to 50 ml with sterile H_2O and store at room temperature. To make Blue Juice/ ethidium bromide, add 50 µl ethidium bromide to 1 ml blue juice.

Luria-Bertaini (LB) broth:

Add 10 g tryptone, 5 g yeast extract, 5g NaCl / L H_2O, pH 7.0.

Luria-Bertaini (LB) agar:

Add 15 g agar bacteriological (Agar No. 1) / L of LB broth. Autoclave and pour into plates when temperature of solution reaches 50°C.

NADPH Cofactor:

Add 0.5 mM NADPH to a total assay volume of 550 µl (i.e. to 430 µl tris-citrate buffer).

Therefore, 0.5 mM x 550/ 430 = 0.639 mM, therefore need to add 6.39×10^{-4} moles or 0.533 g NADPH / L H_2O to get a final concentration of 0.5 mM NADPH.

Phosphate buffer A (200 mM):

Add 6.24 g NaH_2PO_4 / L H_2O.

Phosphate buffer B (200 mM):

Add 28.4 g Na_2HPO_4 / L H_2O.

Reagents, Chemicals and Enzymes

1 Kb DNA ladder (1 μg/ μl)	Gibco
Agarose Ultra Pure	Gibco
Ampicillin (D[-]-Aminoerzylpenicillin, sodium salt)	Sigma
Antibiotics (10,000 mcg/ ml Streptomycin)	CSL
BCA Protein Assay Kit	PIERCE
Bovine Serum Albumin	Boehringer Mannheim
Bromophenol Blue	Merck
Chloroform (Analar®)	BDH
Concert High Purity Plasmid Purification Systems	Gibco
Diethyl Ether (Analar®)	BDH
Diethyl Pyrocarbonate (DEPC)	Sigma
Ethanol (ethyl alcohol, 99.7- 100% v/ v, Analar®)	Merck
Ethylene Glycol	Sigma
Expand™ Reverse Transcriptase (50 U/ μl)	Boehringer Mannheim
Fastart DNA Master SYBR Green I	Roche Molecular
Fetal Bovine Serum	Trace Chemicals
FUGENE™ Transfection Reagent	Promega
Glycerol (99%, GC)	Sigma

Glycogen (20 mg/ ml)	Boehringer Mannheim
LipofectamineTM 2000 Reagent (1 mg/ ml)	Gibco
2-Mercaptoethanol (electrophoresis regent, ≥ 98%)	Sigma
Methanol (Analar®)	BDH
Oligo $(dT)_{12-18}$ Primer (0.5 µg/ µl)	Gibco
pAdvantage™ Vector	Promega
pcDNA3.1(+)	Invitrogen
Pfu DNA Polymerase (2500 U/ ml)	Stratagene
pGEM-T Vector	Promega
pEGFP vector	Clontech
Potassium dihydrogen orthophosphate (KH_2PO_4)	AJAX
QIAEX II Gel Extraction Kit	Qiagen
Quantum Prep Plasmid Miniprep Kit	Bio-Rad
Random Primers (3 µg/ µl)	Sigma Genosys
Restriction endonucleases	Boehringer Mannheim
Reverse Transcriptase AMV	Boehringer Mannheim
(Supplied with 5 x cDNA synthesis buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithiothreitol, pH 8.5)	
RNase Inhibitor (40 U/ µl)	Roche
RNeasy Extraction Kit	Qiagen
Scintillation fluid- EmulsifierSafe™	Packard
Sodium dihydrogen orthophosphate monohydrate (Na_2HPO_4)	BDH
Sodium hydroxide	Sigma
Superscript™ II RNase H⁻ Reverse Transcriptase	Gibco
Taq DNA polymerase (5 U/ µl)	Boehringer Mannheim
Trypsin	CSL

Radiochemicals

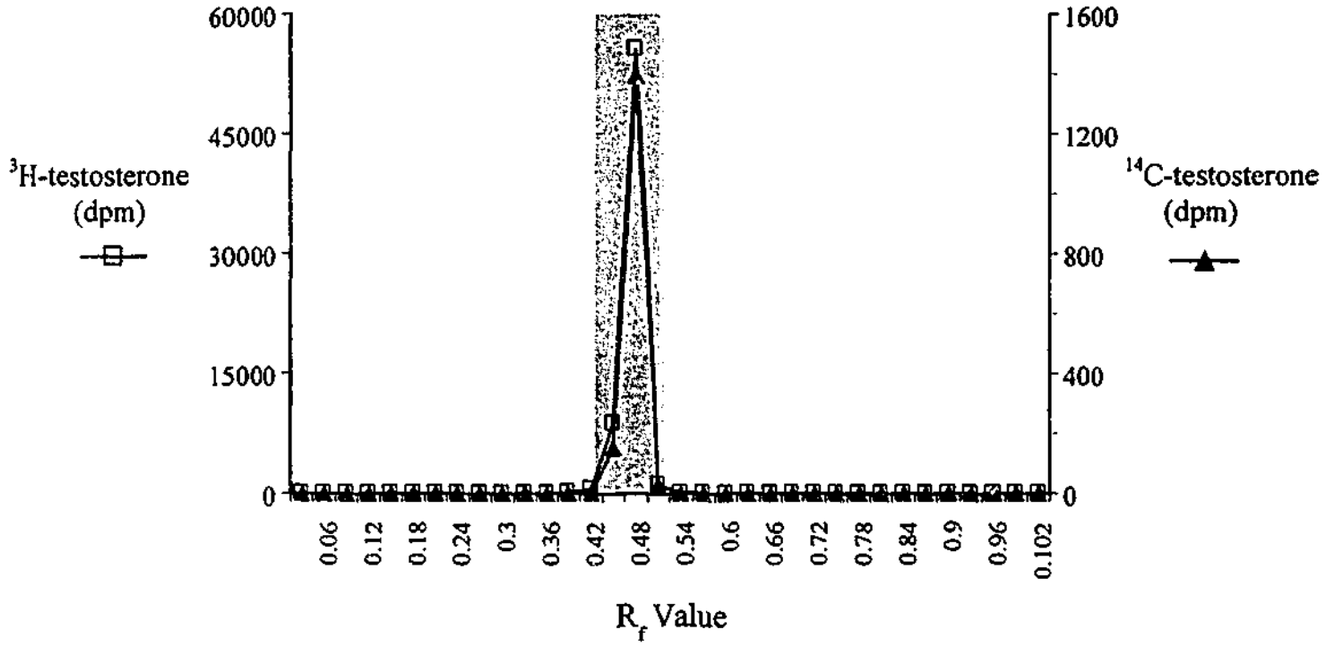
1α, 2α-^{3}H(N)-Testosterone	NEN Life Sciences
4-^{14}C-Testosterone	NEN Life Sciences
4-^{14}C-5α-Androstan-17β-ol-3-one (DHT)	NEN Life Sciences

Software Packages

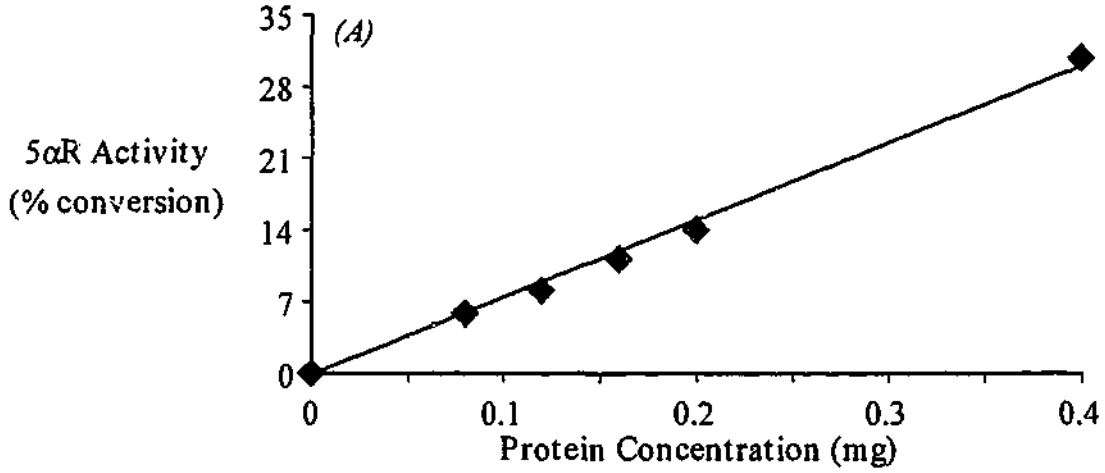
Amplify	(University of Wisconsin)
Graphpad Prism	Graphpad Software, Inc
Gel Doc 2000- gel documentation system (Quantity One 5.1)	BioRad
PlateReader Version 2.10 for Windows	Packard

