

**CHARACTERISATION of ANDROGEN METABOLISM
and
5 α -REDUCTASE ACTIVITY
in
HUMAN PROSTATE CELLS IN VITRO**

by

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ABSTRACT

Androgen metabolism was investigated in the human prostate cancer cell lines (HPC-36M, DU145, PC-3/MA2 and LNCaP), BPH tissue slices, BPH cell suspensions and primary cultures of epithelium and stroma with the aim of developing in vitro systems for the study of androgen metabolism and for evaluating the effects of inhibitors of 5 α -reductase.

Pathways of testosterone metabolism were characterised by identifying metabolites produced after incubation of cells with [³H]-testosterone. BPH slices, cell suspensions, HPC-36M and DU145 formed predominantly dihydrotestosterone (DHT) via 5 α -reductase. In contrast, PC-3/MA2 formed androstenedione, while LNCaP formed only testosterone-glucuronide. LNCaP was the only cell line whose growth and colony-forming ability was stimulated by testosterone and DHT.

COS cells expressing 5 α -reductase 1 and 2 cDNAs were used as sources of 5 α -reductase isozymes. COS cell expressed 5 α -reductase isozymes differed in their optimum pH and sensitivity to the inhibitors finasteride, SKF 105,657, 4-MA and UK117,026. These compounds were used to characterise the DHT-forming activity in prostate cancer cell lines, BPH tissue slices, BPH cell suspensions and BPH primary cultures. The properties of the 5 α -reductase activity in HPC-36M and DU145, resembled those of 5 α -reductase 1, while the properties of 5 α -reductase activity in BPH cells resembled those of 5 α -reductase 2. When human BPH epithelium and stroma were cultured separately, proliferation of the epithelium was accompanied by a gradual decrease in the production of DHT. However, preliminary results from studies with 5 α -reductase inhibitors indicated that cultured epithelial cells lose the expression of 5 α -reductase 2 activity. In contrast, fibroblast cultures converted testosterone to androstenedione and only low levels of DHT were detected.

BPH cell suspensions and tissue slices represent useful systems for studying 5 α -reductase 2 activity while DU145 and HPC-36M provide opportunity to investigate 5 α -reductase 1. The low level of 5 α -reductase activity in cultured cells may indicate the importance of cell-cell interactions in maintaining 5 α -reductase expression.

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CONTENTS

1	THE ROLE OF ANDROGENS AND ANDROGEN METABOLISM IN THE BIOLOGY AND PATHOBIOLOGY OF THE HUMAN PROSTATE GLAND	
1.1	<u>THE ANATOMY, DEVELOPMENT AND MAINTENANCE OF THE PROSTATE GLAND</u>	15
1.1.1	Anatomy	15
1.1.2	Prostate Development	19
1.1.2.i	Normal <u>in utero</u> Development	19
1.1.2.ii	Prostate Development in 5 α -Reductase Deficient Males (Pseudohermaphrodites)	22
1.1.3	Maintenance of the Prostate	23
1.2	<u>BENIGN PROSTATIC HYPERPLASIA (BPH)</u>	26
1.2.1	Natural History	26
1.2.2	Pathogenesis	28
1.2.3	Role of Androgens in the Pathogenesis of BPH	31
1.3	<u>ANDROGEN METABOLISM IN THE PROSTATE</u>	32
1.3.1	Overview of Androgen Metabolising Enzymes	32
1.3.2	<u>In vivo</u> Study of Androgen Metabolism in the Human Prostate	35
1.3.3	<u>In vitro</u> Models for the Study of Prostate Androgen Metabolism	36
1.3.3.i	Tissue Slices, Minces and Organ Cultures	36
1.3.3.ii	Isolated Prostate Cell Studies	37
1.3.3.iii	Primary Cultures of Prostate Cells	38
1.3.4	Concentrations of Androgens in Prostate Tissue	39
1.3.4.i	BPH Tissue	39
1.3.4.ii	Prostate Cancer Tissue	41
1.3.5	Androgen Metabolism in BPH Tissue	42
1.3.6	Androgen Metabolism in Prostate Cancer Tissue	45
1.4	<u>STEROID 5α-REDUCTASE ENZYMES</u>	46
1.4.1	Mechanism of 5 α -Reductase	47
1.4.2	Regulation of 5 α -Reductase Expression	47
1.4.3	5 α -Reductase Isozymes	49
1.4.4	The distribution of 5 α -Reductase in Different Organs	50
1.4.4.i	5 α -Reductase in the Prostate	52
1.4.4.ii	5 α -Reductase in the Liver	53
1.4.4.iii	5 α -Reductase in the Brain	54
1.4.4.iv	5 α -Reductase in the Skin	56

1.5	<u>SURGICAL AND MEDICAL TREATMENT OF BPH</u>	57
1.5.1	Alpha-adrenoreceptor Antagonists	58
1.5.2	Androgen Ablation Therapies	59
1.5.2.i	Luteinizing-Hormone Releasing Hormone (LH-RH) Agonists	59
1.5.2.ii	Androgen Receptor Antagonists (Antiandrogens)	60
1.5.2.iii	5 α -Reductase Inhibitors	61
1.6	<u>THESIS AIMS</u>	65
2	MATERIALS AND METHODS	
2.1	<u>MATERIALS</u>	67
2.2	<u>CELL CULTURE</u>	70
2.2.1	Continuous Cell Lines	70
2.2.2	Routine Maintenance	70
2.2.3	Preparation of a Single Cell Suspension	72
2.2.4	Preparation of Steroid-Depleted Foetal Calf Serum	72
2.3	<u>ANALYSIS OF FCS AND DCC-FCS</u>	73
2.3.1	Radioimmunoassay (RIA)	73
2.3.2	Removal of Tritiated Testosterone and DHT	73
2.4	<u>GROWTH ASSAYS</u>	74
2.4.1	Colony-Forming Assay	74
2.4.2	MTT Cell Proliferation Assay	75
2.4.3	Preparation of Hormones and Drug Solutions	77
2.5	<u>HUMAN PROSTATE TISSUE EXPERIMENTS</u>	77
2.5.1	Collection	77
2.5.2	Preparation of Human BPH Slices	78
2.5.3	Preparation of Human BPH Cell Suspensions	78
2.5.4	Preparation of Human BPH Tissue Homogenates	79
2.5.5	Lowry Assay of Protein Concentration	79
2.6	<u>ANDROGEN METABOLISM EXPERIMENTS</u>	80
2.6.1	BPH Slices	80
2.6.2	BPH Cell Suspensions	81
2.6.3	Prostate Cancer Cell Lines	81
2.6.4	Human BPH Homogenates	82
2.6.5	Transfected COS Cells	82
2.6.6	Primary Cultures of BPH Cells	84

2.6.7	pH Profile Studies	84
2.6.7.i	Homogenates of BPH Tissue	84
2.6.7.ii	Prostate Cancer Cell Lines	85
2.6.7.iii	Transfected COS Cells	85
2.7	<u>5α-REDUCTASE INHIBITOR STUDIES</u>	86
2.7.1	BPH Tissue Slices	86
2.7.2	BPH Cell Suspensions and Prostate Cancer Cell Lines	86
2.7.3	Transfected COS Cells	87
2.7.4	Primary Cultures of BPH Epithelial Cells	87
2.8	<u>SEPARATION AND ANALYSIS OF METABOLITES</u>	88
2.8.1	Thin Layer Chromatography (TLC) Separation of TLC Plates	88
2.8.2	Analysis of TLC Plates	89
2.9	<u>IC₅₀ DETERMINATIONS</u>	89
2.9.1	Colony-Forming Assays	89
2.9.2	5 α -Reductase Assays	89
2.10	<u>PRIMARY CULTURE OF BPH EPITHELIUM AND STROMA</u>	90
2.10.1	Separation of Epithelial Glands (Organoids) from Stromal Elements	90
2.10.2	Culture Conditions for Epithelium	90
2.10.3	Culture Conditions for Stromal Cells	91
2.11	<u>IMMUNOCYTOCHEMICAL CHARACTERISATION OF PRIMARY CELL CULTURES AND PROSTATE CANCER CELL LINES</u>	91
2.11.1	Preparation of Cells	91
2.11.2	Immunoreactivity	92
2.12	<u>STATISTICAL ANALYSIS</u>	92
3	PATHWAYS OF ANDROGEN METABOLISM IN HUMAN BPH TISSUE SLICES AND CELL SUSPENSIONS AND HUMAN PROSTATE CANCER CELL LINES	
3.1	<u>INTRODUCTION</u>	95
3.2	<u>RESULTS</u>	99
3.2.1	Validation of Extraction Procedure for Testosterone and its Metabolites	99

3.2.2	Identification of Testosterone Metabolites	99
3.2.3	Androgen Metabolism in Human BPH Slices	103
3.2.4	Androgen Metabolism in Human BPH Cells	103
3.2.4.i	Testosterone Metabolism	103
3.2.4.ii	Androstenedione Metabolism	107
3.2.5	Androgen Metabolism in Human Prostate Cancer Cell Lines	109
3.2.5.i	Rates of Testosterone Metabolism	109
3.2.5.ii	Testosterone Metabolism in HPC-36M and DU145	109
3.2.5.iii	Testosterone Metabolism in PC-3/MA2	111
3.2.5.iv	Testosterone Metabolism in LNCaP	111
3.3	<u>DISCUSSION AND CONCLUSIONS</u>	116
4	BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERISATION OF 5α-REDUCTASE ACTIVITY IN BPH TISSUE SLICES, CELL SUSPENSIONS AND HUMAN PROSTATE CANCER CELL LINES	
4.1	<u>INTRODUCTION</u>	121
4.2	<u>RESULTS</u>	125
4.2.1	Characterisation of 5 α -Reductase Activity Expressed in COS Cells Transfected with 5 α -Reductase 1 and 2 cDNAs	125
4.2.1.i	Testosterone Metabolism	125
4.2.1.ii	Determination of Optimum pH for Maximum 5 α -Reductase 1 and 2 Activity	129
4.2.2	Comparison of Inhibitors of 5 α -Reductase Isozymes	132
4.2.3	Inhibition of 5 α -Reductase Activity in Human BPH Cells and Prostate Cancer Cell Lines HPC-36M and DU145	135
4.2.4	Inhibition of 5 α -Reductase Activity in Human BPH Slices	137
4.2.5	Determination of the Optimum pH for Maximum 5 α -Reductase Activity in HPC-36M and DU145	141
4.3	<u>DISCUSSION AND CONCLUSIONS</u>	143
5	EVALUATION OF HUMAN PROSTATE CANCER CELL LINES AS MODELS FOR ANDROGEN RESPONSIVE GROWTH <u>IN VITRO</u>	
5.1	<u>INTRODUCTION</u>	147
5.2	<u>RESULTS</u>	150
5.2.1	Determination of Steroid Levels in Foetal Calf Serum	150

5.2.2	The Effects of Testosterone and DHT on Colony-Formation by Prostate cancer Cell Lines	152
5.2.3	The Effect of Testosterone and DHT on LNCaP Proliferation (MTT Assay)	154
5.2.4	Effects of Finasteride on Colony-Formation	156
5.3	<u>DISCUSSION AND CONCLUSIONS</u>	160
6	PRIMARY CULTURE OF EPITHELIAL AND FIBROBLAST CELLS FROM HUMAN BPH TISSUE	
6.1	<u>INTRODUCTION</u>	164
6.2	<u>RESULTS</u>	169
6.2.1	Culture of BPH Epithelium	169
6.2.2	Culture of BPH Fibroblasts	171
6.2.3	5 α -Reductase Activity in Cultured BPH Epithelial Cells	173
6.2.4	5 α -Reductase Activity in Cultured BPH Fibroblasts	175
6.2.5	Characterisation of BPH Epithelial Cell 5 α -Reductase Activity with SKF 105,657 and UK 117,026	179
6.2.6	Characterisation of BPH Cells, Cultured BPH Epithelium and Prostate Cancer Cell Lines	182
6.2.7	Characterisation of BPH Fibroblasts	182
6.3	<u>DISCUSSION AND CONCLUSIONS</u>	185
7	FINAL DISCUSSION AND CONCLUSIONS	192
7.1	Testosterone Metabolism in Human BPH Tissue: Implications for the Treatment of BPH with 5 α -Reductase Inhibitors	193
7.2	Testosterone Metabolism in Human Prostate Cancer Cell Lines: Implications for the Treatment of Prostate Cancer with 5 α -Reductase Inhibitors	196
7.3	Summary	199
7.4	Areas of Further Investigation	199
A1	APPENDIX ONE	200
A2	APPENDIX TWO	200
	REFERENCES	201

LIST OF FIGURES

1.1	The Zonal Anatomy of the Prostate as Described by McNeal (1978)	16
1.2	Development of the Male and Female Internal Genitalia	20
1.3	Pathways of Androgen Metabolism in the Human Prostate	32
1.4	Chemical Mechanism of 5 α -Reductase	48
1.5	Control of Androgen Biosynthesis	55
1.6	Structures of 5 α -Reductase Inhibitors	62
3.1	Pathways of Androgen Metabolism in the Human Prostate	96
3.2a	Radio-TLC Analyser Scan of a Thin Layer Chromatography Separation of [³ H]-Testosterone Radio-Metabolites Produced by Human BPH Cells	100
3.2b	Radio-TLC Analyser Scan of a Thin Layer Chromatography Separation of [³ H]-Testosterone Radio-Metabolites Produced by Human BPH Cells Incubated with Finasteride	102
3.3	Time Course of [³ H]-Testosterone Metabolism by Human BPH Cells	105
3.4	Time Course of [³ H]-Testosterone Metabolism by HPC-36M and DU145	110
3.5	Time Course of Formation of Polar and Non-Polar Metabolites of Testosterone in LNCaP	113
4.1a	Radio-TLC Analyser Scan of a Thin Layer Chromatography Separation of [³ H]-Testosterone Radio-Metabolites Produced by COS Cells Expressing 5 α -Reductase 2 Activity	126
4.1b	Radio-TLC Analyser Scan of a Thin Layer Chromatography Separation of [³ H]-Testosterone Radio-Metabolites Produced by Mock Transfected COS Cells	127
4.2	Effect of Increasing Protein Concentration and Time on the Activity of COS Cell Expressed 5 α -Reductase Isozymes	128
4.3	Effect of pH on the Activity of COS Cell Expressed 5 α -Reductase Isozymes	130
4.4	Effect of pH on 5 α -Reductase Activity in Human BPH Tissue	131

4.5	Dose Response Curves for Inhibition of COS Cell Expressed 5 α -Reductase 1 and 2 Activity	134
4.6	Dose Response Curves for Inhibition of HPC-36M and DU145 5 α -Reductase Activity	138
4.7	Effect of pH on 5 α -Reductase Activity in HPC-36M and DU145	142
5.1	Effect of Androgens on Colony-Formation in Prostate Cancer Cell lines	153
5.2	Effect of Androgens on Cell Proliferation in LNCaP	155
5.3	The Effect of Finasteride on Colony-Formation in Prostate Cancer Cell Lines	158
5.4	The Effect of Finasteride on Androgen Stimulated Colony-Formation In LNCaP Cells	159
6.1	Epithelial Outgrowth from Organoids Isolated by Collagenase Digestion of Human BPH tissue	170
6.2	Primary Cultures of Human BPH Fibroblasts	172
6.3	Time Course of Testosterone Metabolism in Primary Cultures of Human BPH Epithelium	174
6.4	Conversion of Testosterone in Primary Cultures of Human BPH Epithelium with Time	176

LIST OF TABLES

1.1	Concentrations (ng/g Tissue) of DHT in Samples Taken from Normal and Hyperplastic Prostate Tissues	40
1.2	Comparison of the Properties of Human Prostatic 5 α -Reductase Isozymes	51
2.1	Materials and Suppliers	67
2.2	Details of Human Prostate Cancer Cell Lines	71

2.3	Antibody Sources	93
3.1	Metabolites of Testosterone formed by BPH Slices, BPH Cells and Human Prostate Cancer Cell Lines	104
3.2	Rates of Testosterone Metabolism in Human BPH Tissue Slices and Cell Suspensions and in Human Prostate Cancer Cell Lines	106
3.3	Androstenedione Metabolism in Human BPH Cells	108
3.4	Androstenedione Metabolism in PC-3/MA2	112
3.5	Recovery of Radioactivity from LNCaP Cells Before and After Hydrolysis with β -Glucuronidase	115
3.6	Androgen Metabolising Enzyme Activities in Human BPH Tissue and Human Prostate Cancer Cell Lines	117
4.1	Comparison of IC ₅₀ Values for the Inhibition of COS Cell Expressed 5 α -Reductase 1 and 2 Activity	133
4.2	Comparison of IC ₅₀ Values for the Inhibition of 5 α -Reductase Activity in Human BPH Cells and Prostate Cancer Cell Lines	136
4.3	Comparison of Inhibition of Conversion of Testosterone to DHT in Cell Suspensions and Slices of Human Prostate Tissue	140
5.1	Steroid Hormone Concentrations of Foetal Calf Serum (FCS) and Charcoal Stripped Serum (DCC) as Determined by Radioimmunoassay	152
5.2	IC ₅₀ Values (μ M) for the Inhibition of Colony-Formation by Finasteride in Prostate Cancer Cell Lines	157
6.1	Testosterone Metabolism in Human BPH Epithelium After Four and Ten Days in Culture	177
6.2	Testosterone Metabolism in Primary Cultures of Human BPH Fibroblasts	178
6.3	The Effect of SKF 105,657 and UK 117,026 on the Formation of Testosterone Metabolites in Human BPH Epithelium	181
6.4	Characterisation of BPH Epithelium	183
6.5	Characterisation of Prostate Cancer Cell Lines	184

ABBREVIATIONS

BPH:	Benign Prostatic Hyperplasia
BPH Tissue:	Prostate tissue obtained by TURP from BPH patients
DCC:	Dextran Coated Charcoal
DHT:	5α-Dihydrotestosterone
DMSO:	Dimethyl sulphoxide
°C:	Degrees centigrade
EDTA:	Ethylenediaminetetra acetic acid
FCS:	Foetal Calf Serum
g:	Gram
HEPES:	N-(2-Hydroxyethyl)-piperazine-N'-(2-ethane sulphonic acid)
h:	Hour
3α-HSD:	3α-hydroxysteroid dehydrogenase
3β-HSD:	3β-hydroxysteroid dehydrogenase
17β-HSD:	17β-hydroxysteroid dehydrogenase
m:	Milli
M:	Molar
MgCl₂:	Magnesium chloride
min:	Minute
ml:	Millilitre
MTT:	3-4,5-(dimethylthiazole-2-yl)-2,-diphenyltetrazolium bromide
NAD:	Nicotinamide Adenine Dinucleotide
NADPH:	Nicotinamide Adenine Dinucleotide reduced form
n:	Nano (10⁻⁹)
μ:	Micro (10⁻⁶)
OD:	Optical density
p:	Pico (10⁻¹²)
PBS:	Phosphate Buffered Saline
%:	Percent
PAP:	Prostate Acid Phosphatase
PSA:	Prostate Specific Antigen

TLC:	Thin Layer Chromatography
SD:	Standard Deviation
SEM:	Standard Error of the Mean
TRIS:	(Hydroxymethyl) aminomethane
TURP:	Transurethral Resection of the Prostate
UV:	Ultra Violet

Steroids

Androstane-diols:	3α-androstane-diol; 5α-androstane-3α,17β-diol and { 3β-androstane-diol; 5α-androstane-3β,17β-diol
Androstane-dione:	5α-androstane-3,17-dione
Androsterone:	3α-hydroxy-5α-androstane-17-one
Dihydrotestosterone:	17β-hydroxy-5α-androstane-3-one
Epiandrosterone:	3β-hydroxy-5α-androstane-3-one
Finasteride:	N(1,1-dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17-carboxamide
4-MA:	17β-N,N-diethylcarbonyl-4-methyl-4-aza-5α-androstan-3-one
SKF 105,657:	17β-N-<i>t</i>-butylcarboxamide-androst-3,5-diene-3-carboxylic acid
Testosterone:	3β-hydroxy-4-androstene-3-one

CHAPTER ONE

THE ROLE OF ANDROGENS AND ANDROGEN METABOLISM **IN THE BIOLOGY AND PATHOBIOLOGY OF** **THE HUMAN PROSTATE GLAND**

Introduction

The prostate gland is one of the largest exocrine glands and probably the most disease-prone organ in the male human body. The two main diseases affecting the prostate gland, benign prostatic hyperplasia (BPH) and prostate cancer, have an enormous impact in terms of morbidity, mortality and cost of health care. With increasing age, BPH is such a common occurrence that by the age of 70 years 40-50% of the male population have palpable enlargement of the prostate (Ekman, 1989) which may progress to obstruct urine outflow from the bladder. Although BPH is non life-threatening, it has been estimated that between 10 and 20% of 50 year old men will undergo surgery for the relief of the symptoms caused by BPH (Lytton *et al.*, 1968; Birkoff, 1983).

Prostate cancer, in contrast to BPH, is life-threatening and in 1990 deaths from prostate cancer accounted for 10% of all male deaths due to cancer (HMSO Mortality Statistics, 1990) making this the second biggest killer of all cancers in men in England and Wales. In North America it is estimated that prostate cancer is discovered at the rate of more than 300 new cases per day (Labrie *et al.*, 1990). In a survey of autopsy studies it was reported that 15-30% of males over the age 45-50 had histological evidence of prostate cancer (Carter and Coffey, 1990). Prostate cancer is the most prevalent cancer in men. Despite such a high frequency of BPH and prostatic cancer, there is limited understanding as to the causes of these diseases. The development of suitable models of the prostate is

essential to further our understanding of mechanisms involved in controlling the normal biology and the pathogenesis of BPH and prostate cancer and for developing novel medical therapies for the treatment of these diseases.

1.1 THE ANATOMY, DEVELOPMENT AND MAINTENANCE OF THE PROSTATE GLAND

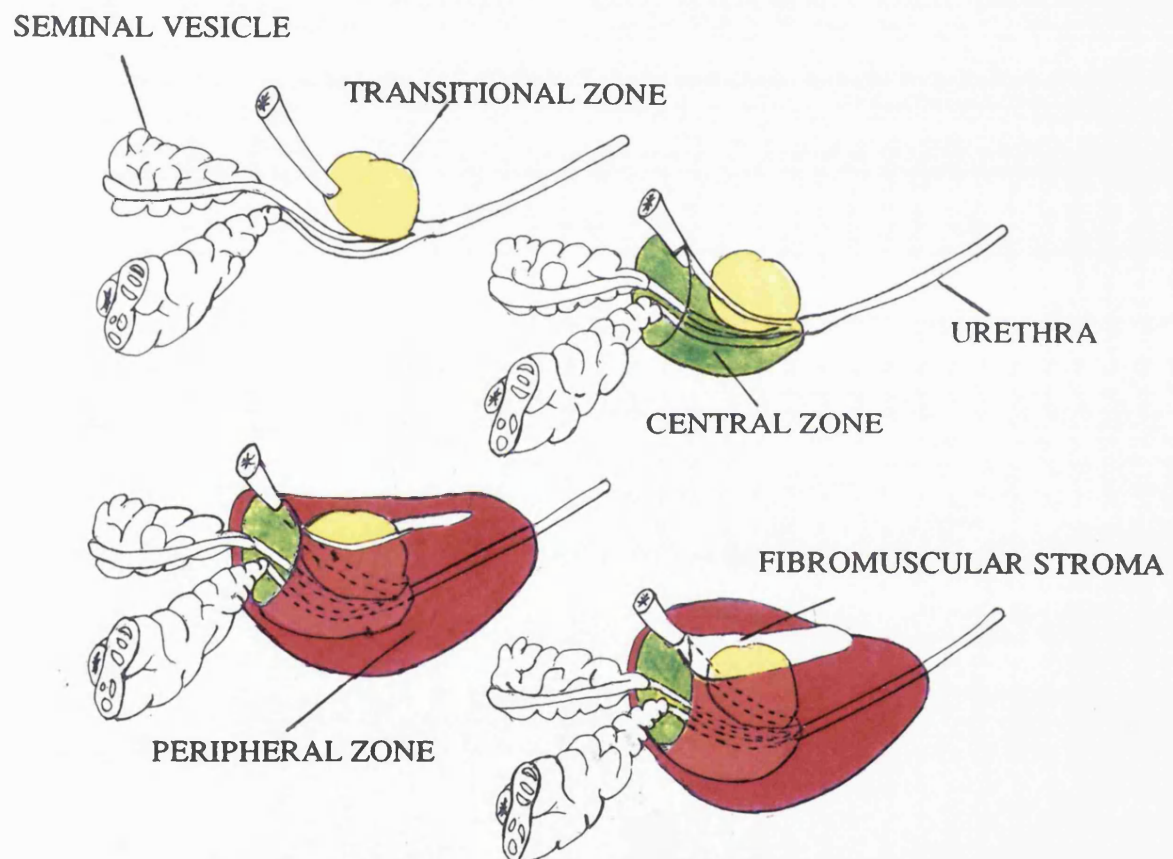
1.1.1 Anatomy

The prostate gland in the human male is found immediately below the bladder surrounding the urethra. Histological studies indicate that it is composed of a series of secretory tubules and acini (ducts) surrounded by fibromuscular stroma. During ejaculation compression of the acini conveys prostatic secretions and seminal fluid, delivered by the vas deferens, into the urethra (Aumüller and Seitz, 1990).

McNeal (1968, 1978, 1980, 1983) has developed an anatomical description of the human prostate based on dissections and embryological considerations of over 500 autopsy prostates of all ages. McNeals' model identifies four areas of the prostate; a zone of fibromuscular stroma and three zones of glandular tissue (Figure 1.1). The fibromuscular stroma constitutes about one-third of the whole prostate, contains no glandular tissue and seems to be without any significant role in prostatic secretory function (McNeal, 1983). The three glandular zones consist of a peripheral zone (representing 65% of the glandular tissue), a central zone (comprising about 25% of the glandular tissue) and a periurethral transitional zone (comprising about 10% of glandular tissue).

FIGURE 1.1

THE ZONAL ANATOMY OF THE HUMAN PROSTATE AS DESCRIBED BY
McNEAL (1978)



The Figure shows the 3 glandular zones (transitional, central and peripheral) and the zone of fibromuscular stroma as described by McNeal (1978) and their relationship to each other in the prostate. Adapted from Greene *et al.* (1991)

McNeal studied the relationship between the different zones and the development of benign prostatic hyperplasia (BPH) and prostate cancer. He proposed that BPH develops in the areas of the prostate that are immediately adjacent to the urethra - the periurethral transitional zone (McNeal, 1978). In contrast, prostate cancer is derived mainly from areas directly related to the fibrous prostate capsule in the periphery of the gland (McNeal, 1968, 1969). McNeal observed that almost 68% of all prostate cancers arise in the peripheral zone, 24% in the transitional zone and 8% in the central zone.

Morphometric analysis of BPH tissue has shown that it consists of 45-60% stromal tissue, 30-34% acinar lumen and 10-20% epithelial tissue (Rohr and Bartsch, 1980). The exact function of the non-secretory epithelial cells is unknown, but they may act as a reserve of stem cells capable of differentiating into secretory cells (see review by Aumüller, 1983).

The secretory epithelial cells are specialised cells responsible for the production and secretion of secretory proteins associated with the functional gland. These secretions (usually less than 1ml per ejaculate) have several functions which include semen gelation, coagulation and liquefaction as well as activation of spermatozoa and protection of spermatozoa from cervical mucus (Aumüller, 1990). Prostatic fluid contains high concentrations of zinc and citrate (Habib, 1978; Costello and Franklin, 1991). Although it is established that both citrate and zinc levels are elevated in BPH and reduced in prostate cancer, the precise physiological function of zinc and citrate in prostatic fluid has yet to be demonstrated.

In addition to citrate, prostatic secretory epithelial cells also produce two characteristic components of prostatic fluid: prostatic acid phosphatase (PAP) and prostatic specific antigen (PSA) (Foti *et al.*, 1977; Choe *et al.*, 1978; Wang *et al.*, 1979; Bates, 1982; Papsidero *et al.*, 1981; Lilja, 1985a).

The exact physiological function of PAP in the prostate is unknown. PSA has chymotrypsin-like and trypsin-like activity (Akiyama *et al.*, 1987; Ban *et al.*, 1984) and cleaves a high molecular weight seminal vesicle protein into smaller proteins causing the seminal fluid to liquify (Lilja, 1985a,b). The production of PSA and PAP by epithelial cells is an important marker of their prostatic origin (Papsidero *et al.*, 1981; Bates *et al.*, 1982).

The term stroma refers to all the cellular and non-cellular elements outside the epithelial basement membrane; these include smooth muscle cells, fibroblasts, blood vessels, connective tissue, nerve terminals and lymphatics (Aumüller, 1983). As already mentioned, 45-60% of the total prostatic tissue is stromal and 10-20% glandular. Siegel *et al.* (1990) showed that of the stromal components, fibrous, vascular and muscular elements contribute 47%, 3% and 7% respectively to the total volume and other structures (nerves, lymphatics, blood cells) the remaining 25% (Siegel *et al.*, 1990).

Prostate stromal cells in the human prostate have an estimated average life span of thirty years based on superoxide dismutase activity, a marker of cell longevity, in comparison to two years for epithelial cells (Tunn *et al.*, 1989). Unlike prostate epithelial cells, stromal cells do not produce either PAP or PSA, instead the stromal elements surround the epithelial acini in a loose collagenous matrix providing a mechanical scaffold consisting of capillaries, lymphatics and nerves providing a supply of oxygen, ions, hormones and transmitter signals to the epithelium (Aumüller and Seitz, 1990).

The stroma surrounding the epithelial ducts contains three or four layers of smooth muscle arranged longitudinally and circularly (Cunha *et al.*, 1987). The stromal prostatic smooth muscle cells are rich in α_1 -adrenergic receptors as are the smooth muscle cells of the bladder neck. The smooth muscle cells of the prostate are responsible for the resting and dynamic tone of the prostate (Caine

et al., 1975; Shapiro and Lepor, 1986; Lepor *et al.*, 1988). Contraction of the prostate smooth muscle, in the presence of alpha-adrenoceptor agonists results in the expulsion of prostatic secretions into the urethra (Caine *et al.*, 1975, Aumüller, 1990). Prolonged contraction of periurethral smooth muscle, for example, in response to cold temperature can produce an acute retention of urine by constricting the bladder neck and prostatic urethra.

1.1.2 Prostate Development

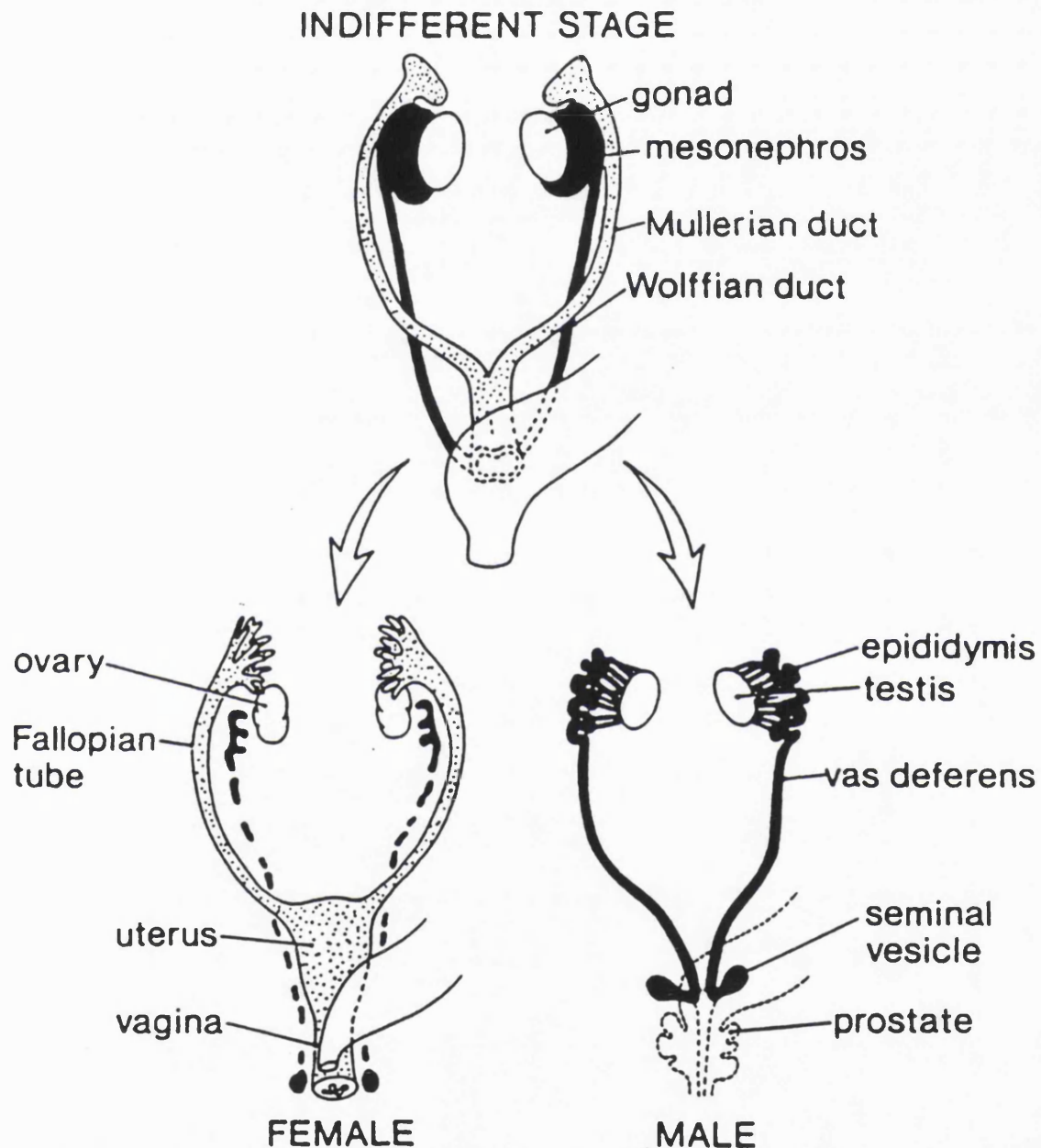
1.1.2.i Normal In Utero Development

Embryogenesis of the urogenital tract in mammals is initially identical in both sexes, this is referred to as the indifferent stage (Figure 1.2). During this stage, the undifferentiated urogenital tract is composed of a dual ductal system (the Wolffian duct and the Müllerian duct), which gives rise to the internal accessory organs of reproduction, and the urogenital sinus and tubercle which form the external genitalia (Wilson, 1979). It is only after the onset of the endocrine function of the testis that anatomic and physiological development of the male and female embryos diverge.

The start of male differentiation is signalled by the regression of the Müllerian ducts which is mediated by a glycoprotein (Müllerian-inhibiting substance) formed in Sertoli cells of the spermatogenic cords and the tubules of foetal and newborn testes (Donahoe *et al.*, 1982). This is followed by synthesis of testosterone in the Leydig cells of the foetal testis and growth and differentiation of the Wolffian duct into the epididymis, vas deferens and seminal vesicle (George and Wilson, 1986). The ductal networks within the prostate are derived from solid epithelial outgrowths (prostatic buds) that emerge from the urogenital sinus immediately below the developing bladder (Cunha ^{*et al.*} 1987).

FIGURE 1.2

DEVELOPMENT OF MALE AND FEMALE INTERNAL GENITALIA



During the indifferent stage (top figure) the undifferentiated urogenital tract is composed of the Wolffian and Müllerian ducts. Synthesis of testosterone by the testes in the male embryo causes the Wolffian ducts to form the epididymis, vas deferens and seminal vesicle (bottom right). In the female embryo the Müllerian duct forms the uterus and fallopian tubes (bottom left). Adapted from George and Wilson, (1986).

The external genitalia develop after virilization of the Wolffian duct and urogenital sinus. Androgens play a vital role in controlling male development and the anatomical and physiological divergence of embryos into male and female occurs only after the onset of endocrine function of the testes (Jost, 1953). Castration of rabbit embryos during the sexually indifferent stage inhibits the development of male accessory organs and results in the embryos developing as females (Jost, 1953). Male development only occurs in the presence of a functional foetal testis.

Testosterone is the principle steroid formed by the testes of human embryos (Wilson and Siiteri, 1973; Siiteri and Wilson, 1974). Its synthesis immediately precedes the initiation of virilization in a variety of mammalian embryos including human embryos emphasising the role of the testes in the development of the male (George and Wilson, 1986). Testosterone produced by the testes mediates virilization of the Wolffian derivatives (epididymis, vas deferens, seminal vesicle and ejaculatory ducts) whereas its 5α -reduced metabolite dihydrotestosterone (17β -hydroxy- 5α -androstane-3-one, DHT) is responsible for virilization of the male urethra, prostate and external genitalia (Wilson and Lasnitzki, 1971). This was deduced from the observation that 5α -reductase activity (the enzyme that converts testosterone to DHT) in mammalian embryos was greatest in the prostate and external genitalia prior to virilization (Wilson and Lasnitzki, 1971; Kelch *et al.*, 1971; Siiteri and Wilson, 1974). In contrast, 5α -reductase activity was undetectable in Wolffian duct derivatives until virilization was advanced (Wilson and Lasnitzki, 1971; Siiteri and Wilson, 1974).

More evidence to support the interpretation that DHT mediates the development of the prostate and the external genitalia in the embryo comes from studying the effects of administration of a 5α -reductase inhibitor to pregnant rats. Treatment of pregnant rats with the 5α -reductase inhibitor finasteride during the period of prostate development in the male embryos, results in the production of offspring

with impaired development of the prostate and virilization of the external genitalia (Clark *et al.*, 1990, 1993). Internal sexual organs, seminal vesicle, vas deferens and epididymis develop normally. Such experiments demonstrate the role of DHT in the development of the prostate and mimic a naturally occurring 5α -reductase deficiency syndrome in man known as male pseudohermaphroditism Type 2 in which the prostate is substantially reduced in size.

1.1.2.ii Prostate Development in 5α -Reductase Deficient Males
(Pseudohermaphrodites)

Male pseudohermaphroditism is an autosomal recessive form of abnormal sexual development in 46XY males (Norwakowski and Lenz, 1961). Affected males are born with ambiguous (often female) external genitalia, a clitoral-like phallus and a blind-ended vaginal pouch in association with bilateral testes and normally virilized Wolffian structures (epididymis, vas deferens, seminal vesicle and ejaculatory duct) (Imperato-McGinley *et al.*, 1974). At puberty virilization occurs and they develop a typical male phenotype. These individuals, when adults, have small prostates, decreased body hair, scant or absent beards and no temporal hair recession (Imperato-McGinley, 1974; Peterson *et al.*, 1977).

Ultrasound and magnetic resonance imaging of prostates in four affected adult males showed that prostate volume is one-tenth that of age matched controls (2cm^2 versus 20cm^2). Furthermore, the prostate tissue contains only stromal tissue and PSA levels were undetectable indicating the absence of secretory epithelial cells (Imperato-McGinley *et al.*, 1992). The abnormal sexual development in male pseudohermaphrodites has been traced to a defect in 5α -reductase (Imperato-McGinley *et al.*, 1974; Moore and Wilson, 1976; Andersson *et al.*, 1991) indicating the importance of DHT in the development of the prostate. However, the partial development of the prostate in these males may be due to the fact that some 5α -reductase activity is present in male pseudohermaphrodites

allowing partial in utero development of the prostate to occur (Imperato-McGinley *et al.*, 1974; 1992).

1.1.3 Maintenance of the Prostate

The importance of the androgens in the maintenance of prostate structure and its function is well recognised. According to Scott (1953) in his review entitled "What makes the prostate grow" it was reported as early as 1786 by John Hunter that removal of the testes in the bull led to a degeneration of the prostate gland, Cowper's gland and the seminal vesicle with little secretions produced. The involution and atrophy of the prostate that occur after castration indicate the importance of the testes in maintaining prostate integrity and implicates the major testicular androgen, testosterone.

The nature of the castration-induced involution of the prostate has been intensively studied using the rat prostate. Following the initial observations by Moore *et al.* (1930) showing that castration results in a breakdown of the cellular structure of the prostate, several other investigators have reported that the rapid onset of cell death results in up to 85% loss of the ventral prostatic cells within 7-10 days of castration (Lesser and Bruchovsky, 1973; Lee, 1981; Isaacs, 1984). The rapid cell death following castration has been suggested by many investigators to be an active process of programmed cell death called apoptosis (Isaacs, 1984; Kyprianou and Isaacs, 1988; Buttyan, 1989). Apoptosis is characterised by increased activity of endonuclease which causes fragmentation of DNA into oligomers (Kyprianou and Isaacs, 1988).

The degenerative changes occur most rapidly and extensively in the epithelial cells (Lee, 1981; Kyprianou and Isaacs, 1988; Rouleau *et al.*, 1990) resulting in the loss of the secretory function of the prostate. In the rat, castration results in a 92%

decrease in ventral prostate epithelial cell number compared to 39% reduction in stromal cells (DeKlerk and Coffey, 1978).

Analysis of human prostate after medical castration (with a LH-RH agonist) has shown a 40% reduction of epithelial and 21% reduction of stromal cells (Peters and Walsh, 1987). Castration-induced regression of the prostate is totally reversible and if testosterone is administered the glandular structure of the prostate gland can be stimulated to regenerate (Moore *et al.*, 1930; Lesser and Bruchovsky, 1973; Isaacs, 1984). The supply of androgen to the disease-free prostate is sufficient to maintain a balance between prostatic cell death and proliferation so that neither involution or overgrowth occurs (Isaacs, 1984). The rapid loss of secretory epithelial cells following castration supports the interpretation that these cells have an absolute dependence upon androgens for stimulation of cell growth. However, some cells in the prostate must be able to survive (but not necessarily proliferate) without androgens so that they are able to respond and regenerate the prostate when androgens are re-administered.

While castration studies indicate the importance of testosterone in the maintenance of the prostate they fail to distinguish which of the potent androgens, testosterone or DHT, is the main mediator of androgenic action in the prostate. With the development of selective 5 α -reductase inhibitors, which inhibit the conversion of testosterone to DHT, it became possible to provide convincing evidence that androgenic effects on the prostate are mediated primarily by DHT.

Treatment of dogs and rats with 5 α -reductase inhibitors, such as finasteride, 4-MA and SKF 105,657, produces a decrease in the DHT concentration of prostate tissue, a reduction in 5 α -reductase activity and degeneration of the prostate (Brooks *et al.*, 1981; Wenderoth *et al.*, 1983; Rittmaster *et al.*, 1991; Lamb *et al.*, 1992). These 5 α -reductase inhibitors also produce an increase in prostate tissue testosterone by as much as five fold (Lamb *et al.*, 1992). The degeneration of

prostate even in the presence of elevated tissue levels of testosterone is good evidence that DHT mediates prostate growth. Furthermore, testosterone stimulated regeneration of the prostate after castration is blocked by 5 α -reductase inhibitors while DHT stimulated regeneration is unaffected (George *et al.*, 1991; Lamb *et al.*, 1992).

Other evidence favouring DHT as the most potent androgen in the prostate is provided by the higher intracellular concentrations of DHT and its greater affinity for the androgen receptor than testosterone. The concentration of testosterone in plasma is approximately 10-fold greater than that in prostate tissue (Ghanadian and Puah, 1981). As no active transport mechanism for testosterone uptake by prostate tissue has been demonstrated it must be assumed that testosterone enters the prostate along its concentration gradient. Once inside the prostate testosterone is converted to DHT by 5 α -reductase so that the concentration of DHT inside the prostate exceeds that in the plasma (Ghanadian and Puah, 1981).

Testosterone and DHT are believed to exert their effects by interacting with a single androgen receptor i.e. there is at present no evidence for different isoforms with different affinities for testosterone and DHT (Goldstein and Wilson, 1972; George and Nobel, 1984). Studies comparing the affinity and dissociation rate of the androgen receptor complex with testosterone and DHT support the interpretation that DHT is the main androgen in the prostate. DHT has a higher affinity (approximately five times) for the androgen receptor than testosterone (Liao *et al.*, 1973; Wilson and French, 1976; Wilbert *et al.*, 1983) and a slower dissociation rate from the receptor than testosterone (Wilbert *et al.*, 1983; Kaufman *et al.*, 1983; Grino *et al.*, 1990). It has also been shown that the DHT-receptor complex is more readily transformed to the DNA binding state than the testosterone-receptor complex (Kovaks *et al.*, 1983).