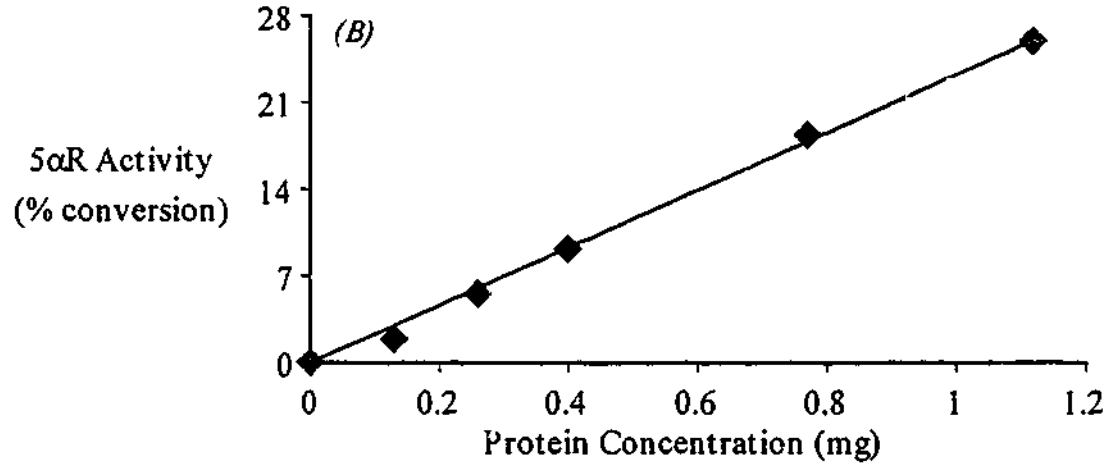
Equipment

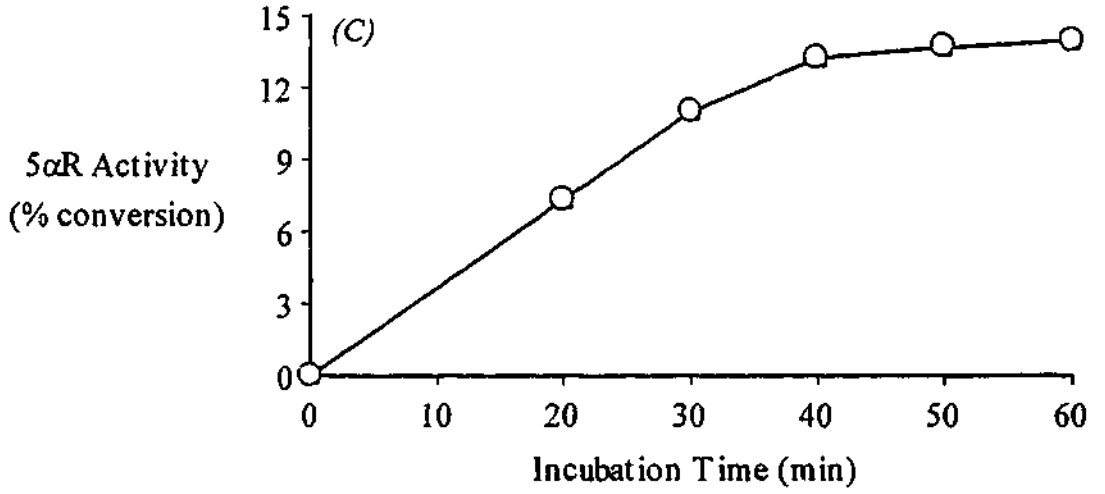
ABIPrism™ 377 DNA Sequencer	Applied Biosystems
Automated Plate Reader Dynatech MR7000	Packard
BioPhotometer (spectrophotometer)	Eppendorf
Eppendorf micro-centrifuge	Crown Scientific
Gel Doc 2000-gel documentation system	Bio-Rad
Liquid Scintillation Analyzer 255TR	Packard
Mini-sub cell GT system	Bio-Rad



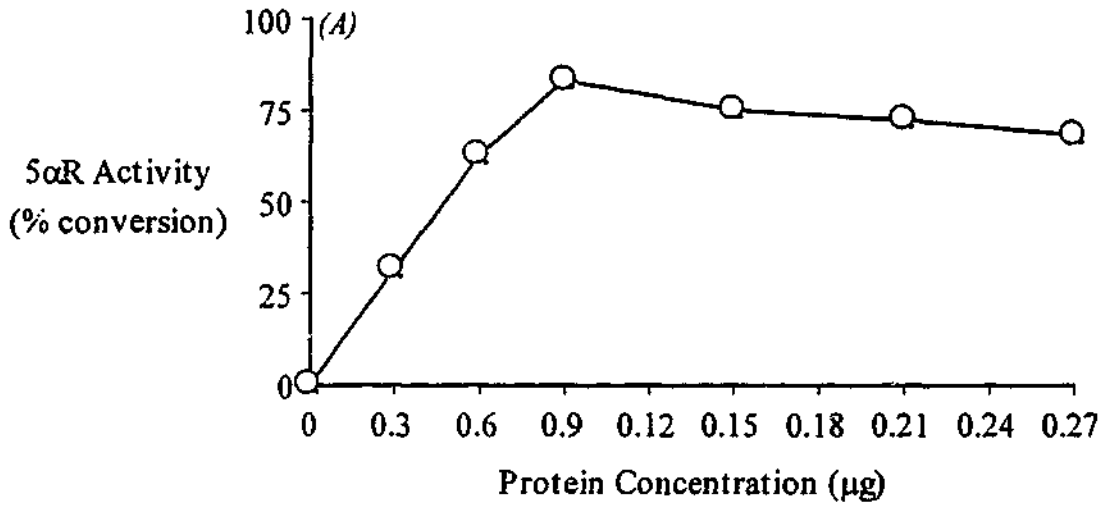
Appendix A: An elution profile of purified ^{3}H-testosterone (open squares). To purify 3H-testosterone, it was spotted on ITLC-SA chromatography plates. In adjacent sample lanes, ^{14}C-T and non-radioactive testosterone were spotted. ^{14}C-Testosterone was detected by cutting the plate into 0.5 cm fractions and counting the radioactivity. Non-radioactive testosterone was located by irradiating with short wave UV light (254 nm). The corresponding area in the ^{3}H-testosterone lane was cut and the radioactivity eluted from the plate with ethanol. Also shown is the area corresponding to ^{14}C testosterone (closed triangles) and non-radioactive testosterone (shaded bar).

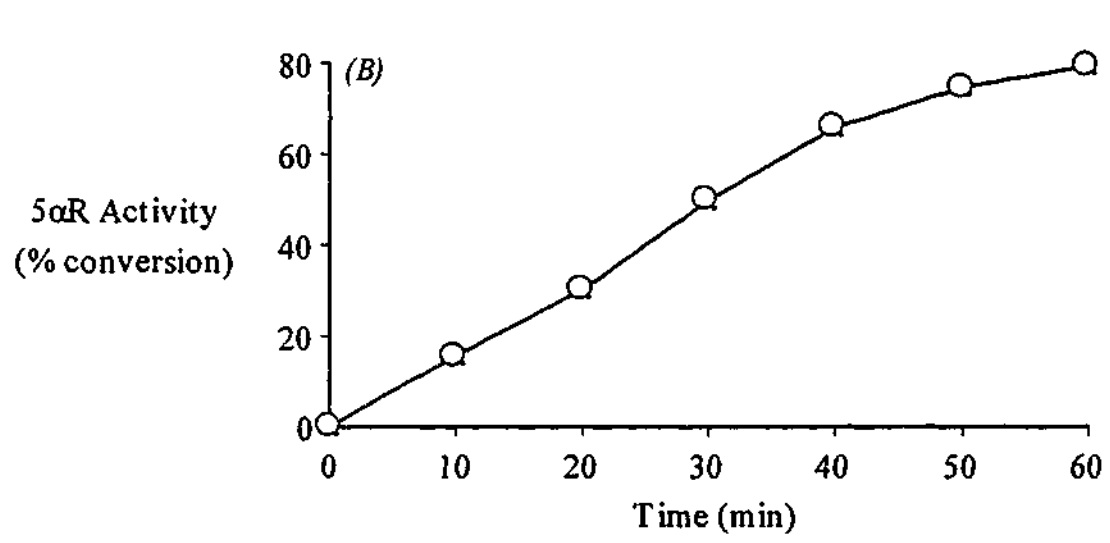




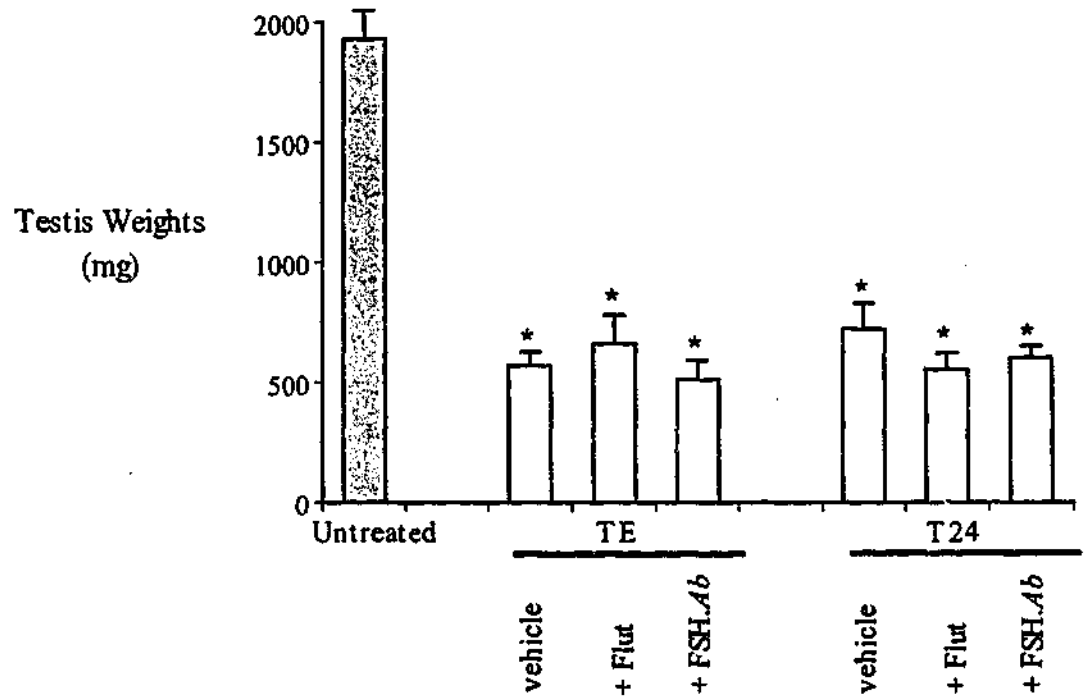


Appendix B: Adult (day 128) rat epididymal homogenate incubated at pH 5.0 for 5αR activity at different protein concentrations for *(A)* 60 min and *(B)* 20 min and *(C)* for different time periods (20 to 60 min) at a fixed enzyme concentration (0.2 mg). Enzyme activity is expressed as percent conversion of substrate. Values are the average of duplicate measurements.

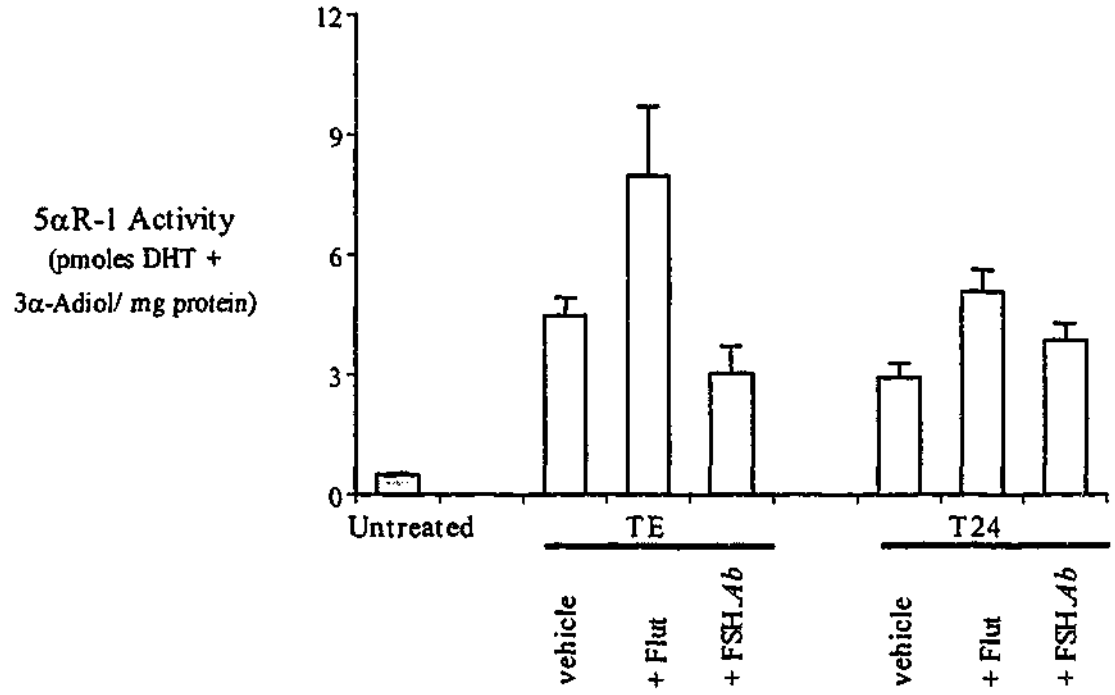




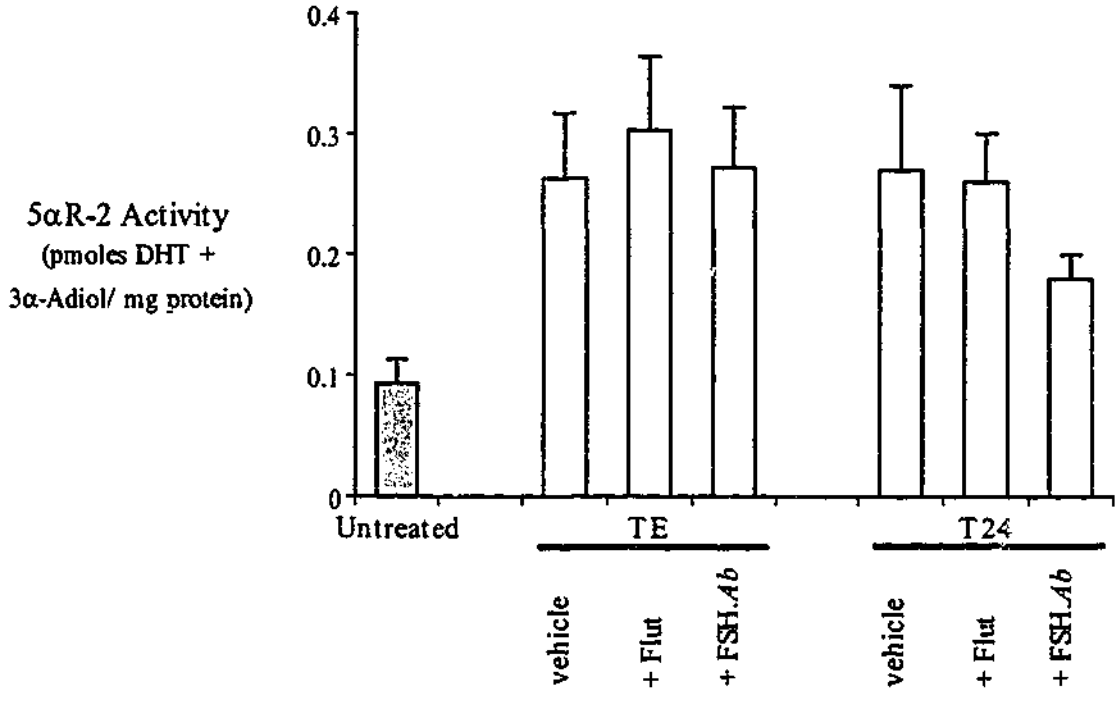
Appendix C: Adult (day 128) male rat liver homogenate incubated at pH 7.0 for 5αR activity for *(A)* 20 min at different protein concentrations and *(B)* with 0.3 mg liver homogenate for 10 to 60 min. Enzyme activity is expressed as percent conversion of substrate.