Studies of the androgen receptor in the Wolffian duct failed to find anything unique that allows it to respond to testosterone preferentially (George and Nobel, 1984). The question that remains to be answered is how two ligands (testosterone and DHT) exert different actions during embryogenesis by binding to one receptor. The weaker androgenic potency of testosterone may reside in its weaker interaction with the androgen receptor as it has been shown that a high concentration of testosterone (20nM) was as effective as ten-fold lower concentration of DHT at promoting the up-regulation of the androgen receptor in fibroblast monolayers from 5 α -reductase deficient patients (Grino *et al.*, 1990). The weaker interaction of testosterone may be compensated for by a local high concentration of testosterone in the Wolffian ducts during embryogenesis, allowing virilization to occur.

In summary, there is much evidence to support the contention that DHT is responsible for mediating many of the androgenic effects in the development of the prostate and in the maintenance of growth of the prostate. However, the separate roles of testosterone and DHT in the virilization of the embryo indicate that testosterone is not simply a precursor for dihydrotestosterone. The suggestion that unregulated DHT formation in the prostate might be involved in the pathogenesis of BPH and prostate cancer has led to many studies focusing on the concentrations of androgens in prostate tissue, and on the pathways and enzymes involved in processing testosterone and DHT in the prostate.

1.2 BENIGN PROSTATIC HYPERPLASIA (BPH)

1.2.1 Natural History

Benign prostatic hyperplasia (BPH) is a common disease affecting middle aged and older men, in which the prostate undergoes enlargement due to increased proliferation of both epithelial and stromal elements. Although there are similarities between BPH and prostate cancer (age of onset and requirement for

androgens) there is no strong evidence to indicate that BPH leads on to the development of prostate cancer. Cancer is found incidentally in approximately 10% of BPH specimens, and approximately 3% of patients treated for BPH by surgery will develop prostate cancer indicating a weak association (Bostwick, 1992).

Histological studies (Rohr and Bartsch, 1980; McNeal ~~et al.~~, 1990; Price *et al.*, 1990) have indicated that prostate enlargement is due to an increase in cell number (hyperplasia), rather than an increase in cell size (hypertrophy). Berry *et al.*, (1984) reviewed two separate autopsy studies comparing the changes in weight of the prostate as a function of age. It was found that at birth the prostate weighs just a few grams and at puberty undergoes an androgen-induced growth spurt to reach a weight of about 20g. This weight represents the average weight for a prostate gland without histologically identifiable BPH. The gland remains stable without significant increase in weight for the next 20 or so years before the onset of hyperplastic growth which can result in the prostate weighing 70g or more. The mean weight of prostate from men considered to have BPH was 33g, while in the 70 years and over age group 3.5% of men had prostates weighing greater than 100g.

An enlarged prostate does not itself cause problems and is not life threatening unless it severely obstructs the outflow of urine from the bladder (Liu *et al.*, 1991). Urinary retention occurs when the bladder can no longer generate enough pressure to overcome outlet resistance. Bladder outlet obstruction in men is composed of a static component related to the bulk of the prostate and a dynamic component related to the tone of smooth muscle (Caine 1988; Shapiro *et al.*, 1992).

If left untreated the complications arising from BPH include urinary tract infection, urinary retention and renal failure (Liu *et al.*, 1991). However, relief

of obstructive symptoms can be achieved before secondary complications occur, most commonly by a surgical technique known as transurethral resection of the prostate (TURP). During this procedure the obstructive bulk of the prostate tissue is cut away using an endoscopic instrument via the urethra. TURP has replaced open-prostatectomy as the standard surgical procedure for BPH with almost 400,000 such procedures performed each year in the US (Mebust and Holtgrew et al., 1989).

1.2.2 Pathogenesis

The pathogenesis of BPH is poorly understood despite the fact that most men will develop it if they live long enough. A great deal of research has been carried out to establish which histological type of prostate tissue (the epithelium or stroma) initiates the hyperplastic growth of the prostate. The most definitive work and much of the knowledge of the origin of BPH has come from the work of McNeal (1968, 1978, 1980, 1983, 1990) who supports the theory that the interaction between the stroma and the epithelium is important in the development of BPH.

McNeal (1978, 1990) observed that in the hyperplastic nodules in the transitional zone were formed by proliferation of glandular tissue rather than stromal elements. These glandular nodules are apparently derived from small duct branches that sprout from existing ducts and give rise to a new ductal system. With the development of BPH, nodular proliferation occurs in the tissues, originating in the transitional zone specifically around the periurethral tissues toward the bladder outlet (McNeal, 1978). This type of new gland formation is rare outside embryonic development. The similarity between glandular morphogenesis in embryonic tissue when androgens induce budding of prostatic ducts into the stroma and the development of BPH led McNeal to postulate that BPH was caused by a process of "embryonic reawakening".

Although there is no direct evidence to support the theory of embryonic reawakening in human BPH, animal studies performed by Cunha *et al.* (1980, 1983, 1984, 1987) have shown that embryonic stroma has an inductive potential on normal prostatic epithelial cells. Embryonic urogenital sinus mesenchyme (embryonic prostatic stroma) of mice can induce adult bladder epithelial cells, which like prostate epithelium is derived from urogenital sinus mesenchyme, from testicular feminized mice (androgen insensitive animals) to form prostate-like glandular structures. This induction is androgen-dependent and will not occur in castrated animals. As the adult bladder epithelium is devoid of androgen-receptors the androgen-dependent growth must be mediated via the stroma (Cunha *et al.*, 1980). Cunha's work supports the interpretation that the stroma has embryonic-like inductive properties which can stimulate prostate epithelium to new growth giving rise to BPH.

An third theory, the stem cell theory, has also been proposed to explain the development of BPH (Isaacs and Coffey, 1989). Stem cells represent a reserve pool of cells capable of extensive renewal and giving rise to short-lived androgen-dependent cells. The stem cell theory proposes that an increase in the number of stem cells has a cascade effect producing an increase in total prostate cell number and is ultimately responsible for an enlarged prostate. This theory is purely hypothetical and Isaacs and Coffey have yet to provide any experimental evidence to support their hypothesis.

It is now clear that other factors other than androgens alone must be involved in the pathogenesis of BPH. Recent studies have focused on the role of growth factors and have identified several growth factors and their receptors in human prostate tissue including: epidermal growth factor (EGF); basic fibroblast growth factor (FGF); transforming growth factor α and β 2 (TGF- α , TGF- β 2), and insulin-like growth factor (IGF) (Elson *et al.*, 1984; Story *et al.*, 1987; Maddy *et al.*, 1987; Mydlo *et al.*, 1989; Serio and Fiorelli, 1991; Yang *et al.*, 1993).

However, a direct link between any one particular growth factor and the development of BPH remains to be established. Furthermore the interaction between androgens and growth factors is unclear. Lawson (1989, 1990) proposes that basic fibroblast growth factor (bFGF) may have a role in the development of BPH. Normal human prostate and BPH tissue have high concentrations of bFGF and cultured fibroblasts also produce and are stimulated by bFGF (Lawson, 1989; Story *et al.*, 1989). Thus, BPH might be caused by overproduction of a growth factor (such as bFGF) causing stromal hyperplasia which then acts like primitive mesenchyme to induce proliferation of the adjacent epithelium.

Until fairly recently, the evidence to support the role of oestrogens in the development of BPH was rather circumstantial. The concept of BPH as a stromal disease and the observation that the concentration oestrogen receptors and 17 β -oestradiol was higher in the stroma seemed to suggest an involvement of oestrogens in BPH (Krieg *et al.*, 1981; Kózak *et al.*, 1982). Some evidence was provided by the observation that DHT alone was insufficient to trigger BPH in the dog prostate (Wilson, 1980). The administration of 3 α -androstanediol (the main breakdown product of DHT in the prostate) and small amounts of 17 β -oestradiol induced a profound enlargement. Further investigation showed that androstanediol acts as a precursor for DHT and treatment of dog prostate with 17 β -oestradiol causes an upregulation of androgen receptors (Moore *et al.*, 1979a, 1979b). More recently stronger evidence to support the role of oestrogens in BPH has been come from studies with atamestane, an aromatase inhibitor, which inhibits the biosynthesis of oestrogens. In animal models of BPH, atamestane is effective in inhibiting oestrogen-induced hyperplasia of the fibromuscular stroma of the prostate (Habenicht and el Etreby, 1991).

In man with increasing age there is an increase in the formation of oestrogens and a decrease in plasma testosterone, with a net result of an increase in the ratio of

plasma 17 β -oestradiol to testosterone (Skoldefors *et al.*, 1978). Although there is no difference in plasma oestrogen in men with BPH compared to controls, oestrogens may be implicated in the pathogenesis of BPH by a synergistic mechanism with androgens. If this is the case then treatment of BPH with aromatase inhibitors may prove to be an effective therapy.

1.2.3 Role of Androgens in the Pathogenesis of BPH

Androgens were implicated in the pathogenesis of BPH when it was observed that at least one intact functional testis is required for development of the disease. In 1944 Moore showed that in the absence of testicular function either due to castration or hypopituitarism prior to the age of 40 years there was no development of BPH in men who lived to greater than 55 years of age (i.e. into the BPH age group). Similarly, the Skoptzys, a Russian sect in which the males undergo ritual castration at the age of 35 years, are reported to have smaller prostates than normal and were lacking in the symptoms of BPH (Zuckerman, 1936). The relationship between intact testes and BPH has been realised for many years and White (1895) and Cabot (1896) reported both a reduction in prostate size and an overall improvement in symptoms following castration for BPH. More recently, Schröder *et al.* (1986) reported a significant decrease in prostatic volume in four patients with BPH treated by surgical castration.

Much of the evidence implicating 5 α -reductase and DHT in the development of BPH is the same as that linking DHT to the development and maintenance of the prostate (Section 1.1). Perhaps the strongest piece of evidence is that 5 α -reductase-deficient males do not develop BPH or prostate cancer (Imperato-McGinley, 1974), and administration of DHT to such individuals can result in an increase in prostate size (Peterson *et al.*, 1977). Plasma testosterone concentrations in male pseudohermaphrodites are normal while plasma DHT concentrations are lower than normal (Imperato-McGinley, 1979, 1991) suggesting

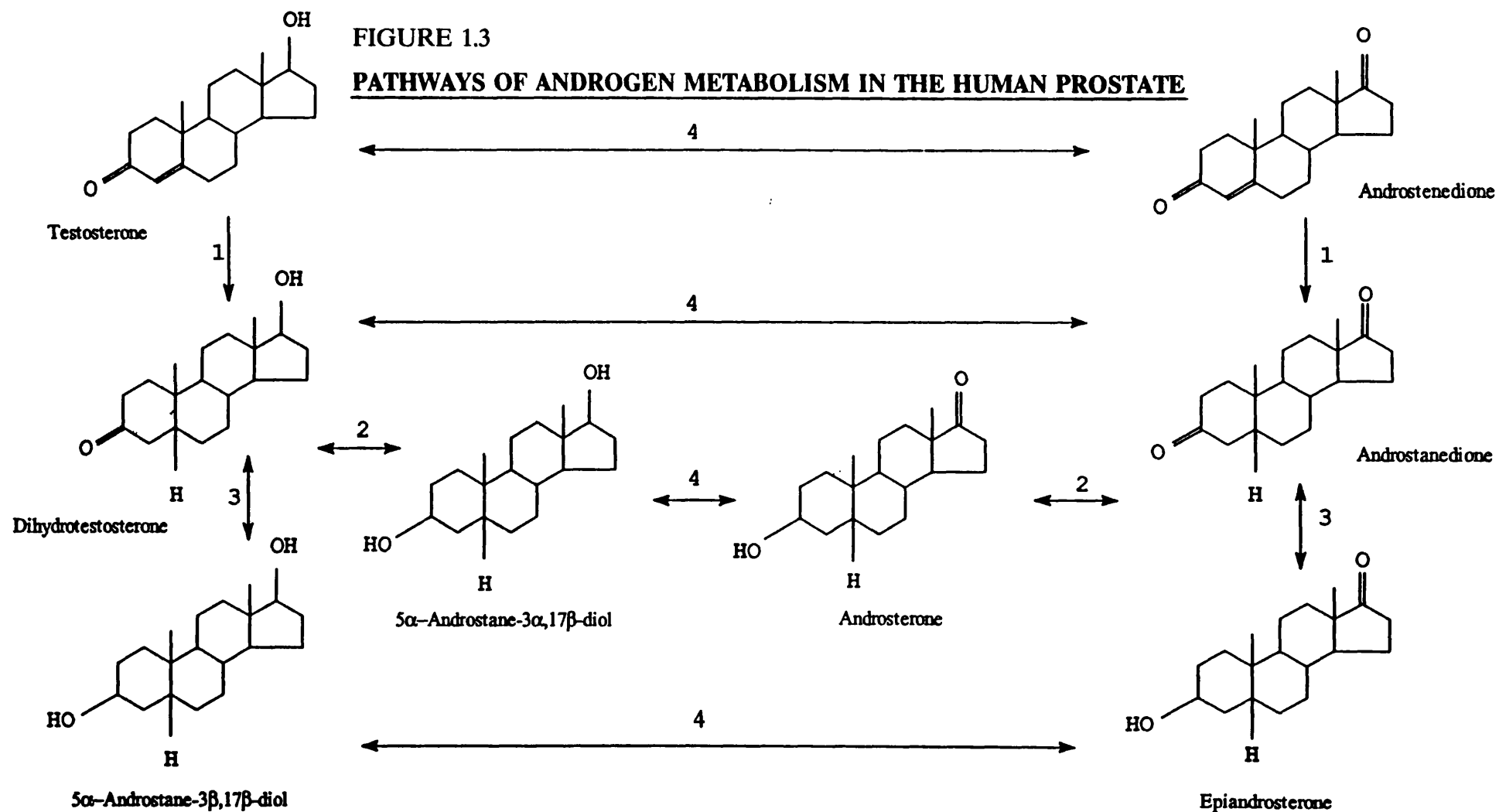
that accumulation of DHT in the prostate might trigger the growth leading to BPH. This is supported by the observation that DHT is more active in stimulating cell hyperplasia of prostate organ cultures than other androgens (Robel *et al.*, 1971). Before considering the mechanisms that may lead to DHT accumulation in BPH it is necessary to briefly consider the pathways involved in DHT production and degradation.

1.3 ANDROGEN METABOLISM IN THE HUMAN PROSTATE

1.3.1 Overview of Androgen Metabolising Enzymes

The metabolic fate of testosterone entering the prostate has been extensively studied in vivo and in vitro by following the conversion of radioactive testosterone to other radioactive steroids. A diversity of procedures have demonstrated the formation of metabolites of testosterone in the prostate of experimental animals and in man. The key transformation in terms of controlling the growth of the prostate is the irreversible conversion of testosterone to DHT by 5 α -reductase (Figure 1.3).

Both in vitro and in vivo studies have identified other metabolites, in addition to DHT, formed in the prostate. These include 4-androstene-3,17-dione (androstenedione), 5 α -androstane-3 α ,17 β -diol (3 α -androstanediol), 5 α -androstane-3 β ,17 β -diol (3 β -androstanediol), 3 α -hydroxy-5 α -androstane-17-one (androsterone), 3 α -hydroxy-5 α -androstane-17-one (epiandrosterone) and 5 α -androstane-3,17-dione (androstanedione) indicating that other androgen metabolising enzymes are present in the prostate (Harper *et al.*, 1974; Becker *et al.*, 1975; Jacobi and Wilson, 1977; Bartsch *et al.*, 1987a, 1990; Trapp *et al.*, 1992).



Numbers refer to the following enzymes:

1: 5α-Reductase 2: 3α-Hydroxysteroid dehydrogenase 3: 3β-Hydroxysteroid dehydrogenase 4: 17β-Hydroxysteroid dehydrogenase

Androgen action in the prostate is regulated by the balance between 5α -reductase activity (androgen activation) and androgen inactivating enzymes. Enzymes which convert DHT to less potent metabolites include 3α -hydroxysteroid dehydrogenase, 3β -hydroxysteroid dehydrogenase ($3\alpha/\beta$ -HSD) and 17β -hydroxysteroid dehydrogenase (17β -HSD). The $3\alpha/\beta$ -HSD enzymes convert DHT to 3α -androstanediol and 3β -androstanediol (androstanediols), while 17β -HSD converts DHT to androstenedione. The major breakdown product of DHT in the prostate is 3α -androstanediol (Mahoudeau *et al.*, 1970; Becker *et al.*, 1975; Jacobi and Wilson, 1977; Geller^{*et al.*}, 1976). The reversible nature of $3\alpha/\beta$ -HSD raises the possibility of potentiating androgenic potency by back-conversion of androstanediols to DHT.

Testosterone and its reduced metabolites possess a 17β -hydroxyl group which may be oxidised by 17β -HSD to a ketone group. The corresponding 17β -oxidised metabolite of testosterone is androstenedione, a weaker androgen than either testosterone or DHT (Dorfman and Dorfman, 1963). Although the formation of androstenedione from testosterone is a minor pathway in the prostate the reverse of this reaction represents activation of androgenic potential. Potentially androstenedione, derived from dehydroepiandrosterone (DHEA) formed in large amounts in the adrenal glands, provides a non-testicular source of testosterone in the prostate which overall represents about 5% of the testosterone formed in the prostate (Lipsett, 1975).

In summary, testosterone enters the prostatic cell via passive diffusion and is irreversibly converted to DHT. The newly formed DHT can then be converted into a complex mixture of oxidised and reduced products. These transformations are catalysed by reversible enzymes which means that all of these metabolites are capable of being converted to DHT and are, therefore, potentially androgenic. Much of the knowledge of the pathways of androgen metabolism in the prostate has come from the identification of metabolites formed in the prostate after in

vivo administration of [^3H]-testosterone or from in vitro incubation of prostate with [^3H]-testosterone.

1.3.2 In Vivo Study of Androgen Metabolism in the Human Prostate

The early studies followed the distribution and accumulation of radioactivity in various tissues and organs after administration of radioactive androgens in the rat. It was found that the radioactivity was rapidly metabolised and removed from circulation and accumulated in androgen-target tissues such as the liver, prostate, kidney, adrenals and seminal vesicle (Pearlman and Pearlman, 1961; Wilson and Gloyna, 1970; Bruchovsky, 1971, Buric *et al.*, 1972). When [^3H]-testosterone was administered in vivo the radioactivity was found to accumulate in the prostate as DHT (Bruchovsky, 1971; Buric *et al.*, 1972). Within minutes of administration of [^3H]-testosterone to rats radioactivity was detected in the prostate as DHT, androsterone and androstanediols with the major metabolite 5α -reduced metabolite being DHT (Bruchovsky and Wilson, 1968a).

In human males it was also demonstrated that DHT is the major metabolite formed in the prostate after in vivo administration of [^3H]-testosterone prior to prostatectomy (Pike *et al.*, 1970; Harper *et al.*, 1974; Becker *et al.*, 1975). Despite the directness of the in vivo approach the possibility exists that radioactive steroids extracted from the prostate may have been produced elsewhere (for example the liver). The prostate tissue-specific formation of [^3H]-DHT was established by the extraction of DHT from prostatic nuclei in hepatectomised rats after administration of [^3H]-testosterone in vivo (Bruchovsky and Wilson, 1968a), a finding independently confirmed in vitro by Baulieu *et al.* (1968) and Anderson and Liao (1968).

1.3.3 In Vitro Models for the Study of Prostate Androgen Metabolism

1.3.3.i Tissue Slices, Minces and Organ Culture Studies

Short-term incubation of prostate tissue with [^3H]-testosterone and analysis of metabolites has provided important information concerning androgen metabolism in the prostate. These studies can be carried out using physiological substrate concentrations and allows kinetic analyses to be made. Using such an approach it was shown that slices and minces of human BPH tissue incubated with [^3H]-testosterone formed mainly DHT by the NADPH-dependent enzyme 5α -reductase (Farnsworth *et al.*, 1963; Shimazaki *et al.*, 1965a; McMahon *et al.*, 1974).

The study of DHT formation in slices of BPH tissue demonstrated that although DHT concentration in BPH tissue is higher than normal tissue, there is no difference in the rate of DHT formation in BPH tissue compared to normal tissue (Siiteri and Wilson, 1970). Homogenates of human prostate tissue used to study the kinetic parameters of 5α -reductase have shown that DHT forming activity of the prostate is greatest at pH 5.0-5.5 (Liang *et al.*, 1985; Andersson *et al.*, 1991; Jenkins *et al.*, 1992). One contentious aspect to these experiments is the addition of NADPH. The enzyme 5α -reductase has an absolute requirement for NADPH (Shimazaki *et al.*, 1965a) and NADPH is an essential additive when the tissue is disrupted, but concentrations of NADPH exceeding 1mM inhibit 5α -reductase activity in separated BPH epithelium and stroma (Bruchovsky *et al.*, 1981). In addition to promoting 5α -reductase activity, added NADH and NADPH will also enhance other reductive pathways in the prostate and therefore the addition of cofactors need careful consideration. This is important in implicating changes in enzyme activity as factors in the pathogenesis of BPH.

Organ culture studies have the advantage of preserving some of the structural integrity of the tissue permitting cell-cell interactions to occur. The tissue is

maintained on a support and metabolites are released into the medium. The tissue is intact and addition of NADPH does not alter the profile of metabolites (Smith *et al.*, 1983). Using this approach it was confirmed that DHT was the principle metabolite of testosterone (Baulieu *et al.*, 1968) and that DHT was the most effective androgen at maintaining the height of epithelial cells in organ culture (Robel *et al.*, 1971).

To overcome the fact that in vitro experiments are not in a dynamic relationship with the radioactive steroids as in vivo, superfusion experiments have been developed. In this procedure tissue explants are maintained in a continuously replenished medium containing a constant amount of radioactive precursor approaching the physiological levels in blood. These studies allow the determination of the extent to which a steroid is released into or removed from the external medium as well (Malathi and Gurpide, 1977). Using this technique it was demonstrated that most of the radioactivity leaves the tissue unmetabolised when the tissue is superfused with [³H]-DHT (Malathi and Gurpide, 1977), while superfusion with [³H]-testosterone results in the formation of three-fold higher levels of [³H]-DHT in the tissue than in the collecting medium (Giorgi *et al.*, 1971).

1.3.3.ii Isolated Prostate Cell Studies

Separation of the main prostatic cell types (epithelial and stromal) offers the potential to study the androgen metabolising properties of the main components of the prostate in isolation. Separation of epithelium and stroma can be achieved by applying pressure to minced tissue held between two glass slides (Franks *et al.*, 1970). Stromal elements are retained by filtration through gauze (150 μ m) (Bruchovsky *et al.*, 1981). Cowan *et al.* (1977) were first to use this approach to demonstrate that 5 α -reductase activity is greater in the stroma than epithelium. This finding has since been supported by several others (Romijn *et al.*, 1980;

Wilkin *et al.*, 1980; Krieg *et al.*, 1981; Bartsch *et al.*, 1982; Rennie *et al.*, 1983; Voigt and Bartsch, 1986; Hudson, 1987; Bruchovsky *et al.*, 1981, 1988; Tunn *et al.*, 1988). Kinetic analyses of stromal and epithelial 5 α -reductase activities led to the speculation, without further substantiation, that these activities represented different isozymes (Bruchovsky *et al.*, 1981, 1988; Rennie *et al.*, 1983; Hudson, 1987). The activity of 17 β -HSD and 3 α/β -HSD is reported to be concentrated in the epithelium (Krieg *et al.*, 1983; Tunn *et al.*, 1987; Bartsch *et al.*, 1987b) although Romijn *et al.* (1980) reported that 17 β -HSD activity was higher in stromal cells.

1.3.3.iii Primary Cultures of Prostate Cells

Despite the large number of studies of androgen metabolism in separated stroma and epithelium there are few studies reporting metabolism in cultured stroma and epithelium. Mechanically separated prostate epithelium was found to be non-viable (Franks *et al.*, 1970) and so other methods for growing isolated prostate cell types were developed.

Fibroblasts, a readily obtainable stromal cell type, can be grown from explants of prostate tissue and maintained for several months. It was shown that fibroblasts cultured from explants of genital and non-genital skin metabolised testosterone to DHT (Wilson, 1975). Greatest activity was found at pH 5.5 and was limited to genital skin, while a lower activity at pH 7-9 was present in all skin types. The activity at pH 5.5 was found to be absent in 5 α -reductase deficient male pseudohermaphrodites (Moore and Wilson, 1976). In contrast, primary cultures of fibroblasts from BPH tissue rapidly metabolised testosterone, but the predominant metabolites were androstenedione, androsterone and androstanedione (Schweikert *et al.*, 1982). Similar results were found in separated stromal and epithelial cultures produced after enzymic (collagenase) digestion of the tissue (Ofner *et al.*, 1984). The different profile of metabolites in separated

stroma and epithelium compared to whole tissue may indicate the importance of interactions between the cells in modulating androgen metabolism.

1.3.4 Concentrations of Androgens in Prostate Tissue

1.3.4.i BPH Tissue

Following the report by Siiteri and Wilson (1970) that the concentration of DHT was higher in hyperplastic prostate (6 ng/g tissue or 20nM) than in normal prostates (1.3 ng/g tissue or 4.5nM) (Table 1.1), investigators sought to implicate changes in the levels of testosterone and DHT and in 5 α -reductase activity to the pathogenesis of BPH. Concentrations of androgens were measured in plasma and prostate tissue of normal and hyperplastic prostates in an attempt to find a link between DHT and BPH.

The finding of elevated DHT in BPH patients was confirmed by several other groups (Albert *et al.*, 1976; Geller *et al.*, 1976; Habib *et al.*, 1976; Hammond, 1978, Meikle *et al.*, 1978; Belis, 1980, Krieg *et al.*, 1979) (see Table 1.2) and it seemed logical to assume that BPH was triggered by the accumulation of DHT in the prostate. However, in 1983, Walsh *et al.* claimed that the low DHT in normal prostate, usually obtained from cadavers, was an artifact due to postmortem cooling of the bodies. In Walsh's study normal tissue obtained at surgery was found to contain similar DHT levels to those in BPH tissue (Table 1.1). Bruchovsky *et al.* (1988) also found a lower level of DHT in prostate tissue from cadavers compared to tissue taken at surgery. However, the normal tissue was from uninvolved regions of malignant prostate from only three patients (aged over 65 years) which raises some doubt as to how representative of BPH tissue these "normal" samples really were.

TABLE 1.1

**CONCENTRATIONS (ng/g Tissue) OF DHT IN SAMPLES TAKEN FROM
NORMAL AND HYPERPLASTIC PROSTATE TISSUE**

Normal (ng/g Tissue)	BPH (ng/g Tissue)	Source of Normal Tissue	Reference
1.3 ± 0.5	6.0 ± 3.0	Autopsy	Siiteri & Wilson, 1970
2.1 ± 0.3	5.6 ± 0.9	Autopsy	Geller <i>et al.</i> , 1976
1.3 ± 0.6	4.0 ± 1.9	Autopsy	Meikle <i>et al.</i> , 1978
1.3 ± 0.3	5.5 ± 0.5	Autopsy	Hammond, 1978
1.6 ± 1.0	4.5 ± 1.4	Autopsy	Krieg <i>et al.</i> , 1979
3.6 ± 0.4	5.7 ± 0.5	Surgery	Belis <i>et al.</i> , 1980
0.7 ± 0.1	1.0 ± 0.2	Autopsy	Walsh <i>et al.</i> , 1983
5.1 ± 0.4	5.0 ± 0.4	Surgery	Walsh <i>et al.</i> , 1983
1.6 ± 0.4	not reported	Autopsy	Bruchovsky <i>et al.</i> , 1988
3.0 ± 0.9	3.6 ± 0.4	Surgery	Bruchovsky <i>et al.</i> , 1988

The Table summarises the concentrations of DHT (in ng/g wet weight of tissue) reported for normal and hyperplastic tissues taken at autopsy and during surgery.

The effects of postmortem body cooling on prostate tissue DHT concentrations were studied by Bolton *et al.* (1986) who found that cooling BPH specimens after surgical removal did not lead to a decrease in DHT. In contrast to Walsh and Hutchins' findings, Belis *et al.* (1980) reported an elevation of DHT in BPH tissues (5.7ng/g tissue) removed at surgery compared to normal tissue (3.6ng/g tissue) removed at surgery. In conclusion, most of the evidence in the literature supports the hypothesis that DHT levels are higher in BPH tissue adding weight to the theory that DHT is implicated in the development of BPH.

1.3.4.ii Prostate Cancer Tissue

The concept that prostate cancer is hormone dependent was first established in 1941 when Huggins and Hodges demonstrated the suppressive effects on the growth of prostate tumours of removing androgens by surgical castration. This understanding that depriving cancer cells of androgens could significantly alter the course of the disease set the gold standard for treatment of prostate cancer and the search for the role of androgens in the pathogenesis of the disease.

Elevated testosterone levels in prostate cancer compared to normal or BPH tissue has been reported by several groups (Farnsworth and Brown, 1976; Krieg *et al.*, 1979; Habib *et al.*, 1976; Hammond, 1978; Ghanadian and Puah, 1981; Voigt and Bartsch, 1986) except for one isolated report that testosterone was higher in BPH tissue (Albert *et al.*, 1976). The increase of testosterone in the prostate tissue occurs in the presence of normal levels of plasma testosterone (Habib *et al.*, 1976; Harper *et al.*, 1976; Saroff *et al.*, 1980; Ghanadian and Puah, 1981). This probably reflects the lower capacity of prostate cancer tissue to metabolise testosterone.

Conflicting data exists as to whether the concentration of DHT is lower in prostate cancer than BPH tissue. Some investigators (Geller *et al.*, 1978a; Habib *et al.*, 1976; Hammond, 1978; Ghanadian and Puah, 1981) found that it was lower,

while Farnsworth and Brown (1976) found it to be higher, with Krieg *et al.* (1979) finding no difference. These contradictory reports may relate to the degree of differentiation of the cancer (Geller *et al.*, 1978a, 1978b, 1979; Belis, 1980). A significant decrease in DHT was shown to relate to loss of differentiation with a tissue DHT concentration of greater than 1.8 ng/g tissue corresponding to a differentiated tumour (Geller *et al.*, 1978b, 1979).

1.3.5 Androgen Metabolism in BPH Tissue

Ultimately, the control of prostatic DHT levels is determined by the relationship between the rate of production and removal. An alteration in enzyme activity might be responsible for the accumulation of DHT in BPH tissue. This might be a change in the activity of the enzyme that forms DHT or in the activity of enzyme(s) which are involved in the catabolism of DHT.

The simplest explanation for elevated DHT levels in BPH tissue is an increase in 5 α -reductase activity. The finding of elevated 5 α -reductase activity in BPH tissue by Farnsworth and Brown (1976) has also been confirmed by other groups of investigators (Djöseland, 1977; Krieg *et al.*, 1979; Wilkin *et al.*, 1980; Isaacs *et al.*, 1983). Bruchovsky and Lieskovsky (1979) compared homogenates of 21 BPH prostate to 8 normal prostates and found formation of 5 α -reduced metabolites to be significantly higher in BPH tissue than normal. Although Siiteri and Wilson (1970) were first to report an elevation of DHT in BPH tissue compared to normal tissue, they failed to find any difference in the rate of DHT formation in the two types of tissue. This finding was supported by Prout ^{*et al.*} (1976), and Morfin *et al.* (1979). Similarly, Morimoto *et al.* (1980) also failed to show a difference in the in vivo rate of conversion of testosterone to DHT in young men compared to elderly men, although there was an accumulation of DHT in BPH tissue. In summary, the case for increased 5 α -reductase activity in the BPH prostate still remains to be proven.

Although 5α -reductase activity is reported to be concentrated in the stroma of BPH tissue (Cowan *et al.*, 1979; Romijn *et al.*, 1980; Krieg *et al.*, 1981; Bartsch *et al.*, 1982; Rennie *et al.*, 1983; Voigt & Bartsch, 1986; Bruchovsky *et al.*, 1981, 1988), there is no difference in the DHT-forming index of BPH stroma compared to normal stroma, while normal epithelial cells actually have a higher DHT-forming index than BPH epithelial cells (Tunn *et al.*, 1988). Several studies have shown that 5α -reductase activity in BPH stromal cells has a higher K_m and V_{max} than normal stromal cells (Rennie *et al.*, 1983; Bruchovsky *et al.*, 1988; Tunn *et al.*, 1988) indicating that the greater amount of 5α -reductase activity in the stroma compensates for its lower affinity for testosterone.

The other simple explanation for DHT accumulation is a decrease in the rate of its degradation. If BPH tissue does not form DHT more efficiently than normal tissue then perhaps it is less efficient at removing it. The formation of 3α -androstenediol is the major reduction product of DHT (Mahoudeau *et al.*, 1970; Becker *et al.*, 1975; Jacobi and Wilson, 1977). Decreased reduction of DHT to androstenediols has been proposed as a mechanism of DHT accumulation in BPH and is supported by the findings of higher levels of 3α -androstenediol in normal prostate tissues than in hyperplastic prostates (Geller *et al.*, 1976; Meikle *et al.*, 1978; Hammond, 1978, Krieg *et al.*, 1979).

Results of studies comparing the activity of $3\alpha/\beta$ -HSD in normal and hyperplastic prostates appear at first sight to be conflicting. Bruchovsky and Lieskovsky (1979) found lower activity of $3\alpha/\beta$ -HSD in tissue homogenates from BPH prostates (21 samples) than that in tissue from 8 normal prostates. An *in vivo* study in man showed that conversion of DHT to 3α -androstenediol was significantly reduced in elderly men with BPH (62-77yrs) compared to young men (21-49yrs) (Morimoto *et al.*, 1980). Becker *et al.* (1975) reported that 3α -androstenediol was converted rapidly to DHT in the prostate and Meikle *et al.*, (1978) reported that the equilibrium of 3α -HSD favoured the formation of DHT. In contrast to these

three studies, Morfin *et al.* (1978, 1979) found no significant difference in the rate of androstenediol formation between minces and slices of BPH tissue and normal prostate tissue minces and slices, while Jacobi and Wilson (1977) found that formation of androstenediols was significantly higher in microsomes and cytosol from human BPH tissue (17 prostates) compared to that obtained from normal prostate (23 prostates).

The differences in these findings by different investigators might relate to reaction conditions and the availability of cofactors to the enzymes. The enzyme $3\alpha/\beta$ -HSD can act as a reductase (converting DHT to androstenediols) and as a dehydrogenase (converting androstenediols to DHT). Experiments carried out in the presence of NADPH (Jacobi and Wilson, 1977; Morfin *et al.*, 1978) favour the reductase activity of the enzyme. The importance of co-factor levels in determining the direction of reaction has been emphasised by Lombardo *et al.* (1992). This group demonstrated that conversion of androstenediols into DHT was completely blocked by the addition of 1mM NADPH to BPH tissue minces. The rate of reduction of DHT to androstenediols could be increased several fold by the presence of NADPH in BPH tissue incubations (Lombardo *et al.*, 1992). Similar experiments by Krieg *et al.* (1979) showed that conversion of DHT to androstenediols was almost completely inhibited in the absence of NADPH. These studies indicate that the concentration of DHT depends on the level of cofactors in the tissue. An increased back-transformation of androstenediols to DHT rather than a decreased formation of androstenediols might explain DHT accumulation. Malathi and Gurpide (1977) found that DHT formation from 3α -androstenediol was favoured in slices of hyperplastic tissue, supporting this idea. However, other investigators failed to show any difference in the conversion of 3α -androstenediol into DHT by normal and hyperplastic prostates (Bruchovsky and Lieskovsky, 1979).

In general, the evidence appears to favour an elevation of DHT in BPH tissue although the mechanism responsible is not well understood. While androgens are implicated in the pathogenesis of BPH it is unlikely that they alone are enough to cause BPH. Other mechanisms involving interactions with oestrogens, and cell-cell interactions may also be implicated.

1.3.6 Androgen Metabolism in Prostate Cancer Tissue

Qualitatively, all the pathways present in normal and hyperplastic tissue are present in prostate cancer tissue (Klein *et al.*, 1988), quantitatively, prostate cancer tissue has a lower metabolic capacity. In particular, 5 α -reductase activity was found to be lower in prostate cancer compared to normal or BPH tissue (Shimazaki *et al.*, 1965a; Bard and Lasnitzki, 1977; Habib *et al.*, 1985; Geller *et al.*, 1978a; Prout *et al.*, 1976; Morfin *et al.*, 1979; Bruchovsky and Lieskovsky, 1979, Hudson *et al.*, 1983; Klein *et al.*, 1988; Bruchovsky *et al.*, 1988). Prout *et al.* (1976) proposes that the reduced ability of prostate cancer tissue to convert testosterone to DHT relates to the loss or repression of 5 α -reductase activity.

Attempts to relate 5 α -reductase activity to tumour differentiation have demonstrated that loss of tumour differentiation is accompanied by lower enzyme activity (Habib *et al.*, 1985, 1989). Geller *et al.* (1978b, 1979) found that DHT concentration actually correlated better with tumour differentiation than 5 α -reductase activity. The loss of 5 α -reductase activity that occurs with loss of tumour differentiation is sometimes accompanied by an increase in the production of 17-oxo metabolites (androstenedione, androstanedione, androsterone and epiandrosterone) (Morfin *et al.*, 1977, 1979; Bard and Lasnitzki, 1977). According to Morfin *et al.* (1979) 5 α -reduction of testosterone in the prostate is related to the androgen-dependent differentiated structures found in normal, BPH and well differentiated tumours.

It is reported that 5 α -reductase in prostate cancer tissue is less efficient at converting testosterone to DHT (Hudson *et al.*, 1983), explaining why DHT levels are lower in prostate cancer tissues than BPH. Later the same group (Hudson and Wherrett, 1990) showed that the activity of the stromal enzyme from prostate cancer was ten-fold lower than that from BPH, but there was no difference in the activity of epithelial enzymes. Stromal 5 α -reductase activity in prostate cancer was also lower than from BPH tissue (Romijn *et al.*, 1980). Since 5 α -reductase activity is concentrated in the stromal component of the prostate the higher 5 α -reductase activity observed in hyperplastic tissue may relate to the higher proportion of stromal tissue in this disease (Lakey *et al.*, 1979).

In summary, the studies of the pathways of androgen metabolism in the prostate clearly support the contention that the prostate enzymes are directed towards the production and accumulation of the potent androgen DHT. In this context the enzyme 5 α -reductase is of prime importance.

1.4 STEROID 5 α -REDUCTASE ENZYMES

Following the demonstration that the prostate contained enzymes capable of reducing the C = C bond in the A-ring of testosterone (Pearlman and Pearlman, 1961) it was shown that the DHT was formed in rat prostate by the action of an NADPH-dependent enzyme called 5 α -reductase (Farnsworth *et al.*, 1963). It is now established that two forms of 5 α -reductase exist in the human and rat prostate (Andersson and Russell, 1990; Andersson *et al.*, 1991). Conversion of testosterone to DHT by 5 α -reductase is non-reversible and produces the most potent androgen and for this reason represents the most important pathway of androgen metabolism in the prostate.

1.4.1 Mechanism of 5 α -reductase

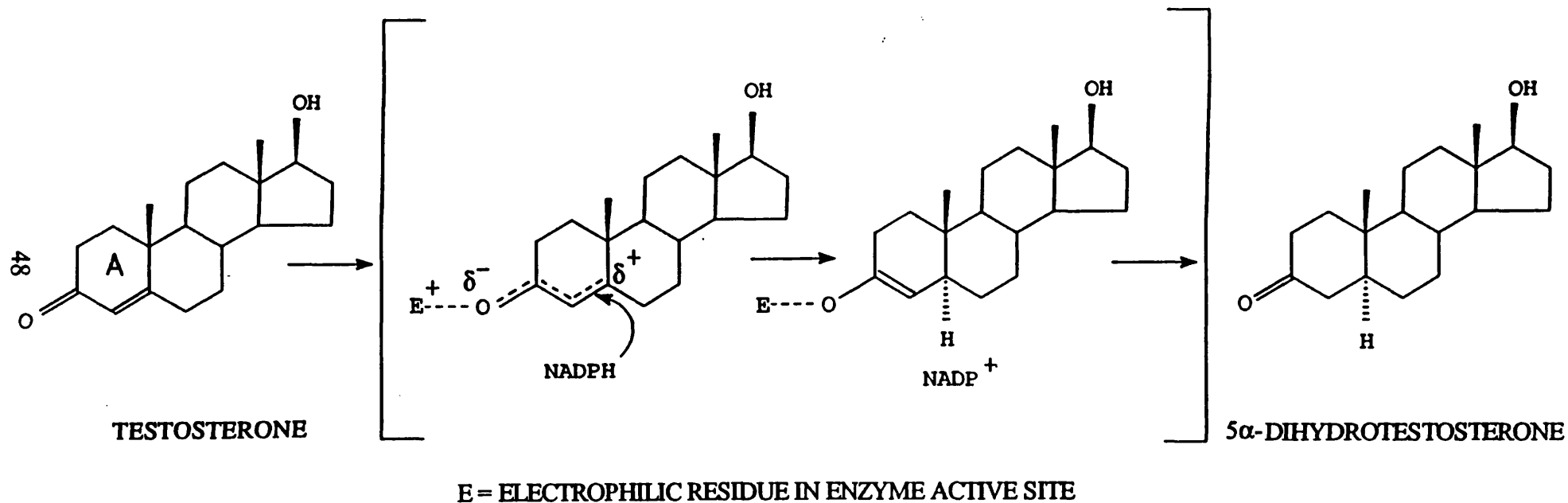
5 α -Reductase isozymes have an absolute dependence on NADPH for its activity (Shimazaki *et al.*, 1965b). Generally, reducing equivalents of NADPH are employed for enzymic hydroxylation or dehydrogenase activities. 5 α -Reductase utilises the NADPH in a unique way as a hydrogen donor for the direct reduction of the C=C bond in the A ring of testosterone (King and Mainwaring, 1974) (Figure 1.4). Kinetic analysis suggests that NADPH binds to the enzyme before testosterone forming an enzyme-NADPH complex. This complex has high affinity for testosterone which binds forming an enzyme-NADPH-testosterone complex. Reduction of the double bond in the A ring of testosterone is followed by the release of DHT leaving an enzyme-NADP⁺ complex. After DHT release NADP⁺ dissociates from the enzyme (Houston *et al.*, 1987; Brandt *et al.*, 1990).

1.4.2 Regulation of 5 α -Reductase Expression

The expression of 5 α -reductase was shown to be under the control of androgens (Moore and Wilson, 1973; Gustafsson *et al.*, 1983) and in particular DHT (Andersson *et al.*, 1989; George *et al.*, 1991, Normington and Russell, 1992). Both testosterone and DHT are able to restore prostate regrowth and induce 5 α -reductase activity in castrated rats (Moore and Wilson, 1973). The observation that testosterone failed to increase 5 α -reductase activity in castrated rats treated with the 5 α -reductase inhibitor, finasteride, indicated that DHT, not testosterone, increased the expression of 5 α -reductase mRNA (George *et al.*, 1991).

FIGURE 1.4

CHEMICAL MECHANISM OF 5 α -REDUCTASE



In the scheme shown above NADPH binds to the enzyme forming an enzyme-NADPH complex with a high affinity for testosterone. Reduction of the C=C bond in the A ring of testosterone is followed by the release of DHT. Adapted from Brandt *et al.* (1990).

The response of the 5 α -reductase genes to DHT represents a feed-forward regulation in which the product of the gene positively affects the expression of its gene (George *et al.*, 1991). In contrast to end-product regulation, where increasing concentration of the product serves to inhibit the enzyme responsible for its production, feed-forward regulation serves to maintain synthesis of product even when its concentration is high. This suggests that the high concentration of DHT in prostate tissue favours 5 α -reductase expression, which in turn will maintain elevated DHT levels in the prostate.

1.4.3 5 α -Reductase Isozymes

Recently, convincing evidence has been provided to support the existence of more than a single form of 5 α -reductase enzyme in the prostate. This has come from the identification of cDNAs encoding two human 5 α -reductase isozymes, designated type 1 and type 2 in the order that they were cloned (Andersson and Russell, 1990, Andersson *et al.*, 1991). The close association of 5 α -reductases to the nuclear envelope and membranes of the endoplasmic reticulum has proved to be a problem in their isolation and purification (Houston *et al.*, 1985a, 1985b; Enderle-Schmitt *et al.*, 1989). Partial purification of 5 α -reductase from prostate was reported but the enzyme preparation was inactive at pH 5.5, the pH at which the major prostatic 5 α -reductase has greatest activity, indicating that perhaps the activity of the enzyme is dependent on the close membrane-association (Sargent and Habib, 1991). However, it is now possible to study the separate isozymes by expression of the cDNA sequences in suitable host cells that do not normally express 5 α -reductase such as human embryonic-kidney 293 cells and simian COS cells (Andersson and Russell, 1990; Andersson *et al.*, 1991). COS cells have low 5 α -reductase activity, however, this is negligible compared with the high levels of activity produced after the expression of human enzymes by transfection with the appropriate cDNAs.