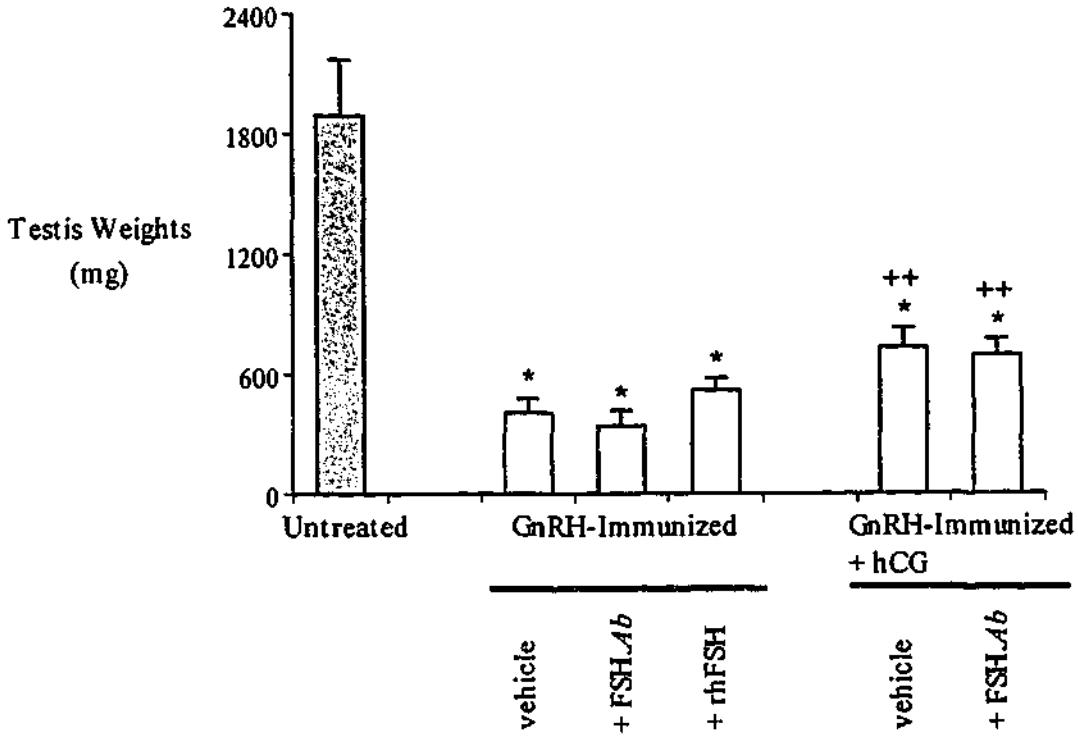
Appendix D: Testis weights (mg) of untreated and TE- and T24-treated rats administered vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*] treatment. Each bar represents mean ± SEM (n = 8). There were no significant differences between any of the treatment groups. * Indicates a significant difference compared to untreated levels.



Appendix E: 5α-Reductase Type 1 (5αR-1) enzyme activity (pmoles DHT + 3α-Adiol/ min/ mg protein testis) in untreated and TE- and T24- treated rats administered vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 8). Results are similar to data when expressed per g testis.



Appendix F: 5α-Reductase Type 2 (5αR-2) enzyme activity (pmoles DHT + 3α-Adiol /min/ mg protein) in untreated and TE- and T24-treated rats administered vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 8). There were no significant differences between any of the treatment groups. Results are similar to data when expressed as per g testis.



Appendix G: Testis weights (mg) of untreated, GnRH-immunized, and GnRH-immunized rats administered human chorionic gonadotrophin (hCG). Animals were treated with either vehicle, flutamide [Flut] or FSH antibody [FSH.*Ab*]. Each bar represents mean ± SEM (n = 7- 8). * Indicates a significant ($p < 0.01$) difference compared to untreated levels. ++ Indicates a significant ($p < 0.01$) difference compared to GnRH controls.

Appendix H

PERGAMON

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Enzyme assay for 5α-reductase Type 2 activity in the presence of 5α-reductase Type 1 activity in rat testis

Kyriakos Pratis, Liza O'Donnell, Guck T. Ooi, Robert I. McLachlan, David M. Robertson *

Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Vic. 3168, Australia

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Abstract

The relative abundance and physiological role of 5α-reductase (5αR) isoforms in rat testis, in particular 5α-reductase Type 2 (5αR2) are poorly understood. Investigation of 5αR2 activity using enzyme kinetic studies was hampered by the high concentrations of 5α-reductase Type 1 (5αR1) in rat testis. Therefore, an assay was developed which exploited the differences in pH optima of the two isoforms. The 5αR assays measured the conversion of ^{3}[H]-testosterone to 5α-reduced metabolites (dihydrotestosterone + 3α-Androstanediol) at pH 5.0 and 7.0. To compensate for the overlap of 5αR1 activity at pH 5.0, the amount of 5αR1 activity at pH 5.0 was determined by measuring recombinant rat 5αR1 expressed in COS-7 cells at pH 5.0 and 7.0. The amount of activity at pH 5.0 that was attributed to 5αR1 was determined to be 12.4 ± 1.4% (mean ± S.D., $n = 14$). The 5αR2 assay was validated by determining recombinant rat 5αR2 activity in the presence of recombinant rat 5αR1 activity in COS cells. A 99.3 ± 14.7% recovery of 5αR2 activity was obtained when comparing 5αR2 activity recovered versus activity added. 5αR1 and 5αR2 activities were then assayed in rat testis extracts from 30, 75 and 147 days. Both isoforms markedly declined (50–100-fold) over this age range, with 5αR1 as the predominant isoform. In conclusion, an enzymatic assay that detects 5αR2 activity in the presence of high concentrations of 5αR1 was developed and is applicable in the measurement of 5αR2 activity in rat testis.

Keywords: 5α-Reductase; Testis; Androgen; Spermatogenesis

1. Introduction

The steroid enzyme 5α-reductase (5αR, E.C. 1.3.99.5) metabolizes testosterone to dihydrotestosterone (DHT) [1]. Two genes encoding 5αR have been identified in rats [2–4] and these isoforms are referred to as 5αR Type 1 (5αR1) and Type 2 (5αR2), based on the chronological order in which they were discovered.

The 5αR isoforms have molecular weights of 28–29 k and are 46% identical in sequence, share similar substrate preferences and have similar gene structures [3,5,6]. They differ however in their pH optima for maximal enzymatic activity (pH 7 for 5αR1 and pH 5 for 5αR2), affinity for steroid substrates (micromolar K_m for 5αR1 and nanomolar K_m for 5αR2), expression levels and sensitivity to certain 4-azasteroid inhibitors [3,5].

The metabolism of testosterone to DHT by 5αR plays an important role in androgen physiology because DHT is a more potent androgen than testosterone [7,8]. The role of DHT in normal spermatogenesis is unknown. However, it has been shown that DHT plays a critical role in spermatogenesis at low testicular testosterone levels during experimentally-induced spermatogenic inhibition in adult rats [9,10].

RNA blotting studies in rat tissues indicate that 5αR1 mRNA predominates in androgen-independent tissues such as the skin and liver, whereas 5αR2 mRNA is the predominant isoform in reproductive tissues [3,4]. Exceptions to this general rule are the prostate and seminal vesicles which express similar amounts of both 5αR isoforms [4].

* Corresponding author. Tel.: [redacted] fax: [redacted]

E-mail address: [redacted] (D.M. Robertson).

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The type of 5αR isoforms expressed in rat testis is controversial [4,11]. Early enzyme activity studies showed a peak in 5αR1 activity in rat testis between days 30 and 35, which decreased to undetectable levels with age [12–15]. More recently two studies using mRNA methodologies have investigated 5αR isoform expression in rat testis. One study [4] using RNA blotting reported the presence of predominantly 5αR2 in the testis (day 49) while the other study showed that only 5αR1 is expressed from days 7 to 91 by Northern blot analysis and immunoblotting [11]. The cellular localization of 5αR1 activity within the testis is also controversial with some studies showing either 5αR1 activity predominantly in the interstitium [16–19], predominantly in the seminiferous tubules [13,20], or in the seminiferous tubules only [14,15,21,22].

Currently, there is no quantitative procedure for measuring 5αR2 enzyme activity in the presence of 5αR1. 5αR2 activity can be assessed by making a ratio of the activities at pH 5.0 and 7.0, however this is only a relative indication of 5αR2 levels. In tissues which express large amounts of 5αR1 and low levels of 5αR2, measurement of 5αR2 activity is difficult because of the overlap of 5αR1 at pH 5. The aim of this study was to measure 5αR1 and 5αR2 enzyme activity in the testicular rat extracts. To the best of our knowledge, 5αR2 enzyme activity has not been investigated in rat testis of any age. In order to measure 5αR2 activity at pH 5 in the presence of high testicular 5αR1 activity, a method was devised to compensate for the overlapping effects of 5αR1 activity at pH 5.

2. Materials and methods

2.1. Reagents

4-Androsten-17β-ol-3-one (testosterone), 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT) and 5α-androstane-3α, 17β-diol (3α-Adiol) were purchased from Sigma (St Louis, MO). [1α, 2α-^{3}H(*N*)]-testosterone (53.5 Ci/mmol), [9,11-^{3}H(*N*)]-3α-Adiol (49.8 Ci/mmol), [4-^{14}C]-testosterone (57.3 mCi/mmol), and [4-^{14}C]-DHT (58.3 mCi/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). [4-^{14}C]-3α-Adiol was prepared by enzymatic reduction of [4-^{14}C]-DHT using 32-day rat testis (10 000 × *g* supernatant) as a source of 3α-hydroxy steroid dehydrogenase activity. The [4-^{14}C]-3α-Adiol produced was isolated by thin layer chromatography (TLC; see below). [4-^{14}C]-3α-Adiol and [1α, 2α-^{3}3H(*N*)]-testosterone were purified by TLC before use. Analytical grade chloroform, toluene, ethanol and diethyl ether (Merck, Kilsyth, Vic., Australia) were used for TLC. The coenzyme β-NADPH was obtained from Sigma (St Louis, MO).

2.2. Animals

Male Sprague–Dawley rats (30, 75 and 147 days) were obtained from the Monash Central Animal House and housed under 12-h light/dark cycle with free access to food and water. The study was approved by the Monash Medical Centre Animal Ethics Committee.

2.3. Tissue preparation

Animals were sacrificed by CO_2 asphyxiation. Testes, epididymis and liver were quickly excised, trimmed of fat, weighed and snap frozen in liquid nitrogen. Tissues were stored at −70°C until required for the 5αR activity assays.

Tissues were stored frozen until homogenization. Epididymis and liver were minced with a razor blade and homogenized (Heidolph Diax 600 homogenizer, Janke & Kunkel, Germany) in 0.25 M sucrose and stored at −70°C. Testes were decapsulated, homogenized in 0.25 M sucrose, centrifuged at 10 000 × *g* and the supernatant stored at −70°C.

2.4. 5α-Reductase activity assay

5α-Reductase activity was determined by measuring the conversion of ^{3}[H]-testosterone to ^{3}[H]-DHT + ^{3}[H]-3α-Adiol, following the protocol of Murono & Payne [23] with several modifications.

5α-Reductase assays were carried out in duplicate in a total volume of 0.55 ml. Radioactive (^{3}H-testosterone; 0.5 μCi) and nonradioactive (9.5 μM) testosterone, 0.5 mM β-NADPH (Sigma) and tissue extract (100 μl) in assay buffer (0.1 M Tris–citrate, pH 5.0 or 7.0) were incubated in a shaking water bath at 37°C for 20 min (epididymis and liver) or 60 min (testis and COS-7 cells). Controls were stored on ice during the incubation period. The assay was terminated by the addition of 2 ml 0.1 M NaOH. The recovery of steroids was monitored by the addition of [4-^{14}C]-testosterone, [4-^{14}C]-DHT and [4-^{14}C]-3α-Adiol. Nonradioactive steroids were also added prior to extraction to aid visualization on the chromatography plate. Samples were extracted with toluene:ether (1:5) and the aqueous phase frozen in an alcohol bath with dry ice. The organic phase containing the steroids was decanted, evaporated under N_2 and the residue dissolved in 10 μl ethanol.

The steroids (testosterone, DHT and 3α-Adiol) were separated by TLC on silica-impregnated glass fiber sheets (ITLC-SA, Gelman Sciences, Sydney, NSW, Australia). The eluting solvent system was chloroform:methanol (98:2, v/v) and the chromatographs were developed for 31 min. Testosterone was visualized by ultraviolet light at 254 nM and DHT and 3α-Adiol by iodine vapor. Visualization of 3α-Adiol was enhanced by spraying the plate with iodine vapor spot

enhancer (Alltech, Sydney, NSW, Australia). The chromatographic zones containing the steroids were cut out and placed into scintillation vials with Packard Emulsifier Safe scintillation fluid (Packard, Meridan, CA). The steroids were quantified by dual isotope liquid scintillation counting to measure both ^{3}H (conversion of testosterone to 5α-reduced metabolites) and ^{14}C (for estimation of steroid recovery) radioactivity.

5α-Reductase activity was expressed as the sum of the 5α-reduced metabolites (DHT + 3α-Adiol) formed from ^{3}H-testosterone after corrections for procedural losses. The recoveries of steroids as indicated by ^{14}C steroids were 47.2 ± 5.1% for testosterone, 47.3 ± 5.0% for DHT and 51.6 ± 4.7% for 3α-Adiol. Contaminating radioactivity in the DHT and 3α-Adiol region of the chromatogram was determined using control incubations, and were subtracted from DHT and 3α-Adiol values for the samples. The average background for the 5αR assays ($n = 6$) was 0.010 ± 0.003 pmol DHT + 3α-Adiol/min which corresponds to less than 0.12% conversion of testosterone.

Protein levels were determined by the BCA method (Pierce Chemical Co., Marrickville, NSW, Australia) using bovine serum albumin as standard.

2.5. Calculation of enzyme characteristics (K_m and V_{max})

The Michaelis–Menten constant (K_m) and V_{max} were determined by measuring 5α-reductase activity at pH 7.0 with 16 different concentrations of testosterone (1.9 nM–9.5 μM). The computer package Graphpad Prism (Graphpad Software, San Diego, CA) was used to analyze the enzyme kinetic data and Eadie–Scatchard plots [24] were used to graphically distinguish the presence of one or two isoforms.

2.6. Expression of recombinant rat 5α-reductase Type 1 and Type 2 in COS-7 cells

The cDNA clones encoding full length rat 5αR1 (pB5αRED1) and partial length 5αR2 (pT801) were kindly provided by Dr D.W. Russell and used as templates to amplify the coding region for subcloning into pcDNA 3.1(+) (Invitrogen, San Diego, CA) expression vectors.