Such studies have revealed that the two 5 α -reductase genes are 46% identical in sequence and share almost identical hydropathy profiles (Andersson *et al.*, 1991), but they can be distinguished by their different kinetics, pH optima, sensitivity to inhibition by finasteride and expression levels within the prostate (see Table 1.3).

The gene for 5 α -reductase 1 is located on chromosome 5 (Jenkins *et al.*, 1991), and the enzyme is optimally active over the pH range 6.0-8.0 (Andersson *et al.*, 1991) and is insensitive to finasteride (Andersson *et al.*, 1991). The gene for 5 α -reductase 2 is located on chromosome 2 (Thigpen *et al.*, 1992a), the enzyme is optimally active at an acidic pH of 5.0 and is 50-100 fold more sensitive to finasteride than the type 1 enzyme (Andersson *et al.*, 1991).

1.4.4 The Distribution of 5 α -Reductase in Different Organs

The prostate is not unique in having 5 α -reductase activity and its presence in the prostate was reported after the initial discovery in liver (Schneider, 1952). Since then 5 α -reductase activity has been found in kidney, skin, brain, adrenals, liver; seminal vesicles and other sexual organs (McGuire *et al.*, 1960; Gomez and Hsia, 1968; Wilson and Gloyna, 1970; Mainwaring and Mangan, 1973; Schweikert and Wilson, 1974; Lephart *et al.*, 1991; Harris *et al.*, 1992; Normington and Russell, 1992). In particular, the prostate, liver and brain have high levels of 5 α -reductase.

TABLE 1.2

COMPARISON OF THE PROPERTIES OF HUMAN PROSTATE
5 α -REDUCTASE ISOZYMES

Property	Type 1	Type 2
Chromosome Location	Chromosome 5	Chromosome 2
Optimum pH	pH 6.0 - 8.0	pH 5.0 - 5.5
Sensitivity to Finasteride	Low: 300 - 600nM	High: 4 - 10nM
Sensitivity to SKF 105,657	0.8 - 7.5 μ M	4.2 - 10nM**
K _m Testosterone	1 - 3 μ M	0.5 - 1.0 μ M
Genetic analysis of MPH*	Normal gene	Mutated
Distribution	Prostate minor form Adrenals ? Liver (rat) - human ? Brain (rat) - human ? Scalp Skin	Major prostatic form. Adrenals ? Liver ? Brain ? Hair follicles Genital Skin

The Table was constructed by summarising the information presented in Section 1.4.3. *MPH = male pseudohermaphrodites ** = prostate homogenates

1.4.4.i 5 α -Reductase in the Prostate

The enzyme present in homogenates of human prostate tissue was found to have a narrow pH optimum centred around pH 5 (Jenkins *et al.*, 1992) and was 30-60 fold more sensitive to finasteride than the 5 α -reductase 1 expressed in 293 cells (Jenkins *et al.*, 1992). These observations support the interpretation that the 5 α -reductase activity of human prostate is predominantly 5 α -reductase 2. Southern blot analysis of DNA from some male pseudohermaphrodites showed that they lacked the 5 α -reductase 2 gene (Andersson *et al.*, 1991), although the gene for 5 α -reductase 1 was present and normal. Mutations in the 5 α -reductase 2 gene appear to underlie the deficiency in 5 α -reductase in male pseudohermaphrodites (Thigpen *et al.*, 1992a).

The mRNA for 5 α -reductase 1 has been identified in human prostate (Andersson and Russell, 1990) and the existence of the active protein reported in microsomes from human prostate (Faller *et al.*, 1993). In the rat ventral prostate 5 α -reductase 1 accounts for approximately 50% of the DHT forming activity (Normington and Russell, 1992). In human prostate this is not the case as indicated by the low amount of DHT formed in 5 α -reductase 2 deficient pseudohermaphrodites.

Several studies have investigated the distribution of 5 α -reductase between the epithelial and stromal components of human prostate. The study of enzyme activity in isolated stroma and epithelium showed that 5 α -reductase activity was localised predominantly in the stroma and had a higher affinity for testosterone than the epithelial enzyme (Cowan *et al.*, 1979; Romijn *et al.*, 1980; Wilkin *et al.*, 1980; Krieg *et al.*, 1981; Bartsch *et al.*, 1982; Rennie *et al.*, 1983; Voigt and Bartsch., 1986; Hudson, 1987; Bruchovsky *et al.*, 1981, 1988). As yet, there is no data published to support the interpretation that the 5 α -reductase activities in

stroma and epithelium correspond to the different 5 α -reductase isozymes identified by Andersson *et al.* (1991).

1.4.4.ii 5 α -Reductase in the Liver

There is a marked difference in the physiological importance of the reductive processes in the liver and prostate. In the liver the process is a part of a general mechanism of detoxification and for lowering the biological activity of steroid hormones. Incubation of [³H]-testosterone and NADPH with slices of liver formed polar products; DHT or androstanediol could not be detected (Wilson and Gloyna, 1970). No specific binding of DHT in liver nuclei has been demonstrated (Tomkins, 1957; Anderson and Liao, 1968; Mainwaring, 1969). In contrast, in the prostate it is a means of amplifying the biological potency of testosterone. The specific activity of 5 α -reductase is higher in male accessory organs than in other tissues, including the liver (Gloyna and Wilson, 1969). Furthermore, the synthesis of 5 α -reductase in the prostate is controlled by DHT (George *et al.*, 1991) while there is no effect on liver 5 α -reductase (Bullock *et al.*, 1971).

The view that the formation of DHT in the prostate is an integral part of the mechanism of androgenic action is strengthened by the high degree of nuclear binding of DHT in the prostate (Bruchovsky, 1971). In contrast the liver does not contain androgen receptors (Thomkins, 1957). Analysis of rat liver 5 α -reductase has shown that it is identical to the cloned rat 5 α -reductase 1 isozyme (Anderson *et al.*, 1989).

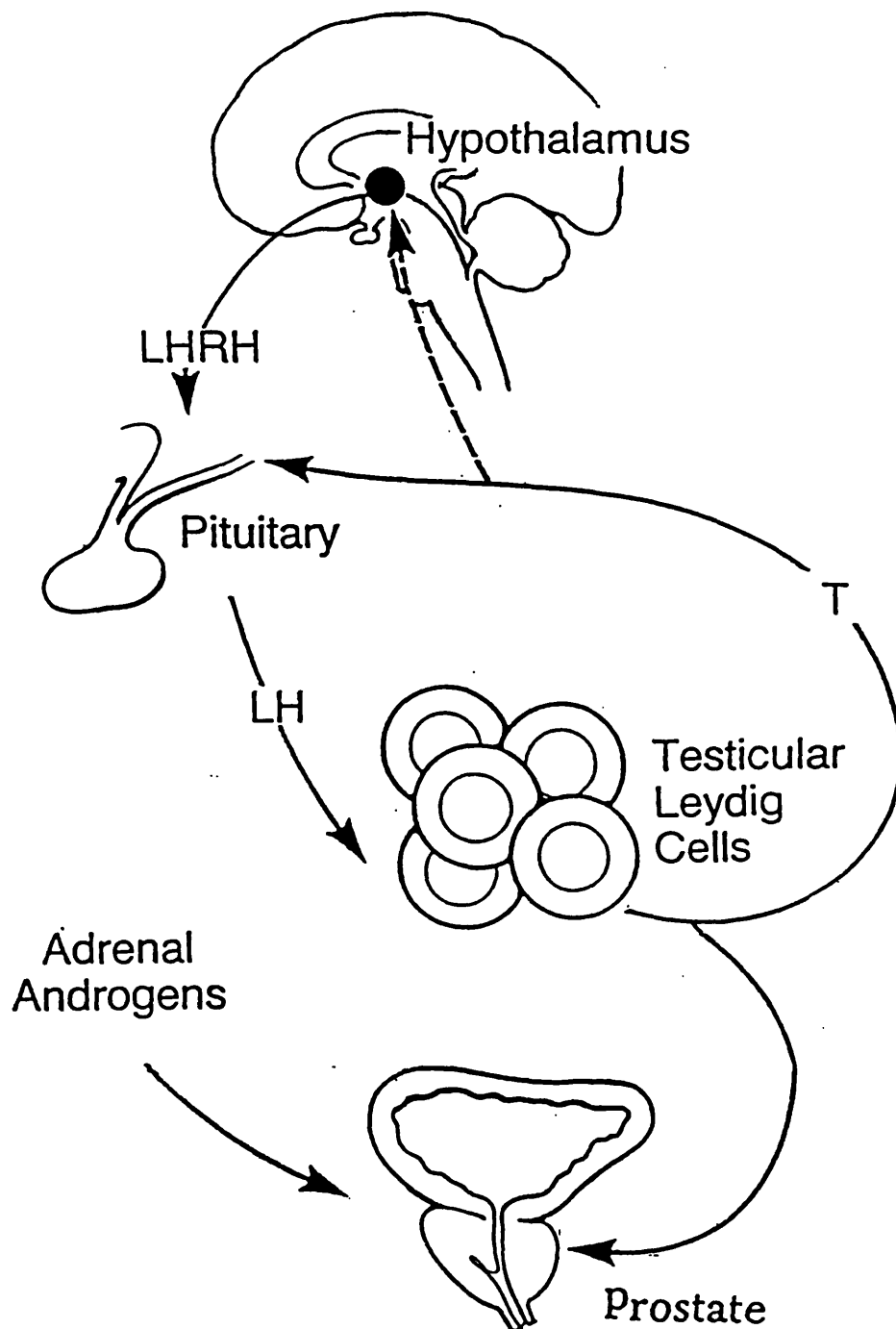
1.4.4.iii 5 α -Reductase in the Brain

5 α -Reductase is found in many areas of the brain. The anterior pituitary, in particular, metabolises testosterone to DHT and 3 α -androstenediol in high amounts. To understand the role of 5 α -reductase in the brain it necessary to briefly consider the mechanism controlling the biosynthesis of testosterone. The production of testosterone by the Leydig cells of the testes is controlled by luteinizing-hormone (LH) released by the pituitary (Figure 1.5). The release of LH is controlled by luteinizing-hormone releasing hormone (LH-RH) or gonadotropin-releasing-hormone (GnRH) produced by the hypothalamus. A negative feed-back mechanism involving androgens and oestradiol inhibits the production of LH-RH and LH (Stewart-Bentley, 1974; Marynick *et al.*, 1979; Schekter *et al.*, 1989; Zoppi *et al.*, 1988).

The role of 5 α -reductase in the regulation of testosterone biosynthesis has been studied in several animals including man and there is evidence to support the interpretation that DHT is more effective than testosterone in inhibiting LH release from the pituitary (Zanisi *et al.*, 1973; Martini, 1982; Kuhn *et al.*, 1984; Liang *et al.*, 1984). Serum LH levels are elevated in male pseudohermaphrodites (Price *et al.*, 1984; Imperato-McGinley ~~et al.~~, 1991) implying that DHT plays a role in the negative feedback mechanism that inhibits the production of luteinizing hormone (LH) by the anterior pituitary gland. However, the fact that LH levels in male pseudohermaphrodites are not as elevated as in castrated men and administration of finasteride to men has no effect on serum LH levels indicates that testosterone may also be directly involved in inhibiting LH production (Vermuelen *et al.*, 1991; Gormley *et al.*, 1992; Rittmaster *et al.*, 1992). The exact function of 5 α -reductase in the brain is not clear but its presence in the pituitary suggests that it may be involved in regulating the production of its major substrate testosterone. 5 α -Reduced metabolites of testosterone could have a psychosexual role.

FIGURE 1.5

CONTROL OF ANDROGEN BIOSYNTHESIS



Production of testosterone by the testicular leydig cells is controlled by the pituitary release of luteinizing hormone (LH). The release of LH is controlled by LH releasing-hormone (LH-RH) produced in the hypothalamus. Androgens (and oestrogens) negatively feedback on the hypothalamus and pituitary to inhibit LH release. Adapted from McConnell, (1990).

1.4.4.iv 5 α -Reductase in the Skin

The conversion of testosterone to DHT has been shown to occur in all skin types tested including genital and non-genital skin (Wilson and Walker, 1969; Mellin *et al.*, 1993). Production of DHT by the skin may represent an important source of non-prostate derived DHT.

Generally, DHT formation is relatively low in most areas of skin but is highest in areas of androgen-mediated growth (Price, 1975). Steroid 5 α -reductase is found primarily in the apocrine and sebaceous glands with lower levels present in the hair follicles and dermis (Fazekas and Lanthier, 1971; Hay and Hodgins, 1978; Takayasu *et al.*, 1980). Excessive 5 α -reductase activity and DHT production have been linked to androgen-dependent disorders such as acne, female hirsutism and male pattern baldness (Hay and Hodgkins, 1974; Kuttenn *et al.*, 1977; Schweikert and Wilson, 1974; Dijkstra *et al.*, 1987). There is evidence to support the interpretation that the enzyme in the human scalp resembles 5 α -reductase 1 and the enzyme in the dermal papilla cells of the human beard resemble 5 α -reductase 2 (Harris *et al.*, 1992; Mellin *et al.*, 1993; Itami *et al.*, 1991). Interestingly, 5 α -reductase deficient males do not display pattern baldness and have scant beard growth (Imperato-McGinley *et al.*, 1974). The scalp enzyme (5 α -reductase 1) in these 5 α -reductase deficient males may be the source of circulating DHT.

Sebaceous glands are rich in 5 α -reductase activity and it has been assumed that DHT is the androgen responsible for sebum production and the development of acne (Dijkstra *et al.*, 1987; Pochi ~~*et al.*~~, 1988). However, the suggestion that 5 α -reductase 2 is involved in sebum production has been challenged by the finding that sebum production in male pseudohermaphrodites was no different from that in normal males. Furthermore, sebum production was not reduced in males treated with finasteride (Imperato-McGinley *et al.*, 1993).

Transurethral resection of the prostate (TURP) has replaced open prostatectomy as the surgical choice for relieving obstructive BPH. It has been estimated that the chance of a 40 year old man needing a TURP during his life time is as high as 29% (Glynn *et al.*, 1985), which makes this operation a very common procedure. The main considerations of TURP are the risk of death, risk of morbidity and risk of reoperation. In general TURP is considered a relatively safe surgical procedure with low mortality (<1%), however, there is significant morbidity (18%) and risk of reoperation with 1 in 10 to 1 in 5 men requiring repeat operation after 8 years (Mebust and Holtgrew^e 1989; Neal ~~*et al.*~~, 1990).

The complications of any form of prostatectomy include retrograde ejaculation, urinary tract infection, impotence and incontinence (Doll *et al.*, 1992). A long-term study of 84 patients after TURP over three years found that while 75% of patients improved, 18% had urge incontinence and 33% had impotence (Bruskewitz *et al.*, 1986). With reoperation there is more likelihood of morbidity.

Some retrospective studies suggest that TURP is associated with a higher long-term mortality than the open procedure with myocardial infarction as the leading cause of death (Roos *et al.*, 1989; Malenka *et al.*, 1990; Andersen *et al.*, 1990). However, the recent study by Concato *et al.* (1992) failed to find an increased risk of mortality due to TURP compared to the open procedure.

The advanced age and presence of other indications of most men undergoing TURP may make them a poor risk for surgery and anaesthesia. For these reasons effective medical treatment for BPH will provide an alternative to surgery and find a place in the treatment of the disease. Medical treatment for BPH is designed either to relieve the dynamic component, related to the tension of smooth muscle in the urethra, prostate and capsule, by blocking alpha adrenergic

stimulation or to reduce the mechanical or static component, related to the volume, consistency and shape of the prostate, by androgen ablation therapies.

1.5.1 Alpha-adrenoceptor Antagonists

Pharmacological agents that cause relaxation of prostatic smooth muscle may be effective for the treatment of BPH by reducing the resistance along the prostatic urethra. Normal prostate tissue has an estimated stromal to epithelial ratio of 2:1 (Bartsch *et al.*, 1979). In BPH tissue this ratio is increased to 5:1 (Barstch *et al.*, 1979; Shapiro *et al.*, 1992) and as stromal tissue contains smooth muscle (Siegel *et al.*, 1990) the treatment of BPH with alpha adrenergic antagonists has been shown in several efficacy trials to be an effective treatment. The pharmacology of the prostatic smooth muscle was first characterised by Caine *et al.* (1975) who also demonstrated that the prostatic adenoma and capsule contracts in the presence of the adrenergic agonist nor-adrenaline. Further characterisation has revealed that alpha₁-adrenergic receptors are abundant in the smooth muscle of the bladder base, the prostate and the prostate capsule but sparse in the bladder body (Hieble *et al.*, 1985; Shapiro and Lepor, 1986; Shapiro and Lepor, 1987; Lepor *et al.*, 1988). Alpha₁ adrenergic antagonists reduce bladder outlet resistance without affecting the bladder contractility.

The prevalence of histologic BPH does not correspond to the prevalence of clinical (symptomatic) BPH (Barry, 1990) neither does the size of the gland relate to the severity of symptoms (Bartsch *et al.*, 1979). However, a higher stromal to epithelial cell ratio does seem to be related to the development of symptomatic BPH (Shapiro *et al.*, 1992) suggesting that increased smooth muscle tone represents a significant contribution to symptomatic BPH. The first alpha-adrenergic blocking agent to be tested in BPH was phenoxybenzamine, a non-selective alpha adrenergic receptor antagonist (Caine *et al.*, 1978). Significant improvements in flow rates and irritative symptoms were reported but the high

frequency of side effects (dizziness and tiredness) and concern regarding possible carcinogenicity has limited widespread use of phenoxybenzamine in the treatment of BPH (Caine 1988).

The search for a selective α_1 blocking agent led to the introduction of prazosin for the treatment for BPH (Shapiro *et al.*, 1981). The negligible side-effects of prazosin make it well tolerated, however, it is less effective than phenoxybenzamine especially in reducing irritative symptoms (Hedlund and Andersson, 1988). Terazosin and doxazosin are a new α_1 antagonists currently being evaluated for treating BPH. The results of a recent phase three study involving 313 patients showed that terazosin significantly reduced symptoms and increased peak flow rate compared to the placebo group (Lepor and Laddu, 1992). Terazosin and doxazosin are well-tolerated and have the advantage of a once-a-day dosage requirement (Christensen *et al.*, 1993).

1.5.2 Androgen Ablation Therapies

Treatments that interfere either with normal androgen biosynthesis or androgen action have been designed to reduce the obstructive component of BPH. This is based on the assumption that androgens are required to maintain hyperplastic growth and that androgen withdrawal will result in regression of the tissue and an improvement in urine flow.

1.5.2.i Luteinizing-Hormone Releasing Hormone (LH-RH) Agonists

LH-RH agonists block LH release by the pituitary by desensitisation of the pituitary LH-RH receptor complex (McConnell, 1990). Treatment with LH-RH agonists produces a 90% reduction in prostatic DHT, a 75% reduction in prostatic testosterone and a decrease in 5α -reductase activity (Forti, *et al.*, 1989; Salerno *et al.*, 1988). Morphometric analysis of prostate tissue from patients treated with

nafarelin, a LH-RH agonist, demonstrated a 40% reduction in epithelial cell volume, and a 21% reduction in stromal cells (Peters and Walsh, 1987). LH-RH agonists are effective in reducing the size and weight of the prostate (Schröder *et al.*, 1986; Peters and Walsh, 1987; Keane ~~*et al.*~~, 1988) but this is not always accompanied by improvement in obstructive symptoms (Peters and Walsh, 1987; Keane *et al.*, 1988). Furthermore, treatment needs to be maintained in order to suppress prostatic size and all patients treated become impotent (Peters and Walsh, 1987) limiting the use of LH-RH agonists in treatment of BPH.

1.5.2.ii Androgen Receptor Antagonists (Antiandrogens)

The first antiandrogen to be tested in BPH was cyproterone acetate (Scott and Wade, 1969). Cyproterone acetate is a progestational antiandrogen (Sufrin and Coffey, 1973) able to inhibit canine prostatic hyperplasia (Tunn *et al.*, 1980). The drug inhibits the release of LH-RH from the pituitary in addition to its major action as an androgen-receptor antagonist (Sufrin and Coffey, 1973). Although cyproterone acetate was shown to have some effect in a small trial of 13 men with BPH (Scott and Wade, 1969), the side effects of impotence and decreased libido render it an undesirable therapy for BPH.

Flutamide is a non-steroidal pure antiandrogen, which after metabolism to a hydroxylated derivative, competes for the androgen receptor (Peets *et al.*, 1974; Sufrin and Coffey, 1976). Flutamide is devoid of progestational and antigonadotrophic activity and produces regression of canine hyperplasia (Neri and Monahan, 1972). In a study of 30 BPH patients Caine *et al.* (1975) reported improvement in urine flow rates, but no difference in residual urine, prostatic size or histological change compared to controls. Stone ~~*et al.*~~ (1989) reported significant increases in flow rate and significant decreases in prostatic volume in patients treated with flutamide. However, although no cases of impotence occurred over half the patients reported gynaecomastia and breast pain.

Treatment with flutamide has the potential to be a therapy for BPH if the side effects could be reduced. One concern, important in prostate cancer therapy, is that the increase in LH and plasma testosterone that results from blocking androgen receptors in the pituitary may overcome the androgen blockade reducing the effectiveness of flutamide. However, flutamide in combination with an LH-RH agonist produces total androgen ablation and is the gold standard for treating prostate cancer (Labrie *et al.*, 1990). This kind of approach might prove to be an effective therapy for BPH in poor surgical risk patients.

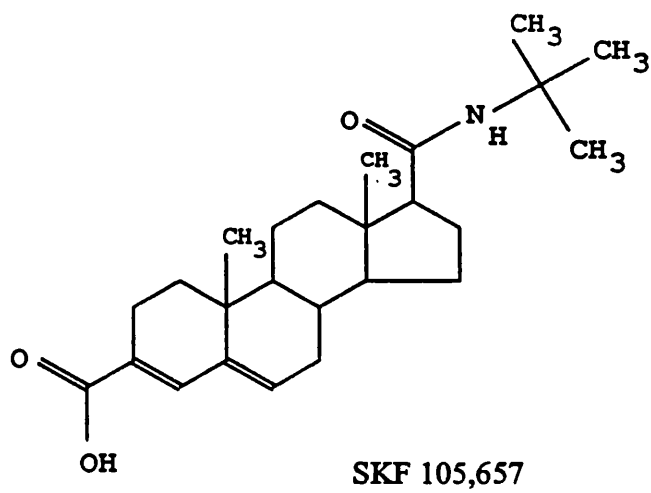
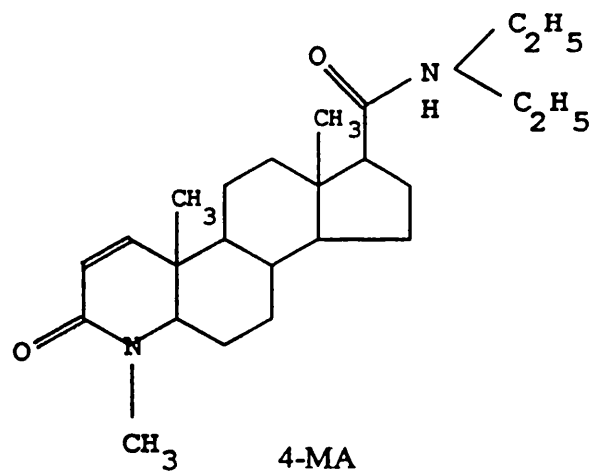
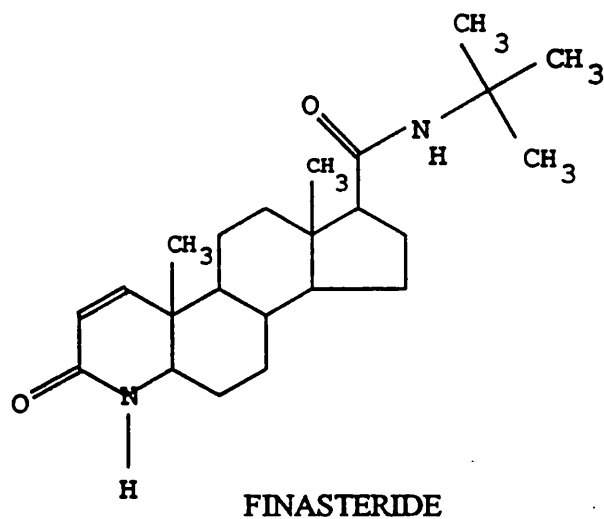
1.5.2.iii 5 α -Reductase Inhibitors

The concept of treating BPH with 5 α -reductase inhibitors arose from the observation that male pseudohermaphrodites have poorly developed prostates due to a deficiency in 5 α -reductase activity (Imperato-McGinley *et al.*, 1974). Administration of DHT to one such individual produced an increase in the size of his prostate (Peterson *et al.*, 1977) emphasising that DHT is the main mediator of prostate growth. The demonstration that these males have erections, ejaculations and libido indicates that inhibition of DHT production should not interfere with sexual function. This avoids the undesirable side effects associated with classical androgen ablation therapies and makes treatment of BPH with 5 α -reductase inhibitors an attractive therapy.

The structures of three potent 5 α -reductase inhibitors; finasteride, SKF 105,657 and 4-MA are shown in Figure 1.6. All three compounds are effective in preventing testosterone induced growth of the rat ventral prostate either as competitive (4-MA and finasteride) or uncompetitive inhibitors (SKF 105,657) (Brooks *et al.*, 1981; Lamb *et al.*, 1992; Liang *et al.*, 1983).

FIGURE 1.6

STRUCTURES OF 5 α -REDUCTASE INHIBITORS



As 4-MA has some affinity for the androgen receptor and suffers from extensive first pass metabolism it is not being developed for human use. In contrast, finasteride is the first 5 α -reductase inhibitor to be approved by the FDA for the treatment of BPH. In addition, SKF 105,657 is currently undergoing phase III trials. Administration of finasteride in man produces a 5 α -reduced steroid metabolite profile similar to that seen in male pseudohermaphrodites. Plasma DHT levels are reduced and there is an accompanied elevation of serum testosterone but no reported elevation of serum LH (Imperato-McGinley, 1991; Vermuelen *et al.*, 1991).

The results of a one year trial of finasteride in men with BPH demonstrated that serum DHT levels were suppressed to castrate levels while serum testosterone rose by 10%. There was a 19% reduction in total gland size and some improvements in flow rate. No significant side effects were reported (Gormley *et al.*, 1992). There is no doubt that the improvement in flow rate achieved with finasteride is not as great as that achieved by TURP. However, the initial results are promising and further development of these types of inhibitors and greater understanding of the roles of 5 α -reductase isozymes in the prostate should lead to the development of a safe and effective medical treatment of BPH.

Finasteride and SKF 105,657 are selective 5 α -reductase 2 inhibitors (Andersson *et al.*, 1991; Jenkins *et al.*, 1992; Mellin *et al.*, 1993; Jones *et al.*, 1993; Harris *et al.*, 1992) and although the contribution of 5 α -reductase 1 isozyme to the overall DHT production in the prostate appears to be minimal it may be that inhibitors of both enzymes will prove to be more effective at producing a total blockade of DHT production and greater efficacy. Plasma levels of DHT rise to normal levels in male pseudohermaphrodites treated with high doses of testosterone, indicating that under certain conditions the ability of 5 α -reductase 1 isozyme to form DHT may be significant (Price *et al.*, 1984).

A potential use for 5 α -reductase inhibitors is in the treatment of prostate cancer. Although finasteride, alone, was found to have no beneficial effects on stage D cancers (Presti *et al.*, 1992), treatment of prostate cancer may be more effectively treated by a combination of a 5 α -reductase inhibitor and an androgen receptor antagonist with the advantage of retaining sexual potency (Labrie *et al.*, 1991). The combination of flutamide and finasteride was shown to be as effective at reducing rat ventral prostate weight as flutamide and a LH-RH agonist (Fleshner and Trachtenberg, 1992). This combination has been evaluated in ten patients with stage C or D1 disease and the preliminary data is encouraging (Trachtenberg, 1993). No increase in PSA was observed in any patient, and a fall of within 20% was noted in three patients. Eight out of ten patients remained potent and side effects were few. Such treatment might be effective in preventing cancers in high risk groups (Petrov, 1986; Gormley, 1990; Ross *et al.*, 1992) and in cancers which have 5 α -reductase activity and are androgen dependent; the more differentiated tumours (Geller *et al.*, 1978b, 1979). Identification of the 5 α -reductase isozymes in prostate cancer tissue will be necessary to fully evaluate the use of 5 α -reductase inhibitors in this area.

1.6 THESIS AIMS

The overall aim of this thesis was to identify and develop suitable systems for the study of androgen metabolism in the human prostate in vitro. To achieve this pathways of androgen metabolism were studied in prostate cells using human BPH tissue slices and cells, human prostate cancer cell lines and primary cultures of separated BPH epithelium and stroma. The main motivation for developing in vitro models of human prostate cells is to produce systems in which 5 α -reductase is present for the evaluation of compounds designed as 5 α -reductase inhibitors for the treatment of BPH and possibly prostate cancer.

In vitro systems involving the short-term incubation of tissue are needed to evaluate the efficacy of compounds designed as 5 α -reductase inhibitors. In developing such systems it was necessary to first characterise the profile of metabolites formed when prostate cells are incubated with [³H]-testosterone. It is well established that slices of BPH tissue retain the ability to metabolise testosterone to a pattern of metabolites characteristic of prostate tissue in vivo. For this reason the ability of prostate cell suspensions (prepared by collagenase digestion of human BPH tissue) to metabolise [³H]-testosterone was compared with the metabolism observed in BPH tissue slices. Similarly, testosterone metabolism in prostate cancer cell lines was studied to determine if the origin of the cells altered their ability to metabolise testosterone.

It is accepted that 5 α -reductase 2 is the predominant 5 α -reductase isozyme in human BPH tissue, however, 5 α -reductase activity in human prostate cancer tissue has yet to be characterised. A useful tool for characterising 5 α -reductase activity is the observation that some 5 α -reductase inhibitors differ in their potency against the two 5 α -reductase isozymes. This approach has not been employed to characterise the 5 α -reductase activity in the human prostate cancer cell lines and,

therefore, one of the aims in this thesis was to use such compounds as tools to characterise the 5 α -reductase activity in human prostate cancer cell lines.

Compounds designed as 5 α -reductase inhibitors are currently evaluated in vitro by measuring their ability to inhibit the conversion of testosterone to DHT. An alternative in vitro approach is to measure the ability of such compounds to block DHT-stimulated cell growth. Such an approach has yet to be reported in the literature. In this thesis the ability of finasteride to inhibit androgen stimulated growth of LNCaP cells was investigated with the aim of developing a more appropriate system for testing 5 α -reductase inhibitors than simply measuring inhibition of DHT production.

Primary cultures of separated BPH epithelium and stroma provide the opportunity to investigate the distribution of 5 α -reductase isozymes in different cell types of the prostate and may also provide a model to study androgen-responsive growth. For these reasons primary cultures of separated BPH epithelium and stroma were studied with the aim of developing an in vitro system combining DHT production and DHT-stimulated growth, key properties of human prostate cells.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

Details of materials and their suppliers are listed in Table 2.1. laboratory reagents and chemicals not listed were obtained from Sigma Chemicals and BDH, Merck.

TABLE 2.1
MATERIALS AND SUPPLIERS

<u>SUPPLIER</u>	<u>MATERIAL</u>
Amersham International PLC Amersham, UK	Testosterone / DHT RIA Kit (TRK 600)
BDH, Merck Ltd. Leics. UK	Absolute Ethanol
	Acetone (Analytical grade)
	Dichloromethane (Analytical grade)
	Diethylether (Analytical grade)
	Giemsa's Stain
	Methanol (Analytical grade)
	Toluene (Analytical grade)
	TLC plates 60F ₂₅₄ Glass Silica
	TLC plates F ₂₅₄ Type E Aluminium Oxide
	Univert Fixative
Costar Ltd. High Wycombe, Bucks, UK	0.2 μ Sterile Filters

Dako Ltd.
High Wycombe, Bucks, UK

Antibodies see Section 2.11

Diagnostic Products Corporation
Los Angeles, USA

Insulin RIA Kit (TK INI)

Oestradiol RIA Kit (TK E21)

Progesterone RIA Kit (TKPG1)

Falcon, Becton Dickinson
Marathon Lab. Supplies,
London, UK.

Primaria 6-well and 24-well plates

Sterile 50ml Centrifuge Tubes

Fisons Ltd
Leics. UK

Hydrogen Peroxide (100 vols)

Gibco BRL
Paisley, Scotland

Gentamycin Solution (10mg/ml)

L-Glutamine (0.2M sterile solution)

Nunc tissue culture flasks (25cm²)

Nunc 96-well plates

Penicillin and Streptomycin Solution ()

Phosphate buffered saline (PBS)

RPMI 1640 medium

RPMI 1640 phenol red-free medium

Trypan Blue Solution

Trypsin/EDTA 1x sterile solution

Imperial Laboratories
Andover, Hants. UK

Foetal calf serum (Heat Inactivated)

NEN, DuPont
Stevenage, Herts. UK

[1 α -2 α -³H]-Testosterone (1mCi/ml)

[1 α -2 α -³H]-Androstenedione

Pfizer Central Research
Sandwich, Kent, UK

COS cells expressing 5 α -reductase 1 and 2
isozymes

5 α -Reductase inhibitors (Finasteride, 4-MA,
SKF 105,657, UK 117,026)

Pharmacia Biosystems Ltd.
Milton Keynes, UK

Dextran T70

Sigma Chemicals,
Poole, Dorset, UK

AEC (3-amino-9-ethyl carbazole)

5 α -Androstane-3 α ,17-dione

5 α -Androstane-3 α ,17 β -diol

5 α -Androstane-3 β ,17 β -diol

Androst-4-ene-3,17-dione

Androsterone

Antifade Fixative

Collagenase Type 1A (C9891)

Diethylformamide

5 α -Dihydrotestosterone

Epiandrosterone

Folin Ciocalteu's Reagent

Glucose-6-phosphate

Glucose-6-phosphate dehydrogenase

Hyaluronidase (H3506)

NADPH

Norit A Charcoal

MTT (3-(4,5-dimethylthiazol-2-yl)-2,
diphenyltetrazolium bromide)

Testosterone

Universal Biologicals Ltd
London, UK

Basement membrane Matrigel

2.2 CELL CULTURE

2.2.1 Continuous Cell Lines

Details of the origins of the human prostate cancer cell lines HPC-36M, DU145, PC-3/MA2 and LNCaP are shown in Table 2.2. HPC-36M is a subline developed at the ICRF (Lincoln's Inn Fields, London) from HPC-36 which was a gift from Dr. David Lubaroff (University of Iowa, USA). DU145 was a gift from Dr. Mickey Stone (Duke Medical Centre, North Carolina, USA). PC-3/MA2, a subline of PC-3, was a gift from Dr. Edward Kaighn (Pasadena Foundation, California, USA). LNCaP was a gift from Dr. Julius Horoszewicz (State University, New York, USA).

2.2.2 Routine Maintenance

All cell lines were maintained under identical conditions as monolayers in 25cm² flasks in 5ml of RPMI 1640 medium supplemented with 5% foetal calf serum and 2mM L-glutamine at 37°C in a humidified atmosphere of 5% CO₂ in air. Each new 500ml bottle containing foetal calf serum was heated to 56°C for 1 h to inactivate complement prior to storage in 25ml aliquots at -20°C. At least once a week, or sooner if required, the medium from the culture flasks was removed and replaced with 5ml fresh medium. Cells were subcultured when they reached 70-80% confluency. This was done by first washing the monolayers with phosphate-buffered saline (PBS) and then incubating with 1x trypsin / EDTA solution (600µl) for 5 min at 37°C. The cells were detached from the culture surface by a gentle tap on each side of the flask which was also sufficient to produce a crude single-cell suspension. The detached cells were then taken up into 20ml of medium and distributed into four 25cm² flasks. The medium in the flasks was replaced with fresh medium 24 h after passaging to remove unattached cells.

TABLE 2.2

DETAILS OF HUMAN PROSTATE CANCER CELL LINES

Cell Line	Derivation	Reference
HPC-36M	Subline of HPC-36 established at the ICRF laboratories following disaggregation of xenografted tumour of HPC-36 grown in nude mice. HPC-36 was derived from a primary prostatic adenocarcinoma (Lubaroff, 1977).	Metcalf <i>et al.</i> , (1983)
DU145	Established from a metastatic CNS lesion of prostatic adenocarcinoma.	Stone <i>et al.</i> (1978)
PC-3/MA2	Cloned subline of xenograft of PC-3 line grown in nude mice. PC-3 was established from a metastatic bone lesion of prostatic adenocarcinoma.	Kaighn, (1979)
LNCaP	Established from a metastatic lymph node lesion of prostatic adenocarcinoma.	Horoszewicz <i>et al.</i> (1983)

2.2.3 Preparation of a Single-Cell Suspension

When cells were required for experiments, exponentially growing cells (70-80% confluent) were detached from the culture surface by first washing with phosphate-buffered saline (PBS) and incubating with 1x trypsin / EDTA (600 μ l) for 5 min at 37°C. The detached cells were taken up in 10ml of medium and a fine single-cell suspension was produced by passing the cells through a 19 gauge needle prior to counting. Viable cell number was assessed using a haemocytometer by counting the number of cells in a 100 μ l aliquot excluding trypan blue (Freshney, 1986). A minimum of 100 cells were counted. The number of cells per millilitre was calculated as follows:

$$\text{average number of viable cells} \times (2 \times 10^4) = \text{number of cells/ml.}$$

An appropriate dilution of the cells was made to produce the final cell number required for experiments.

2.2.4 Preparation of Steroid-Depleted Foetal Calf Serum

Dextran-coated charcoal (DCC) was prepared by incubating Norit A charcoal and dextran T70 (0.25% and 0.0025% w/v respectively) in 500ml of Hepes buffer (10mM, pH 7.4 at 4°C) containing 1.5mM MgCl₂ and 0.25M sucrose. The buffer was prepared and the pH adjusted to 7.4 before adding the charcoal and dextran. The suspension was left stirring continuously overnight at 4°C (Leake *et al.*, 1987).

The DCC-suspension was centrifuged (1000g for 10 min) to pellet the charcoal and the supernatant was discarded. 500ml of heat-inactivated FCS was then added to the charcoal pellet and the mixture left stirring for 12 h at 4°C. After this time the charcoal was removed from the serum by centrifugation (1000g for 20 min) and the resulting charcoal-treated serum (DCC-FCS) was filter sterilised through a 0.2 μ filter and stored in 25ml aliquots at -20°C until required.

2.3 ANALYSIS OF FCS AND DCC-FCS

2.3.1 Radioimmunoassay (RIA)

The concentrations of testosterone, DHT, 17 β -oestradiol, progesterone and insulin were measured by RIA using commercially available kits (Table 2.1). The technique of RIA is based on the competition between unlabelled antigen (in this case testosterone, DHT, etc.) and a fixed quantity of radioactively labelled antigen for binding to an antiserum specific for the antigen being measured. The amount of labelled antiserum is inversely related to the amount of antigen in the sample (Cook and Beasall, 1987). Testosterone and DHT were measured using the Amersham Kit which combines the use of tritiated DHT and an antibody which is specific for testosterone and DHT. The assay involved a two-step procedure in which total testosterone plus DHT is measured and then, following oxidation of testosterone, DHT was measured. The concentrations of 17 β -oestradiol, progesterone and insulin were determined using single step procedures. RIAs were carried out according to the manufacturers instructions using triplicate samples of FCS and DCC-FCS.

2.3.2 Removal Of Tritiated Testosterone and DHT

Aliquots of FCS (10ml) were spiked by adding 1 μ Ci of tritiated testosterone or DHT with 6 samples for each androgen. The spiked FCS samples were left stirring overnight at 4°C to allow exchange of the labelled steroids for endogenous steroids at the normal bindings sites. After this time a small aliquot (100 μ l) was removed for liquid scintillation counting and mixed with 3ml of Ecoscint A. Samples were left to stand for 1 h before counting using an LSE Beckman Counter. The remaining FCS was subjected to dextran-coated charcoal treatment as described in Section 2.2.4. Following this treatment 100 μ l was removed for liquid scintillation counting as described before. The amount of

tritiated testosterone or DHT removed was calculated by dividing the amount of radioactivity remaining in the serum after dextran-coated charcoal treatment by the amount of radioactivity in the serum before treatment.

2.4 GROWTH ASSAYS

2.4.1 Colony-Forming Assay

Each of the cell lines was adapted to growth in steroid-depleted medium by a minimum of three passages in RPMI supplemented with 5% DCC-FCS. These cells were designated as HPC-36M-DCC, DU145-DCC, PC-3/MA2-DCC and LNCaP-DCC; their use was restricted to studies using DCC-FCS supplemented medium only. For colony-forming assays a single-cell suspension was prepared from cells, grown as described in Section 2.2.2, in medium containing either 5% FCS or 5% DCC-FCS.

Cells which had been growing in FCS supplemented medium were plated into 5cm² petri dishes containing 5ml of medium supplemented with 5% FCS and cells which had been growing in 5% DCC-FCS were plated into dishes containing 5ml of medium supplemented with 5% DCC-FCS. Different numbers of cells were plated out depending on the cell line to compensate for differences in colony-forming efficiency between the cell lines. Preliminary experiments had determined the number of cells needed for each cell line to produce approximately 150 colonies after 14-20 days growth. For HPC-36M and DU145 this was 500 cells per dish, for PC-3/MA2 this was 3000 cells per dish and for LNCaP this was 5000 cells per dish. The cells were plated out with three dishes per drug concentration and five dishes for controls and left in the incubator at 37°C to plate down for 24 h.

Following the 24 h period allowed for cell attachment drugs and hormones were added to the cells with control dishes receiving 0.5% (v/v) vehicle (absolute ethanol). The cells were then incubated for a further 14-20 days depending on the rate of growth. Once colonies comprising more than 50 cells were clearly visible in control dishes the medium was removed (with care) from the dishes and the colonies were fixed by adding 70% methanol (3-4ml) to each dish which was left to stand for 15 min. The methanol was removed and the colonies were stained with 10% Giemsa (3-4ml per dish) for 15 min. After this time, colonies stained a blue/purple colour and those comprising more than 50 cells were counted with a binocular microscope.

The mean number of colonies at each drug concentration was expressed as a percentage of controls. The results from a minimum of three separate experiments were combined and averaged and a graph of the percentage colony formation versus drug concentration was plotted on semi-log paper.

2.4.2 MTT Cell Proliferation Assay

This is a colorimetric assay based upon the ability of mitochondrial dehydrogenases in viable cells to reduce a tetrazolium dye (MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) into water insoluble MTT-formazan crystals of dark blue colour (Mosmann, 1983). The blue crystals are solubilised in DMSO and the intensity measured colorimetrically. The assay measures the ability of drug-treated cells to reduce MTT compared to the ability of untreated cells to reduce MTT, and provides a rapid measure of cell survival. Preliminary experiments were carried out with LNCaP to determine the optimum conditions (cell number and MTT concentration) for the MTT assay that would result in an absorbance at 540nm of 1-1.5 absorbance units in a semi-confluent well after seven days incubation.

To determine the optimum assay conditions, duplicate 96 well plates were set up containing a range of cell numbers (2000-8000) in 200 μ l of medium in rows 2-12, and medium alone in row 1. Plates were incubated for 7 days at 37°C in a humidified atmosphere of 5% CO₂ in air. Viable cell number was determined by adding 50 μ l of MTT solution (1 mg/ml or 5mg/ml) to each well (one concentration per plate). The MTT solution was prepared fresh on the day of assay by dissolving the appropriate amount in PBS and the solution was left to stir for at least 3 h to ensure that it had completely dissolved. After addition of MTT solution to the cells, the plates were incubated at 37°C for 3 h and then the medium containing the MTT solution was carefully removed from the cells and replaced with DMSO (100 μ l) to solubilise the blue formazan crystals. The plates were gently shaken to aid solubilisation and the absorbance was measured at 540nm using a Titertek Multiskan MCC spectrophotometer (Flow). The appropriate cell number and MTT concentration was selected from the blank-corrected absorbances. The optimum cell number selected was 3000 cells per well and MTT concentration was 1mg/ml (0.2mg/ml final concentration).

For hormone response assays, a single-cell suspension of 2x10⁴ LNCaP cells (3000 cells / well) was prepared in either 5% FCS or 5% DCC-FCS supplemented medium. The cells (150 μ l) were added to rows 2-10 of a 96 well plate using a multichannel pipette and medium alone (150 μ l) was added to row 1 as a blank. After 24 h incubation at 37°C, medium (50 μ l) was added to row 1 and 2 (blank and controls), and 50 μ l of medium (50 μ l) containing testosterone or DHT (to give final assay concentrations of 1pM-10 μ M) was added to rows 3 to 10. After a further six days incubation, MTT solution (50 μ l of 1 mg/ml; final assay concentration of 1mg/ml) was added to every well and the plates incubated at 37°C for 3 h. Medium containing MTT was removed and DMSO added and the plates treated as described above.

Absorbance at 540nm was measured and the mean absorbances in hormone-treated wells relative to the controls (row 2) were calculated using an on-line Amstrad computer and a program supplied by Flow Laboratories. The MTT program records the absorbance in every well, then subtracts the mean absorbance in row 1 (blanks) from the absorbances in rows 2-10. It then calculates the percentage absorbance in each of the drug-treated rows relative to the controls (which are taken as 100%). Graphs of percent control absorbance versus androgen concentration were plotted from two experiments.

2.4.3 Preparation of Hormones And Drug Solutions For Growth Assays

Testosterone and dihydrotestosterone (DHT) were dissolved in absolute ethanol to produce stock solutions of 20mM which were stored at -20°C until required. Stock solutions were diluted in medium prior to use so that the final solvent concentration in colony-forming assays was 0.5% (v/v) and for MTT assays 0.1% (v/v). For colony-forming assays a stock solution of finasteride was made fresh in absolute alcohol for each experiment. Final solvent concentration was 0.5% for colony forming assays (v/v).

2.5 HUMAN PROSTATE TISSUE EXPERIMENTS

2.5.1 Collection

Specimens were obtained from patients undergoing transurethral resection of the prostate (TURP) for the relief of obstructive symptoms due to BPH. This tissue is referred to as "BPH tissue" even though potentially it may have contained some normal prostate tissue. Immediately following surgery, TURP chips were immersed in medium consisting of RPMI 1640 (phenol-red free) supplemented with 2mM L-glutamine, penicillin (100IU/ml), streptomycin (100µg/ml) and gentamycin (50µg/ml) for transportation to the laboratory. Upon reaching the

laboratory, the tissue was washed in fresh medium and the tissue was trimmed to remove blood clots and charred areas. The tissue was washed several times to remove debris and blood cells before further use.

2.5.2 Preparation of Human BPH Slices

Fresh tissue was collected and washed as described in Section 2.5.1. Thin slices of prostate (1-2mm thick) were prepared by sandwiching a TURP chip between two microscope slides, one of which was attached to a flat-sided bottle. Once the tissue was secured length-wise serial sections of the tissue were cut using a razor blade held by a pair of Spencer Wells forceps. Slices of tissue from the edges of each TURP chip were discarded, and the remaining slices were kept moist in a petri dish containing medium containing antibiotics until further use.

2.5.3 Preparation of Human BPH Cell Suspensions

Prostate tissue obtained as described in Section 2.5.1 was diced into cubes (approximately 1mm³) using sterile scalpel blades. The tissue was washed several times in medium containing antibiotics and then transferred to a 50ml centrifuge tube containing 25ml PBS. The volume of PBS displaced by the tissue was recorded. The tissue was allowed to settle and the PBS was decanted off and discarded. For every 1ml of PBS displaced by the tissue 5-6 volumes of dissociating solution were used to digest the tissue. Dissociation solution consisted of RPMI 1640 (phenol-red free), 5% FCS, 2mM L-glutamine, 225 units/ml collagenase type 1 and 125 units/ml hyaluronidase based on the method described by Deshpande *et al.* (1989).

The mixture was left stirring gently at 37°C for 12-18 h (overnight) until the cubes of tissue had been digested to form a suspension of epithelial and fibroblast cells. For preparation of cell suspensions to be used for primary culture

experiments (Section 2.10) the digestion was allowed to proceed until a suspension consisting of small clumps of epithelial cells and single fibroblasts were visible when examined under the light microscope. For metabolism studies and IC_{50} determinations the digestion was allowed to proceed for approximately 2 h longer, with slightly more vigorous stirring, so that the suspension consisted of mostly single cells. Following the digestion period the suspension was washed twice with medium by centrifugation (200g, 5 min) and resuspended in fresh medium. Any large undigested lumps of tissue remaining after digestion were removed by filtration of the suspension through sterile gauze.

2.5.4 Preparation of Human BPH Tissue Homogenates

Human BPH tissue was obtained as described in Section 2.5.1 and an homogenate was prepared by first pulverising the tissue frozen at -80°C . The pulverised tissue was then resuspended in approximately 30ml of freshly prepared sodium phosphate buffer (20mM, pH 6.5) containing sucrose (0.32M), dithiothreitol (1mM) and NADPH (50 μM) (Liang *et al.*, 1985) and homogenised using a Polytron blender (3 x 5 sec, setting 6). The homogenate was then filtered through sterile gauze to remove any large pieces of tissue and the resulting filtrate was stored at -80°C until further use.

2.5.5 Lowry Assay of Protein Concentration

An aliquot of sample to be assayed (200 μl) was first digested by adding an equal volume of 6M NaOH (200 μl) and incubating for 30 min at 40°C . The digested sample was then diluted to 12ml with distilled water. Protein concentration was estimated by a modification of the method described by Lowry *et al.* (1951). This method involves the use of two solutions (A and B) which were prepared as follows: solution A was prepared by adding 2ml of sodium potassium tartrate (1% w/v in 0.5% w/v $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, pH 7.0) to 100ml of Na_2CO_3 (2% w/v in 0.1M

NaOH); solution B was prepared by adding 5ml Folin Ciocalteu reagent to 5ml distilled water.

The assay was carried out as follows: solution A (2ml) was added to duplicate sample tubes containing a total volume of 500 μ l (various volumes of sample plus distilled water to 500 μ l). The tubes were vortex mixed and left to stand at room temperature for 15 min. After this time reagent B (200 μ l) was added, the tubes were remixed and left to stand at room temperature for 30 min. The absorbance was read at 700nm (Uvikon, spectrophotometer) against a blank of distilled water. Protein concentration in the samples was determined from a calibration curve prepared by assaying bovine serum albumin (0-100 μ g). Protein observed in 500 μ l sample (y) = 2y x 60mg/ml.

2.6 ANDROGEN METABOLISM EXPERIMENTS

2.6.1 BPH Slices

Tissue slices were set up in 5cm petri dishes using a modified Trowell technique (1959). Three organ culture grids covered by lens tissue were placed into each petri dish. Slices of tissue (1-2 per grid) were placed on the lens tissue and 3ml of serum free and phenol-red free RPMI 1640 containing [1 α ,2 α -³H]-testosterone (1 μ Ci) were added to each dish. The substrate (testosterone) was prepared by evaporating the ethanol solvent from the appropriate amount of labelled plus unlabelled testosterone to produce a final concentration of 20nM in the assay. The tissue was incubated for 24 h at 37°C. Control dishes of tissue and medium containing unlabelled testosterone were prepared in parallel with, and treated identically to, the test dishes.

The incubation was stopped by transferring the medium to a fresh tube containing two volumes of ethylacetate plus trace steroids (10 μ g/ml): testosterone; DHT;

androstenedione; androstenedione; androstenediols; androsterone and epiandrosterone. Each tube was vortex mixed and centrifuged (500g, 10 min). The ethylacetate phase (upper) was transferred to a fresh tube and the aqueous phase was re-extracted with ethylacetate as before. Metabolites were extracted from the tissue and kept separate from the medium extracts. The tissue was first minced finely in 1ml of medium and then extracted twice with 3ml ethylacetate. The ethylacetate phases were stored at 4°C until analysed by thin layer chromatography (TLC).

2.6.2 BPH Cell Suspensions

BPH cell pellets prepared as described in Section 2.3.1 were resuspended in serum-free and phenol-red free RPMI (5ml medium per 1ml PBS displaced by the undigested tissue). An aliquot of the cell suspensions was retained for protein estimation (Section 2.5.5). Tritiated testosterone (1μCi) plus unlabelled testosterone, or [1α,2α-³H]-androstenedione (1μCi) plus unlabelled androstenedione to give final concentrations of 20nM were added to glass screw-top tubes and the ethanol was evaporated off under a stream of nitrogen gas. The reaction was started by adding 1ml of cell suspension, in 15 sec intervals, to each tube containing substrate. The tubes were vortex mixed, sealed and incubated at 37°C in a shaking water bath for 1 h. The reaction was terminated by adding 2ml of ethylacetate containing trace steroids as described in Section 2.6.1.

2.6.3 Prostate Cancer Cell Lines

Prostate cancer cell lines were grown in monolayer culture in 25cm² tissue culture flasks to approximately 80% confluency (as judged by eye) and 24 h prior to the addition of [³H]-testosterone the monolayers were washed with PBS and 5ml serum-free RPMI 1640 was added. The medium was removed after 24 h and replaced with 5ml of the same medium containing [³H]-testosterone (20nM,

1 μ Ci). The flasks were then incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 3 h. After this time the medium was removed and transferred to a fresh tube containing 5ml ethylacetate plus trace steroids. The cells were scraped from the flask into 2ml PBS and extracted with 2ml ethylacetate. Extractions were performed as described in Section 2.6.1.

2.6.4 Human BPH Homogenates

Homogenates of human BPH tissue prepared as described in Section 2.5.4 were defrosted and diluted in citrate phosphate buffer (40mM, pH 5.2) containing dithiothreitol (1mM). The homogenate was diluted so that a 200 μ l aliquot turned over approximately 15% of substrate during a 30 min incubation period with [³H]-testosterone (20nM, 1 μ Ci). This corresponded to a final protein concentration of 0.1mg/ml.

The assay tubes were prepared by adding labelled and unlabelled testosterone to produce a 20nM final concentration (in 1ml) in each tube and evaporating off the ethanol solvent under a stream of nitrogen gas. The reaction was started by adding BPH homogenate, in 15 sec intervals (200 μ l), to tubes containing substrate, citrate phosphate buffer (40mM, pH 5.2) and an NADPH-regenerating system of NADPH (5mM), glucose-6-phosphate (50mM) and glucose-6-phosphate-dehydrogenase (5 μ units/ml). Tubes were vortex mixed, sealed and incubated at 37°C for 30 min in a shaking water bath. The reaction was terminated by adding 2ml of ethylacetate containing trace steroids as described in Section 2.6.1.

2.6.5 Transfected COS Cells

Separate batches of COS cells transiently expressing the complementary DNA (cDNA) sequences coding for the human 5 α -reductase 1 and 2 isozymes were a gift from the Molecular Genetics Department (Pfizer Central Research). These

cells were prepared by the Molecular Genetics Department by transfecting separate populations of COS cells with the cDNA sequences identical to those coding for 5 α -reductase 1 and 2 isozymes (Andersson *et al.*, 1991).

Transfected cells were harvested 48 h following calcium phosphate transfection, washed in PBS and stored as a pellet at -20°C until required. Cell pellets were resuspended in 8ml of sodium phosphate buffer (20mM, pH 6.5) containing sucrose (0.32M), dithiothreitol (1mM) and NADPH (50 μ M) made fresh before homogenisation. The resuspended COS cells were homogenised using a Polytron blender (3 x 5 sec on setting 6) and the activity and protein concentration measured so that 50 μ l homogenate metabolised approximately 15% of [³H]-testosterone (20nM, 1 μ Ci) when incubated at 37°C for 30 min. For COS cells expressing 5 α -reductase 1 enzyme this was a protein concentration of 2.5 μ g/ml and for the COS cells expressing 5 α -reductase 2 enzyme this was 2 μ g/ml. Homogenates of mock transfected COS cells (no DNA inserted into the cells) were prepared and analysed in parallel with COS cells transfected with 5 α -reductase 1 and 2 cDNAs.

Experiments with COS cells expressing 5 α -reductase 1 were carried out with sodium phosphate buffer (40mM, pH 7.4) and experiments with COS cells expressing 5 α -reductase 2 were carried out using citrate phosphate buffer (40mM, pH 5.2). The activity (conversion of testosterone to DHT) was measured using similar conditions to BPH homogenates (Section 2.6.4), with the inclusion of 50 μ l of BSA (2mg/ml). The reaction was started by adding cell homogenate (50 μ l), in 15 sec intervals, to tubes containing substrate, buffer, BSA and an NADPH-regeneration system (Section 2.6.4). Total volume was 1ml and the tubes were vortex-mixed, sealed and incubated at 37°C for 30 min in a shaking water bath. The reaction was stopped by adding 2ml of ethylacetate and extraction of metabolites was performed as in Section 2.6.1.

2.6.6 Primary Cultures of BPH Cells

Testosterone metabolism in primary cultures of human BPH epithelial and fibroblast cells was analysed in the 24-well plates in which the cells were growing. Prior to incubation with [^3H]-testosterone the cells were washed with pre-warmed PBS. For time course experiments with cultured epithelial cells pre-warmed RPMI containing 5% FCS and [^3H]-testosterone ($1\mu\text{Ci}$, 20nm) was prepared and added to each well. The plates were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 h. After this time the medium was removed, under sterile conditions, from the cells and added to 2ml of ethylacetate and treated as described in Section 2.6.1. The cells were then washed with PBS and fresh medium containing [^3H]-testosterone was added to the cells which were then incubated for a further 24 h. This process was repeated after each 24 h period until the end of the experiment.

Experiments with fibroblasts were performed using similar conditions to those described for epithelial cells except that incubation times of 1 h and 24 h were used.

2.6.7 pH Profile Studies

2.6.7.i Homogenates of BPH Tissue

The conditions for the pH profile assay of 5α -reductase activity in human BPH tissue were identical to those described in Section 2.6.4 except that a series of buffers (citrate phosphate, 40mM) with different pH values (4.0-8.5) were used instead of a single pH buffer. Solutions of NADPH and substrate were also prepared at each pH value using buffer at the appropriate pH.

2.6.7.ii Prostate Cancer Cell Lines

Separate 10ml single-cell suspensions (6×10^6 cells / ml) of the prostate cancer cell lines HPC-36M and DU145 were prepared as described in Section 2.2.3. The cells were collected as a pellet by centrifugation (1000g, 10 min) and resuspended in 4ml of sodium phosphate buffer (40mM, pH 6.5) containing sucrose (0.32M, dithiothreitol (2mM) and NADPH (50 μ M). The cell suspensions were homogenised using a Polytron blender (3 x 5 sec on setting 6) and the homogenate was stored on ice.

Duplicate tubes containing [3 H]-testosterone (20nM, 1 μ Ci) were prepared and the ethanol solvent was evaporated under a stream of nitrogen gas. Total assay volume was 2ml, with each tube containing 25 μ l of an NADPH-regeneration system (Section 2.6.4) and citrate phosphate buffer (40mM) covering the pH range 4-9. The reaction was started by adding the cell homogenate in 15 sec intervals to each tube (100 μ l) containing substrate, buffer and an NADPH-regeneration system. The tubes were then vortex-mixed, sealed and incubated for 3 h at 37°C in a shaking water bath. The reaction was terminated by adding 4ml ethylacetate and extraction of metabolites was performed as in Section 2.6.1.

2.6.7.iii Transfected COS Cells

Homogenates of COS cells expressing 5 α -reductase 1 and 2 isozymes were prepared as described in Section 2.6.5. Duplicate tubes were prepared containing [3 H]-testosterone (20nM, 1 μ Ci), an NADPH regeneration system (Section 2.6.4), BSA (2mg/ml) and citrate phosphate assay buffer (40mM) at different pH values. The reaction was started by adding cell homogenates to tubes containing substrate, buffer, BSA and an NADPH-regeneration system and the assay was carried out as described in Section 2.6.5.

2.7 5 α -REDUCTASE INHIBITOR STUDIES

2.7.1 BPH Tissue Slices

Petri dishes containing BPH slices were set up as described in Section 2.6.1 with three dishes per 5 α -reductase inhibitor. Medium (3ml of serum-free RPMI) containing [3 H]-testosterone (20nM, 1 μ Ci) and 100nM compound (ethanol solvent was evaporated before adding the medium) was added to each dish. Stock solutions of inhibitors: finasteride; SKF 105,657; 4-MA and UK 117,026; were freshly prepared in absolute ethanol at 200 times the final concentration before each experiment.

Control dishes containing tissue slices and substrate (no inhibitor) and blank dishes containing tissue and medium (no substrate) were prepared. The tissue was incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. The reaction was terminated by adding ethylacetate. Metabolites were extracted as described in Section 2.6.1.

2.7.2 BPH Cell Suspensions and Prostate Cancer Cell Lines

Single-cell suspensions (3 x 10⁶ cells/ml) prepared from exponentially growing prostate cancer cell lines (Section 2.2.3) and freshly prepared BPH cell suspensions (2-4mg/ml protein) (Section 2.5.3) were added to screw-top glass tubes containing [3 H]-testosterone (1 μ Ci, 20nM) in 15 second intervals. The tubes were sealed, vortex mixed and incubated for 1 h at 37°C in a shaking water bath. Total assay volume was 1ml in serum-free RPMI 1640 and compounds (5 μ l of stock solution, Section 2.7.1) and [3 H]-testosterone were added to the tubes and the ethanol solvent was evaporated before starting the reaction by adding the cell suspension. Control (no inhibitor) and blank (no cell suspension) tubes were

prepared for each experiment. The reaction was stopped by adding 2ml of ethylacetate and metabolites were extracted as described in Section 2.6.1.

2.7.3 Transfected COS Cells

Substrate was prepared by drying down the appropriate amount of labelled and unlabelled testosterone (to produce a final concentration of 20nM in the assay) and adding 5ml assay buffer. The assay buffer for 5 α -reductase 1 was sodium phosphate buffer (40mM, pH 7.4) and citrate-phosphate buffer (40mM, pH 5.2) for 5 α -reductase 2. Each tube was prepared in duplicate containing 100 μ l of substrate solution, NADPH regeneration system (Section 2.6.4), BSA (2mg/ml), assay buffer (sodium phosphate 40mM, pH 7.4 for 5 α -reductase 1 and citrate phosphate 40mM, pH 5.2 for 5 α -reductase 2) and compound (5 μ l of stock solution, Section 2.7.1).

The reaction was started by adding the cell homogenate (50 μ l) to each tube in 15 sec intervals and vortex mixing. Tubes were sealed and incubated for 30 min at 37°C in a shaking water bath. The reaction was stopped by adding 2ml ethylacetate containing trace steroids and extracting as in Section 2.6.1.

2.7.4 Primary Cultures of BPH Epithelial Cells

Experiments were performed using epithelial growing in 24-well plates as described in Section 2.6.6. Substrate was prepared by drying down the appropriate amount of labelled and unlabelled testosterone (to produce a final concentration of 20nM in the assay) and adding 30ml of serum-free RPMI. An appropriate amount of stock solution of inhibitor (Section 2.7.1) was added to the medium to produce the required final concentration. The medium was thoroughly mixed and placed in a 37°C water bath until needed. The medium from the cells was removed and the cells were washed with PBS prior to adding 1ml of warm

medium containing [^3H]-testosterone and inhibitor. The cells were then returned to the incubator and left for 24 h. After this time the medium was removed and added to 2ml of ethylacetate and treated as described in Section 2.6.1.

2.8 SEPARATION AND ANALYSIS OF METABOLITES

2.8.1 Thin Layer Chromatography (TLC) Separation of Metabolites

Ethylacetate extracts containing [^3H]-testosterone and its metabolites were evaporated to dryness under nitrogen gas in glass tubes. The residue was redissolved in absolute ethanol (70 μl) and the tubes vortex mixed. The dissolved residues were applied to glass silica gel TLC plates (60F₂₅₄) using an automated TLC spotter (Desaga). Samples were spotted onto the plate at an origin of 20mm with a spacing of 18mm. A blank from each experiment was included on each plate.

Separation of metabolites was carried out by thin layer chromatography (TLC) using a solvent system of dichloromethane : acetone (12.3:1 v/v) (Schweikert *et al.*, 1982). This system does not completely resolve DHT, androsterone and epiandrosterone and in order to achieve separation of these metabolites the appropriate area on the plate was scraped off after it had been identified by co-chromatography with standards (following development in an iodine vapour tank). The metabolites were extracted from the silica gel by two elutions in 3ml of dichloromethane : acetone (2:1 v/v) which was evaporated to dryness under nitrogen gas. The residue was dissolved in ethanol (80 μl) and applied to an aluminium oxide plate (F₂₅₄ Type E). Metabolites were separated by two developments in a solvent system consisting of toluene : diethylether (1:1 v/v) (modification of the method of Schweikert *et al.*, 1982).

2.8.2 Analysis of TLC Plates

The profile of separated radioactive metabolites was quantified using a radio-TLC plate reader (Raytest). Metabolites were identified by comparing their TLC mobilities with authentic standards which were located by UV light absorption and by development in an iodine vapour tank (R_f values in Appendix 1). Following the second TLC separation the areas of silica gel corresponding to DHT, androsterone and epiandrosterone were located by co-chromatography with standards, scraped off and quantified by liquid scintillation counting (R_f values in Appendix 2).

2.9 IC₅₀ DETERMINATIONS

2.9.1 Colony-Forming Assays

The results from individual colony-forming assays were plotted on semi-log paper. Points which fell on the straight part of the curve were used for linear regression analysis to determine the IC₅₀ concentration. The IC₅₀ concentration for a colony-forming assay is defined as the concentration of drug required to inhibit colony-formation to 50% of that produced in controls. Data from three separate experiments was used to calculate the mean IC₅₀ concentration \pm standard error of the mean (SEM).

2.9.2 5 α -Reductase Assays

The IC₅₀ values for the inhibition of 5 α -reductase activity \pm the standard deviation (SD) were determined from sigmoidal dose-response curves which were fitted to the combined data from 3 separate experiments using the program Sigfit (De Lean *et al.*, 1978). The IC₅₀ concentration for a 5 α -reductase assay is defined as the concentration of drug required to inhibit the production of DHT by 50% of that produced in controls.

2.10 PRIMARY CULTURE OF BPH EPITHELIUM AND STROMA

2.10.1 Separation of Epithelial Glands (Organoids) from Stromal Elements

Tissue was collected from patients undergoing TURP and processed as described in Section 2.5.3. The tissue was left stirring overnight until small clumps (organoids) were remaining. Following the overnight digestion, the cell suspension was washed twice in RPMI (phenol red-free) containing antibiotics (penicillin, 100IU/ml; streptomycin, 100µg/ml and gentamycin 50µg/ml). Washing was performed by discarding the supernatant and resuspending the pellet after a short period of centrifugation (200g, 5 min). Following the second wash, the pellet of epithelial glands and stromal elements was resuspended in 50ml of RPMI containing 5% FCS, 2mM L-glutamine and antibiotics (as above) and transferred to a 50ml centrifuge tube.

The resuspended mixture was then centrifuged (50g, 5 min) and the supernatant was transferred to a separate sterile vessel. The pellet was resuspended, mixed by inversion on a rotating carousel for 5 min and left to stand for 30 min after which the supernatant was then removed and combined with the first supernatant. The pellet was resuspended, mixed by inversion and allowed to stand for a further 30 min. This process was repeated as many times (in practice usually 5-6 times) as required to remove most of the stromal elements and enrich the pellet with epithelial glands (Deshpande *et al.*, 1989).

2.10.2 Culture Conditions For Epithelium

Epithelial organoids were plated into 24-well Primaria plates at 10 per well. The surface of Primaria plates is specially treated to favour the attachment and proliferation of epithelial cells over fibroblasts. The number of organoids per millilitre was estimated by counting (with a light microscope, magnification x40)

the number in a 50 μ l aliquot. Initially organoids were plated out in 500 μ l of medium (RPMI containing 5% FCS, 2mM L-glutamine and antibiotics) and left at 37°C in a humidified 5% CO₂ incubator for 24 h to attach. Plating out in larger volumes reduced the number of organoids that attached. The following day the medium containing unattached material was removed and 2ml of fresh medium was added to the cultures. Thereafter fresh medium was added to the cultures every three days.

2.10.3 Culture Conditions for Stromal Cells

The stromal cells in the combined supernatants were pelleted by centrifugation (200g, 5 min) and resuspended in RPMI supplemented with 10% FCS, L-glutamine and antibiotics (as in Section 2.10.2). The stromal cells were resuspended in 10ml of medium for every 1ml of PBS initially displaced by the diced tissue (Section 2.4.2) and plated into 25cm² standard tissue culture flasks. The cells were left in a 37°C incubator for at least two days to allow attachment. Fresh medium was added to the cultures every three days. Confluent cultures were passaged as described in Section 2.2.2, with the fibroblasts from a single 25cm² flask being distributed into three new 25cm² flasks.

2.11 IMMUNOCYTOCHEMICAL CHARACTERISATION OF PRIMARY CELL CULTURES AND PROSTATE CANCER CELL LINES

2.11.1 Preparation of Cells

Single-cell suspensions (2 x 10⁴ cells/ml) of each of the human prostate cancer cell lines were prepared as described in Section 2.2.3. Each cell line was processed separately by adding 300 μ l of cell suspension to the cytopsin chambers and centrifuging at 400 rpm for 10 min. The slides were then fixed immediately

by immersion in ice cold methanol:acetone (1:1 v/v) for 10 min. After fixation the slides were air dried for 30 min and then stored at -70°C until required. Cytospin preparations of BPH cell suspensions (Section 2.5.3) were prepared in the same way. Primary cultures of BPH cells growing in 6-well and 24-well plates were washed in PBS and fixed in ice-cold methanol:acetone (1:1 v/v) and stored at -70°C until required.

2.11.2 Immunoreactivity

The antibodies, sources and specificities are shown in Table 2.3. For detection of antibody-binding a two-step procedure was used with second layer antibodies being conjugated to either peroxidase or fluorescein isothiocyanate (FITC). The slides and cells prepared in Section 2.11.1 were removed from storage at -70°C and air-dried for 30 min, before addition of the primary antibody (30µl for slides; 100µl or 200µl for wells). After incubation of the cells with the primary antibody for at least 1 h at room temperature the slides were washed in tap water for 10-15 min and the second layer antibody was applied (50µl for slides; 200µl or 300µl for wells) and left for a further 1 h. The slides were then washed in tap water as before.

TABLE 2.3

ANTIBODY SOURCES

Antibody Name	Antigen Detected	Source Reference
6B10	Cytokeratin 4	van Muijen <i>et al.</i> (1986)
LP1K	Cytokeratin 7	Lane <i>et al.</i> (1985)
LE41	Cytokeratin 8	Lane <i>et al.</i> (1982)
LH2	Cytokeratin 10	Lane <i>et al.</i> (1985)
IC7	Cytokeratin 13	van Muijen <i>et al.</i> (1986)
LOO1	Cytokeratin 14	Purkis <i>et al.</i> (1990)
LL025	Cytokeratin 16	Lane <i>et al.</i> , in press
E3	Cytokeratin 17	Gulstein <i>et al.</i> , (1988)
LE61	Cytokeratin 18	Lane <i>et al.</i> (1982)
LP2K	Cytokeratin 19	Stasiak <i>et al.</i> (1989)
Vimentin	Vimentin	Dako Product Information
PSA	Prostate Specific Antigen	Dako Product Information
PSAP	Prostate Specific Acid Phosphatase	Dako Product Information
Desmin	Desmin	Dako Product Information

All antibodies to cytokeratins were gifts from the Experimental Dermatology Laboratory, at the London Hospital. PSA, PAP, Desmin were obtained from Dako Ltd. Primary antibodies were all mouse anti-human except LL025 which was rabbit anti-human. Secondary antibodies (peroxidase conjugated and FITC-conjugated rabbit anti-mouse and mouse anti-rabbit were obtained from Dako Ltd.

Cells which had been incubated with FITC-conjugated second layer antibodies were counter stained with propidium iodide (1% v/v in distilled water). These slides were cover-slipped and fixed in Antifade (BDH) to retard fading of fluorescence. A Zeiss microscope equipped with appropriate filters (red and green) was used to examine the slides. Peroxide substrate was made fresh by dissolving 3-amino-9-ethyl carbazole (AEC) in diethylformamide (4mg/ml). The volume was made to 15ml with sodium acetate buffer (0.1M, pH 6.2) after which 13.6 μ l of hydrogen peroxide solution was added.

Cells which had been incubated with peroxidase-conjugated second layer antibodies were developed by adding enough peroxide substrate solution to completely cover the cells. The cells were left for 10 min and then washed in tap water as before. The slides were cover-slipped and fixed in Univert. The percentage of positive cells was calculated from examining a minimum of 200 cells.

2.12 STATISTICAL ANALYSIS

Where appropriate statistical differences between sample means were analysed using the Student's t-test where $t = \frac{\bar{x}_1 - \bar{x}_2}{\text{s.e}}$

and s.e = standard error of $\bar{x}_1 - \bar{x}_2$.

CHAPTER THREE

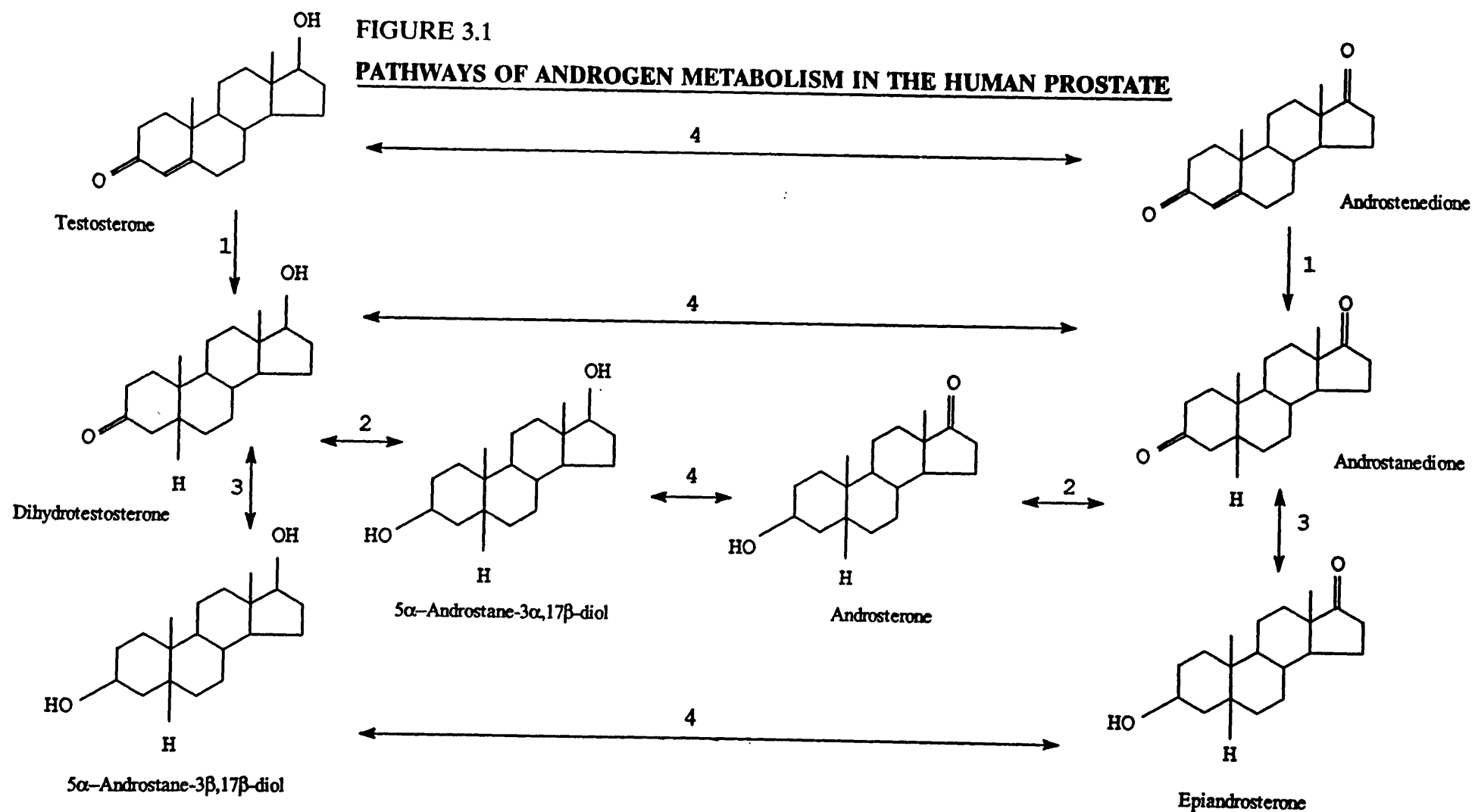
PATHWAYS OF ANDROGEN METABOLISM IN HUMAN BPH **TISSUE SLICES AND CELL SUSPENSIONS AND** **HUMAN PROSTATE CANCER CELL LINES**

3.1 INTRODUCTION

Studies of androgen metabolism *in vivo* and *in vitro* have established the fact that the prostate contains steroid metabolising enzymes capable of transforming testosterone and androstenedione entering the prostate into a range of different products (Farnsworth *et al.*, 1963; Pike *et al.*, 1970; Jenkins and McCaffery, 1974; Harper *et al.*, 1974; Becker *et al.*, 1975). These products differ in their ability to stimulate the growth of the prostate. Enzymes capable of producing more potent androgens include: 5 α -reductase, which irreversibly converts testosterone to DHT (Farnsworth *et al.*, 1963); 17 β -hydroxysteroid dehydrogenase (17 β -HSD), which reversibly converts androstenedione to testosterone, (Harper *et al.*, 1974; Bartsch *et al.*, 1987b; and 3 α /3 β -hydroxysteroid dehydrogenase (3 α / β -HSD), which reversibly converts the androstanediols to DHT (Becker *et al.*, 1975) (Figure 3.1).

The reversible nature of these enzymes (except 5 α -reductase) means that they are also responsible for converting potent androgens into less potent androgens. Thus, 17 β -HSD converts testosterone to androstenedione (Harper *et al.*, 1974) and DHT to androstanedione (Malathi and Gurpide, 1977) while 3 α / β -HSD converts DHT to androstanediols (Jacobi and Wilson, 1977). Androgen action within the prostate is, therefore, regulated by the balance between 5 α -reductase (androgen activation) and androgen inactivating enzymes.

FIGURE 3.1

PATHWAYS OF ANDROGEN METABOLISM IN THE HUMAN PROSTATE

Numbers refer to the following enzymes:

1: 5 α -Reductase 2: 3 α -Hydroxysteroid dehydrogenase 3: 3 β -Hydroxysteroid dehydrogenase 4: 17 β -Hydroxysteroid dehydrogenase

Human benign prostatic hyperplasia (BPH) tissue is reported to have a higher metabolic capacity than prostate cancer tissue, in particular, greater 5 α -reductase activity (Bard and Lasnitzki, 1977; Habib *et al.*, 1985; Geller *et al.*, 1978a; Prout *et al.*, 1976; Morfin *et al.*, 1979; Bruchovsky and Lieskovsky, 1979; Hudson *et al.*, 1982; Klein *et al.*, 1988; Bruchovsky *et al.*, 1988). The reduction in the metabolic capacity of prostate cancer tissue may also be accompanied by a shift towards the production of 17-oxo metabolites such as androstenedione, androstanedione, androsterone and epiandrosterone, which some investigators suggest is related to the degree of differentiation of the tissue (Morfin *et al.*, 1977, 1979; Bard and Lasnitzki, 1977; Geller *et al.*, 1978). This shift to an oxidative metabolic pathway does not occur in BPH and several *in vitro* studies have shown that incubation of BPH tissue with [³H]-testosterone leads predominantly to the formation of DHT rather than androstenedione. Studies carried out using slices of tissue (McMahon *et al.*, 1974; Morfin *et al.*, 1979), minces (Jenkins and McCaffery, 1974; Prout *et al.*, 1976), homogenates (Harper *et al.*, 1974; Bruchovsky and Lieskovsky, 1979; Klein *et al.*, 1988; Andersson *et al.*, 1991; Jenkins *et al.*, 1992) and organ culture (Robel *et al.*, 1971; Lasnitzki, 1979; Smith *et al.*, 1983) have all confirmed that DHT is the predominant metabolite produced when prostate tissues are incubated with [³H]-testosterone.

The majority of studies following testosterone metabolism in prostate tissue have included the addition of NADPH and non-physiological concentrations of substrate (concentrations of testosterone exceeding 30nM in this study are considered to be non-physiological). The absolute dependence of 5 α -reductase on NADPH necessitates its inclusion with disrupted tissue preparations, but NADPH supplementation of prostate tissue will also affect 3 α / β -HSD activity (Jacobi and Wilson, 1977; Morfin *et al.*, 1978; Lombardo *et al.*, 1992) favouring its reductase activity and giving an altered profile of metabolites. Studies involving short-term incubation of intact BPH cell suspensions and tissue slices in physiological concentrations of testosterone without added cofactors (as

performed in this study) should reflect the tissue's actual pattern of metabolism.

The aim of this study was to identify suitable systems for the study of androgen metabolism in human prostate cells in vitro and assess their potential use in evaluating novel 5α -reductase inhibitors designed for the treatment of BPH. This was approached by characterising androgen metabolism in BPH tissue slices and cell suspensions of BPH tissue (produced by collagenase digestion). BPH cell suspensions were investigated as a potential system in which the cells remain intact, in the presence of physiological concentrations of testosterone, and without the addition of cofactors to provide an alternative to using prostate homogenates. It has previously been reported that prostate cancer cell lines DU145, PC-3 and LNCaP have 5α -reductase activity and are able to metabolise testosterone (Castagnetta *et al.*, 1990; Farley *et al.*, 1990; Damassa *et al.*, 1991; Bélanger *et al.*, 1991). In addition, the recently established prostate cancer cell line ALVA-31 is reported to convert testosterone to DHT (Loop *et al.*, 1993). For this reason testosterone metabolism in some of the prostate cancer cell lines was investigated.

3.2 RESULTS

3.2.1 Validation of Extraction Procedure for Testosterone and its Metabolites

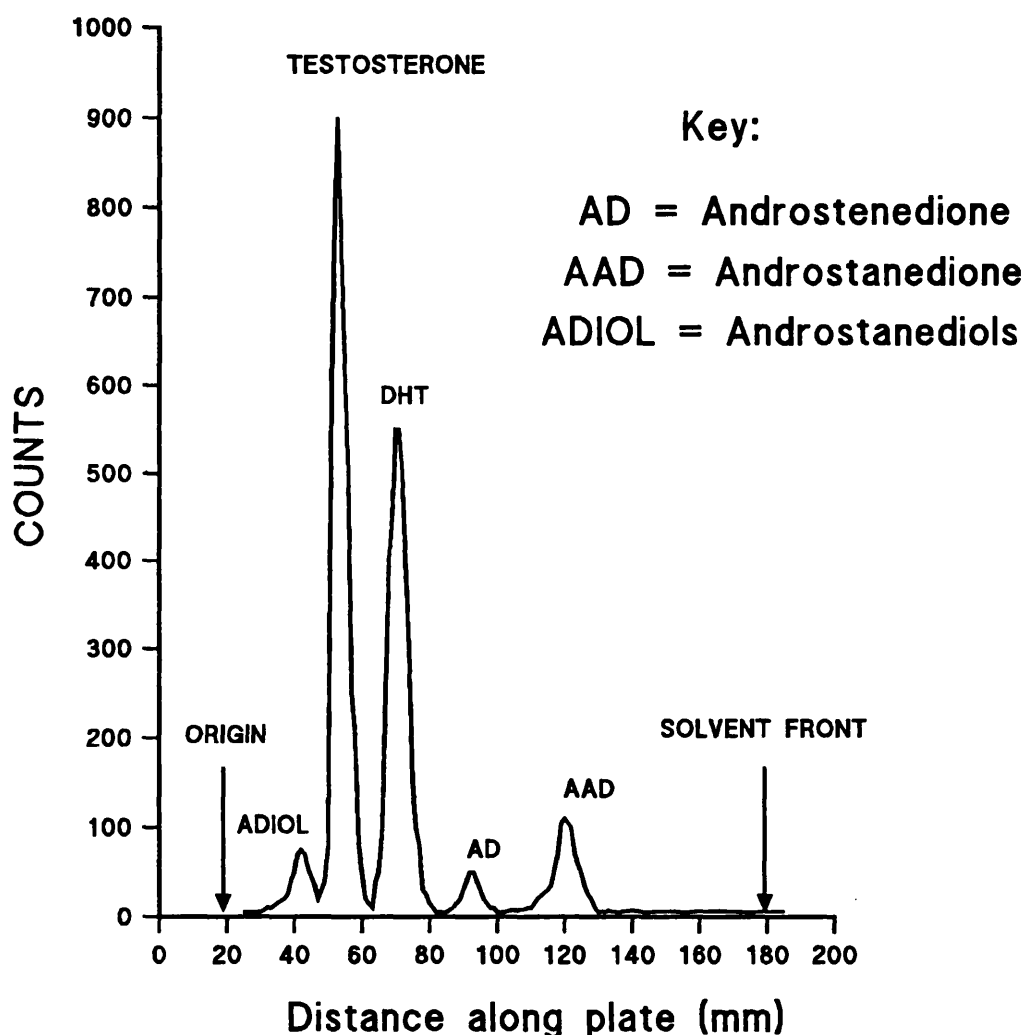
The recovery of 1 μ Ci of [3 H]-testosterone obtained by ethylacetate extraction of 1ml and 5ml RPMI medium after 1 h and 3 h incubations respectively at 37°C was 90-95%. TLC analysis of this extracted radioactivity demonstrated that it was 99.5% testosterone, showing that there was no significant conversion of testosterone by the medium. In each experiment blank incubations (in the absence of cells or tissue) were performed and analysed in the same way as the samples containing cells. The amount of radioactivity detected in the blanks (by the TLC plate analyser) associated with each individual metabolite was subtracted from that detected in the samples. Individual metabolites detected in blank incubations did not exceed 0.1% of the total radioactivity recovered.

3.2.2 Identification of Testosterone Metabolites

The identity of products formed by metabolism of [3 H]-testosterone in the prostate and the enzymes responsible for their formation have been extensively studied and characterised in the literature. For this reason it was considered acceptable to identify metabolites produced in the studies with prostate cells primarily by co-chromatography with authentic standards. The metabolites produced during incubation of prostate cells with [3 H]-testosterone were extracted with ethylacetate and separated using thin layer chromatography (TLC). TLC plates were analysed by a Radio-TLC-Analyser which measured the radioactivity associated with each separated TLC band. Figure 3.2a shows a typical radio-TLC trace of separated metabolites produced by BPH cell suspensions incubated for 1h with [3 H]-testosterone (20nM, 1 μ Ci).

FIGURE 3.2a

**RADIO-TLC ANALYSER SCAN OF A THIN LAYER CHROMATOGRAPHIC
SEPARATION OF [³H]-TESTOSTERONE RADIO-METABOLITES
PRODUCED BY HUMAN BPH CELLS**



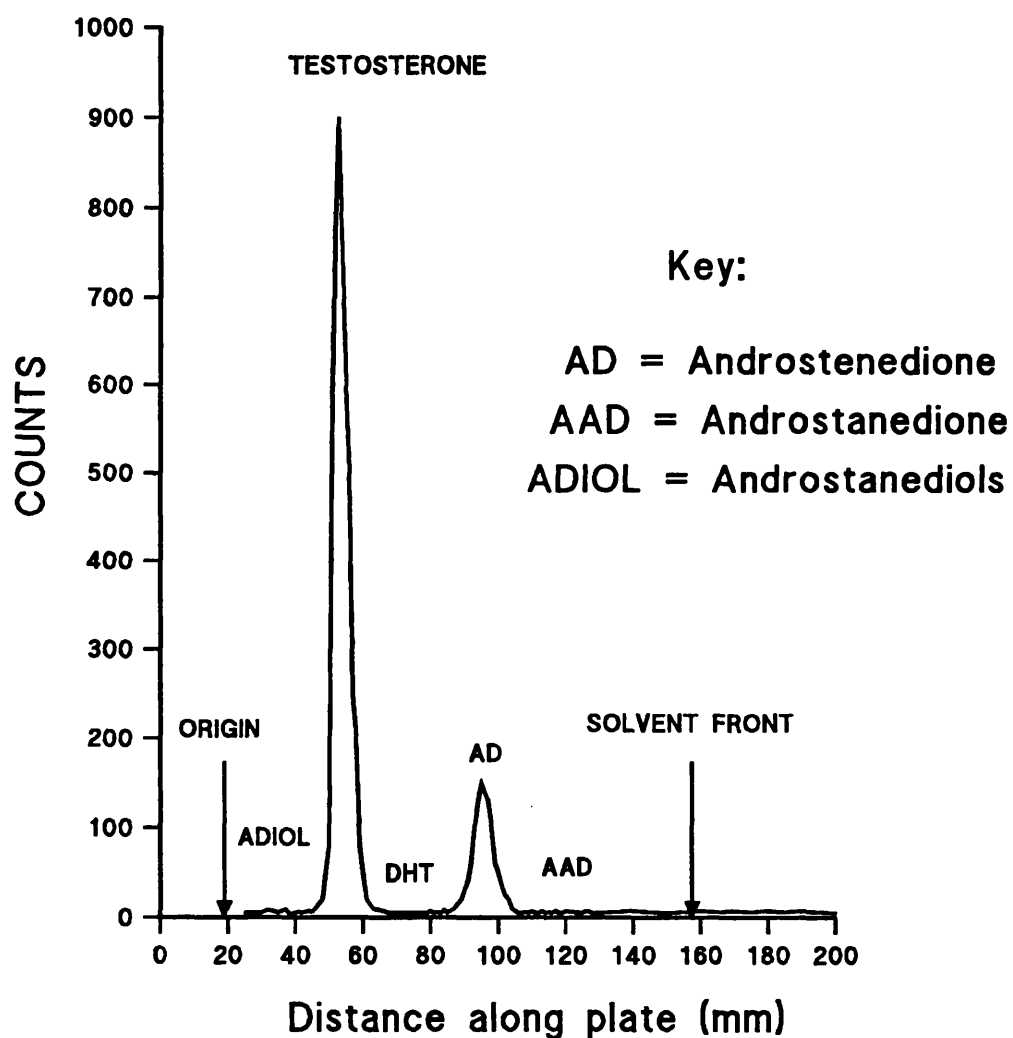
Suspensions of human BPH cells (protein 2-4mg/ml) were incubated with [³H]-testosterone (20nM, 1 μ Ci) for 1h. Metabolites were extracted with ethylacetate and separated by TLC using a solvent system of dichloromethane:acetone (12.3:1 v/v). The metabolites were identified by co-chromatography with authentic standards and uv absorption characteristics (testosterone and androstenedione positive; 5 α -reduced metabolites negative).