Oligonucleotides synthesized by GIBCO (GIBCO Life Technologies, Melbourne, Vic., Australia) were designed with relevant restriction sites; *Eco*R1 and *Xho*1 for 5αR1, *Eco*R1 and *Xba*1 for 5αR2 (Boehringer and Mannheim, Melbourne, Vic., Australia). Forward and reverse primers for 5αR1 were [(5′-GCAGGAATTCAC CTC AGCTATGGAGTTGGATGAG-3′) and (5′-CAGACTCGAGTCACCAGGGA GACAGACAGAC-3′)] and forward and reverse primers for 5αR2 were [(5′-ACCAGAATTCAC-CACAG GCGAGATGCAGATTG-3′) and (5′-AG-CATCTAGACAGTTCCTCCACA GAAACTTTG-CTC-3′)]. Restriction sites are underlined.

Polymerase chain reaction (PCR) was used to amplify the coding region with 2.5 U Pfu DNA polymerase (Stratagene, Sydney, NSW, Australia), 1.25 mM dNTP, 50 pmol specific primers and 100 ng cDNA template. The amplification consisted of an initial denaturation step (94°C/2 min), 39 PCR cycles (annealing [55°C/2 min], extension [72°C/2 min], and denaturation [94°C/1 min]) and a final annealing (55°C/2 min) and extension step (72°C/7 min). The PCR products were cut with the appropriate restriction enzymes, purified and separated by electrophoresis on a 1.5% agarose gel and extracted using the QIAEX II Gel Extraction Kit (Qiagen, Clifton Hill, Vic., Australia).

The PCR product was subcloned into the pcDNA 3.1(+) expression vector using the Rapid DNA Ligation Kit (Boehringer and Mannheim) and transformed into DH5α competent cells (GIBCO). Recombinant plasmids were purified using the Concert High Purity Plasmid Purification System (GIBCO). Nucleotide sequencing of double-stranded plasmid DNA was performed with the Automated DNA Sequencer 377 (Perkin Elmer Biosystems, Scoresby, Vic., Australia) using the T7 priming site and the pcDNA 3.1(+)/Bovine Growth Hormone reverse priming site (TAGAAGGCACAGTCGAGGC).

COS-7 cells (SV40-transformed monkey kidney cells) were plated at 5×10^6 cells per cm^2 in 175-cm^2 culture flasks and grown in DMEM containing 10% (v/v) fetal bovine serum and supplemented with 7.5% sodium bicarbonate, 200 mM L-glutamine and 100 mM sodium pyruvate. The day before transfection, cells were plated in 100 mm culture dishes in serum-free medium to give ~50% confluency on the day of transfection. The transfection reagent Fugene-6 (Promega, Annandale, NSW, Australia) was used to transfect either vector alone (pcDNA 3.1(+)) or recombinant rat 5αR1 or 5αR2 into COS-7 cells. The pAdvantage vector, containing the adenovirus VA1 gene was cotransfected with the individual cDNA's to enhance expression [25]. Transfection efficiency was measured by transfecting the pCMV-β-galactosidase plasmid. The media was replaced with serum-containing medium on days 2 and 3 and cells were harvested for 5αR activity assays on day 4. The cells were washed twice and frozen in 0.25 M sucrose. Cells were thawed and homogenized before use in the 5αR activity assay. The assay for recombinant 5α-reductases was carried out for 60 min at pH 7.0 (5αR1) and pH 5.0 (5αR2). All other conditions were as described above for the tissues.

2.7. Statistics

Differences between groups were assessed by independent t-tests at the level of $P < 0.05$. All data are expressed as mean ± S.D.

3. Results

3.1. Eadie–Scatchard plots to differentiate between 5α-reductase Type 1 and 2 enzyme activities

The 5αR isoforms can be differentiated at pH 7.0 based on their differences in affinity (K_m) for testosterone. The Eadie–Scatchard plots for male rat liver (Fig. 1A) and epididymis (Fig. 1B) showed the presence of two enzyme activities. The K_m value (Table 1) for 5αR1 in liver (2.41 μM) and epididymis (2.82 μM) is comparable to that obtained for recombinant rat 5αR1 (rec 5αR1, 1.64 μM).

Eadie–Scatchard plots from 30-day rat testis (Fig. 1E) showed the presence of both 5αR1 ($K_m = 2.25$ μM) and 5αR2 ($K_m = 0.13$ μM) activity, whereas 75-day rat testis (Fig. 1F) showed the presence of only 5αR1 ($K_m = 3.06$ μM). In rat testis at day 30, the V_{max} value for 5αR1 is 15-fold that of 5αR2 (Table 1), indicating that 5αR1 is the predominant isoform in rat testis. However, the V_{max}/K_m ratio for the two enzymes suggests that the conversion of testosterone to 5α-reduced metabolites would be attributed equally to both isoforms (1.66 and 1.98 for 5αR1 and 5αR2, respectively; Table 1).

3.2. pH profiles for 5α-reductase isoforms in rat testis

In order to develop an enzymatic assay for measuring 5αR2 activity in the presence of high concentrations of 5αR1, as is the case in rat testis, studies were undertaken to assess if differences in activities at their respective pH optima could be utilized to differentiate the two isoforms.