Radiolabelled metabolites were identified by comparing their TLC mobilities with those of authentic standards (R_f values are shown in Appendix 1) and by their uv absorption characteristics (testosterone and androstenedione positive; the 5α -reduced metabolites negative).

The solvent system of dichloromethane : acetone (12.3:1 v/v) separated DHT, androstenedione, androstanedione and androstane diols (but not the isomers 3α -androstanediol and 3β -androstanediol). This solvent system only partially resolved DHT from androsterone and epiandrosterone. These metabolites were therefore separated on aluminium oxide plates in a second solvent system of consisting toluene : diethylether (1:1, v/v) and identified by co-chromatography with authentic standards (R_f values in Appendix 2) and quantified by liquid scintillation counting. However, epiandrosterone was not formed in any of the in vitro systems examined and androsterone was present only in PC-3/MA2 as a minor metabolite. The selective 5α -reductase inhibitor finasteride was used to confirm the identity of 5α -reduced metabolites; DHT, androstanedione and androstane diols. The formation of these metabolites was completely inhibited by 100nM finasteride (Figure 3.2b)

FIGURE 3.2b

**RADIO-TLC ANALYSER SCAN OF A THIN LAYER CHROMATOGRAPHIC
SEPARATION OF [³H]-TESTOSTERONE RADIOMETABOLITES
PRODUCED BY HUMAN BPH CELLS INCUBATED WITH FINASTERIDE**



Suspensions of human BPH cells (protein 2-4mg/ml) were incubated with [³H]-testosterone (20nM, 1 μ Ci) in the presence of finasteride (100nM). Metabolites were separated and identified as in Figure 3.2a.

3.2.3 Androgen Metabolism in Human BPH Slices

Small slices of human BPH tissue converted approximately 50% of added testosterone to metabolites during a 24 h incubation period. At this time point DHT accounted for 70% of total metabolites; other 5α -reduced androgens, androstenedione and androstenediols constituted 25% of total metabolites (Table 3.1). The remaining 5% was androstenedione.

Approximately 80-85% of added radioactivity was recovered from the medium and only 5-10% from the tissue. The amount of unmetabolised testosterone in the tissue was always lower than in the medium, indicating that the high metabolic capacity of the tissue prevented testosterone accumulating to the same level as in the external medium.

3.2.4 Androgen Metabolism in Human BPH Cells

3.2.4.i Testosterone Metabolism

The suspensions of prostate cells produced by collagenase digestions of human BPH tissue were found to have a high capacity for conversion of [^3H]-testosterone in the absence of exogenously added NADPH (Figure 3.2a). Metabolism of [^3H]-testosterone (20nM, 1 μCi) by cell suspensions (protein concentration (2-4mg/ml) was linear during the first hour (Figure 3.3) with an estimated conversion rate of 6.4pmoles testosterone / mg protein / h (Table 3.2).

TLC analysis of metabolites indicated that most of the radioactivity was associated with DHT. This was confirmed by the use of finasteride (100nM) which inhibited the production of DHT by over 90% (Figure 3.2b). Total recovery of radioactivity after a 1 h incubation was 80-85%.

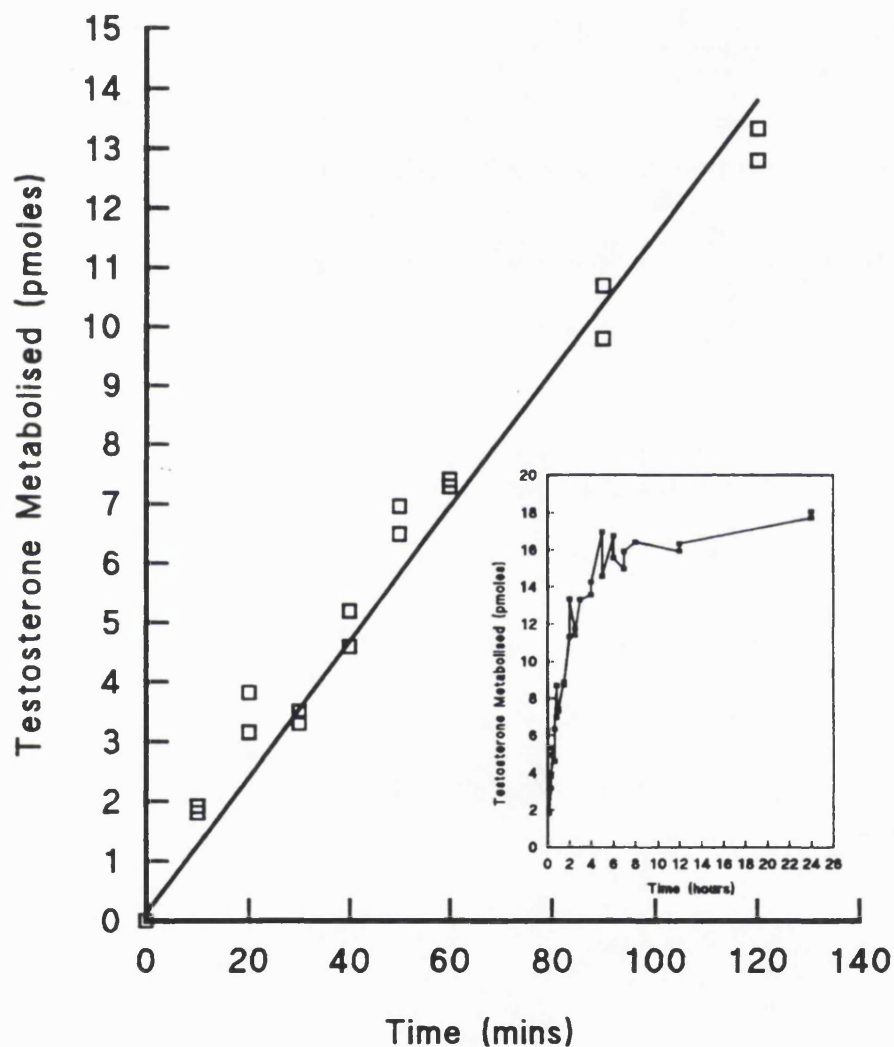
TABLE 3.1 METABOLITES OF TESTOSTERONE FORMED BY BPH SLICES, BPH CELLS AND HUMAN PROSTATE CANCER CELL LINES

In Vitro System	% TOTAL METABOLITES					
	ADIOLS	DHT	AEDIONE	AADIONE	A	T-G
BPH SLICES	11 ± 6	71 ± 7	2 ± 3	16 ± 5	(-)	(-)
BPH CELLS (1h)	4 ± 0.4	88 ± 1	2 ± 0.2	6 ± 0.9	(-)	(-)
BPH CELLS (24h)	8 ± 1.2	75 ± 3	5 ± 2.4	12 ± 2.4	(-)	(-)
HPC-36M	8 ± 1.4	82 ± 2	8 ± 3	2 ± 1	(-)	(-)
DU145	23 ± 4	68 ± 5	9 ± 4	(-)	(-)	(-)
PC-3/MA2	(-)	(-)	84 ± 2	15 ± 1	1 ± 0.2	(-)
LNCaP	(-)	(-)	(-)	(-)	(-)	100

BPH slices (24h incubation, n=10), BPH cells (protein 2-4mg/ml, 1h and 24h incubation, n=11), and prostate cancer cell lines (monolayers in 25cm² flasks, 3h incubation, n=4) were incubated with [³H]-testosterone (20nM, 1μCi). Metabolites in solvent extracts were separated by TLC in a solvent system consisting of dichloromethane:acetone (12.3:1 v/v) and quantified using a radio-TLC plate reader. The identity of metabolites was confirmed by co-chromatography with authentic standards and uv absorption (testosterone and androstenedione). Results are expressed as percentage total metabolites (mean ± SD). (-) = not detected; ADIOLS = androstanediols; AEDIONE = androstenedione; AADIONE = androstanedione; A = androsterone; T-G = testosterone-glucuronide.

FIGURE 3.3

TIME COURSE OF [³H]-TESTOSTERONE METABOLISM BY HUMAN BPH CELLS.



Human BPH cell suspensions (protein 2-4mg/ml) were incubated with [³H]-testosterone (20nM, 1 μ Ci) and at each time point metabolites were extracted and separated by TLC. The TLC plate was scanned using a radio-TLC plate analyser and the results were expressed as percent conversion of total substrate added. The figure shows the results of one experiment of two replicate incubations. The insert shows the time course over 24h.

TABLE 3.2

RATES OF TESTOSTERONE METABOLISM IN HUMAN BPH TISSUE SLICES AND CELL SUSPENSIONS AND IN HUMAN PROSTATE CANCER CELL LINES

In Vitro System	Rate Testosterone conversion (pmoles /mg protein/h)
BPH Cells	6.4 ± 0.2
HPC-36M	0.046 ± 0.001
DU145	0.089 ± 0.017
PC-3/MA2	12.8 ± 1.2
LNCaP	7.4 ± 1.5

Rates ± S.D were estimated using data from 4 separate experiments in HPC-36M and DU145, from 6 separate 1h incubation experiments in BPH cell suspensions and from single time course experiments in PC-3/MA2 and LNCaP. Testosterone concentration in all experiments was 20nM (1 μ Ci).

The profiles of metabolites produced after 1 h and 24 h incubation times are shown in Table 3.1. Turnover of substrate during 1h incubations varied from 40% to 70% in different experiments, but the overall profiles of metabolism remained consistent between experiments, with DHT accounting for greater than 85% of total metabolites. Increasing the incubation time from 1 h to 24 h resulted in the production of more of the secondary metabolites, androstanediols and androstenedione, formed by further metabolism of DHT and androstenedione (see Figure 3.1). Nevertheless, DHT still accounted for 75% of total metabolites.

3.2.4.ii Androstenedione Metabolism

The back-metabolism of androstenedione by 17 β -hydroxysteroid dehydrogenase (17 β -HSD-reductase) results in the formation of a more potent androgen, testosterone, which in BPH tissue would be rapidly converted to DHT. For this reason BPH cell suspensions were incubated with androstenedione to investigate the potential contribution of this pathway to the overall profile of metabolism in the prostate.

Table 3.3 shows that the 5 α -reduced metabolite, androstenedione, was the major product when cell suspensions were incubated with [3 H]-androstenedione (20nM, 1 μ Ci) for 1 h; testosterone and DHT accounted for less than 1% of the total radioactivity recovered, indicating relatively low levels of 17 β -HSD-reductase activity. Approximately 20% of the [3 H]-androstenedione was converted in untreated BPH cells but in the presence of finasteride (100nM) this was reduced to only 1% turnover (Table 3.3) confirming that the pathway of metabolism of androstenedione in prostate cell is almost exclusively via 5 α -reductase.

TABLE 3.3

ANDROSTENEDIONE METABOLISM IN HUMAN BPH CELLS

BPH Cells	% Total Radioactivity Recovered.			
	Testosterone	DHT	AEDIONE	AADIONE
Untreated	0.6 ± 0.1	0.4 ± 0.1	82 ± 2	17 ± 0.7
Finasteride (100nM)	0.3 ± 0.1	0.3 ± 0.1	99 ± 0.4	0.4 ± 0.1

BPH cells (protein 2-4mg/ml) were incubated with [³H]-androstenedione (20nM, 1μCi) for 1h. Metabolites in solvent extracts were separated by TLC using a solvent system consisting of dichloromethane:acetone (12.3:1 v/v). Radioactive metabolites were quantified using a radio-TLC plate analyser. The results (mean ± SD) are from 2 experiments each consisting of 3 replicates.

AEDIONE = androstenedione; AADIONE = androstanedione.

3.2.5 Androgen Metabolism in Human Prostate Cancer Cell Lines

3.2.5.i Rates of Testosterone Metabolism

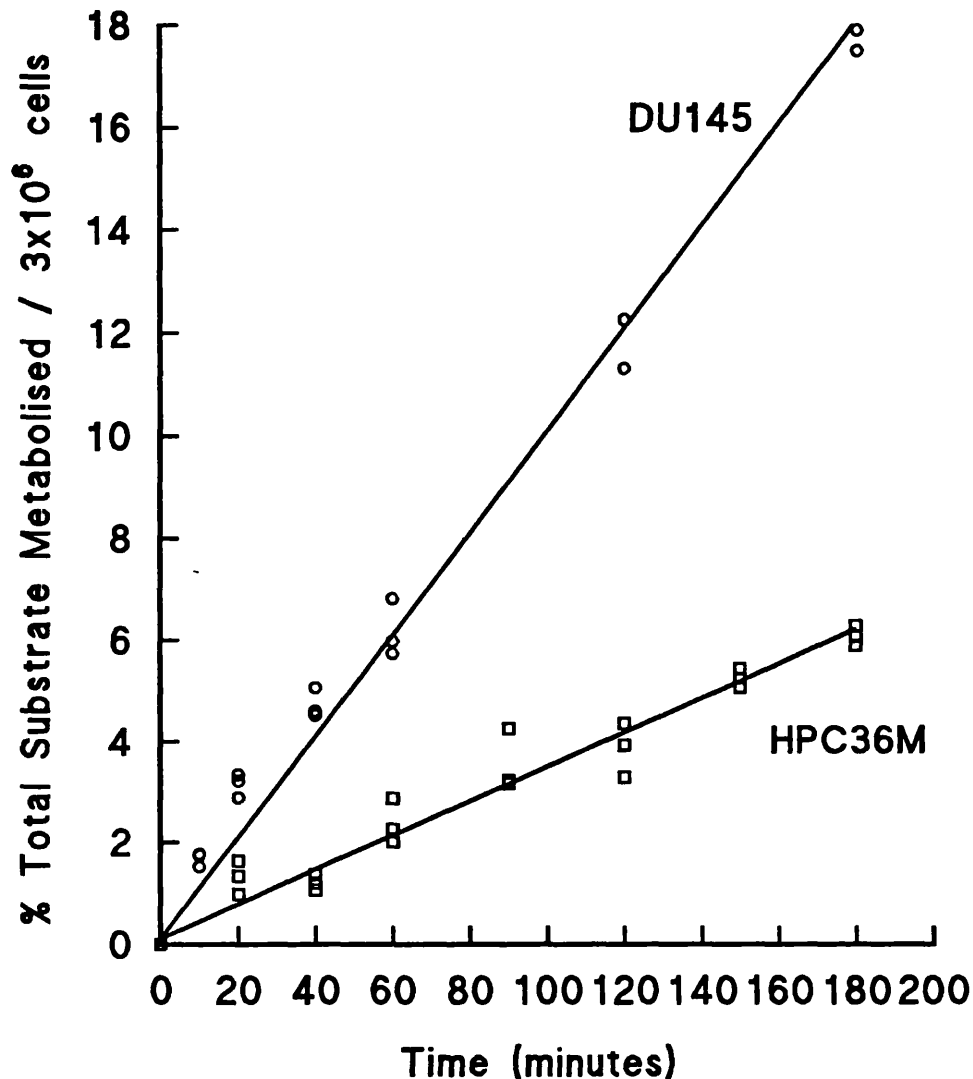
Table 3.2 shows that the two cell lines with the highest rates of testosterone metabolism were PC-3/MA2 (12.8 ± 1.2 pmoles testosterone / mg protein / h) and LNCaP (7.4 ± 1.5 pmoles testosterone / mg protein / h). In contrast, DU145 and HPC-36M had lower rates of metabolism (0.089 ± 0.001 and 0.046 ± 0.017 pmoles testosterone / mg protein / h) respectively. The rate of metabolism in PC-3/MA2 was twice the rate in BPH cell suspensions, while the rate of metabolism in LNCaP was approximately equivalent to that in BPH cells. In comparison, the rates of testosterone metabolism in DU145 and HPC-36M cells were 80 and 150 times lower than that in BPH cells.

3.2.5.ii Testosterone Metabolism in HPC-36M & DU145

The conversion of testosterone to [^3H]-labelled products was linear with time up to 180 min (Figure 3.4). In both cell lines DHT was the major metabolite accounting for 80% and 68% of total metabolites in HPC-36M and DU145 respectively. HPC-36M also converted testosterone to androstenedione and the secondary metabolites, androstanedione and androstanediols, were also detected (Table 3.1); these accounted for the remaining 20% of metabolites. DU145 formed more androstanediols than HPC-36M (23% of total metabolites compared with 8%) but androstanedione was not detected.

FIGURE 3.4

**TIME COURSE OF [³H]-TESTOSTERONE METABOLISM BY HPC-36M
AND DU145**



The prostate cancer cell lines HPC-36M and DU145 (3×10^6 cells/ml) were incubated with [³H]-testosterone (20nM, 1 μ Ci) and metabolites were extracted and separated by TLC in a solvent system of dichloromethane:acetone (12.3:1 v/v). The TLC plate was scanned using a radio-TLC plate analyser and the sum of the metabolites formed used to calculate percentage [³H]-testosterone conversion. The figure shows the results of one experiment of three replicate incubations.

3.2.5.iii Androgen Metabolism in PC-3/MA2

PC-3/MA2 metabolised testosterone primarily to androstenedione. This contrasts with the formation of DHT as the major metabolite in BPH cells, HPC-36M and DU145. After a 3h incubation period of PC-3/MA2 with [³H]-testosterone (20nM, 1μCi) androstenedione accounted for 84% of the total metabolites, androstanedione for 15%, and androsterone for 1% of total metabolites (Table 3.1). DHT was not detected when PC-3/MA2 was incubated with [³H]-testosterone.

During a 1 h incubation period PC-3/MA2 converted [³H]-androstenedione (20nM, 1μCi) primarily to androstanedione (6% of total radioactivity), while conversion of androstenedione to testosterone and androsterone was low (less than 1% total radioactivity). When finasteride (100nM) was included in the incubation mixture, less than 1% of the radioactivity recovered was associated with androstanedione (Table 3.4), confirming the presence of 5α-reductase in the PC-3/MA2 cell line.

3.2.5.iv Testosterone Metabolism in LNCaP

Following 3 h incubation of LNCaP with [³H]-testosterone, the majority of product formed was non-extractable with ethylacetate and less than 5% of added radioactivity was recovered from the medium. This was in contrast to 80-85% in the other cell lines. A time course experiment demonstrated the rapid loss of ethylacetate-extractable radioactivity from the medium and a concomitant increase in the radioactivity in the aqueous phase (Figure 3.5).

TABLE 3.4

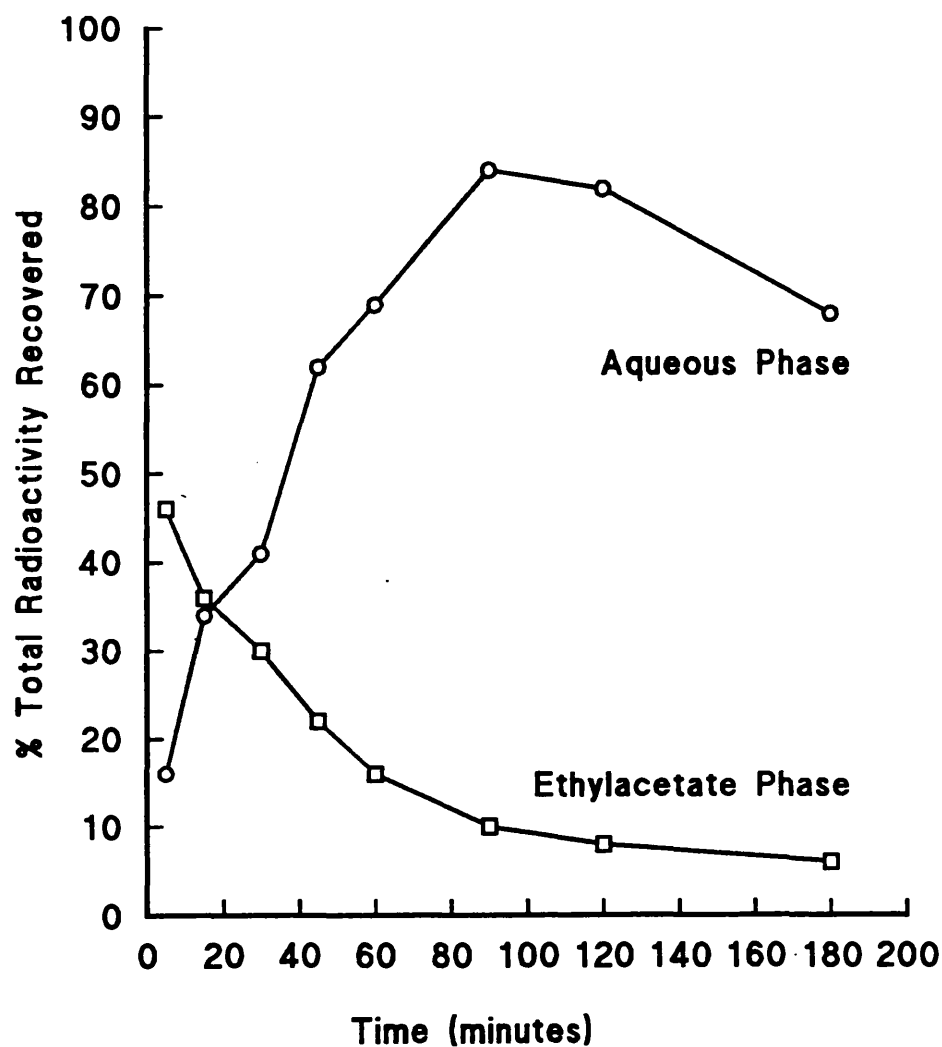
ANDROSTENEDIONE METABOLISM IN PC-3/MA2

PC-3/MA2 cells	% Total Radioactivity Recovered.			
	Testosterone	DHT	AEDIONE	AADIONE
Untreated	0.7 ± 0.1	0.7 ± 0.7	90.8 ± 0.2	7.8 ± 0.2
Finasteride (1µM)	0.8 ± 0.1	0.3 ± 0.7	98.2 ± 0.3	0.8 ± 0.3

PC-3/MA2 cells (3×10^6 /ml) were incubated with [^3H]-androstenedione (20nM, 1µCi) for 1h at 37°C. Metabolites in solvent extracts were separated by TLC using a solvent system consisting of dichloromethane:acetone (12.3:1 v/v). Metabolites were quantified using a radio-TLC plate analyser. Results (mean ± SD) are expressed as percentage total radioactivity recovered from 2 experiments. AEDIONE = androstenedione; AADIONE = androstanedione.

FIGURE 3.5

TIME COURSE OF FORMATION OF POLAR AND NON-POLAR METABOLITES OF TESTOSTERONE IN LNCaP



Monolayers of LNCaP cells in 25cm² flasks were incubated with [³H]-testosterone (20nM, 1μCi) for 3h. After ethylacetate extraction of the medium plus cells the percentage total radioactivity recovered in ethylacetate (non-polar) and aqueous (polar) fractions was determined by liquid scintillation counting. Polar = combined recoveries from aqueous fractions after ethylacetate extraction of the cells and medium. Non-polar = combined recoveries from ethylacetate fractions of cell and medium extractions.

Treatment of the aqueous phase with β -glucuronidase (400 units/ml) resulted in approximately 50% of the radioactivity being converted to an ethylacetate extractable-form (Table 3.5), which after TLC separation was found to consist of one compound which co-chromatographed with testosterone. In an attempt to saturate the glucuronidation pathway and reveal other enzyme activities in LNCaP the substrate concentration (testosterone) was increased by 100 fold to $2\mu\text{M}$. At this concentration the recovery of radioactivity was similar to that obtained at 20nM substrate (less than 5%) and no other metabolites were detected. This indicated that the rate of glucuronide formation at $2\mu\text{M}$ was approximately 100 fold greater than at 20nM, that is, had increased in direct proportion to the substrate concentration.

When LNCaP monolayers were incubated with [^3H]-androstenedione (20nM, $1\mu\text{Ci}$), which is not a direct substrate for conjugation, most of the radioactivity was recovered in the ethylacetate fraction as unconverted androstenedione. The 5α -reduced metabolite, androstanedione, accounted for approximately 10% of the radioactivity.

TABLE 3.5

RECOVERY OF RADIOACTIVITY FROM LNCaP CELLS BEFORE AND AFTER HYDROLYSIS WITH β -GLUCURONIDASE

	% Radioactivity Recovered From	
	Cells	Medium
<u>PRE-HYDROLYSIS:</u>		
Aqueous Fraction	6 \pm 1	76 \pm 8
Ethylacetate Fraction	4 \pm 0.6	2 \pm 0.8
<u>POST-HYDROLYSIS:</u>		
Aqueous Fraction	< 0.5	33 \pm 20
Ethylacetate Fraction	4 \pm 1	26 \pm 10

Monolayers of LNCaP (T25) were incubated with [3 H]-testosterone (100nM, 1 μ Ci) for 3h. After ethylacetate extraction of cells and medium the recovery of radioactivity was determined before and after treatment with β -glucuronidase (400units/ml, 20h, pH4.5) by liquid scintillation counting. Recoveries are expressed as percent of total radioactivity initially added to the monolayers as [3 H]-testosterone recovered from the medium and cells (mean \pm S.D., 4 replicates).

The aim of this chapter was to study testosterone metabolism in BPH tissue and prostate cancer cell lines and identify suitable in vitro systems for the study of androgen metabolism by human prostate cells. Analysis of metabolites and relative rates of formation were used to construct Table 3.6 which shows the relative levels of steroid metabolising enzyme activities in each of the cell systems and BPH slices.

The first major finding from the present study is that cell suspensions produced by collagenase digestion of human BPH tissue retained a high capacity for conversion of testosterone to 5α -reduced metabolites (DHT, androstanediols and androstanedione). The profile of metabolites, dominated by DHT, resembles that reported in human prostate after in vivo administration of [^3H]-testosterone (Becker *et al.*, 1975; Pike *et al.*, 1970, Harper *et al.*, 1974), and is also similar to that to that previously reported from studies in several human prostate in vitro systems (Farnsworth *et al.*, 1963; Robel *et al.*, 1971; Jenkins and McCaffery, 1974; McMahon *et al.*, 1974; Prout *et al.*, 1976; Morfin *et al.*, 1978; Bard and Lasnitzki, 1979; Smith *et al.*, 1983). In addition, the present study demonstrated 5α -reductase activity, for the first time, in the prostate cancer cell line HPC-36M as well confirming its existence in DU145, PC-3/MA2 and LNCaP (Table 3.6) (Castagnetta *et al.*, 1990; Kaighn *et al.*, 1980; Farley *et al.*, 1990; Damassa *et al.*, 1991 and Bélanger *et al.*, 1991).

This study also showed that the ability of the prostate cancer cell lines to form DHT was much lower than BPH cells. Furthermore, the only two cell lines to form DHT as the primary metabolite, DU145 and HPC-36M, had the lowest rate of testosterone metabolism.

TABLE 3.6

ANDROGEN METABOLISING ENZYME ACTIVITIES IN HUMAN BPH TISSUE
AND HUMAN PROSTATE CANCER CELL LINES

	5 α - Reductase	17 β -HSD	3 α / β - HSD	Glucuronyl- Transferase
Prostate <u>in vivo</u> *	+++	++	++	not reported
BPH cells	+++	++	++	ND
BPH Slices	+	+	+	ND
HPC-36M	+	+	+	ND
DU145	+	+	+	ND
PC-3/MA2	++	+++	+	ND
LNCaP	+	(-)	(-)	+++

The relative activities were assessed as follows: 5 α -reductase: from the sum of 5 α -reduced metabolites formed; 17 β -HSD: from the sum of androstenedione and androstanedione formed and 3 α / β -HSD: from the amount of androstanediols formed.

17 β -HSD = 17 β -hydroxysteroid dehydrogenase; 3 α / β = 3 α / β -hydroxysteroid dehydrogenase. * from Harper *et al.* (1974). (-) = not detected; ND = not determined

Key: + = less than 2% total radioactivity recovered
 ++ = 2-20% total radioactivity recovered
 +++ = more than 20% total radioactivity recovered

In the present study a low rate of testosterone metabolism was not a characteristic of all prostate cancer cell lines investigated. The rate of testosterone metabolism in LNCaP was equivalent to that in BPH cells, while the rate in PC-3/MA2 was twice that in BPH cells. In contrast to BPH cells, a high rate of metabolism in PC-3/MA2 and LNCaP did not result in the production of DHT.

The balance of steroid metabolising enzymes in PC-3/MA2 and LNCaP favours the rapid removal of potent androgens (testosterone and DHT). This is in contrast the situation in BPH where the balance of enzyme activity favours the formation and accumulation of DHT in the tissue (Siiteri and Wilson, 1970; Geller *et al.*, 1976; Hammond, 1978; Krieg *et al.*, 1979; Belis, 1980), and prostate cancer where the reduced ability of the tissue to form DHT leads to the accumulation of testosterone (Farnsworth *et al.*, 1976; Voigt and Bartsch, 1986; Krieg *et al.*, 1979; Habib *et al.*, 1976; Hammond, 1978; Ghanadian and Puah, 1981).

Formation of androstenedione by PC-3/MA2 demonstrates a shift from a reductive pathway of metabolism to an oxidative pathway. Such a shift has been observed in poorly differentiated tumours and tumours treated with oestrogens (Jenkins and McCaffery, 1974; Bard and Lasnitzki, 1977; Morfin *et al.*, 1979; Smith *et al.*, 1983). It was an interesting observation that in this study both PC-3/MA2 cells and BPH cells did not convert androstenedione to testosterone suggesting that under the present experimental conditions the conversion of adrenal-derived androstenedione to the more potent androgens testosterone and DHT is not a major pathway.

The production of testosterone glucuronide by LNCaP demonstrates the existence of an enzyme activity (UDP-glucuronyltransferase) more commonly associated with the liver, kidneys and lung (Mackenzie *et al.*, 1992). The finding from this study supports the previous report of a high rate of DHT metabolism and the

formation of polar metabolites by Berns *et al.* (1986) and the formation of DHT glucuronide and androsterone glucuronide by Damassa *et al.* (1991) and Belanger *et al.* (1991) respectively.

Glucuronidation of steroids is a process involving the covalent addition of a bulky sugar acid to the steroid that renders it less biologically active and more readily eliminated in bile and urine (Mackenzie *et al.*, 1992). Several forms of the enzyme UDP-glucuronyltransferase, that catalyses this reaction, exist. Two forms are known to act on steroids: one glucuronidates testosterone, DHT and oestradiol at position 17 and the other glucuronidates androsterone and epiandrosterone at position 3. The data from the present study lends support for the existence, in LNCaP, of the form of enzyme that glucuronidates at position 17.

UDP-glucuronyl transferase exists in human and rat prostate where formation of glucuronides is a minor route of metabolism (Chung and Coffey, 1977, 1978). The formation of androgen glucuronides accounts for 1.5% of total metabolites produced after 1 h incubation of BPH tissue with [³H]-testosterone (Chung and Coffey, 1978). Testosterone-glucuronide is the predominant glucuronide formed by normal tissue, while BPH tissue forms mainly DHT-glucuronide (Chung and Coffey, 1978). In LNCaP formation of testosterone-glucuronide is much greater than that reported for normal and BPH tissue and contrasts the high level of DHT formed in BPH tissue.

In summary, the present study demonstrated that cell suspensions obtained from BPH tissue by collagenase digestion were intact, viable and metabolically active. The assay is carried out in the presence of physiological concentrations of testosterone without added cofactors. This, and a number of other features, indicates the suitability of the BPH cell system for studying androgen metabolism and testing 5 α -reductase inhibitors in vitro. Firstly, because the digested tissue

retains such high activity multiple assays (investigating the effects of inhibitors) can be performed with a relatively small amount of tissue, also the separation of the cells allows rapid uptake of substrate and release of products. In contrast, the use of BPH tissue slices requires a larger amount of tissue and longer incubation times to allow uptake of substrate and exit of products to occur through the layers of cells. However, tissue slices are useful for determining the effectiveness of inhibitors at penetrating several layers of prostate tissue. As all of the TURP specimen is used to prepare the cell suspensions, each assay replicate contains similar material, providing a reproducible system. This is not the case for tissue slices which may have different proportions of stromal and epithelial cells.

In spite of a low rate of DHT formation in HPC-36M and DU145 compared to BPH cells, these cell lines are useful in vitro models for studying androgen metabolism and testing 5 α -reductase inhibitors. They have the advantage of being readily available and totally reproducible. PC-3/MA2 and LNCaP are less useful cell lines for testing 5 α -reductase inhibitors because DHT is not the primary metabolite. This study showed that HPC-36M and DU145 have 5 α -reductase activity but did not identify which form(s) of 5 α -reductase enzyme may be responsible. It is important to determine which 5 α -reductase isozymes are present in DU145 and HPC-36M cells so that they may be fully evaluated as models for the testing 5 α -reductase inhibitors. This question forms the basis of the next chapter.

CHAPTER FOUR

BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERISATION OF 5 α -REDUCTASE ACTIVITY IN HUMAN BPH TISSUE SLICES, CELL SUSPENSIONS AND HUMAN PROSTATE CANCER CELL LINES

4.1 INTRODUCTION

The prostate contains two forms of 5 α -reductase enzyme, designated 5 α -reductase 1 and 5 α -reductase 2, which are coded for by two different genes (Andersson *et al.*, 1991). These isozymes have not been purified from human prostate but have been characterised by studying the protein expressed following transfection of their complementary DNA (cDNA) sequences into cells which do not normally express 5 α -reductase such as simian COS cells (Andersson and Russell, 1990) and human embryonic kidney 293 cells (Andersson *et al.*, 1991).

Using this approach comparative studies have shown that the two isozymes differ in their biochemical and pharmacological as well as genetic properties. The two isozymes have greatest DHT-forming activity at different pH values. Greatest 5 α -reductase 1 activity occurs over a broad range of pH values from 6.0 - 8.5, while activity of 5 α -reductase 2 is greatest over a narrow pH range of 5.0 - 5.5 (Andersson *et al.*, 1991; Jenkins *et al.*, 1992). In comparison, homogenates of human prostate tissue have greatest DHT-forming activity at pH 5.0 - 5.5 (Liang *et al.*, 1985; Jenkins *et al.*, 1992).

The competitive 5 α -reductase inhibitor finasteride is a potent inhibitor of DHT production in human prostate homogenates with an IC₅₀ value of approximately 10nM (Liang *et al.*, 1985; Harris *et al.*, 1992; Jenkins *et al.*, 1992; Mellin *et al.*, 1993). Studies have shown that finasteride is a selective inhibitor of 5 α -reductase 2 with an IC₅₀ value of 30nM (Andersson *et al.*, 1991) compared to an IC₅₀ value

in the range 1-10 μ M for 5 α -reductase 1 (Andersson and Russell, 1990; Andersson *et al.*, 1991; Harris *et al.*, 1992; Jenkins *et al.*, 1992). The most potent inhibitors of 5 α -reductase seem to include those which have a steroid moiety and a lipophilic side chain on carbon-17 such as finasteride, SKF-105,657 and 4-MA shown in Figure 1.6 (Liang *et al.*, 1985; Harris *et al.*, 1992; Lamb *et al.*, 1992; Mellin *et al.*, 1993; Thigpen *et al.*, 1992b).

Studies relating the structure of the compound to the ability to inhibit 5 α -reductase indicates that the bulkier tertiary-butyl group on carbon-17 of inhibitors such as finasteride and SKF 105,657 determines the ability to inhibit 5 α -reductase 1 or 2 isozyme (Thigpen *et al.*, 1992b). Compounds with small aliphatic substituents at carbon-17, such as 4-MA, are more effective inhibitors of 5 α -reductase 1 (Harris *et al.*, 1992; Thigpen *et al.*, 1992b; Mellin *et al.*, 1993). However, inhibition of 5 α -reductase activity is not restricted to steroidal structures and non-steroidal 5 α -reductase inhibitors, such as ONO-3805 (Nakai *et al.*, 1985) have also been developed. A novel class of agents, the benzoquinolinones, have recently been reported as potent and selective inhibitors of 5 α -reductase 1 by Jones *et al.* (1993). The development of these various compounds provides useful tools for investigating the role of 5 α -reductase isozymes in different androgen-target tissues and the means to achieve a more effective, and perhaps a total inhibition of DHT production.

Biochemical and pharmacological evidence indicates that 5 α -reductase 2 is the major form of 5 α -reductase in the human prostate (Andersson and Russell, 1990; Andersson *et al.*, 1991). This interpretation was confirmed when genetic analysis of 5 α -reductase cDNAs isolated from some 5 α -reductase deficient male pseudohermaphrodites showed that there were defects (mutations and deletions) in the gene for 5 α -reductase 2 but not 5 α -reductase 1 (Andersson *et al.*, 1991; Thigpen *et al.*, 1992a).

The situation in human prostate where 5 α -reductase 2 is the main generator of DHT is in contrast to the rat ventral prostate where the 5 α -reductase 1 isozyme appears to account for 60% of the total DHT-forming activity (Normington and Russell, 1992). Until recently there was little experimental evidence to support the interpretation that human prostate actually expresses 5 α -reductase 1. The demonstration that human prostate is capable of expressing 5 α -reductase 1 has come from the work of Hirsch *et al.* (1993) who compared the sensitivity of 5 α -reductase in freshly isolated prostate cells to finasteride and LY191704 (a selective inhibitor of 5 α -reductase 1) with that in cultured prostate cells. Freshly isolated cells were sensitive to finasteride (IC_{50} = 16nM) and insensitive to LY191704 (IC_{50} = >1 μ M). In contrast, cultured prostate cells were more sensitive to LY191704 (IC_{50} = 12nM) than finasteride (IC_{50} = 102nM) leading Hirsch to conclude that the human prostate is capable of expressing both 5 α -reductase isozymes.

The approach of using the differential sensitivity of 5 α -reductase isozymes to inhibitors has also been used to characterise the 5 α -reductase activity in the human scalp (Harris *et al.*, 1992). The results, that finasteride and SKF, 105657 were over 100 and 500 fold respectively more selective for 5 α -reductase activity in prostatic homogenates than scalp homogenates and COS cells expressing 5 α -reductase 1, indicated that scalp 5 α -reductase resembled 5 α -reductase 1. In contrast, 4-MA was a potent inhibitor of 5 α -reductase activity in prostate, scalp homogenates and COS cell expressed 5 α -reductase 1.

Human prostate cancer cell lines all contain 5 α -reductase activity, and like BPH cells, have the potential to convert testosterone to DHT. However, although it is well established that the biochemical and pharmacological characteristics of 5 α -reductase activity in BPH resembles that of 5 α -reductase 2, the 5 α -reductase activity in the prostate cancer cell lines has not been characterised. The aim of this chapter was, therefore, to characterise the 5 α -reductase activity in HPC-36M

and DU145 using selective inhibitors of 5 α -reductase 1 and 5 α -reductase 2. Selective inhibitors were identified and profiled in COS cells expressing 5 α -reductase 1 and 2 isozymes. The sensitivity of the 5 α -reductase activity in DU145 and HPC-36M to the selective inhibitors was then compared to that of BPH cells.

The inhibitors used were finasteride, SKF 105,657, 4-MA and one non-steroidal compound, known to be potent inhibitor of 5 α -reductase 1, but a weak inhibitor of 5 α -reductase 2 isozyme, UK 117,026 (Pfizer compound, unpublished data).

4.2 RESULTS

4.2.1 Characterisation of 5 α -reductase Activity Expressed in COS Cells Transfected with 5 α -Reductase 1 and 2 cDNAs

4.2.1.i Testosterone Metabolism

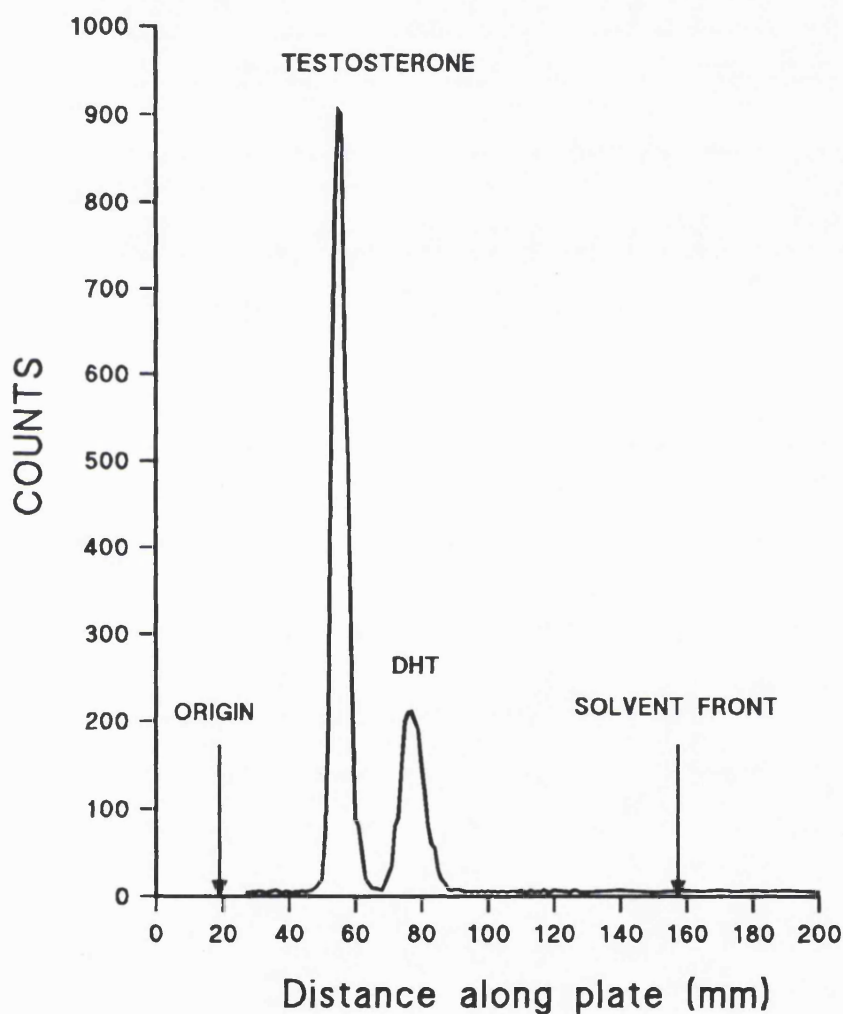
Separate homogenates of simian COS cells transfected with expression vectors containing identical sequences to the cDNAs coding for the human 5 α -reductase 1 and 2 isozymes (Andersson and Russell, 1990; Andersson *et al.*, 1991) were prepared 48 h after transfection. The homogenates had high levels of 5 α -reductase activity as indicated by conversion of testosterone to DHT. Incubation of cell homogenates with [3 H]-testosterone (20nM, 1 μ Ci) in the presence of an NADPH regeneration system produced only one metabolite which after TLC analysis was shown to be DHT (Figure 4.1a).

No metabolites were detected when homogenates of mock transfected cos cells were incubated with [3 H]-testosterone under identical conditions to 5 α -reductase cDNA transfected cells (Figure 4.1b).