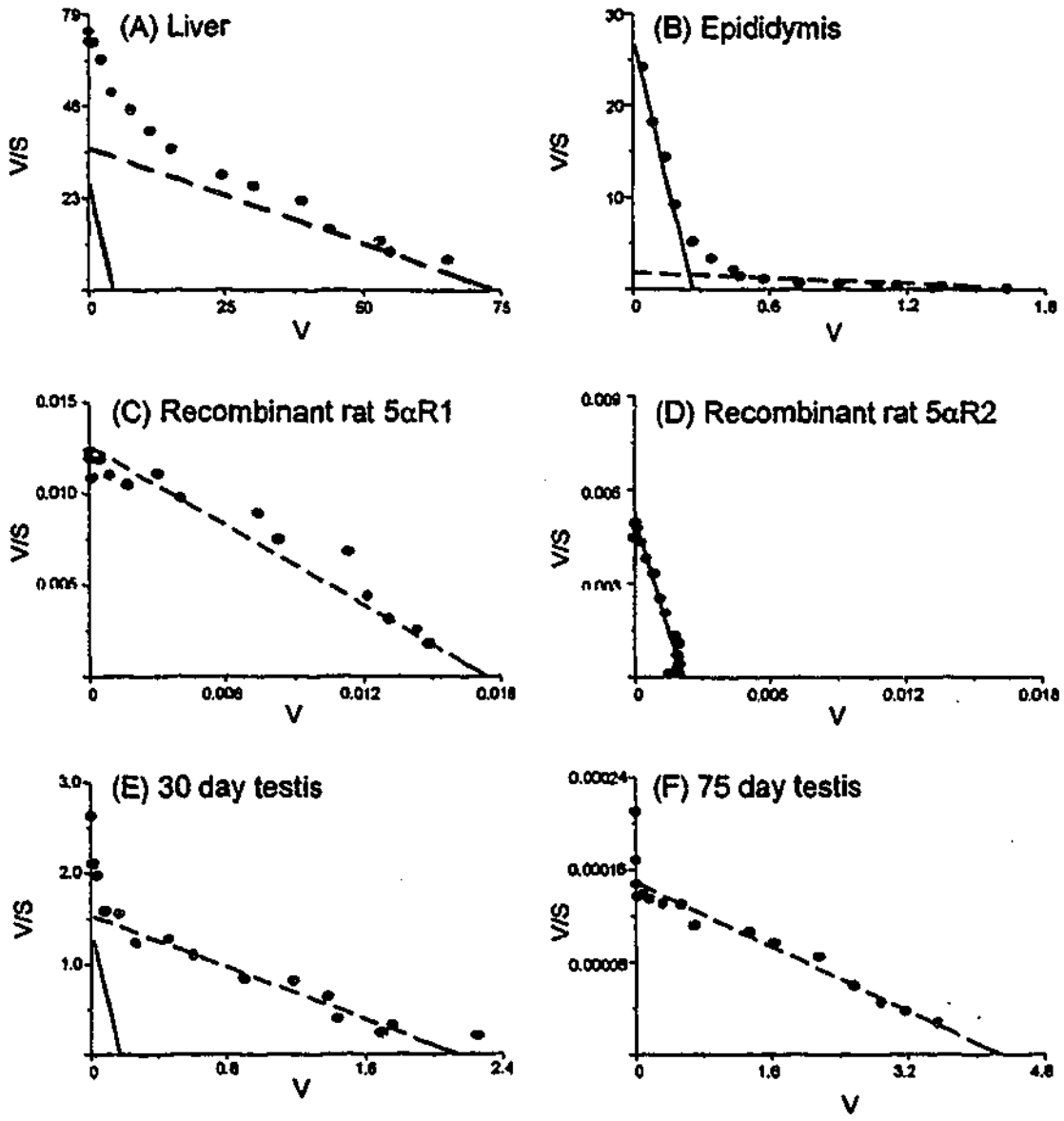

Fig. 1. Eadie–Scatchard plots for (A) liver, (B) epididymis, (C) recombinant rat 5α-R1, (D) recombinant 5α-R2, (E) 30 day testis and (F) 75 day testis. These graphs are representative of three separate experiments. Enzyme parameters (K_m, V_{max}, and K_m/V_{max}) for these analyses are presented in Table 1. Enzyme activity is presented as fmol DHT + 3α-Adiol per min per mg protein.

Table 1
Enzyme characteristics (K_m, V_{max}, and V_{max}/K_m ratio) for 5α-reductase activity in 30- and 75-day rat testis, epididymis, liver and recombinant rat 5αR Type 1 (5αR1) and Type 2 (5αR2) at pH 7.0[a]

Rat tissue/cells	5αR1			5αR2		
	K_m (μM)	V_{max} (fmol/min per mg protein)	V_{max}/K_m ($\times 10^{-12}$)	K_m (μM)	V_{max} (fmol/min per mg protein)	V_{max}/K_m ($\times 10^{-12}$)
30-day Rat testis	2.25 ± 0.96	3.73 ± 1.44	1.66	0.13 ± 0.09	0.26 ± 0.2	1.98
75-day Rat testis	3.06 ± 0.97	0.36 ± 0.14	0.12	ND	ND	
Rat epididymis	2.82 ± 1.39	1.35 ± 0.11	0.48	0.03 ± 0.03	0.35 ± 0.16	11.8
Rat liver (male)	2.41 ± 0.66	104 ± 21.6	43.2	0.11 ± 0.03	4.74 ± 0.86	43.9
Recombinant rat 5αR	1.64 ± 0.40	13.7 ± 4.84	8.32	0.27 ± 0.02*	3.16 ± 2.17	11.9

[a] Details of enzyme assays and analysis are presented in the Section 2. Values are the mean ± S.D. from three separate experiments. ND denotes nondetectable and * denotes enzyme kinetics performed at pH 5.0.

The pH activity profile for 5αR1 in rat liver (Fig. 2A) showed a broad neutral pH range (pH 5.5–7.5). The corresponding pH profile for 5αR2 in rat epididymis had an acidic pH optimum at pH 5.0 (Fig. 2A).

The 5αR pH activity profiles for 30 and 75-day testis (Fig. 2B) show similar patterns of activity across a broad neutral pH range (pH 5.5–7.5), although the activity at day 30 was approximately 40-fold higher than at day 75. Differences in activity at pH 5.0 suggest higher levels of 5αR2 in testis extracts from 30 day compared with 75 day rats. The activity of 5αR2 at pH 5.0, in the presence of 5αR1 activity, can be determined provided the contribution of 5αR1 activity at pH 5.0 is established.

3.3. Measurement of 5α-reductase Type 2 activity in the presence of 5α-reductase Type 1

To determine the enzyme activity at pH 5.0 that can be attributed to 5αR1, rec 5αR1 was measured at pH 5.0 in the absence of any 5αR2 activity. Rec 5αR1 was transiently expressed in COS-7 cells, which expressed no endogenous 5αR activity (Table 2). The proportion of activity at pH 5.0 compared with pH 7.0 (Table 2) was determined to be 0.124 ± 0.014 ($n = 14$).

Given that the contribution of 5αR1 to 5αR activity at pH 5.0 was established, the following formula for measuring 5αR2 activity in the presence of 5αR1 was devised '5αR2 Activity = [(pH 5.0/7.0 sample) − 0.124] × [pH 7.0 sample]'.

3.4. Validation of assay method for measuring 5α-reductase Type 2 activity in the presence of 5α-reductase Type 1

The assay was validated using the following criteria.

1. The sensitivity of the 5αR2 assay was defined as the activity calculated at two times the S.D. above the pH 5.0/7.0 ratio for rec 5αR1 (sensitivity, 0.152; Table 2). Tissue samples with a pH 5.0/7.0 ratio of less than or equal to this value were defined as nondetectable and given an activity equal to this sensitivity value.

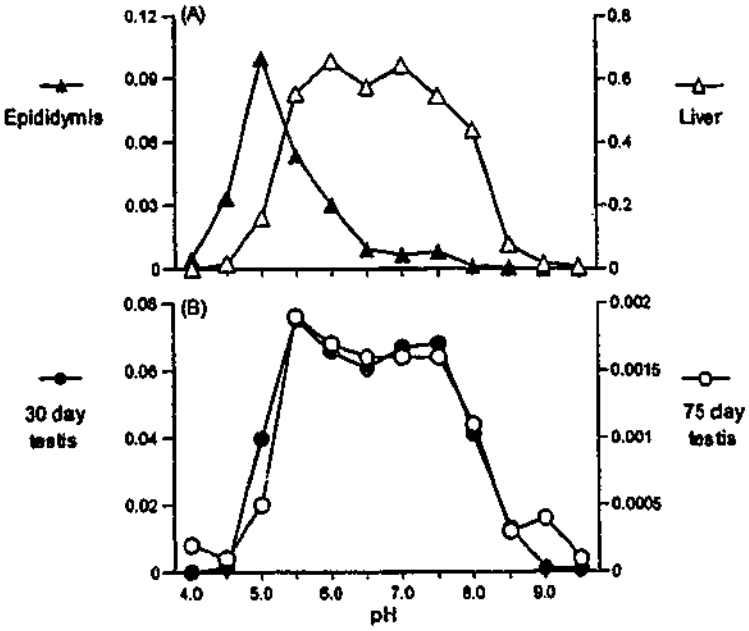

Fig. 2. 5α-Reductase activity (μmol DHT + 3α-Adiol per min per mg protein) as a function of pH for (A) 75-day epididymis (▲) and liver (△), and (B) 30 (●) and 75- (○) day rat testis. Note the different scales on the two y-axes for each graph.

Table 2
Recombinant rat 5αR1 enzyme activity at pH 5.0 and 7.0[a]

Expression plasmid	Recombinant 5αR1 activity (fmol DHT + 3α-Adiol per min per mg protein)		
	pH 5.0	pH 7.0	pH 5.0/7.0 ratio
Vector	<0.3[b]	<0.3[b]	–
Recombinant 5αR1	35.3 ± 10.2	283 ± 56.3	0.124 ± 0.014

[a] Values are mean ± S.D. from five separate transfections and a total of 14 different measurements.

[b] At the detection limits of the assay (~0.2% conversion of substrate).