Using a 30 min incubation time conversion of [3 H]-testosterone to DHT by both isozymes was still linear with increasing protein concentration up to 2.5 μ g/ml (Figure 4.2). Homogenates of transfected COS cells were diluted so that a 50 μ l aliquot of homogenate converted 15-20% of the substrate when incubated with [3 H]-testosterone (20nM, 1 μ Ci) at 37°C for 30 min. For COS cells expressing 5 α -reductase 1 activity this translated to 2.5 μ g protein/ml in pH 7.5 buffer and for COS cells expressing 5 α -reductase 2 activity this was 2 μ g protein/ml in pH 5.2 buffer.

FIGURE 4.1a

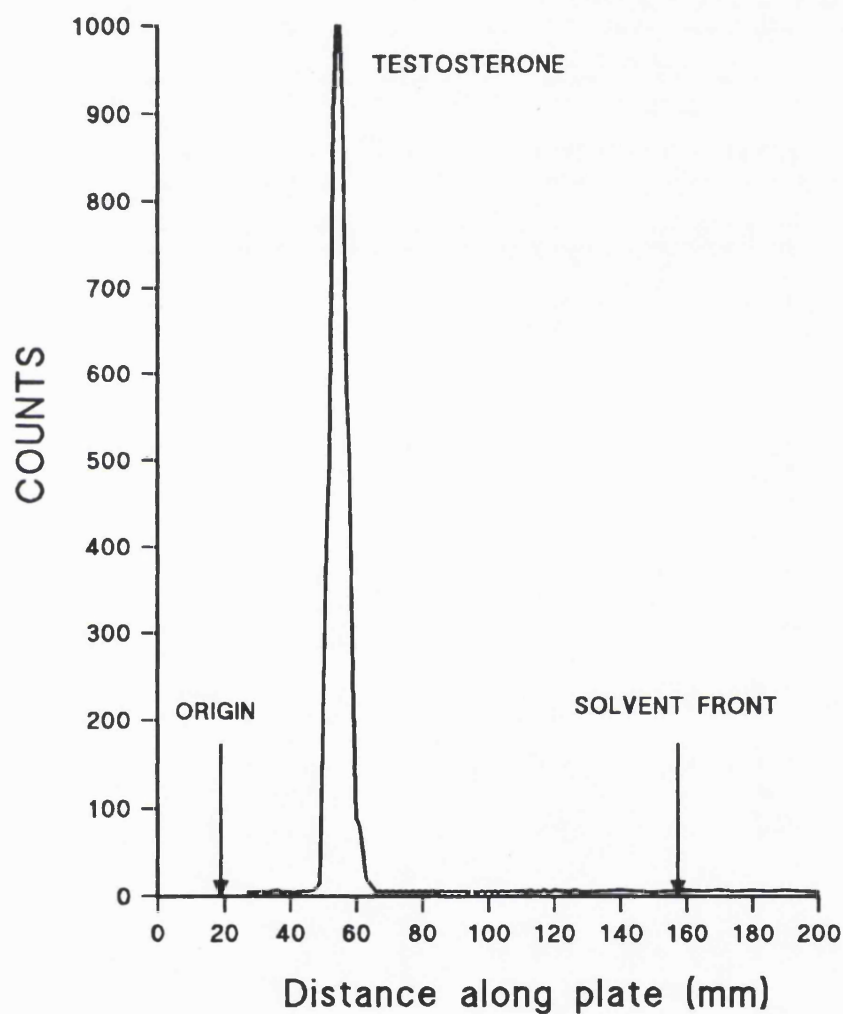
**RADIO-TLC ANALYSER SCAN OF A THIN LAYER CHROMATOGRAPHIC
SEPARATION OF [³H]-TESTOSTERONE RADIO METABOLITES PRODUCED
BY COS CELLS EXPRESSING 5 α -REDUCTASE 2 ACTIVITY**



Homogenates of transfected COS cells expressing 5 α -reductase 2 activity (2 μ g protein/ml) were incubated at 37°C with [³H]-testosterone (20nM, 1 μ Ci) and an NADPH-regeneration system for 30min at pH 5.2. Testosterone and DHT were extracted with ethylacetate and separated by TLC using a solvent system of dichloromethane: acetone (12.3:1 v/v).

FIGURE 4.1b

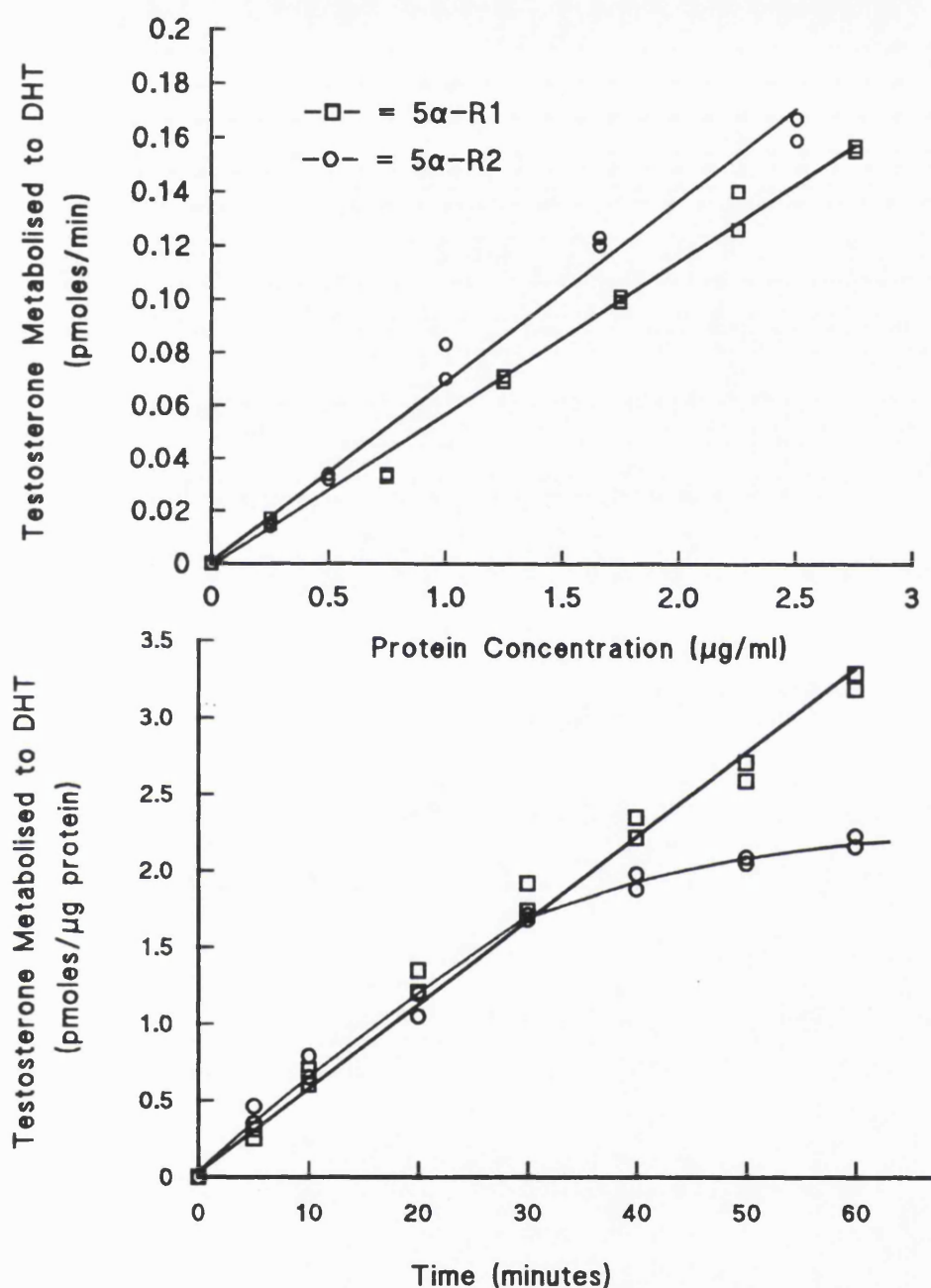
**RADIO-TLC ANALYSER SCAN OF A THIN LAYER CHROMATOGRAPHIC
SEPARATION OF [³H]-TESTOSTERONE RADIO-METABOLITES PRODUCED
BY MOCK TRANSFECTED COS CELLS**



Homogenates of mock transfected COS cells (2 μ g protein/ml) were incubated at 37°C with [³H]-testosterone (20nM, 1 μ Ci) and an NADPH-regeneration system for 30min at pH 5.2. Radioactivity was extracted with ethylacetate and separated by TLC using a solvent system of dichloromethane: acetone (12.3:1 v/v).

FIGURE 4.2

**EFFECT OF INCREASING PROTEIN CONCENTRATION AND TIME ON THE
ACTIVITY OF COS CELL EXPRESSED 5 α -REDUCTASE ISOZYMES**



Homogenates of COS cells expressing 5 α -reductase (5 α -R) 1 or 2 activity were incubated with [3 H]-testosterone (20nM, 1 μ Ci) and an NADPH-regeneration system at 37°C using different protein concentrations (top) and different incubation times (bottom). A 30min incubation time was used for the top graph and protein concentrations for the time course were 2 μ g and 2.5 μ g protein/ml for 5 α -R1 and 2 respectively. Testosterone and DHT were extracted with ethylacetate and separated by TLC. The TLC plates were scanned with a radio-TLC plate reader to determine the amount of testosterone converted to DHT. Data shown are from single experiments with 2 replicates.

Conversion of DHT by 5 α -reductase 1 (protein concentration 2.5 μ g/ml) was linear with time for at least 60 min while 5 α -reductase 2 conversion (protein concentration 2 μ g/ml) was only linear with time until 30 min (Figure 4.2).

4.2.1.ii Determination of the Optimum pH for Maximum 5 α -Reductase 1 and 2 Activity

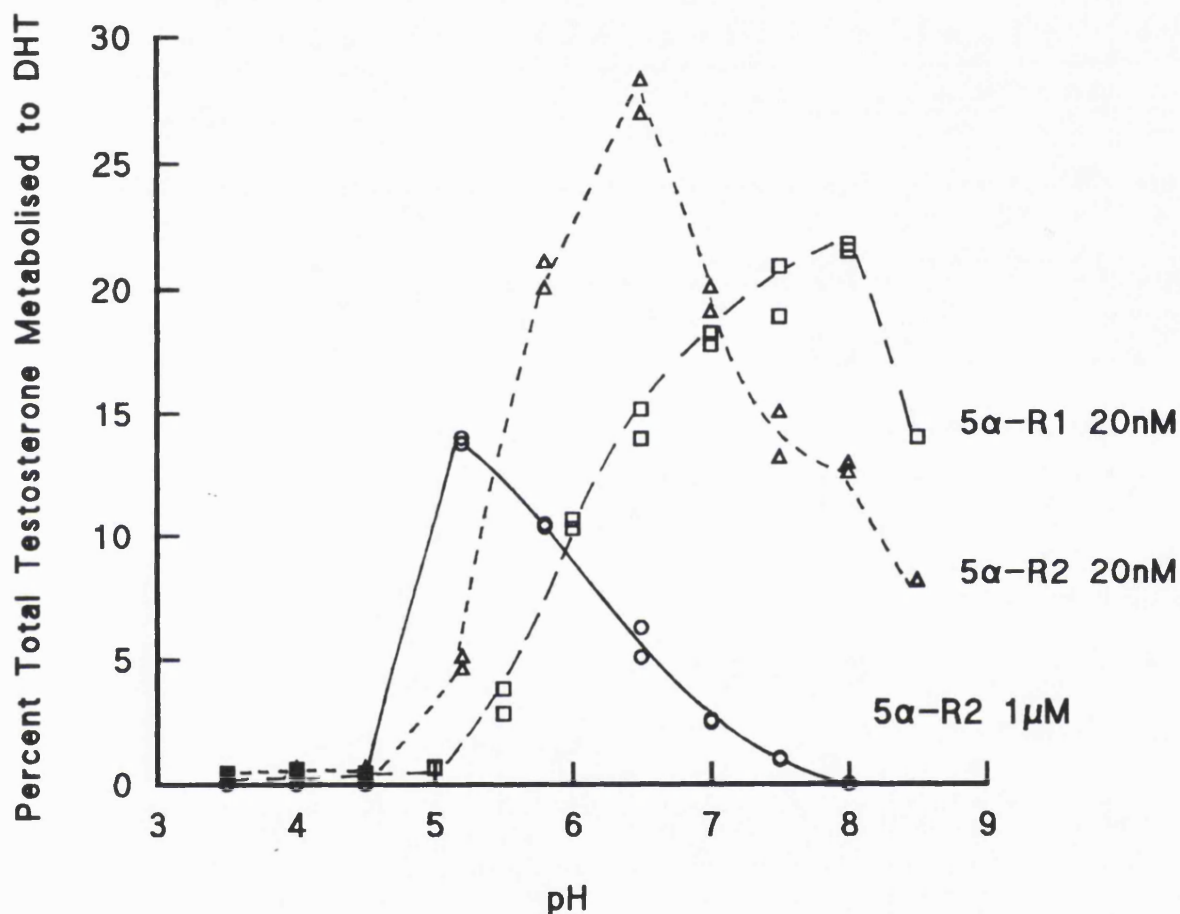
The pH at which conversion of testosterone to DHT was greatest was determined by incubating homogenates of COS cells expressing 5 α -reductase 1 and 2 activity over a range of different pH values (3.5-8.5).

Activity of 5 α -reductase 1 was greatest over the range 6.5-8.5 when incubated with 20nM testosterone (Figure 4.3). The pH profile was similar to that reported using 10 μ M testosterone substrate (Andersson *et al.*, 1991; Jenkins *et al.*, 1992). In contrast, lowering the substrate concentration altered the pH optimum of 5 α -reductase 2 isozyme. Using 1 μ M testosterone, maximum activity of 5 α -reductase 2 occurred between pH 5 and 5.5, while the activity was greatest between pH 6.0 and 7.0 when the substrate concentration was lowered to 20nM (Figure 4.3).

No difference in the pH optimum for maximum activity was observed in BPH tissue homogenates incubated with 20nM testosterone compared to 1 μ M testosterone (Figure 4.4)

FIGURE 4.3

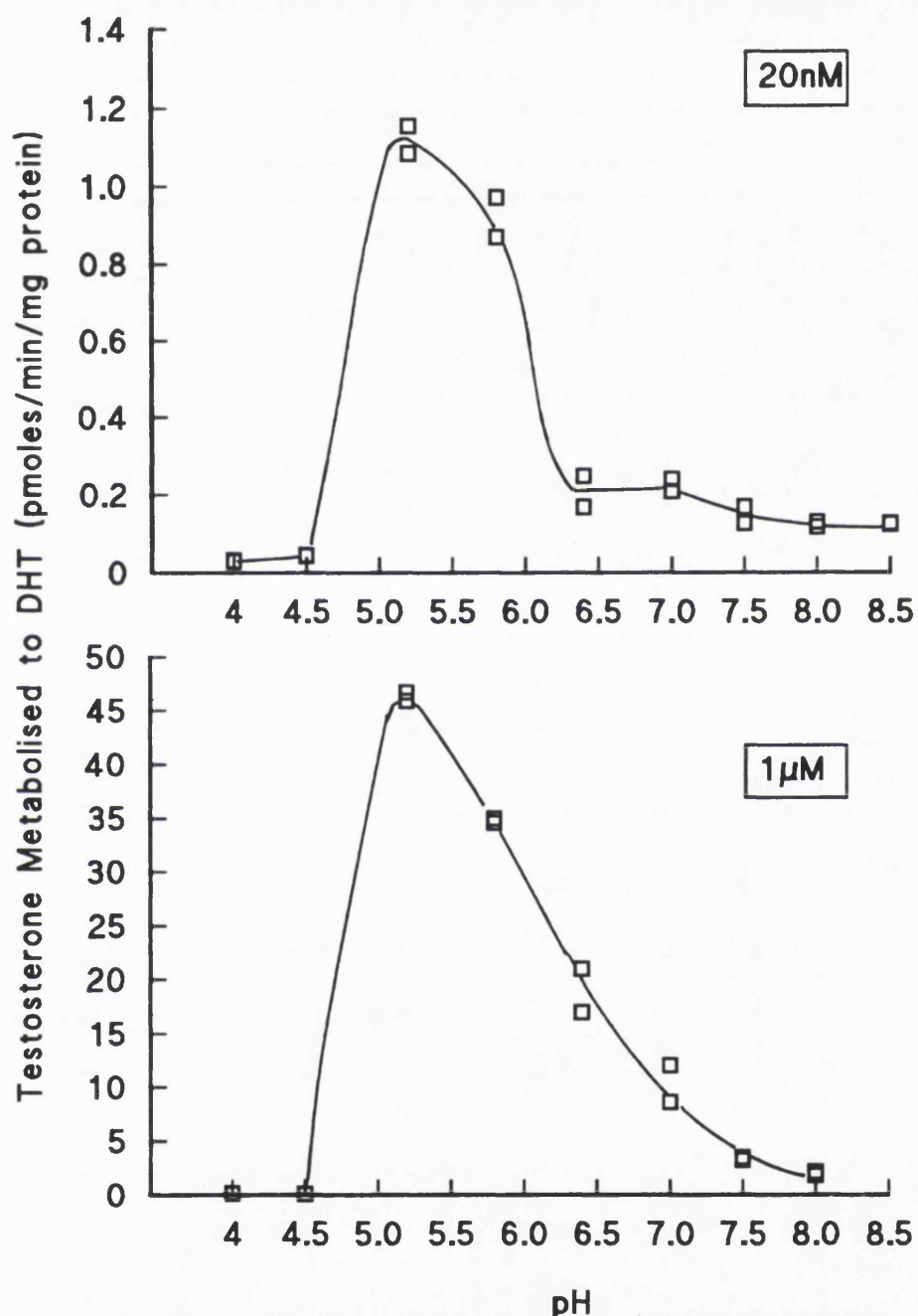
EFFECT OF pH ON THE ACTIVITY OF COS CELL EXPRESSED
5 α -REDUCTASE ISOZYMES



Homogenates of COS cells expressing 5 α -reductase (5 α -R) 1 (2.5 μ g protein/ml) or 2 activity (2 μ g protein/ml) were incubated with 20nM and 1 μ M (5 α -R2 only) [3 H]-testosterone (1 μ Ci) and an NADPH-regeneration system at 37°C for 30min at different pH values in a citrate-phosphate buffer (40mM). Testosterone and DHT were extracted with ethylacetate and separated by TLC. The TLC plates were scanned with a radio-TLC plate reader to determine the percent of testosterone converted to DHT. Data shown are from single experiments with 2 replicates.

FIGURE 4.4

EFFECT OF pH ON 5 α -REDUCTASE ACTIVITY IN HUMAN BPH TISSUE



Homogenates of human BPH tissue (0.1mg protein/ml) were incubated with 20nM (top) and 1 μ M (bottom) [3 H]-testosterone (1 μ Ci) and an NADPH-regeneration system at 37°C for 30min at different pH values in a citrate-phosphate buffer (40mM). Testosterone and DHT were extracted with ethylacetate and separated by TLC. The TLC plates were scanned with a radio-TLC plate reader to determine the amount of testosterone converted to DHT. Data shown are from single experiments with 2 replicates.

The potency of finasteride, SKF 105,657, UK 117026 and 4-MA against COS cell expressed 5 α -reductase 1 and 2 isozymes was determined using the concentration of 20nM testosterone reported for normal adult male plasma (Imperato-McGinley, 1979). Inhibitor studies with 5 α -reductase 1 were carried out at pH 7.5, while those involving 5 α -reductase 2 were performed at its reported optimum of pH 5.2. As initial experiments with 5 α -reductase 2 indicated that there was no difference in IC₅₀ values obtained at pH 6.5 (the observed optimum pH at 20nM testosterone) compared to pH 5.2 (Table 4.1) subsequent experiments were performed using 5 α -reductase 2 at pH 5.2.

Finasteride had an IC₅₀ value of 0.8nM against the 5 α -reductase 2 isozyme compared to 238nM for the 5 α -reductase 1. This represented a selectivity by finasteride for 5 α -reductase 2 of approximately 300 fold compared to 5 α -reductase 1 (Figure 4.5a). A greater selectivity for 5 α -reductase 2, was found with SKF 105,657. This inhibitor had an IC₅₀ value of 4.9nM against 5 α -reductase 2 compared to 21,730nM for 5 α -reductase 1 (Figure 4.5b). This represented a 4000 fold selectivity for 5 α -reductase 2 by SKF 105,657.

In contrast to finasteride and SKF 105,657 the compound UK 117,026 was found to be approximately 95 fold more potent an inhibitor of 5 α -reductase 1 than of 5 α -reductase 2 activity. UK 117,026 had an IC₅₀ value of 199nM against 5 α -reductase 1 compared to 19,100nM against 5 α -reductase 2 enzyme (Figure 4.5c).

TABLE 4.1

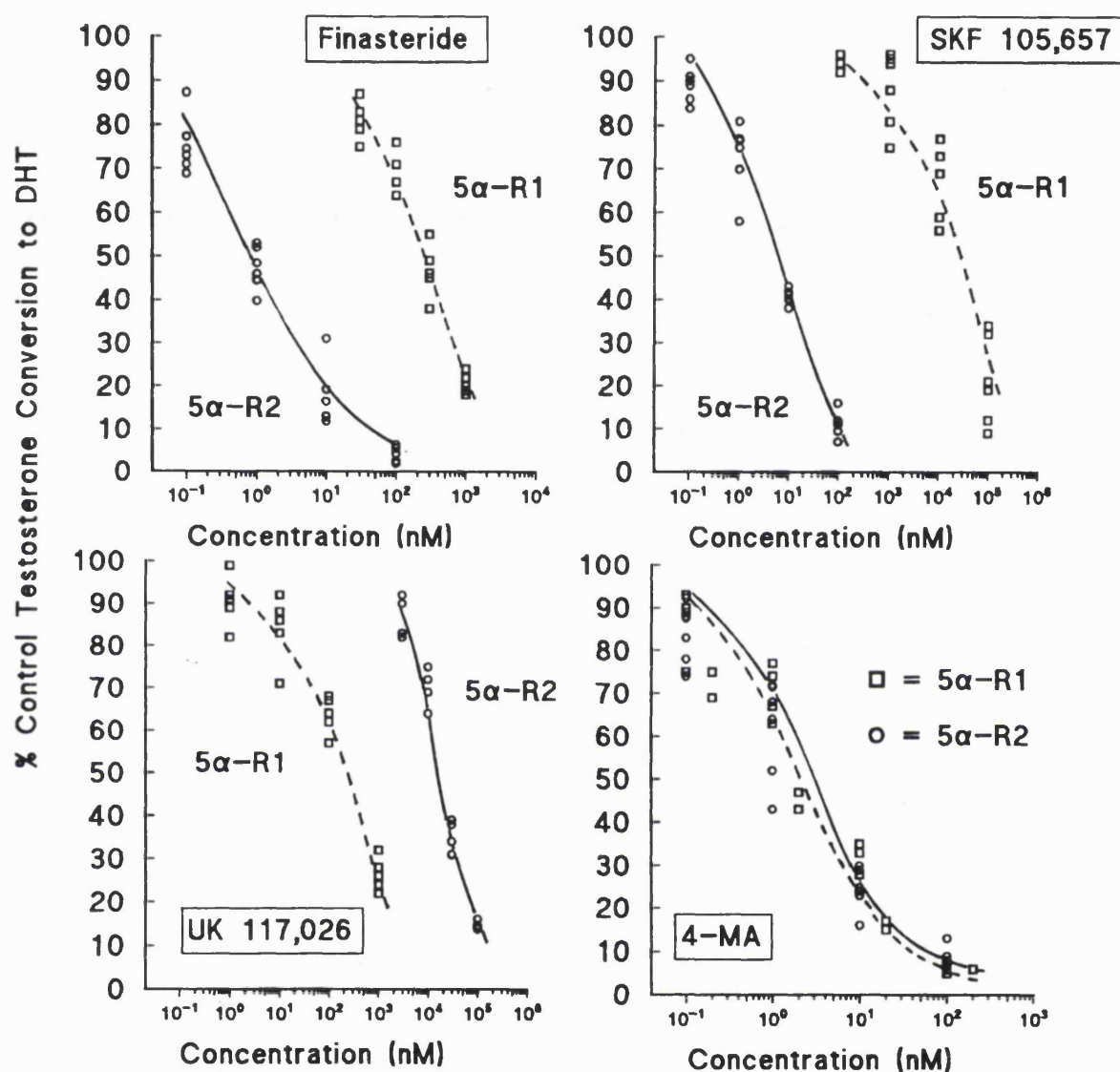
**COMPARISON OF IC₅₀ VALUES FOR THE INHIBITION OF
COS CELL EXPRESSED 5 α -REDUCTASE 1 AND 2 ACTIVITY**

Compound	IC ₅₀ Concentration (nM) For Inhibition Of DHT Formation		
	5 α -R1 pH 7.4	5 α -R2 pH 5.2	5 α -R2 pH 6.5
Finasteride	238 \pm 15	0.8 \pm 0.1	0.6 \pm 0.2
SKF 105,657	21,730 \pm 2,600	4.9 \pm 0.7	2.8 \pm 0.7
4-MA	1.7 \pm 0.3	1.9 \pm 0.2	0.8 \pm 0.1
UK 117,026	199 \pm 24	19,100 \pm 1,400	15,000 \pm 0.7

Homogenates of COS cells expressing either 5 α -reductase (5 α -R) 1 or 2 activity (2.5 and 2.0 μ g protein/ml respectively) were incubated with [³H]-testosterone (20nM, 1 μ Ci) and an NADPH-regeneration system for 30min in the presence of the inhibitors and at pH values indicated in the Table. Testosterone and DHT were extracted with ethylacetate, separated by TLC and analysed by a radio-TLC plate reader. The IC₅₀ (\pm SD) value is the concentration of compound required to give a 50% reduction in DHT formation compared to untreated cells. This was obtained from sigmoid dose-response curves fitted to the combined data from 3 separate experiments (except for 5 α -reductase 2 at pH 6.5, 1 experiment only) using the programme Sigfit (Delean et al., 1978).

FIGURE 4.5

DOSE RESPONSE CURVES FOR INHIBITION OF COS CELL EXPRESSED 5 α -REDUCTASE 1 AND 2 ACTIVITY



Homogenates of COS cells expressing either 5 α -reductase (5 α -R) 1 or 2 activity were incubated with [3 H]-testosterone (20nM, 1 μ Ci) and an NADPH-regeneration system for 30min in the presence of increasing concentration of the inhibitors shown on each graph. Assays were carried out at pH 7.4 for 5 α -R1 (2.5 μ g protein/ml) and pH 5.2 for 5 α -R2 (2 μ g protein/ml). Testosterone and DHT were extracted with ethylacetate, separated by TLC and analysed by a radio-TLC plate reader. Results were expressed as a percentage of DHT formed in control incubations and sigmoid dose-response curves were fitted to the combined data from 3 separate experiments using the programme Sigfit (Delean et al., 1978).

As expected 4-MA was equally effective at inhibiting conversion of testosterone to DHT by both 5 α -reductase isozymes with no difference in IC₅₀ values for inhibition of 5 α -reductase 1 (1.7nM) compared to 5 α -reductase 2 enzyme (1.9nM) (Figure 4.5d).

The results indicated that the compounds could be classified as selective inhibitors of 5 α -reductase 1 activity (UK 117026), selective inhibitors of 5 α -reductase 2 activity (finasteride and SKF 105,657) and inhibitors of both isozymes (4-MA).

4.2.3 Inhibition of 5 α -reductase Activity in Human BPH Cells and Prostate Cancer Cell Lines HPC-36M and DU145

The concentration of BPH cells used in 5 α -reductase assays to determine IC₅₀ values was selected so that the turnover of substrate was less than 50% after 1h incubation at 37°C (protein concentration 2-4mg/ml). The IC₅₀ values for each compound were the concentration of inhibitor required to give a 50% reduction in DHT formation after 1h incubation compared with untreated cells.

The ability of compounds to inhibit conversion of testosterone to DHT in the prostate cancer cell lines HPC-36M and DU145 was determined using cell suspensions (3x10⁶ cells/ml). Cell suspensions were used in preference to monolayers to reduce the variability between replicates. In the absence of inhibitors the cell lines converted approximately 10% of the [³H]-testosterone (20nM, 1 μ Ci) to 5 α -reduced products during a 3h incubation.

The most effective inhibitor of 5 α -reductase activity in human BPH cells, HPC-36M and DU145 was 4-MA with IC₅₀ values of 1.6nM, 1.3nM and 3.1nM respectively (Table 4.2).

TABLE 4.2

**COMPARISON OF IC₅₀ VALUES FOR THE INHIBITION OF
5 α -REDUCTASE ACTIVITY IN HUMAN BPH CELLS AND
PROSTATE CANCER CELL LINES**

Compound	IC ₅₀ Concentration (nM) For Inhibition Of Formation of 5 α -Reduced Metabolites		
	BPH Cells	HPC-36M	DU145
Finasteride	3.2 \pm 0.4	68.4 \pm 4.3	75.7 \pm 6.3
SKF 105,657	1.0 \pm 0.2	1,250 \pm 230	1,936 \pm 175
4-MA	1.6 \pm 1.0	1.3 \pm 1.4	3.1 \pm 1.9
UK 117,026	8,900 \pm 1,800	29.0 \pm 5.6	55.5 \pm 8.5

[³H]-Testosterone (20nM, 1 μ Ci) was incubated with BPH cells (1h, 2-4mg protein/ml) and suspensions of HPC-36M and DU145 (3h, 3x10⁶cells/ml) at 37°C in the presence of the inhibitors shown in the Table. Testosterone and metabolites were extracted with ethylacetate and separated by TLC and analysed by a radio-TLC plate reader. The IC₅₀ value (\pm SD) is the concentration of compound required to give a 50% reduction in the formation of 5 α -reduced metabolites compared to untreated cells. This was obtained from sigmoid dose-response curves fitted to the combined data from 3 separate experiments (except for UK 117,026 n=1 in BPH cells) using the programme Sigfit (Delean et al., 1978).

However, in contrast to BPH cells, which were sensitive to finasteride with an IC_{50} value of 3.2nM, the 5 α -reductase activity in HPC-36M and DU145 was less sensitive to finasteride with IC_{50} values of 68.4nM and 75.7nM respectively. (Table 4.2).

This trend was also observed with SKF 105,657 which was a potent inhibitor of the 5 α -reductase activity in BPH cells with an IC_{50} of 1nM but a weak inhibitor of 5 α -reductase activity in HPC-36M and DU145 with IC_{50} values of 1,250nM and 1,936nM respectively (Figure 4.6).

As with COS cell expressed 5 α -reductase 2 activity, UK 117,026 was an equally poor inhibitor of 5 α -reductase activity in BPH cells with an IC_{50} value of 8,900nM. In contrast, UK 117,026 was a more potent inhibitor of 5 α -reductase activity in DU145 and HPC-36M with IC_{50} values of 29.0nM and 55.5nM respectively (Figure 4.6).

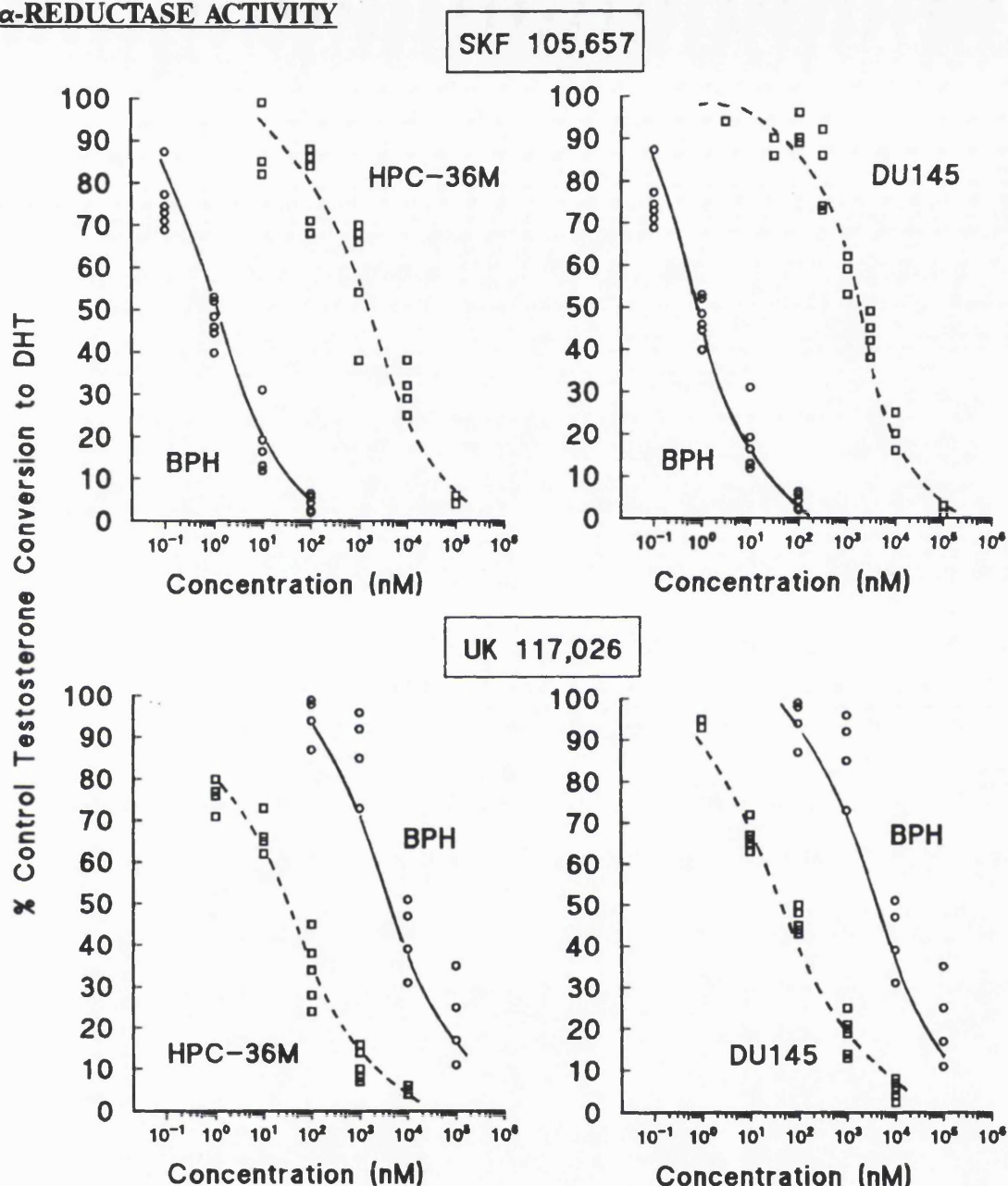
4.2.4 Inhibition of 5 α -Reductase Activity in Human BPH Tissue Slices

The aim of these experiments was to determine if BPH tissue slices displayed the same pattern of sensitivity to the 5 α -reductase inhibitors as suspensions of BPH cells. It is quite possible that the potency of compounds may be increased by disrupting prostate tissue (as in cell suspensions) simply by allowing easy access to the cells. Such disruption could potentially also alter the activity of the 5 α -reductase isozymes by releasing it from its normal environment. Experiments using slices of BPH tissue enable the ability of a compound to penetrate through several layers of cells to be assessed.

FIGURE 4.6

DOSE RESPONSE CURVES FOR INHIBITION OF HPC-36M AND DU145

5 α -REDUCTASE ACTIVITY



Cell suspensions (3×10^6 cells/ml) of HPC-36M and DU145 were incubated with [3 H]-testosterone (20nM, 1 μ Ci) for 3h in the presence of increasing concentrations of SKF 105,657 (top) and UK 117,026 (bottom). Assays were carried out in serum-free and phenol-red free RPMI. Testosterone and metabolites were extracted with ethylacetate, separated by TLC and analysed by a radio-TLC plate reader. Results were expressed as a percentage of 5 α -reduced metabolites formed in control incubations and sigmoid dose-response curves were fitted to the combined data from 3 separate experiments using the programme Sigfit (Delean et al., 1978).

As these experiments required a larger amount of tissue than was available from TURP specimens, full dose responses were not carried out. Instead a single concentration of finasteride, SKF 105,657 and UK 117,026 (100nM) was added to tissue slices and incubated in the presence of [³H]-testosterone (20nM) and the amount of DHT produced after 24 h was measured and compared to untreated tissue slices. Experiments using 4-MA were not performed.

The inhibitor concentration of 100nM was chosen so that 5 α -reductase 2 activity would be inhibited by over 90% with finasteride and SKF 105,657 while 5 α -reductase 1 activity would be inhibited by less than 30% by finasteride and not significantly by SKF 105,657 (Table 4.3). Conversely, 100nM UK 117,026 was chosen so that 5 α -reductase 2 activity would not be significantly inhibited while 5 α -reductase 1 activity should be inhibited by approximately 40% (Table 4.3).

There was no difference between the inhibition of DHT production produced by finasteride (100nM) or SKF 105,657 (100nM) in BPH slices and BPH cells (Table 4.3). In both BPH slices and BPH cells the observed inhibition in response to finasteride or SKF 105,657 was similar to that predicted from COS cells expressing 5 α -reductase 2. The inhibition of DHT production by UK 117026 in BPH cells was greater than predicted from the COS cell studies. Whereas 100nM UK 117026 had no effect on COS cell expressed 5 α -reductase 2 activity, the same concentration of UK 117,026 inhibited the production of DHT in BPH slices by 15% (Table 4.3).

TABLE 4.3

COMPARISON OF INHIBITION OF CONVERSION OF TESTOSTERONE TO DHT IN CELL SUSPENSIONS AND SLICES OF HUMAN PROSTATE TISSUE

Compound (100nM)	Predicted Inhibition (%) Of 5 α - Reductase Activity		Observed Inhibition (%) Of 5 α - Reductase Activity	
	5 α -R1	5 α -R2	BPH Cells	BPH Slices
Finasteride	29	96	93 \pm 3	95 \pm 5
SKF 105,657	5	89	93 \pm 4	96 \pm 2
UK 117,026	26	0	6 \pm 5	15 \pm 7

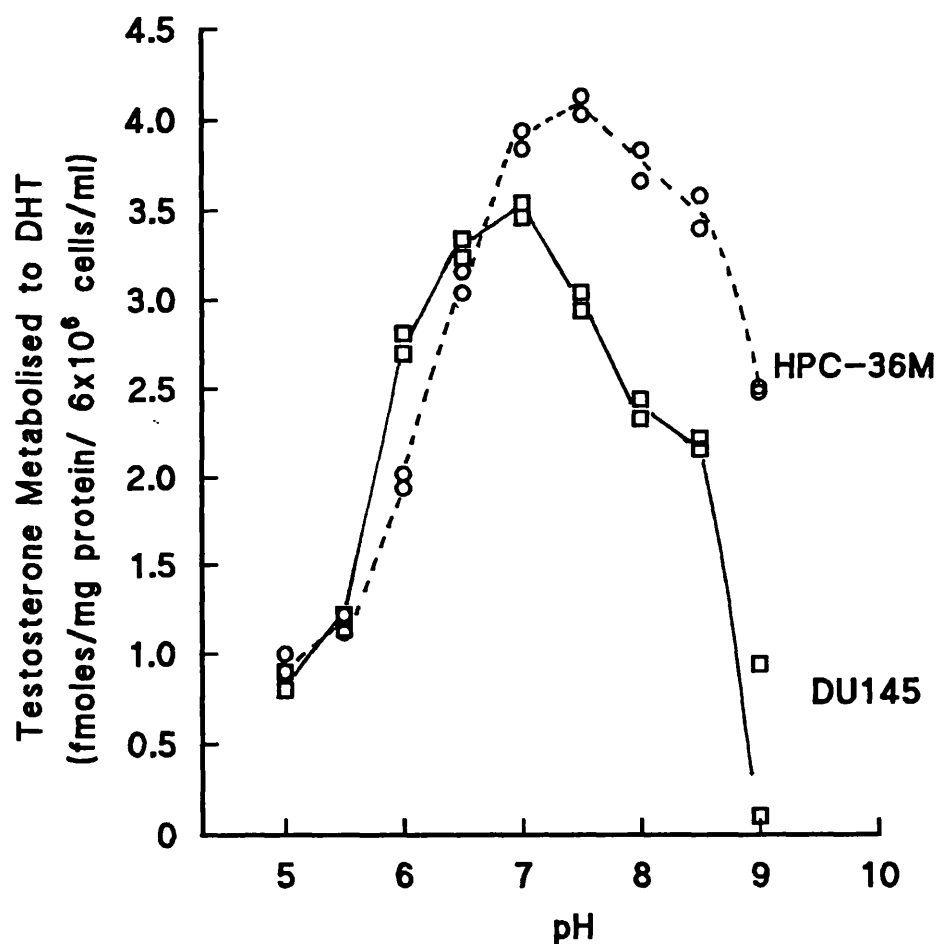
BPH cell suspensions (1h incubation, 2-4mg protein/ml), BPH tissue slices (24h incubation) were incubated with [3 H]-testosterone (20nM, 1 μ Ci) at 37°C in the presence of the inhibitors (100nM) shown in the Table. Predicted inhibitory effects on 5 α -reductase 1 and 2 isozymes were estimated from dose-response curves used to construct Table 4.1. Metabolites were extracted with ethylacetate, separated by TLC and analysed by a radio-TLC plate analyser. The percentage inhibition of 5 α -reductase (5 α -R) activity (\pm SD) was calculated from 3 separate experiments by comparing the amount of DHT formed in treated cells with that of untreated cells.

4.2.5 Determination of Optimum pH for Maximum 5 α -Reductase Activity in HPC-36M and DU145

The pH profile of 5 α -reductase activity in HPC-36M and DU145 was carried out as a further step in identifying the 5 α -reductase isozymes present in these cell lines. Cell homogenates were incubated with [³H]-testosterone over pH range 5-9 using a citrate buffer (40mM) and an NADPH-regeneration system. Under these conditions the only metabolite detected was DHT. In contrast to homogenates of BPH tissue (Figure 4.4), the 5 α -reductase activity in HPC-36M and DU145 was greatest between pH 6 - 8 (Figure 4.7).

FIGURE 4.7

EFFECT OF pH ON 5 α -REDUCTASE ACTIVITY IN HPC-36M AND DU145



Homogenates of HPC-36M and DU145 (6×10^6 cells/ml) were incubated with [3 H]-testosterone (1μ Ci) and an NADPH-regeneration system at 37°C for 3h at different pH values. Testosterone and DHT were extracted with ethylacetate and separated by TLC. The TLC plates were scanned with a radio-TLC plate reader to determine the amount of testosterone converted to DHT. Data shown are from single experiments with 2 replicates.

The 5α -reductase activity present in human hyperplastic prostate has identical properties to COS cell expressed 5α -reductase 2 (Andersson and Russell, 1990; Andersson *et al.*, 1991; Jenkins *et al.*, 1992). In contrast, extensive characterisation of the 5α -reductase activity in prostate cancer tissue has not been performed. Using the differential sensitivity of 5α -reductase isozymes to 5α -reductase inhibitors as a method of characterising 5α -reductase activity, the present study has shown that the 5α -reductase activity in prostate cancer cell lines is different to that in BPH tissue and is similar to 5α -reductase 1.

In this study four compounds have been characterised with respect to their potency as inhibitors of 5α -reductase 1 and 5α -reductase 2 isozymes. The finding that finasteride and SKF 105,657 are poor inhibitors of 5α -reductase 1 is consistent with previous reports for COS cell expressed 5α -reductase 1 and human scalp (Andersson and Russell, 1990; Harris *et al.*, 1992; Mellin *et al.*, 1993). The existence of selective 5α -reductase 1 selective inhibitors has previously been reported (Harris *et al.*, 1992; Jones *et al.*, 1993; Mellin *et al.*, 1993) but this is the first study to indicate that UK 117,026 also belongs to this class of inhibitor.

The ability to express 5α -reductase isozymes in COS cells provides the means to study and characterise the isozymes in isolation. However, it is important to be aware that the information obtained about any enzyme expressed from its cDNA is only relevant if it truly reflects what is known about that enzyme *in vivo*. For 5α -reductase 1 and 2 isozymes this is difficult to assess because neither isozyme has been isolated and purified, indeed, most attempts to isolate and purify 5α -reductase from the human prostate have been only partially successful (Houston *et al.*, 1985a, 1985b; Enderle-Schmitt *et al.*, 1986; Sargent and Habib, 1991).

In this study, the properties of COS cell 5 α -reductase 2 activity resembled those of the 5 α -reductase activity present in BPH tissue, the only difference being the change in the pH optimum of COS cell expressed 5 α -reductase 2 from 5.2 at 1 μ M testosterone to pH 6.5 at 20nM testosterone. The higher activity at pH 6.5 did not alter the pattern of sensitivity to inhibitors compared to that at pH 5.2. This pH optimum of 6.5 of 5 α -reductase 2 with 20nM testosterone has not previously been reported and does not occur with homogenates of BPH tissue (Figure 4.4). At this low substrate concentration the whole-cell environment (in the COS cell) favours optimal activity of the enzyme around a physiological pH contrasting with the observation that optimal activity of BPH homogenates with the same testosterone concentration occurs at pH 5.2. Other factors present in the tissue, but absent in COS cells, may be present that serve to regulate the activity of 5 α -reductase enzyme. The observation that the pH optimum of 5 α -reductase 2 was 5.2 when testosterone concentration was increased to 1 μ M suggested that perhaps the activity of the enzyme is regulated by its substrate concentration.

The properties of 5 α -reductase activity in BPH tissue homogenates or BPH cell suspensions, as used in this study, appear to be almost identical to 5 α -reductase 2 leaving the question of how does 5 α -reductase 1 fit into the overall profile of 5 α -reductase activity in the prostate and are there any circumstances when its expression becomes important. The mRNA for 5 α -reductase 1 is present in human prostate (Andersson and Russell, 1990) and so the potential for the 5 α -reductase 1 protein to be expressed by human prostate cells exists. However, 5 α -reductase 1 activity has to date only been detected in cultured human prostate cells and not freshly isolated cells (Hirsch *et al.*, 1993).

The data from this study supports the existence of 5 α -reductase-1 like activity in the prostate cancer cell lines HPC-36M and DU145. Whether this is a true reflection of prostate cancer cells in vivo or is this a result of some change that has occurred as a result of maintenance in long-term culture is open to

speculation. In this study the prostate cancer cell lines had lower 5 α -reductase activity than BPH cells, prostate cancer tissue also has lower 5 α -reductase activity than BPH tissue. Perhaps loss of 5 α -reductase 2 in prostate cancer tissue unmasks 5 α -reductase 1. One might speculate that as prostate cancer is epithelial in origin the 5 α -reductases may be differently distributed between epithelial and stromal tissue and the 5 α -reductase activity in HPC-36M and DU145 reflects this distribution.

Both HPC-36M and DU145 cell lines were established from poorly differentiated prostate tumours (Lubaroff, 1977; Stone *et al.*, 1978) and the results from this study support the interpretation that these cells have lost expression of the major prostatic form of 5 α -reductase but have retained expression of the minor prostatic form of 5 α -reductase. However, this interpretation is only valid providing that prostate epithelial cells normally express both 5 α -reductase isozymes.

The finding that the properties of the 5 α -reductase activity in HPC-36M and DU145 resemble those of 5 α -reductase 1 indicates that these cells are not useful in predicting potent inhibitors of the major form of 5 α -reductase present in BPH tissue. Clearly, an effective inhibitor of HPC-36M 5 α -reductase activity would not be effective in treating of BPH. Conversely, if HPC-36M and DU145 5 α -reductase activity is representative of that in prostate cancer cells *in vivo* then treatment of prostate cancer with potent inhibitors of 5 α -reductase 2 would also be ineffective. In this situation potent inhibitors of both isozymes, such as 4-MA, would provide total inhibition of 5 α -reductase activity. The cell lines would be useful in predicting potent inhibitors of 5 α -reductase 1 activity which maybe useful in combination with 5 α -reductase 2 inhibitors or a single agents for treating male pattern baldness. Studies aimed at characterising 5 α -reductase activity in prostate cancer tissue need to be carried out to determine if HPC-36M and DU145 are good models of prostate cancer 5 α -reductase *in vivo* and to examine the role of 5 α -reductase inhibitors in treating this disease.

Having characterised 5 α -reductase activity and identified the predominant pathways of metabolism in the prostate cancer cell lines, the aim of the next chapter was to determine if the pathway of testosterone metabolism was related to the sensitivity of the cell lines to androgens.

CHAPTER FIVE

EVALUATION OF HUMAN PROSTATE CANCER CELL LINES AS MODELS FOR ANDROGEN RESPONSIVE GROWTH IN VITRO

5.1 INTRODUCTION

Human prostate cancer cell lines provide a continuous and reproducible supply of identical cells which may be useful in studying the biological characteristics of the prostate. To date only a few prostate cancer cell lines have been established and these include HPC-36M (Lubaroff, 1977), DU145 (Stone *et al.*, 1978), PC-3/MA2 (Kaighn *et al.*, 1979) and LNCaP (Horoszewicz *et al.*, 1983). Three of the cell lines DU145, PC-3/MA2 and LNCaP were established from tissue taken from metastatic prostate cancers while HPC-36M was established from tissue taken from a primary prostatic tumour (see Table 2.1). Recently, Loop *et al.* (1993) have reported the establishment of a new human prostate cancer cell line, the ALVA-31 line, from tissue taken from a primary prostatic tumour. Growth of this cell line is reported to be modulated by androgens, however, the cell line is not androgen-dependent. Further characterisation of the properties of this cell line are necessary to establish how relevant it is as a model of prostate cancer cell growth *in vivo*.

Prostatic specific markers, such as PSA and PAP, are useful in establishing that the origin of a cell line is the prostate. The presence of androgen receptors is another characteristic often used. HPC-36M, DU145, PC-3/MA2 and LNCaP all express PAP but LNCaP is the only line to express PSA and PAP (Lubaroff, 1977; Stone *et al.*, 1978; Kaighn *et al.*, 1979; Horoszewicz *et al.*, 1983). Furthermore, LNCaP is the only line retaining androgen-responsive growth (Horoszewicz *et al.*, 1983). While growth of androgen insensitive cells (HPC-36M, DU145 and PC-3/MA2) occurs in the absence of androgens the LNCaP cells require the presence of a critical level of androgens in order to proliferate (van Steenbrugge *et al.*, 1989).

Prostate epithelial cells in vivo are dependent on androgens and do not proliferate when androgens are removed this characteristic is retained by LNCaP cells. However, most prostate cancers eventually progress to an androgen-insensitive state and acquire the ability to proliferate in the absence of androgens, a trait reflected in HPC-36M, DU145 and PC-3/MA2. The mechanism by which prostate cancer cells progress to androgen independent growth is not understood.

Several studies have shown that LNCaP cells display a characteristic biphasic response when testosterone or DHT is added to cells growing in medium containing steroid-depleted serum (DCC-FCS). At low androgen concentrations cell proliferation increases in a dose-dependent manner reaching maximum stimulation between 0.1nM and 10nM while higher concentrations inhibit proliferation (Horoszewicz *et al.*, 1983; Berns *et al.*, 1986; Schuurmans *et al.*, 1988; Sonnenschein *et al.*, 1989; Wilding *et al.*, 1989; Olea *et al.*, 1990; Eaton *et al.*, 1991; Smith *et al.*, 1993). Paradoxically, the growth of LNCaP cells is also stimulated by oestrogens, progestins and antiandrogens (Wilding *et al.*, 1989; Olea *et al.*, 1990). This stimulation appears to be the result of a point mutation in the steroid binding domain of the androgen receptor gene of LNCaP which confers aberrant binding affinity (and subsequent activation) of the receptor for other non-androgen molecules (Trapman *et al.*, 1990).

DHT has a higher relative binding affinity, than testosterone, for the LNCaP androgen receptor (Veldscholte *et al.*, 1990), but whether the growth of LNCaP cells is dependent on DHT, rather than testosterone, remains to be established. If DHT is the major growth-stimulus then LNCaP cells will be a useful model for testing the ability of 5 α -reductase inhibitors to inhibit testosterone-stimulated growth by preventing its conversion to DHT.

The main aim of this chapter was relate the pattern of sensitivity to testosterone and DHT to the metabolic capacity of the prostate cancer cell lines. In addition this study aimed to investigate the usefulness of the LNCaP cell line as a model of androgen responsive growth for the evaluation of 5 α -reductase inhibitors. Finasteride has been evaluated in a small phase III clinical trial for the treatment of prostate cancer (Presti *et al.*, 1992) and so it is of interest to determine the effects of 5 α -reductase inhibitors on the growth of androgen-responsive and unresponsive cell lines. To do this, it was first necessary to define the conditions required to demonstrated androgen-responsive growth.

Human prostate cancer cell lines are routinely cultured in medium containing 5% foetal calf serum (FCS) a complex mixture of biomolecules including growth factors, hormones, attachment and spreading factors, transport proteins and several other less well characterised components (Barnes, 1980). Serum-containing medium is therefore of limited value for testing the effects of hormones and growth factors on prostate cells. Treatment with dextran-coated charcoal (DCC) strips steroid hormones from serum (Leake *et al.*, 1982) and produces steroid-depleted serum (DCC-FCS). In this study, androgen levels in FCS and DCC-FCS sera were defined before the proliferative effects of testosterone and DHT on LNCaP cells were investigated. MTT and colony-forming assays were employed in contrast to previous studies which measured changes in cell number by [³H]-thymidine uptake, direct counting or DNA measurement (Horoszewicz *et al.*, 1983; Berns *et al.*, 1986; Schuurmans *et al.*, 1988; Wilding *et al.*, 1989; Olea *et al.*, 1990; Eaton *et al.*, 1991). The MTT assay and colony-forming assays are simple to perform and avoid the use of radioactivity. The MTT assay is able to distinguish between metabolically active and non-active cells, unlike assays involving direct cell counts or thymidine uptake (Romijn *et al.*, 1988). Once the conditions for androgen-responsive growth were defined the ability of finasteride to inhibit androgen-responsive growth was investigated in LNCaP cells.

5.2 RESULTS

5.2.1 Determination of Steroid Levels in Foetal Calf Serum (FCS)

The concentrations of the steroid hormones, testosterone, DHT, 17 β -oestradiol and progesterone and one peptide hormone, insulin, (all reported to stimulate growth of prostate cells) were measured using radioimmunoassay (RIA) and the results are shown in Table 5.1. The detection limit of the Amersham RIA for total androgen concentration i.e testosterone plus DHT is 0.45nM, this is lower than the detection limit for DHT which is 0.8nM. Initially the assay measures total androgen concentration in the samples. For untreated FCS this was 0.75nM.

The second stage of the assay removes testosterone, by oxidation, leaving DHT which is then measured. After oxidative removal of testosterone, from FCS, DHT could not be detected. The results from RIA could only be used to establish the upper limit of testosterone plus DHT (total androgen concentration) in medium containing 5% FCS as approximately 0.04nM.

Dextran-coated charcoal treatment of FCS reduced the concentration of all steroid hormones measured to below the detection limit of RIA (Table 5.1). However, as expected the stripping process did not remove the peptide hormone insulin. The concentration of testosterone and DHT in DCC-FCS was below the detection limit (0.45nM).

The efficiency of the stripping process was determined by following the removal of radio-labelled testosterone and DHT from FCS. Following the addition of tritiated testosterone or DHT (1 μ Ci) to 10ml FCS it was left stirring overnight (4°C) to allow the exchange of radioactive androgen for non-radioactive androgen in normal binding sites of FCS.

TABLE 5.1

STEROID HORMONE CONCENTRATIONS OF FOETAL CALF SERUM (FCS)
AND CHARCOAL-STRIPPED SERUM (DCC) AS DETERMINED BY
RADIOIMMUNOASSAY

Hormone	FCS (nM)	DCC-FCS (nM)	Detection Limit
Total T + DHT ¹	0.75	< 0.45	0.45
Testosterone ¹	≤ 0.75	< 0.45	0.45
DHT ¹	< 0.8	< 0.8	0.8
17β-Oestradiol ²	0.06 ± 0.06	< 0.035	0.035
Progesterone ²	13.5 ± 1.1	< 0.15	0.015
Insulin ² (μIU/ml)	6.9 ± 1.2	5.3 ± 3.3	5

Concentrations of steroid hormones and insulin were determined by radioimmunoassay of two samples of foetal calf serum (FCS) and charcoal stripped FCS (DCC-FCS). ¹: Amersham RIA; ²: Diagnostic Products RIA.

Dextran-coated charcoal treatment of FCS spiked with tritiated testosterone and DHT ($1\mu\text{Ci}$, 2nM) removed 98% and 97% of the respective steroids. Thus the total concentration of testosterone and DHT in DCC-FCS was estimated from the amount of tritiated testosterone and DHT removed during the stripping process. This was estimated as 1.1pM .

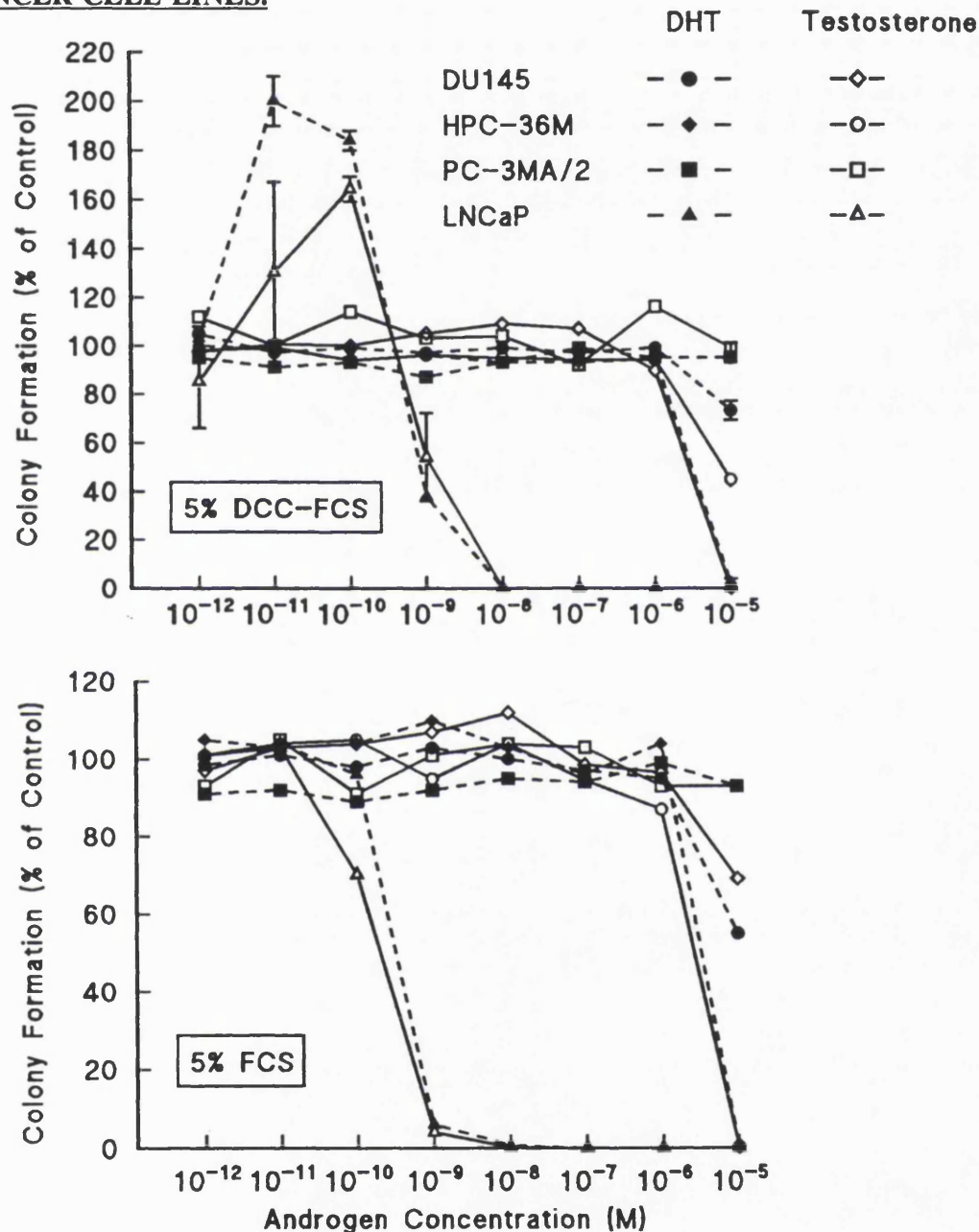
5.2.2 The Effects Of Testosterone and DHT on Colony-Formation by Prostate Cancer Cell Lines

The prostate cancer cell lines HPC-36M, DU145, PC-3/MA2 and LNCaP were adapted to growth in steroid-depleted medium by passaging them in 5%-DCC-FCS RPMI 1640 for at least three passages. The growth of HPC-36M, DU145 and PC-3/MA2 and their ability to passage in DCC-FCS medium was similar to that in untreated FCS supplemented medium. In contrast, when LNCaP cells were passaged at a low density (for example one 25cm^2 flask into six 25cm^2 flasks) in DCC-FCS containing medium they appeared to grow at a slower rate than when maintained in FCS supplemented medium. Frozen stocks of all 4 cell lines were prepared after they had been maintained in DCC-FCS and these became designated as HPC-36M-DCC, DU145-DCC, PC-3/MA2-DCC and LNCaP-DCC.

The addition of testosterone or DHT to DCC-FCS supplemented medium caused stimulation only in LNCaP-DCC, with maximum stimulation at 0.1nM testosterone and 0.01nM DHT respectively (Figure 5.1). Testosterone and DHT did not stimulate colony-formation in HPC-36M-DCC, DU145-DCC or PC-3/MA2-DCC at any concentration tested. Interestingly, testosterone and DHT concentrations of 1nM produced 50% inhibition of LNCaP-DCC colony-formation, while at 10nM , which is below the lower level of normal plasma testosterone in men (Ghanadian and Puah, 1981), colony-formation was completely inhibited.

FIGURE 5.1

**THE EFFECT OF ANDROGENS ON COLONY-FORMATION IN PROSTATE
CANCER CELL LINES.**



Prostate cancer cell lines (HPC-36M, DU145, PC-3/MA2 and LNCaP) were plated in 5% DCC-FCS (top graph) and 5% FCS (bottom graph) supplemented medium and left 24h to attach. Testosterone and DHT (10^{-12} - 10^{-5} M) were added and the cells incubated at 37°C for 14 days. Colonies were fixed in 70% methanol and stained with Giemsa's stain. Results are expressed as percentage of colony formation in controls (mean \pm S.D, 3 experiments). For clarity error bars are only plotted for LNCaP.

In HPC-36M-DCC and DU145-DCC colony-formation was not inhibited until androgen concentrations exceeded $1\mu\text{M}$. Even this concentration had no effect on colony-formation in PC-3/MA2-DCC, which was not inhibited by $10\mu\text{M}$ testosterone or DHT.

In medium containing 5% FCS, testosterone and DHT (1pM - $1\mu\text{M}$) inhibited colony formation in LNCaP in a concentration dependent manner (Figure 5.1) and concentrations exceeding 1nM inhibited colony-formation by over 90%. The effect of testosterone and DHT on colony-formation by HPC-36M, DU145 and PC-3/MA2 was unchanged by replacing FCS with DCC-FCS in the medium.

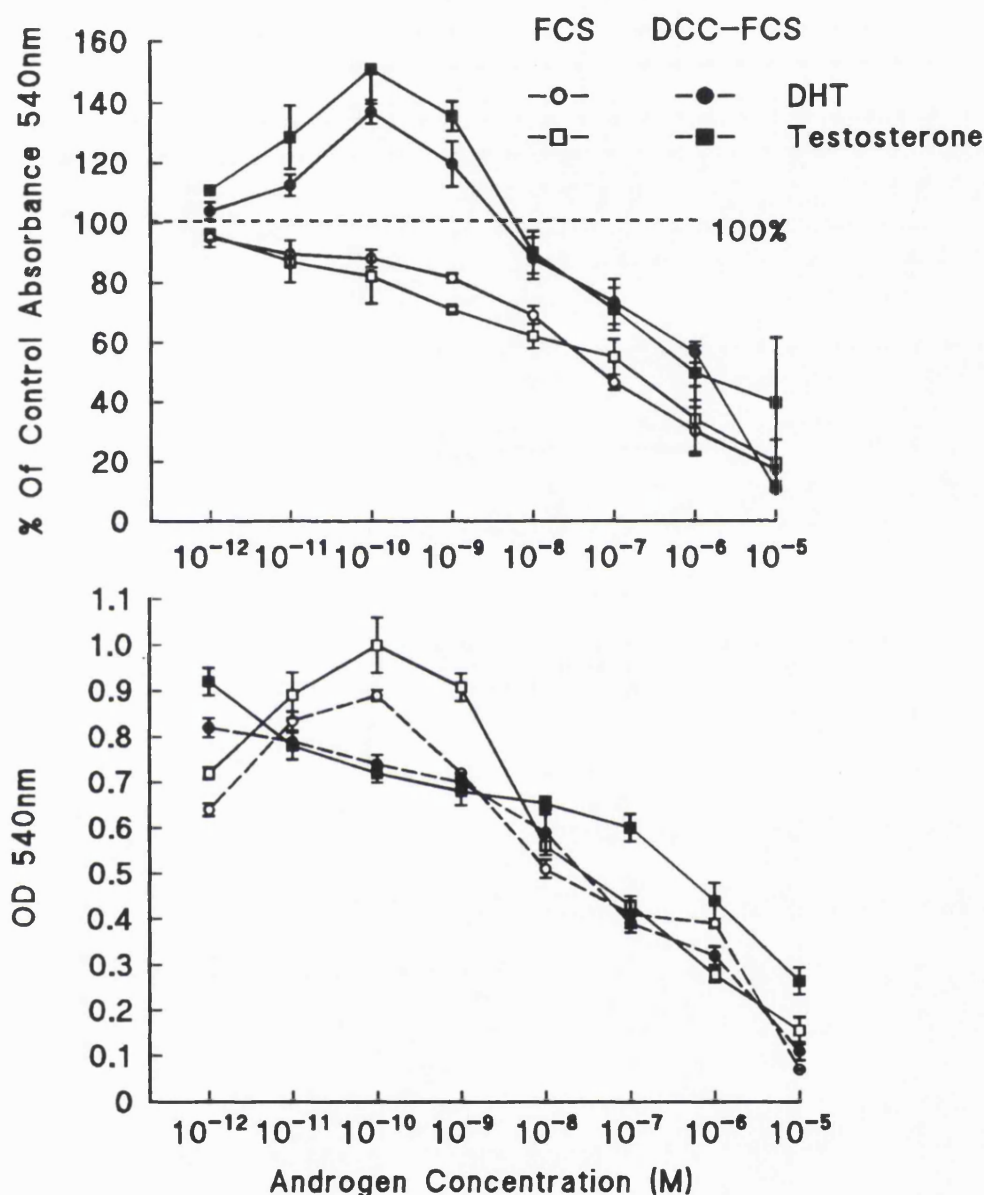
5.2.3 The Effect of Testosterone and DHT on LNCaP Cell Proliferation (MTT Assay)

In medium containing 5% DCC-FCS there was stimulation of LNCaP-DCC cell proliferation by testosterone and DHT between 1pM and 1nM . Maximum stimulation was produced in response to 0.1nM testosterone and DHT. Proliferation was inhibited by concentrations of testosterone and DHT exceeding 10nM . In medium supplemented with untreated serum there was a dose-dependent inhibition of LNCaP cell proliferation (Figure 5.2).

The relative growth rates of LNCaP in medium containing DCC-FCS and FCS in the presence of testosterone and DHT were determined in a single MTT experiment. Figure 5.2 shows the absolute absorbance values (as opposed to converting the data to % control absorbance) recorded from a single MTT experiment. In the absence of testosterone and DHT, cell proliferation was faster in medium containing FCS than DCC-FCS. The absolute absorbance values showed that the maximum stimulation of LNCaP-DCC cells produced in response to 0.1nM testosterone or DHT results in a rate of growth that is equivalent to the growth of the cell in medium supplemented with untreated serum (Figure 5.2).

FIGURE 5.2

THE EFFECT OF ANDROGENS ON CELL PROLIFERATION IN LNCAP



LNCaP cells were plated (3000/well) in 5% FCS or 5% DCC-FCS supplemented medium and left 24h to attach. Testosterone and DHT (10^{-12} - 10^{-5} M) were added and the plates incubated at 37°C for 7 days. MIT (0.25mg/well) was incubated with the cells for 3h at 37°C and the absorbance of DMSO-solubilised formazan measured at 540nm. Top graph shows the results expressed as percentage of control absorbance (mean \pm S.D, 3 experiments) and bottom graph shows the raw absorbances from one experiment.

MTT assays were not performed on HPC-36M, DU145 and PC-3/MA2 as no stimulation of growth was produced in colony-forming assays.

5.2.5 Effects of Finasteride on LNCaP Colony-Formation

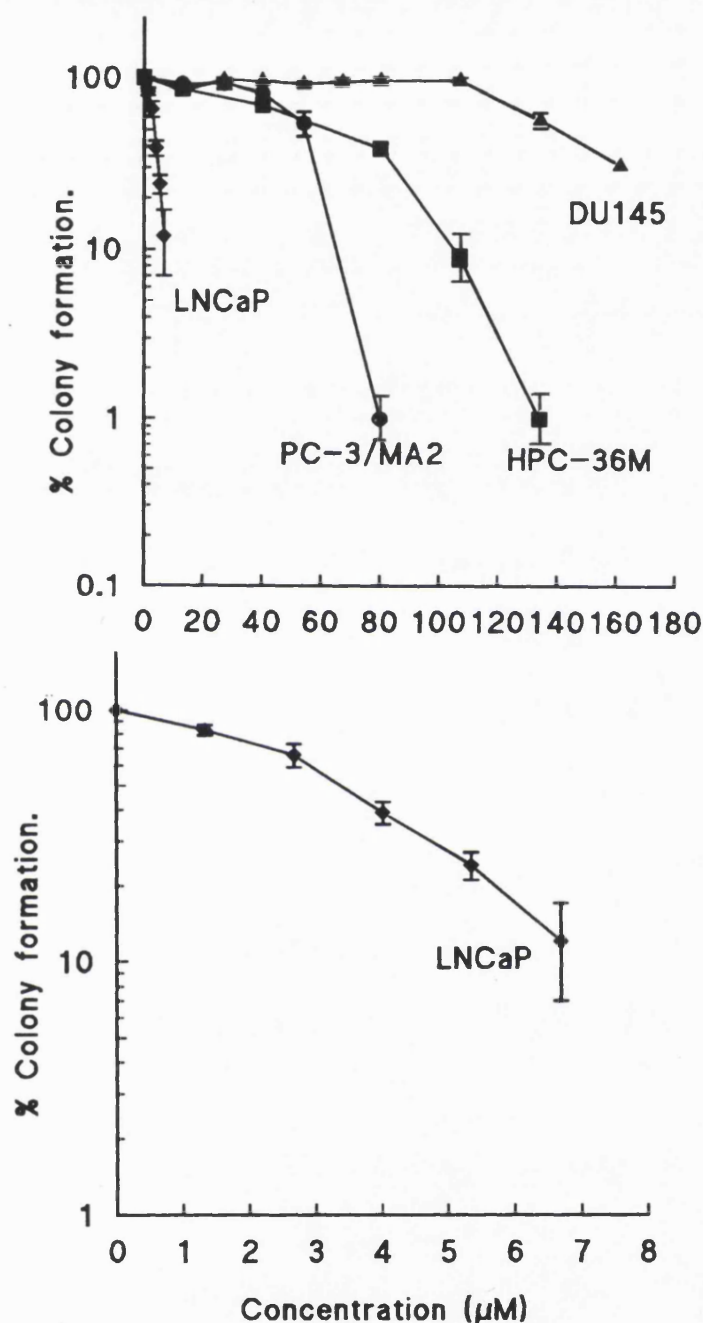
Initially, finasteride was tested for its ability to inhibit colony-formation in LNCaP, HPC-36M, DU145 and PC-3/MA2 in medium containing untreated FCS. This was designed to assess the effect of inhibiting 5 α -reductase activity on the ability of the cells to proliferate and form colonies under conditions of optimal growth (i.e. in the presence of serum). In addition, it was necessary to determine the highest concentration of finasteride that would completely inhibit both 5 α -reductase enzymes without inhibiting growth.

Figure 5.3 shows that LNCaP was between 15 and 42 times more sensitive to finasteride than the other cell lines. The IC₅₀ value for inhibition of colony-formation in LNCaP by finasteride was 3.4 μ M, compared to 53 μ M in PC-3/MA2, 56 μ M in HPC-36M and 140 μ M in DU145 (Table 5.2). The concentration of 500nM finasteride was considered to be the maximum concentration that was without any growth inhibitory effects on LNCaP cells. From the data presented in Chapter 4 it is known that 500nM finasteride inhibits BPH 5 α -reductase completely and DU145 and HPC-36M 5 α -reductase activity by over 85%.

Figure 5.4 shows that finasteride had no effect on either testosterone or DHT stimulated growth on LNCaP cells (growing in DCC-FCS supplemented medium).

FIGURE 5.3

**THE EFFECT OF FINASTERIDE ON COLONY-FORMATION IN PROSTATE
CANCER CELL LINES**



Prostate cancer cell lines (HPC-36M, DU145, PC-3/MA2 and LNCaP) were plated in 5% FCS supplemented medium and left 24h to attach. Increasing concentrations of finasteride was added and the cells incubated at 37°C for 14 days. Colonies were fixed in 70% methanol and stained with Giemsa's stain. Results are expressed as percentage of colony formation in controls (mean \pm S.D, 3 experiments). The lower graph shows the dose-response for LNCaP plotted on a smaller scale.

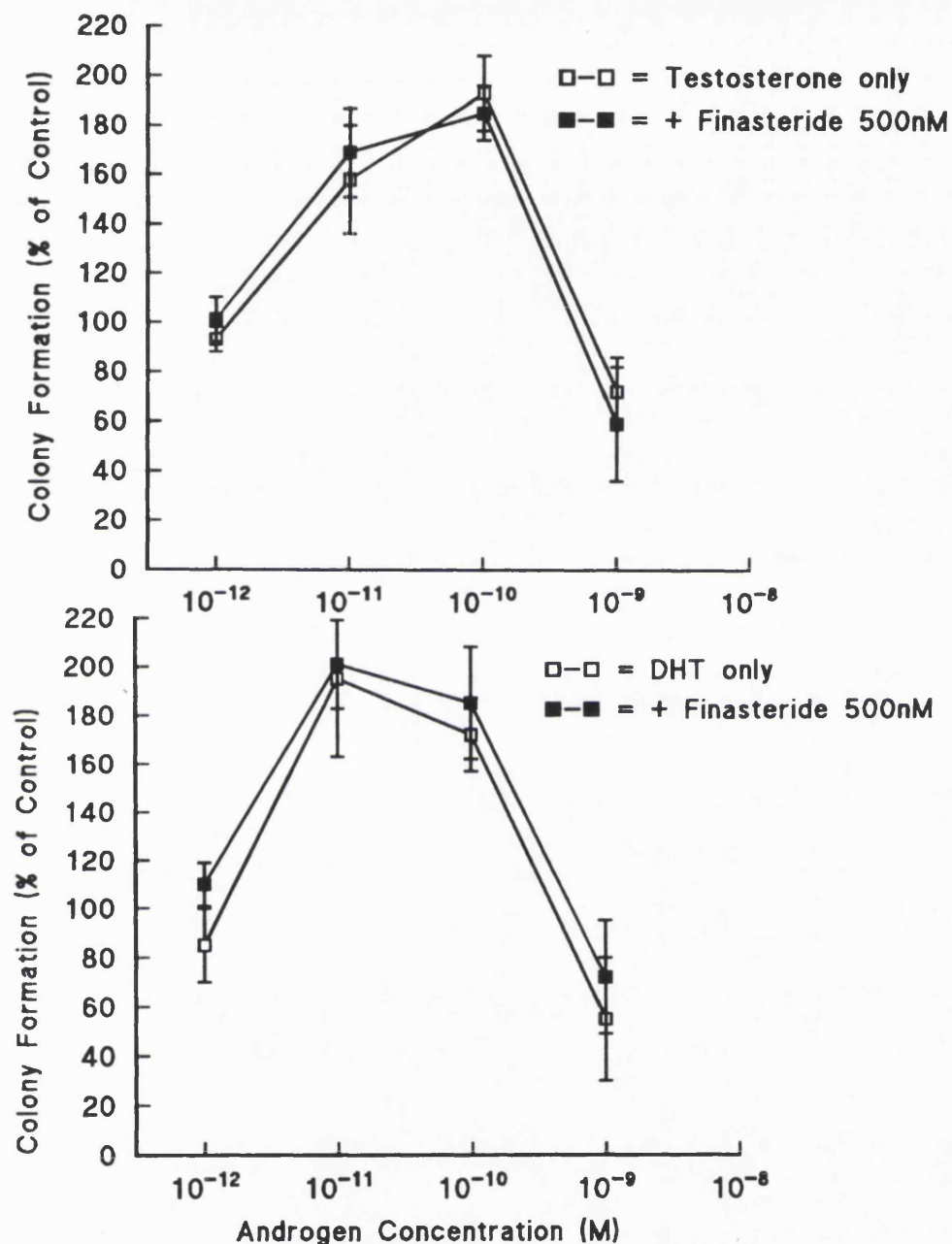
TABLE 5.2

**IC₅₀ VALUES (μ M) for the INHIBITION of COLONY-FORMATION by
FINASTERIDE in PROSTATE CANCER CELL LINES**

Cell Line	IC ₅₀ (μ M)
LNCaP	3.4 \pm 0.3
PC-3/MA2	53 \pm 5
HPC-36M	56 \pm 3
DU145	140 \pm 5

Experiments were carried out as described in the legend for Figure 5.3. The concentration required to inhibit colony-formation by 50% of controls (IC₅₀) was determined from 3 separate experiments. The results are the mean \pm SD.

FIGURE 5.4 **THE EFFECT OF FINASTERIDE ON ANDROGEN**
STIMULATED COLONY-FORMATION IN LNCaP CELLS



LNCaP cells (2000 / 5ml petri dish) were plated in 5% DCC-FCS supplemented medium and left for 24h to attach. Testosterone or DHT (10^{-12} - 10^{-9} M) was added to dishes which contained either 500nM finasteride or vehicle (ethanol 0.5% v/v). Cells were incubated at 37°C for 14 days. Colonies were fixed in 70% methanol and stained with Giemsa's stain. Results are expressed as percentage of colony-formation in controls (means \pm SD, 3 experiments).

As predicted, in this study androgen-stimulated growth was only observed in the androgen-receptor positive cell line LNCaP. In addition, LNCaP was more sensitive to testosterone and DHT and finasteride than the other cell lines.

The concentration of total androgens in FCS in the present study was estimated as 0.75nM which is similar to reported value for testosterone of 0.5 - 1.0nM by van Steenbrugge *et al.* (1991) and to the value of 1nM for total androgens reported by Berns *et al.* (1986). The concentration of testosterone in DCC-FCS is reported to be 0.15nM (Berns *et al.*, 1986) and 0.072nM (Horoszewicz *et al.*, 1983). No actual values for DHT concentrations in FCS or DCC-FCS are reported in the literature indicating that, as in this study, the concentration of DHT in FCS is too low for detection.

Stimulation of LNCaP-DCC colony-formation and cell proliferation occurred at testosterone and DHT concentrations between 1pM and 1nM. In MTT assays DHT was 10-fold more potent at stimulating cell proliferation than in colony-forming assays. This suggests that DHT is more potent than testosterone and may reflect the greater sensitivity of the MTT assay to accurately detect increases in cell number.

The concentrations of testosterone and DHT that stimulated LNCaP-DCC growth were in the same order of magnitude as those present in medium containing unstripped FCS. Medium supplemented with 5% FCS had a total androgen concentration of 40pM which was reduced to an estimated 1pM by charcoal treatment. This supports the interpretation that the maximum stimulation of LNCaP-DCC growth by the addition of 1pM to 100pM testosterone or DHT is simply due to replacement of the androgens removed from FCS by charcoal stripping.

The lack of androgen-stimulated growth in the presence of FCS is consistent with the interpretation that androgen concentrations are sufficient to maintain maximum proliferation of LNCaP and is in agreement with the observations of Horoszewicz *et al.* (1983) and Berns *et al.* (1986). Growth rates of LNCaP-DCC in MTT assays in which the absolute absorbances were plotted (Figure. 5.2) confirmed that the stimulation of growth by testosterone and DHT in LNCaP-DCC was no greater than that in the presence of untreated FCS. Further evidence for this is provided by the work of Sonnenschein *et al.* (1989) who observed that DHT (3pM and 30pM) increased LNCaP cell yields in medium containing 10% untreated human female serum but not in untreated male human serum. Cell yields only increased in response to DHT when male serum was charcoal-treated.

The inhibition of LNCaP cell growth by concentrations of androgens exceeding 10nM is an interesting observation considering that the concentration of DHT in prostate tissue from adult human males is approximately 20nM. The ability of androgens to inhibit cell proliferation has been proposed as a normal mechanism by which androgens control the proliferation of prostate cells and indicates two roles for androgens in controlling the growth of androgen sensitive prostate cells described by de Launoit *et al.* (1991) as a proliferative process and an antiproliferative process. The characteristics of the biphasic response of LNCaP cells to androgens in relation to cell cycle kinetics were studied by De Launoit *et al.* (1991). They reported that the maximal stimulatory concentration of 0.1nM DHT increased the number of cycling cells with an increase in the S and G₂+M fractions. With 1nM DHT there was a decrease in the G₀-G₁ fraction and a return of cell proliferation to control levels suggesting that the inhibitory effect is due to a change in cell cycle parameters rather than a cytotoxic effect of high concentrations of DHT. The ability of the androgen receptor negative cell lines HPC-36M, DU145 and PC-3/MA2 to proliferate in high concentrations of

androgens may indicate an escape from the normal mechanism by which androgens regulate the proliferation of prostate cells in vivo.

It was an interesting finding LNCaP, the only cell line to form testosterone-glucuronide in large amounts, was the most sensitive of the cell lines to exogenously added testosterone and DHT. Perhaps intracellular accumulation glucuronide (testosterone or DHT) is responsible for the sensitivity of LNCaP to testosterone and DHT. The other cell lines which did not form testosterone-glucuronide in such large amounts as LNCaP were able to tolerate much higher concentrations of testosterone and DHT.

The results from the colony-forming assay with LNCaP cells are not identical to those obtained from the MTT assay. For example, the MTT assay indicated that with 10 μ M testosterone and DHT there were more than 60% viable LNCaP cells while the same concentrations in the colony-formation assay were completely inhibitory. The difference in results between the colony-forming assay and the MTT assay reflects the ability of the MTT assay to detect total viable cell number compared to the "more-demanding" nature of the colony-forming assay which requires each individual plated cell to give rise to a colony of at least 50 cells. Thus, small colonies (less than 50 cells) are not recorded despite the fact that they contain viable cells.

Having established that optimal growth of LNCaP cells occurred in the presence of serum supplemented medium, the ability of finasteride to inhibit testosterone-stimulated growth in LNCaP was investigated. LNCaP was between 15 and 42-fold more sensitive to finasteride than HPC-36M, PC-3/MA2 and DU145 when growing in medium containing unstripped serum. From this data it is possible to conclude that the ability of finasteride to inhibit growth is not a direct consequence of inhibiting 5 α -reductase since both testosterone and DHT are present in FCS supplemented medium. The sensitivity of LNCaP to finasteride

must, therefore, be an indirect consequence of its ability to inhibit 5 α -reductase. The ability of 5 α -reductase inhibitors to inhibit cell proliferation can only be demonstrated under conditions where growth of the cells is dependent on DHT. For this reason the study by Bologna *et al.* (1992) examining the effect of finasteride on DU145 and PC-3 proliferation is of no direct relevance to the role of 5 α -reductase inhibitors in controlling the proliferation of androgen-responsive cells in prostate cancer.

The present study failed to demonstrate that finasteride is able to block testosterone stimulated growth of LNCaP cells in DCC-FCS supplemented medium. This supports the interpretation that both testosterone and DHT are able to stimulate growth of LNCaP cells but is in contrast to the report that the LNCaP androgen receptor has a higher relative binding affinity for DHT (Veldscholte *et al.* 1990).

In terms of developing an androgen-responsive cell culture model for testing 5 α -reductase inhibitors the LNCaP is, therefore, a poor model. Culture of androgen-responsive primary human BPH cells should lead to the development of suitable models for testing the growth-inhibiting properties of 5 α -reductase inhibitors.

CHAPTER SIX

TESTOSTERONE METABOLISM IN PRIMARY CULTURES OF HUMAN BPH EPITHELIAL AND FIBROBLAST CELLS

6.1 INTRODUCTION

The culture of prostate epithelium and stroma provides the opportunity to investigate the individual properties of these cell types, such as androgen dependence, patterns of androgen metabolism and distribution of steroid metabolising enzymes. The advantage of cell culture is that the cells may be studied in isolation, under precisely controlled conditions. However, a potential drawback is the possibility of selecting a cell type, perhaps in a different stage of differentiation, that gives rise to a population of cells that are not representative of the original tissue.

Establishing pure cultures of prostate epithelium and stroma first requires a method for isolating the cells. Pure cultures of fibroblasts are readily obtained from explants of tissue (Wilson, 1975; Schweikert *et al.*, 1979; 1982) and, while this technique has also been used to obtain epithelium, contamination by fibroblasts can be a problem (Stonington and Hemmingsen, 1971; Sanford *et al.*, 1977; Clark and Merchant, 1990_b, Syms *et al.*, 1982; Merchant, 1987; Jones and Harper, 1992). This drawback with explant cultures led to the use of enzymic dissociation of the tissue as a basis of separating the epithelial cells from the stromal components prior to culture.

Lasfargues (1957) pioneered the use of collagenase for the primary culture of mammary epithelium and this technique has successfully been used to culture prostatic epithelium (Webber, 1979). Crude collagenases of bacterial origin, e.g. from Clostridium histolyticum / C. perfringens, are the most effective at dissolving collagen by peptide bond cleavage (Mandl, 1972). Normal prostatic

epithelium sits on a basement membrane which separates it from the underlying stromal elements of smooth muscle, fibroblasts, connective tissue, blood vessels, nerve cells and lymphatics (Aumüller, 1983). When prostate tissue is exposed to collagenase the basement membrane is one site of action, in addition to collagen in the stromal elements (Webber, 1979). The epithelial acini are stripped of all supportive stroma without substantial disruption of the acini themselves. These glandular structures are viable and proliferate in culture in contrast to mechanically separated prostate acini which are non-viable and do not proliferate in culture (Franks *et al.*, 1970).

Studies involving primary culture of human prostate epithelium and fibroblasts have tended to focus on the effects of growth factors (Chaproniere and Webber, 1985; Peehl and Stamey, 1986) and agents designed to inhibit growth (Deshpande *et al.*, 1989; Hallowes *et al.*, 1991; Peehl *et al.*, 1991; Mitchen *et al.*, 1993) rather than pathways of androgen metabolism and 5 α -reductase activity. Schweikert *et al.* (1982) reported that fibroblasts derived from explants of BPH tissue metabolised testosterone predominantly to androstenedione and androsterone, rather than DHT. Similar findings were reported by Ofner *et al.* (1984) who observed that the predominant 5 α -reduced metabolite in human BPH fibroblasts and epithelial cells (derived by collagenase digestion) was androsterone. The major metabolite formed by fibroblasts was androstenedione, and not DHT.

The first study to characterise the 5 α -reductase activity in primary cultures of human prostate cells has been reported by Hirsch *et al.* (1993). These authors found that the 5 α -reductase activity in cultured human BPH cells has similar properties to 5 α -reductase 1 in terms of the sensitivity to selective inhibitors of 5 α -reductase 1 and 2. The cultures were predominantly stromal cells and the authors report that DHT was formed at a low level, but details of other metabolites formed are not provided. This study by Hirsch *et al.* provides the first evidence to indicate that under certain conditions human prostate cells express 5 α -reductase 1.

An inherent problem with cultured prostate cells, in particular the epithelium, is the rapid loss of differentiated function (Fong *et al.*, 1991). Fully differentiated prostate epithelial cells in vivo are highly specialised expressing androgen receptors, secreting prostatic acid phosphatase (PAP) and prostate specific antigen (PSA), and able to metabolise androgens. These characteristics are markers of differentiated prostate epithelial cells and to date no one has identified satisfactory culture conditions that preserve all of these features.

One approach to preserving the differentiated function of the cells has been to examine the effects of the basement membrane on production of PSA. In their normal in vivo environment prostate epithelial cells sit on a basement membrane and it is likely that this has some influence on the functioning of the epithelial cells. Culture of prostatic epithelial cells (both primary BPH epithelial cells and LNCaP cells) on reconstituted basement membrane protein (Matrigel), a commercially available preparation containing extracellular components such as laminin, fibronectin and collagens, has been found to enhance the secretion of PAP and PSA compared to identical cells growing on tissue culture plastic (Fong *et al.*, 1991; Fong *et al.*, 1992). This supports the interpretation that the extracellular matrix can modulate the differentiation of epithelial cells.