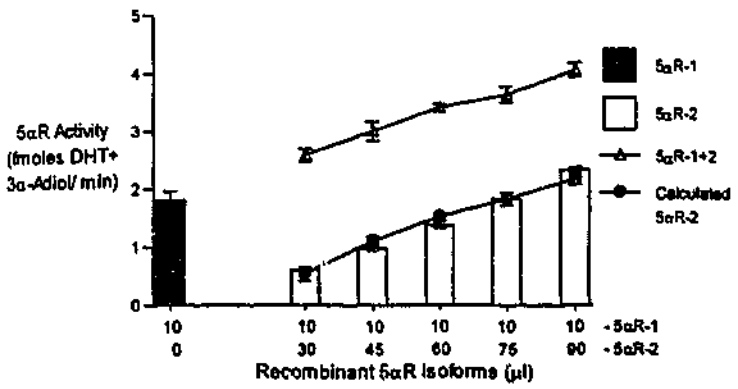

Fig. 3. Measurement of 5αR2 activity in the presence of 5αR1 activity. Increasing amounts of recombinant rat 5αR2 were measured alone (open bars) or in combination with a constant amount of recombinant 5αR1 (open triangles). The levels of 5αR2 were determined by the proposed 5αR2 assay (closed circles). Enzyme activity is presented as fmol DHT + 3α-Adiol per min.

2. To determine the accuracy of the assay, increasing concentrations of recombinant rat 5αR2 (rec 5αR2) were measured in the presence of a fixed concentration of rec 5αR1. A representative experiment (Fig. 3A) demonstrated that by correcting for the overlap of 5αR1 activity at pH 5.0 with the formula mentioned above, 5αR2 can be accurately measured. The correlation of added rec 5αR2 activity versus calculated 5αR2 activity from three separate experiments (data not shown) gave a linear response, with a correlation coefficient of $r = 0.98$ and a recovery of $99.3 \pm 14.7\%$ ($n = 12$).
3. The within-assay variation was assessed from the average CV within replicates for samples assayed and was 4.0% ($n = 56$). The between-assay variation was assessed from the reproducibility of measurement of a testicular extract which was included as a QC in every assay and was 3.6% ($n = 14$).

3.5. Measuring 5α-reductase Type 1 and 2 activity in rat testis extracts

5αR1 activity at pH 7.0 and 5αR2 activity at pH 5.0 using the method described above were determined in 10 000 × g testicular supernatants from rats aged 30, 75 and 147 days (Table 3). The pH 5.0/7.0 ratio is also shown for comparison, as this method is often used as an estimate of 5αR2 activity [26]. 5αR1 activity declined with age to reach < 1% the levels of day 30 at 147 days. The corresponding values for 5αR2 showed a similar decline with age to reach values < 2% that of day 30 by 147 days. 5αR2 levels in three of the six animals at day 75 were nondetectable and have been given an activity value at the level of detection of the assay. The levels of 5αR2 at 147 days were readily detectable, despite the similar or even lower pH 5.0 activity compared with 75 days. This is due to the fact that there is lower 5αR1 activity at day 147, resulting in a higher pH 5.0/7.0 ratio at 147 days (0.335) compared with 75 days (0.136–0.187; Table 3).

4. Discussion

Several studies have examined 5αR1 activity in rat testis, focusing mainly on the immature rat testis where DHT levels are high and thus suggested to play a role in androgen physiology [14–20]. To the best of our knowledge there are no studies that have investigated 5αR2 activity in rat testis. With the intention of investigating the regulation of 5αR, the type of 5αR activity expressed in rat testis at various ages was first investigated. Enzyme kinetics was initially used to investigate 5αR isoform activity in rat testis. Both isoforms were found to be expressed at day 30, and the K_m values obtained for the isoforms were comparable to those obtained for the liver, epididymis and rec 5αR1 and rec 5αR2. At 75 days, however, only 5αR1 activity was detected. Comparison of V_{max} values at day 30 showed that 5αR1 was quantitatively (> 93%) the predominant isoform. The inability to detect 5αR2 at day 75 by enzyme kinetic analysis is attributed to the lack of sensitivity of this method to differentiate between the 5αR isoforms when 5αR2 levels are very low.

A new method was developed in order to measure low levels of 5αR2 activity in the presence of high 5αR1 levels in rat testis. The pH 5.0/7.0 ratio has commonly been used as a relative measure for 5αR2 activity when a sample expresses both 5αR isoforms [26]. This measurement, however, is an estimate and thus is not

Table 3
Measurement of 5αR1 and 5αR2 activities in 30, 75, and 147-days rat testis (pmol DHT+3α-Adiol per min per mg protein)[a]

Rat testis	N	5αR1	5αR2		
		pH 7.0	pH 5.0	pH 5.0/7.0 ratio	Calculated 5αR2
30 days	11	46.1 ± 9.67	15.4 ± 3.23	0.339 ± 0.069	3.44 ± 1.49
75 days	3	2.70 ± 1.54	0.49 ± 0.24	0.187 ± 0.013	0.16 ± 0.04
	3	1.90 ± 0.16	0.26 ± 0.03	0.136 ± 0.006	0.05 ± 0.01*
147 days	7	0.25 ± 0.05	0.08 ± 0.03	0.335 ± 0.112	0.06 ± 0.02

[a] Mean ± S.D.;*, Denotes below the sensitivity of the assay; pH 5.0/7.0 ratio <0.152.

quantitative. In order to measure 5αR2 at pH 5.0 in the presence of 5αR1 activity, the contribution of 5αR1 to the activity at pH 5.0 needs to be known. Therefore, the amount of 5αR1 activity at pH 5.0 and 7.0 was measured in COS-7 cells transiently transfected with a cDNA encoding rat 5αR1. These cells did not contain endogenous 5αR activity. The pH 5.0/7.0 ratio for rec rat 5αR1 was 0.124 ± 0.014 (i.e. 12.4% of the activity detected at pH 5.0 can be attributed to 5αR1 activity). Thus when pH 5.0 activity is used to measure 5αR2 activity, the 12.4% attributed to 5αR1 must be corrected to accurately measure 5αR2. Hence, a formula was devised to take into account the pH 5.0/7.0 ratio of rec 5αR1, removing the activity due to 5αR1.

To test this formula a constant amount of rec 5αR1 was combined with varying known concentrations of rec 5αR2. These samples were assayed at pH 5.0 and 7.0 and the formula used to measure 5αR2 activity. These values were then compared with the actual amount of rec 5αR2 added, and a linear correlation was obtained, indicating that this method allowed an accurate determination of 5αR2 activity in the presence of 5αR1.

This method was then applied to assay 5αR1 and 5αR2 activities in rat testis at days 30, 75 and 147. 5αR1 activity declined with increasing age. This is consistent with previous findings that showed a peak expression of 5αR1 activity [13,14,17,18] and mRNA [4] at day 30, with very low levels of 5αR1 activity at day 75 [17,18]. In our study, 5αR2 activity also showed a similar pattern of expression with 5αR1. 5αR2 was detected in the testis of some animals at day 75, which was previously not detectable by enzyme kinetics.

Based on the V_{max} values (i.e. enzyme concentration) there is approximately three times more 5αR1 in the epididymis than there is 5αR2. The V_{max}/K_m ratio is an estimation of the potential in vivo isoform activity when the endogenous T concentration is much lower than the K_m value [26,27]. Based on V_{max}/K_m values the higher efficiency ratio for 5αR2 in the epididymis compared with 5αR1 (25-fold) suggests that even though 5αR1 is present in higher concentrations, the potential in vivo activity at pH 7.0 could be attributed mainly to 5αR2. Similarly in rat testis at day 30, even though the V_{max} indicated that there is approximately 15 times higher concentration of 5αR1 than there is 5αR2, the potential in vivo activity could be attributed equally to both isoforms.

In summary, by removing the contribution of 5αR1 activity at pH 5.0, 5αR2 activity can be measured accurately in the presence of high levels of 5αR1 activity. This method was used to examine 5αR isoform expression in rat testis to show that 5αR1 is the predominant isoform expressed, and that the activity of both 5αR isoforms decline with age. The sensitivity and specificity of this assay has allowed the investigation of testicular 5αR isoforms at low expression levels (> 70 days). The regulation of the 5αR isoforms in the testis is central to our understanding of their role in spermatogenesis, especially in the contraceptive setting. Current studies are focusing on the hormonal regulation of the 5αR isoforms in rat testis by measuring enzyme activity and using PCR methods to quantitate mRNA levels.

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