Prostate epithelium also has a characteristic pattern of cytokeratin expression. The cytokeratins are a class of cytoskeletal intermediate filaments found in epithelial cells (Moll *et al.*, 1982). The pattern of cytokeratins can be used to distinguish cells of simple and complex epithelia and also identify the tissue of origin (Moll *et al.*, 1982; Brawer *et al.*, 1985; Sherwood *et al.*, 1990, 1991). In the prostate, cytokeratins 5, 7, 8, 15, 18 and 19 have been observed in fresh specimens of normal and BPH tissue (Achstätter *et al.*, 1985; Nagle *et al.*, 1987; Sherwood *et al.*, 1990, 1991). Prostate epithelium growing on tissue culture plastic has been found to assume a pattern of cytokeratin expression reflecting that of simple epithelium accompanied by the expression of vimentin, a cytokeratin normally

expressed by mesenchymal cells (Sherwood *et al.*, 1989; 1990). Although the expression of vimentin can be suppressed by growing cells on Matrigel, the characteristic profile of cytokeratin expression is not preserved (Fong *et al.*, 1991), indicating that other, as yet unidentified, factors play a role in the differentiation of prostate epithelial cells.

Since prostate cells *in vivo* are androgen dependent, the androgen responsiveness of cultured cells is another important consideration. Cultured cells which do not respond to androgens but do express 5 α -reductase activity will only be suitable for testing the effects of 5 α -reductase inhibitors on enzyme activity and not on growth. Despite the large number of studies reporting primary culture of prostate epithelial cells, only a few have looked at the direct effects of androgens on growth. In a study on normal prostate epithelial cultures, Webber (1980) found that DHT (0.34 μ M) stimulated epithelial cell growth, as measured by total area density. A much lower concentration of testosterone (0.3nM) was found to stimulate primary cultures of BPH epithelium when total DNA was measured (Hallowes *et al.*, 1991). In a study involving 50 BPH specimens Syms *et al.* (1982) reported that growth of epithelial cells was stimulated by testosterone and DHT as measured by total cell count and area of outgrowth from encapsulated explants (small pieces of tissue which have been kept in suspension to encourage epithelial cell proliferation, prior to plating). When DHT (10nM) was added to BPH epithelial cells cultured on plastic no response was seen, in terms of enhanced PSA or PAP secretion, however, the secretion of PSA and PAP by epithelial cells growing on basement membrane was increased approximately 2-fold by DHT (10nM) (Fong *et al.*, 1991) indicating that the basement membrane may have a role in the way epithelial cells respond to androgens.

The development of isolated prostate epithelial and stromal cell cultures expressing 5 α -reductase activity would provide the means to investigate the expression of 5 α -reductase isozymes and the contribution of the epithelial and

stromal cell types to the overall production of DHT in the prostate. Cultured prostate epithelial and stromal cells which display androgen dependent growth would also be valuable models in testing the potency and efficacy of growth inhibition by 5 α -reductase inhibitors and androgen receptor antagonists.

The aim of this chapter was to develop cultures of separated epithelium and fibroblasts from fresh human BPH tissue and to investigate pathways of testosterone metabolism and 5 α -reductase activity in these cell types. Cultured cells were characterised, along with the prostate cancer cell lines, by using the expression of cytokeratins and production of PSA and PAP as markers of differentiated function.

6.2 **RESULTS**

6.2.1 **Culture of BPH Epithelium**

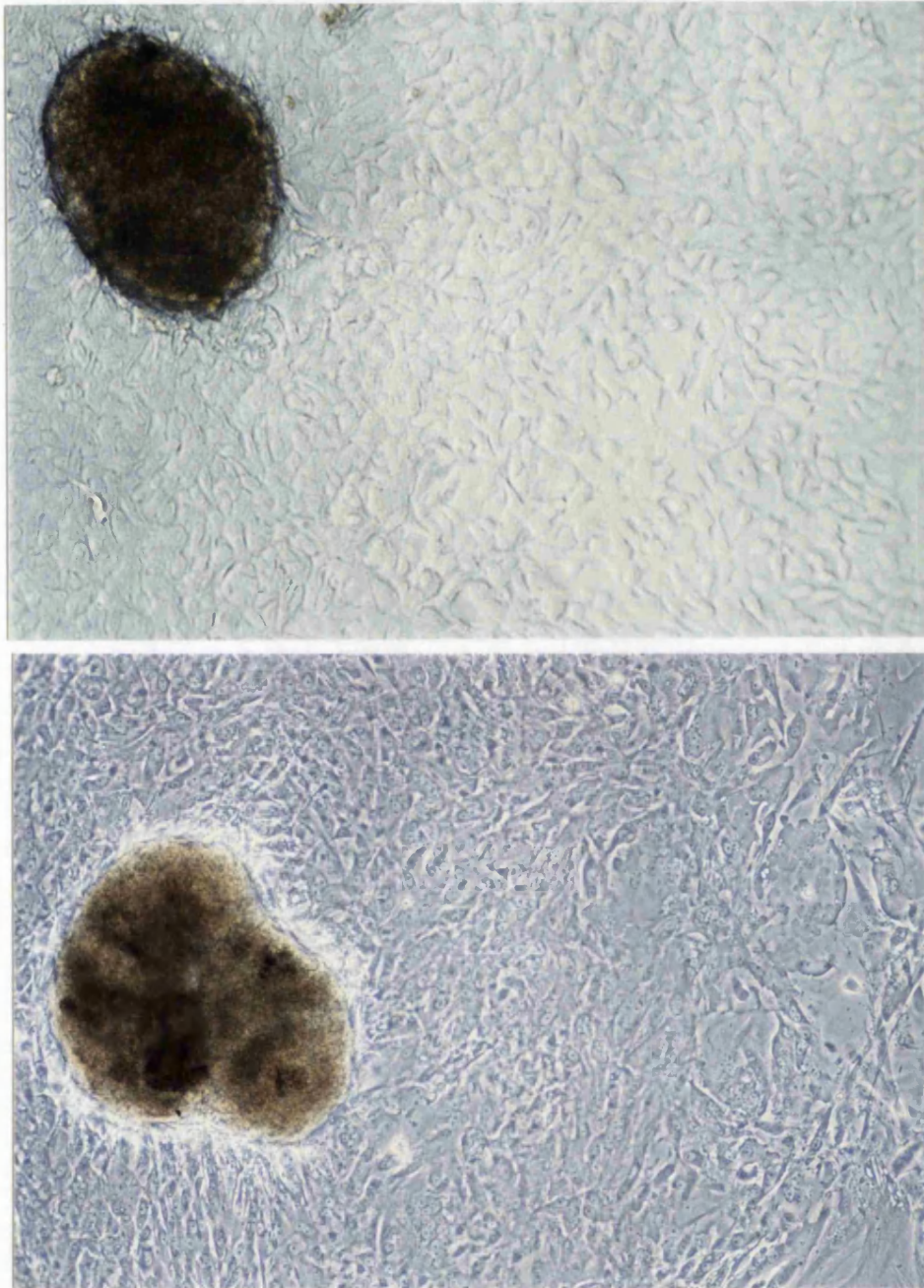
Treatment of minced BPH tissue with collagenase and hyaluronidase overnight (Section 2.7) produced a suspension consisting of small clumps of epithelial cells (organoids) and stromal cells. Organoids were separated from stromal cells by differential sedimentation under gravity and then plated into 24-well Primaria (Falcon) plates containing phenol-red free RPMI 1640 supplemented with 2mM glutamine and 5% FCS. Primaria plates have been developed to support the primary culture of epithelial cells while suppressing proliferation of fibroblasts. The organoids were plated out at approximately ten per well in 0.5ml medium and left for 24 h to attach, after which the medium was replaced with 2ml of fresh medium.

Outgrowth of new cells from the original clump of cells was observed after a further two or three days incubation at 37°C. These polygonal shaped cells grew in close association with each other and had a "cobblestone" morphology characteristic of epithelial cells in vitro (Figure 6.1). The organoids readily attached themselves to Primaria tissue culture plates, however, in contrast to the findings by Fong *et al.* (1991), no attachment of organoids was observed when ordinary tissue culture plastic was coated with Matrigel.

Tissue from a total of 14 patients (ages ranged from 62 - 86 years), whose clinical diagnosis of BPH had been confirmed by microscopic examination as pathological BPH, was used to establish primary cultures. Primaria coated tissue culture plastic was used for epithelial cultures.

FIGURE 6.1

**EPITHELIAL OUTGROWTH FROM ORGANOIDS ISOLATED BY
COLLAGENASE DIGESTION OF HUMAN BPH TISSUE**



Epithelial cells growing out from organoids isolated from different preparations of human BPH tissue as seen under the phase contrast microscope (x250) after 5 days in culture. The cells are growing on in a 24-well Primaria plate in RPMI 1640 supplemented with 5% FCS and 2mM L-glutamine.

In 8/14 attempts to establish cultures epithelial cells grew; in 3/14 attempts the cultures were lost to contamination (fungal and/or bacterial) within 24 h of plating and in 3/14 attempts no cells grew despite the absence of any obvious (microscopic) contamination. Epithelial cells grew for approximately 18 days (range 16-20 days) after which they stopped proliferating. At this stage the cells remained attached to the culture surface for a further two to three days without further division before detaching from the culture surface.

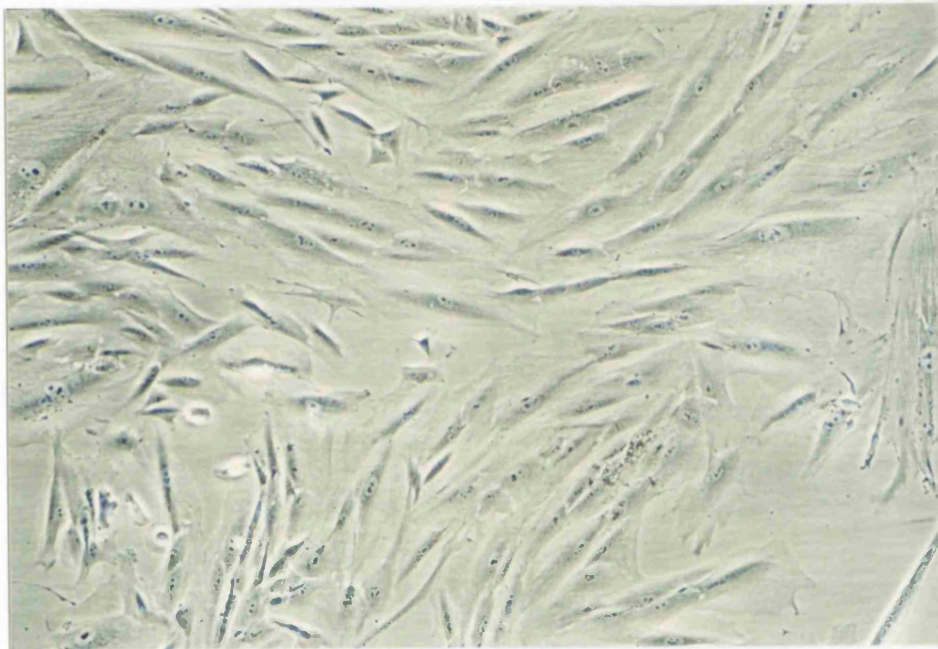
Passaging the epithelial cells was difficult and they required 20-30 minutes incubation at 37°C with trypsin (1x) in order to detach the cells from the culture surface and from each other. Replating after trypsinisation was extremely poor. Fibroblast contamination was rare and when fibroblasts were observed in the cultures they were dealt with by removing the medium from the cultures and incubating with 100µl trypsin (1x) for 1-2 minutes. This treatment followed by gentle tapping of the culture vessel readily detached the fibroblasts from the culture surface, leaving the epithelial cells attached.

6.2.2 Culture of Fibroblasts

The combined supernatants generated during the sedimentation of epithelial clumps were used to generate cultures of fibroblasts by plating into 25cm² standard tissue culture plastic flasks. Growth of fibroblasts followed after a lag period of between five and seven days. The growth of the fibroblasts (as determined by the time to reach confluency) was moderately improved over that in 5% FCS supplemented RPMI by plating the cells in 10% FCS supplemented medium. In contrast to the epithelial cells, fibroblasts had an elongated spindle-shaped morphology (Figure 6.2). At low density the cells were loosely associated while at higher density they grew in characteristic whorls.

FIGURE 6.2

PRIMARY CULTURES OF HUMAN BPH FIBROBLASTS



Fibroblasts isolated by collagenase digestion of human BPH tissue as seen under the phase contrast microscope (x250) after 12 days in culture. The cells are growing on standard tissue culture plastic in RPMI 1640 supplemented with 5% FCS and 2mM L-glutamine.

The success rate for establishing fibroblast cultures (5/14) was lower than that for epithelial cells indicating that the collagenase digestion was more deleterious to the stromal elements than the epithelium. It was found that if the tissue was digested for only two hours with a higher concentration of collagenase (750 units/ml) and then washed and left to attach the lag period was reduced to two or three days.

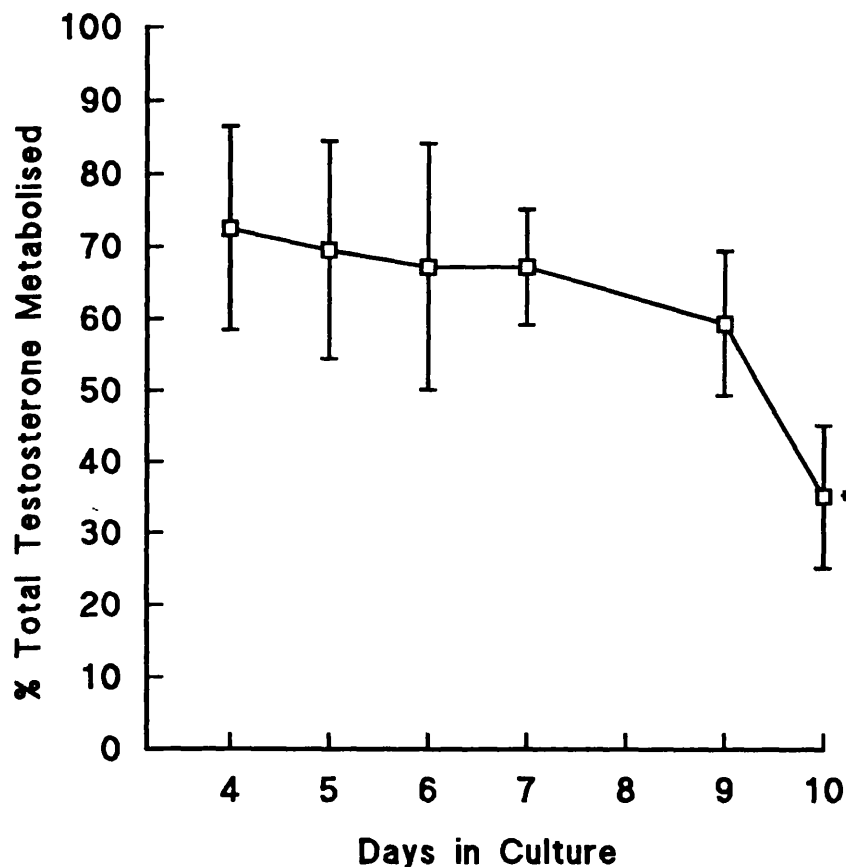
Once initial growth was established the cells grew rapidly (reaching confluency in approximately seven days) and passaged easily with high plating efficiency using trypsin (1x). The fibroblasts survived between 10 and 12 passages before ceasing to proliferate; the longest surviving cultures were maintained for four months.

6.2.3 5 α -Reductase Activity in Cultured BPH Epithelial Cells

The ability of the epithelial cells to metabolise [^3H]-testosterone was investigated as soon as outgrowth of cells could be seen (four day old cultures) and this was followed for a further six days. Despite an apparent increase in the number of cells (no formal measurements were made) the overall conversion of testosterone did not increase and remained constant until day 9. The overall conversion of testosterone on days 5, 6, 7, and 9 was not significantly lower than that measured on day 4 (Figure 6.3). On day 4 approximately 70% of the added substrate was converted to metabolites and the overall conversion was still approximately 60% on day 9. However, the overall conversion on day 10 (35%) was significantly lower than on day 4 ($p < 0.05$). The greatest fall in the overall conversion was between day 9 (60%) and day 10 (35%).

FIGURE 6.3

TIME COURSE OF TESTOSTERONE METABOLISM IN PRIMARY CULTURES OF HUMAN BPH EPITHELIUM



Primary cultures of BPH epithelial cells growing in 24-well Primaria plates were incubated for successive 24h periods with [^3H]-testosterone (20nM, 1 μCi). After every 24h the medium was removed, the cells washed with PBS, and replaced with fresh medium containing [^3H]-testosterone. Metabolites released into the medium were extracted with ethylacetate, separated by TLC and quantified using a radio-TLC plate reader. Metabolites were identified by co-chromatography with authentic standards and by uv light absorption. The results are taken from one time course experiment. Each data point is the mean \pm SD from 24 wells.

(n/d = not detected). * Significantly different ($p < 0.05\%$) from day 4 using Student's t-test.

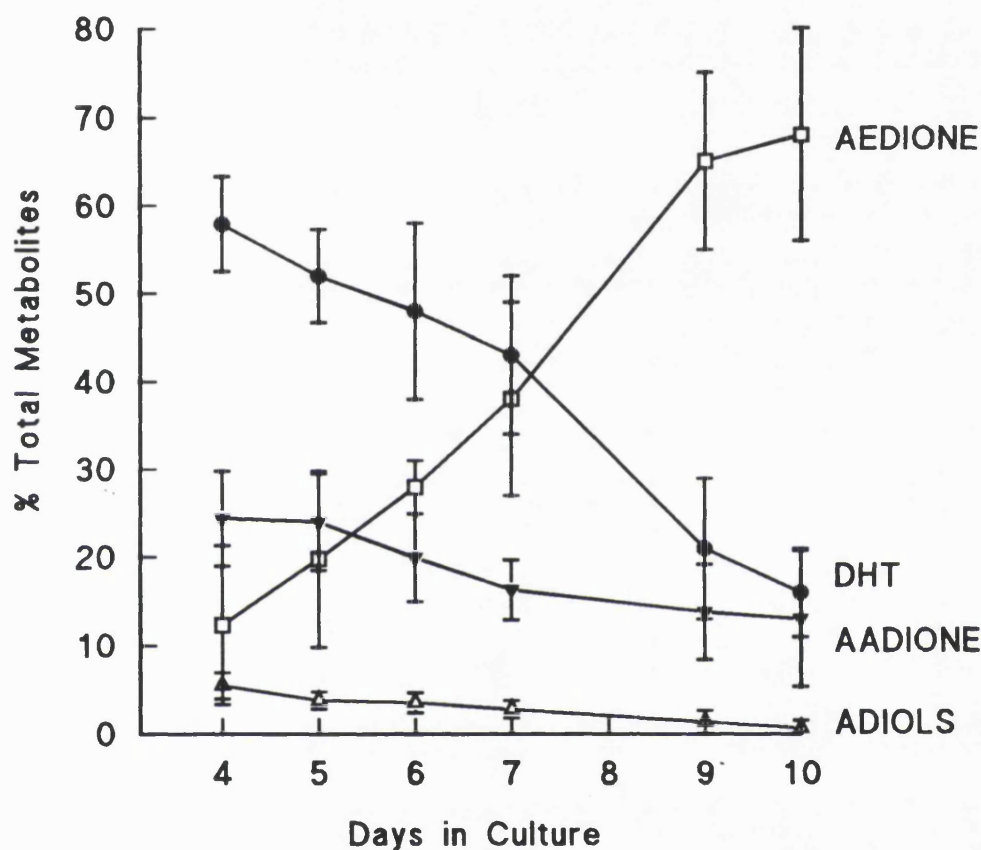
Figure 6.4 shows that during the early stages of culture the predominant metabolite formed by the cells was DHT accounting for approximately 60% of the total metabolites formed. With increasing time there was a steady decline in DHT production which was accompanied by an increase in androstenedione production. The decrease in the production of androstenedione was not as great as that for DHT and androstanediols. By day seven DHT and androstenedione were produced in almost equal amounts and by day ten androstenedione was the major metabolite accounting for approximately 70% of the total metabolites formed (Table 6.1). Epithelial cells older than 15 days produced only androstenedione when incubated with [³H]-testosterone (data not shown).

6.2.4 5 α -Reductase Activity in Cultured BPH Fibroblasts

Owing to the lag period in growth of fibroblasts, at least ten days in culture were required before metabolism of testosterone could be investigated. Suspensions of fibroblasts were prepared and plated into 24-well plates (8×10^3 cells/well) and left for three days to proliferate to approximately 80% confluency (as judged by eye). After this time the medium was removed and replaced with 1ml RPMI (serum-free) containing [³H]-testosterone (20nM, 1 μ Ci) and the cells incubated for 1 h and 24 h. The turnover of substrate after 1 h was approximately 20% in comparison to 60% after 24 h. However, there was no difference in the profile of metabolites formed by fibroblasts after 1 h or 24 h (Table 6.2). At both time points testosterone was converted predominantly to androstenedione with only minor amounts of DHT and androstenedione (less than 5% total metabolites) produced.

FIGURE 6.4

CONVERSION OF TESTOSTERONE IN PRIMARY CULTURES OF HUMAN BPH EPITHELIUM WITH TIME



Primary cultures of BPH epithelial cells growing in 24-well Primaria plates were incubated for successive 24h periods with 1ml of medium containing [^3H]-testosterone (20nM, 1 μCi). After every 24h the medium was removed, the cells washed with PBS, and replaced with fresh medium containing [^3H]-testosterone. Metabolites released into the medium were extracted with ethylacetate, separated by TLC and quantified using a radio-TLC plate reader. Metabolites were identified by co-chromatography with authentic standards and by uv light absorption. The results are taken from one time course experiment. Each data point is the mean \pm SD from 24 wells.

TABLE 6.1

**TESTOSTERONE METABOLISM IN HUMAN BPH EPITHELIUM AFTER
FOUR AND TEN DAYS IN CULTURE**

	% Total Metabolites	
Metabolite	4 Day Cultures	10 Day Cultures
DHT	58 ± 5	16 ± 5
Androstanedione	25 ± 5	14 ± 8
Androstenedione	11 ± 7	69 ± 12
Androstanediols	5 ± 2	0.6 ± 0.9
Total Testosterone Metabolised	72 ± 14%	35 ± 20%

BPH epithelial cells growing in 24-well Primaria plates were incubated for 24h with [³H]-testosterone (20nM, 1μCi) at 4 and 10 days after initial plating. Metabolites released into the medium were extracted by ethylacetate, separated by TLC and quantified using a radio-TLC plate reader. Metabolites were identified by co-chromatography with authentic standards and by uv light absorption. The results are taken from one time course experiment and each data point is the mean ± SD from 24 wells.

TABLE 6.2

TESTOSTERONE METABOLISM IN PRIMARY CULTURES OF HUMAN BPH FIBROBLASTS

Metabolite	% Total Metabolites Formed	
	1h Incubation	24h Incubation
DHT	1 ± 0.8	1 ± 1
Androstenedione	96 ± 8	98 ± 3
Androstanedione	3 ± 5	2 ± 3
Androstanediols	n/d	n/d
Total Testosterone Metabolised	19 ± 6%	61 ± 15%

Primary cultures of BPH fibroblasts (passages 2, 4 and 5) growing in 24-well plates were incubated for 1 and 24h with [³H]-testosterone (20nM, 1μCi). Metabolites released into the medium were extracted with ethylacetate, separated by TLC and quantified using a radio-TLC plate reader. Metabolites were identified by co-chromatography with authentic standards and by uv light absorption. The results are the mean ± SD of data from 3 experiments. (n/d = not detected)

6.2.5 Characterisation of BPH Epithelial Cell 5 α - Reductase Activity with SKF 105,657 and UK 117,026

The compounds UK 117,026 and SKF 105,657 were selected for these experiments because in Chapter 4 they were found to have the greatest difference in selectivity for 5 α -reductase 1 and 2 isozymes respectively.

Initially, low concentrations of the inhibitors (10nM SKF 105,657, 30nM UK 117,026) were used as it was not known if there would be any cytotoxic effects, that were unrelated to 5 α -reductase inhibition, on the cells. Based on the data from Chapter 4 (Table 4.1) these concentrations were predicted to give 0% inhibition of 5 α -reductase 1 by SKF 105,657 and 10% inhibition of 5 α -reductase 1 by UK 117,026. Similarly, these concentrations were predicted to give 70% inhibition of 5 α -reductase 2 for SKF 105,675 and 0% inhibition of 5 α -reductase 2 by UK 117,026.

Incubation of the cells with 10nM SKF 105,657 and 30nM UK 117,026 for 24 h did not have any major cytotoxic effects as determined by the appearance of the cells under the microscope or by cells lifting up from the culture surface. Higher concentrations were then used with the aim of completely inhibiting 5 α -reductase 2 with SKF 105,657 (90nM) but without inhibiting 5 α -reductase 1. It was considered that concentrations of UK 117,026 that would completely inhibit 5 α -reductase 1 would probably be toxic to the cells so a concentration of UK 117,026 (300nM) was chosen that would inhibit 5 α -reductase 1 by more than 50%. This concentration was not expected to inhibit 5 α -reductase 2. No obvious cytotoxic effects were evident when the cells were incubated with 90nM SKF 105,657 or 300nM UK 117,026.

The effects of SKF 105,657 and UK 117,026 on the production of the metabolites DHT, androstenedione and androstanedione by cultured prostate epithelial cells are shown in Table 6.3. The inhibition of DHT production by 90nM SKF 105,657 was no greater than that produced with 10nM. In both cases the production of DHT was inhibited by approximately 60% which was greater, but not statistically significant (Student's t-test), than the decline in DHT produced in no-inhibitor control cells (40%).

Increasing the concentration of UK 117,026 from 30nM to 300nM did not result in greater inhibition of DHT production. Furthermore, the reduction of DHT production with 300nM UK 117,026 was no different to that measured in cells incubated without inhibitor. Both SKF 105,657 and UK 117,026 inhibited the production of androstanedione by approximately 50%. The reduction in androstanedione produced by both concentrations of UK 117,026 was statistically significant ($p < 0.1$). However, while the reduction in androstanedione by 10nM SKF 105,657 was statistically different ($p < 0.1$) the reduction in androstanedione by 90nM SKF 105,657 was not significantly different from no-inhibitor control cells. Androstenedione production was only significantly increased ($p < 0.1$) above no-inhibitor control in cells incubated with 90nM SKF 105,657.

TABLE 6.3

THE EFFECT OF SKF 105,657 AND UK 117,026 ON THE FORMATION OF TESTOSTERONE METABOLITES IN HUMAN BPH EPITHELIUM

Concentration of Inhibitor	Increase or Decrease in Metabolite Production		
	DHT	AEDIONE	AADIONE
No Inhibitor	-40 ± 16	+21 ± 7	-20 ± 8
SKF 105,657 10nM	-66 ± 5	+31 ± 3	-55 ± 10*
90nM	-62 ± 18	+62 ± 38*	-43 ± 16
UK 117,026 30nM	-55 ± 5	+26 ± 3	-56 ± 5*
300nM	-40 ± 10	+23 ± 13	-57 ± 8*

The effect of SKF 105,657 and UK 117,026 on the production of the metabolites shown in the Table was determined in BPH epithelial cells which had been cultured for 7 days. Each well served as its own control by determining the amounts of metabolites produced when the cells were incubated only with [³H]-testosterone (20nM, 1μCi) for 24h in the absence of inhibitor. The same wells were then incubated for 24h in the presence of [³H]-testosterone. Metabolites released into the medium were extracted by ethylacetate, separated by TLC and quantified using a radio-TLC plate reader. The results shown are the mean ± SD from one experiment consisting of 5 wells per inhibitor and 12 wells in the absence of inhibitor. + = increased production ; - = decreased production; AEDIONE = androstenedione; AADIONE = androstanedione. * Significantly different (p<0.1, Student's t-test) from no-inhibitor control cells.

6.2.6 Characterisation of BPH Cells, Cultured BPH Epithelium and Prostate Cancer Cell Lines

Cultured BPH epithelium (either at four or ten days) did not express PSA and PAP; expression was only observed in the epithelial cells in BPH tissue digests (Table 6.4). All four of the prostate cancer cell lines expressed PAP, while LNCaP was the only cell line to express PSA (Table 6.5). Vimentin was expressed by cultured epithelium (after 4 and 10 days in culture) as well as all four prostate cancer cell lines; it was not detected in freshly isolated BPH epithelial cells. As expected, desmin and α -actin (smooth muscle markers) were not expressed by the epithelial cultures or the cell lines; only the stromal components in the BPH digest expressed desmin and α -actin.

Epithelial cells in the BPH tissue digest were strongly positive for cytokeratins 4, 7, 8, 13, 14, 18 and 19 (Table 6.4). Epithelial cells cultured for four days retained expression of all of these, although cytokeratins 7, and 14 were expressed at a lower level by the cultured cells. Ten day old cultured epithelium did not express cytokeratins 4 and 13 but retained expression of the other cytokeratins. Expression of cytokeratins 8, 18 and 19 was much stronger in cultured epithelium than in freshly isolated cells and cultured epithelium expressed cytokeratins 10 and 17 which were not detected in freshly isolated cells.

6.2.7 Characterisation of BPH Fibroblasts

In contrast to freshly isolated prostate epithelial cells, cultured BPH fibroblasts did not express PSA or PAP. Fibroblasts were positive only for the expression of vimentin and did not express desmin, α -actin or any of the other cytokeratins tested. In freshly isolated stromal cells a small number of desmin and α -actin positive cells were observed but these cells did not grow in culture.

TABLE 6.4

CHARACTERISATION OF BPH EPITHELIUM

Antibody	Epithelium in Digest	Epithelium 4 Days	Epithelium 10 Days
PSA	++	-	-
PAP	++	-	-
Desmin	-(+s)	-	-
Vimentin	-(+s)	+++	+++
α -actin	-(+s)	-	-
Cyk 4	++	++	-
Cyk 5	n/d	n/d	n/d
Cyk 7	++	+/-	+/-
Cyk 8	++	+++	+++
Cyk 10	-	++	++
Cyk 13	++	+/-	-
Cyk 14	++	+/-	+
Cyk 15	n/d	n/d	n/d
Cyk 16	-	-	-
Cyk 17	-	+++	+++
Cyk 18	++	+++	+++
Cyk 19	++	+++	+++

The Table summarises the immunoreactivity of BPH cell suspensions and cultured BPH epithelial cells (4 and 10 day old cultures) with antibodies to prostate specific antigen (PSA), prostatic acid phosphatase (PAP), desmin, vimentin, α -actin and a panel of cytokeratins (cyk). Peroxidase and FITC-conjugated secondary antibodies were used to detect binding. Cultured BPH epithelium and cytopsin preparations of BPH cell suspensions were fixed in methanol:acetone (1:1 v/v) prior to staining. Key: - = no positive cells; +/- = <10% cells positive; + = 10-20% cells positive; ++ = 30-50% cells positive; +++ = 50-100% cells positive; +s = stromal cells (only) positive.

TABLE 6.5

CHARACTERISATION OF PROSTATE CANCER CELL LINES

Antibody	HPC-36M	DU145	PC-3/Ma2	LNCaP
PSA	-	-	-	++
PAP	+	+	+	++
Desmin	-	-	-	-
Vimentin	+++	+++	+++	+++
α -actin	-	-	-	-
Cyk 4	-	+	-	-
Cyk 7	-/+	-	+/-	-
Cyk 8	+++	+++	+++	+++
Cyk 10	++	++	++	++
Cyk 13	-	-	-	-
Cyk 14	+	-	+	++
Cyk 16	-	-	-	-
Cyk 17	-	-	-	-
Cyk 18	+++	+++	+++	+++
Cyk 19	-	+++	+++	+

The Table summarises the results of immunoreactivity of prostate cancer cells with antibodies to prostate specific antigen (PSA), prostatic acid phosphatase (PAP), desmin, vimentin, α -actin and a panel of cytokeratins (cyk). Peroxidase and FITC-conjugated antibodies were used to detect binding. Cytospin preparations of cell suspensions were fixed in methanol:acetone (1:1 v/v) prior to staining. Key: - = no positive cells; +/- = <10% cells positive; + = 10-20% cells positive; ++ = 30-50% cells positive; +++ = 50-100% cells positive.

Cell suspensions prepared from BPH tissue by collagenase digestion have a high capacity for converting testosterone to DHT and the results shown in Chapter 4 support the interpretation that 5 α -reductase 2 is the enzyme responsible for producing DHT in these cells. The aim of this study was to investigate testosterone metabolism and characterise 5 α -reductase activity in cultured BPH epithelial and stromal cells. The exact contribution of 5 α -reductase 1 to the production of DHT in the prostate has not yet been determined and the culture of prostate epithelial and stromal cells provide an approach to investigate the distribution of 5 α -reductase isozymes in prostate cell types.

In this study, cultured BPH epithelial and stromal cells metabolised testosterone, but the overall profile of metabolites produced was quite distinct from that produced by freshly isolated BPH cells. Furthermore, whilst the profile of metabolites produced by cultured epithelial cells (at least during the first few days of culture) resembled that of the tissue from which it had been derived, the profile of metabolites produced by cultured fibroblasts was quite different. This may have been due to the long lag time before testosterone metabolism could be measured in the fibroblasts.

The major metabolite produced by cultured epithelial cells until day 7 was DHT. Other 5 α -reduced metabolites such as androstenedione and androstenediols were also formed, indicating that other enzyme activities including 3 α / β -hydroxysteroid dehydrogenase and 17 β -hydroxysteroid dehydrogenase (17 β -HSD) were present in these cells. Up to day 8 of culture the sum of the 5 α -reduced metabolites exceeded the production of androstenedione indicating that the profile of metabolites in the cultured epithelial cells is similar to the tissue they were derived from. However, by day 9 of culture androstenedione was the

predominant metabolite produced and exceeded production of 5α -reduced metabolites.

The change in profile of metabolites produced by the epithelial cells is difficult to interpret without a corresponding cell number or protein concentration at each time point to relate the enzyme activities to. Androstenedione production increased approximately six fold from day 4 to day 10. If the cell number also increases six fold from day 4 to day 10, which would seem likely, then there is no increased expression of 17β -HSD on a per cell basis. However, if the cell number does not increase by this much then it would appear that there is an increased expression of 17β -HSD on a per cell basis. In contrast to androstenedione, DHT production fell by almost four fold from day 4 to day 10. If 5α -reductase activity was constant in each cell with time and 17β -HSD was using up all the testosterone then the production of androstenedione would be expected to increase. This was not the case and the production of androstenedione also decreased with time. The possibility exists that the 5α -reductase activity was gradually diluted with each cell division so that 17β -HSD was the predominant enzyme expressed in the cells. Whatever the mechanism the implication from this experiment is that the cell in culture are expressing or producing much less active 5α -reductase than those in the prostate.

In this study, the predominant metabolite formed by cultured fibroblasts was androstenedione, DHT was produced only in low amounts. In this respect, the profile of testosterone metabolism in cultured fibroblasts did not resemble that seen in BPH cell suspensions or tissue slices (Chapter 3). Androstenedione was the major metabolite detected in 1 h and 24 h incubations confirming the published data that androstenedione is the major metabolite produced after 1 h in cultured BPH fibroblasts but contrasting with the observation that longer incubation times (24 h) result in androsterone as the major metabolite (Schweikert *et al.*, 1982). These results contrast the finding from freshly isolated

BPH stroma and epithelium that 5 α -reductase activity is concentrated in the stroma of BPH tissue (Cowan *et al.*, 1979; Romijn *et al.*, 1980; Krieg *et al.*, 1981; Bartsch *et al.*, 1982; Rennie *et al.*, 1983; Voigt *et al.*, 1986; Bruchovsky *et al.*, 1981, 1988). Fibroblasts, maintained in culture, like epithelial cells, do not retain their ability to metabolise testosterone to 5 α -reduced metabolites after 10 days in culture under the conditions used in this study.

An inherent problem with the method used in this study for culturing the fibroblasts was the lag time taken for the cells to actively commence proliferating. By the time enough cells were available for assay only a low level of 5 α -reductase activity could be detected. Although further modification of the method (Section 6.2.2) resulted in a quicker production of fibroblasts, DHT was still only produced at a low level.

Experiments aimed at characterising the 5 α -reductase activity in cultured BPH epithelial cells using SKF 105,657 indicated that the 5 α -reductase activity in these cells was less sensitive to SKF 105,657 than 5 α -reductase activity in freshly isolated BPH cell suspensions. This observation supports the recent findings by Hirsch *et al.* (1993) who demonstrated that primary cultures of BPH cells are less sensitive to inhibition by finasteride than freshly isolated cells. However, Hirsch *et al.* state that their primary cultures were predominantly stromal whereas in the present study described the cells were predominantly epithelial. If 5 α -reductase 2, the major prostatic form of 5 α -reductase, was responsible for producing DHT in the primary epithelial cells then 10nM SKF 105,657 should have reduced DHT production by more than 50% and 90nM SKF 105,657 should have inhibited DHT production totally. This was not the case and the inhibition produced by both concentrations of SKF 105,657 was not significantly different from that being produced in no control cells. This suggests that DHT was not being produced solely by 5 α -reductase 2.

If 5 α -reductase 1 was responsible for producing the DHT detected in the presence of SKF 105,657, then addition of UK 117,026 should have inhibited the production of DHT. However, this was not the case and no inhibition of DHT production was observed when the cells were incubated with 300nM UK 117,026. The IC₅₀ for inhibition of 5 α -reductase 1 by UK 117,026 is approximately 200nM. The concentration of 300nM UK 117,026 in this study was selected so that it was high enough to produce a significant inhibition of 5 α -reductase 1 but low enough not to be toxic to the cells. Inhibition of DHT production in the epithelial cells may have been produced if this concentration had been raised or if UK 117,026 had been used in combination with SKF 105,657. The selective 5 α -reductase 1 inhibitor, LY 191,704, used in the study by Hirsch *et al.* (1993) is a more potent inhibitor of 5 α -reductase 1 with an IC₅₀ of less than 10nM. Hirsch *et al.* (1993) were able to show what this present study failed to, i.e. that 5 α -reductase 1 selective inhibitors are more potent against 5 α -reductase expressed in cultured prostate cells than in freshly isolated cells. However, Hirsch used 10-12 μ M testosterone and since the K_m of 5 α -reductase 1 is 1.7 μ M and 5 α -reductase 2 is less than 0.2 μ M (Thigpen *et al.*, 1993) his assay was heavily biased toward demonstrating 5 α -reductase 1 while the experiments in this chapter with 20nM testosterone would favour 5 α -reductase 2 activity. The experiments in this Chapter are more physiologically relevant and would detect 5 α -reductase 2 activity which Hirsch would fail to detect.

One limitation with the experiments described in this Chapter relates to the fact that the effects of the inhibitors had to be assessed against a background of falling DHT production. Ideally, a constant level of DHT production is needed to determine how effective a compound is at inhibiting the production of DHT. An improved approach might be to remove the cells from the culture surface and divide the cells from each well into two with one half treated with inhibitor and the other used to establish the control level of 5 α -reductase activity. In addition, the toxicity of the compounds on cell proliferation needs greater consideration.

It is important to establish that a decrease in DHT production, during inhibition studies, is a result of 5 α -reductase inhibition and not due to the cells being affected by a non-selective toxic mechanism.

In this study the expression of PSA and PAP could not be detected in cultured BPH epithelial cells. In addition, the cells had an altered profile of cytokeratin expression compared to that observed in freshly isolated epithelial cells and also that previously reported for BPH epithelium in situ. Expression of PSA and PAP was detected in freshly isolated BPH cells but was not present in epithelial cells which had been cultured for 4 days. As expected, all of the prostate cancer cell lines expressed PAP, but LNCaP was the only prostate cancer cell line to express both PSA and PAP.

Freshly isolated epithelial cells and cultured epithelial cells expressed cytokeratins 7, 8, 18, and 19 characteristic of simple epithelial cells. (Barwick and Mardi, 1983; Achst tter *et al.*, 1985; Sherwood *et al.*, 1989, 1990). The prostate cancer cell lines also displayed cytokeratin profiles characteristic of simple epithelium. The induction of cytokeratins 10 and 17 in cultured BPH epithelial is consistent with the previous findings of Sherwood *et al.* (1989, 1990); Hallowes *et al.* (1991) and Mitchen *et al.* (1993). Cytokeratins 10 and 17 are usually associated with epidermis (Moll *et al.*, 1982) and their expression in cultured BPH epithelium is, therefore, an unexpected finding and indicates an alteration in the normal pattern of cytokeratin expression that can occur when cells are maintained in culture. Vimentin was also expressed by cultured BPH cells and all of the prostate cancer cell lines. Vimentin is generally present in mesenchymal cells (Gown and Vogel, 1982) and is not expressed by epithelial cells in situ. Induction of vimentin expression in cultured BPH epithelial cells is reported to be suppressed by basement membranes of collagen and laminin (Hallowes *et al.*, 1991; Fong *et al.*, 1991) indicating the importance of the extracellular matrix in influencing the differentiation of the cells.

Previous reports have shown that prostate epithelial cells cultured on Matrigel have enhanced production of PSA and PAP (Fong *et al.*, 1991), however, the influence of the extracellular matrix on the expression of 5 α -reductase by primary prostate epithelial cells in culture has not been examined by these authors. One of the aims of this study was to examine this question, however, the failure of the cells to attach to culture surface coated with Matrigel prevented further investigation.

The production of PSA and PAP by prostate epithelial cells has been shown to be modulated by androgens (Huggins and Hodges, 1941; Goldfarb *et al.*, 1986; Montgomery *et al.*, 1992). Interestingly, in LNCaP the production of PAP is down-regulated in response to androgens (Henttu *et al.*, 1992) and the mechanism responsible for this is unknown. Two possible factors may relate to the lack of PSA and PAP expression in cultured prostate cells: the first is the loss of androgen receptor expression, and the second is the absence of stromal elements. Considering the former, the only cell line to express high levels of androgen receptor, LNCaP (Tilley *et al.*, 1990), is the only cell line to also express PSA. In cultured BPH cells the lack of PSA expression may relate to a loss in androgen receptor expression although this cannot be definitely concluded as this parameter was not measured. The report that the production of PSA and PAP in primary epithelial cells grown on Matrigel could be increased by DHT and by stromal cell conditioned medium (Fong *et al.*, 1991) supports the interpretation that both factors (androgen receptors and stromal cell interactions) are involved. Some evidence for this has been provided by the observation that cultured stromal cells from rat prostate can induce protein secretion in the rat epithelial cell line PA-III (Djakiew *et al.*, 1990). Further evidence for a role of the stroma in supporting the stimulation of epithelial cells was provided by Kabalin *et al.* (1989) who demonstrated that the growth of human prostate epithelial cells was greater when cocultured with human fibroblasts than when cultured alone.

Cultured fibroblasts expressed vimentin but none of the other cytokeratins. Weak staining with antibodies to desmin and α -actin was observed in the freshly digested BPH tissue and indicated that smooth muscle cells were present (Shapiro *et al.*, 1992), but the lack of desmin and α -actin expression in cultured stromal cells suggested that only fibroblasts had been cultured.

The data from this study supports the interpretation that the cells that emerge from prostatic acinar tissue in culture are undifferentiated and may represent basal cells (simple non-secretory epithelium), in contrast to luminal secretory cells (Merchant *et al.*, 1983, 1990a, 1990b, Heatfield *et al.*, 1980; Jones and Harper, 1992). The time-dependent decline of 5 α -reductase activity in cultured epithelial cells despite an apparent cell proliferation is indicative of reduced expression compared with cells in vivo. In addition, the data from experiments using the 5 α -reductase inhibitors, SKF 105,657 and UK 117,026, indicate a difference in sensitivity of 5 α -reductase in cultured cells compared to freshly isolated BPH cells which may indicate a change in the expression of 5 α -reductase enzymes during culture.

The results from this study provide the background and scope for further investigation into the expression of 5 α -reductase 1 and 2 in primary cultures of human prostate cells.

CHAPTER SEVEN

FINAL DISCUSSION AND CONCLUSIONS

The main objective of this thesis was to identify and develop suitable systems for the study of androgen metabolism in the human prostate in vitro. Pathways of androgen metabolism were studied in prostate cells using human BPH slices and cell suspensions, human prostate cancer cell lines and primary cultures of separated BPH epithelium and stroma. The rationale for developing in vitro models of human prostate cells was to produce systems for the evaluation of compounds designed as 5 α -reductase inhibitors for the treatment of BPH and possibly prostate cancer.

It is well established that the major metabolite of testosterone produced by human prostate tissue is DHT. This consistent finding has led to the suggestion that DHT is implicated in the development of BPH and prostate cancer. Perhaps the strongest evidence to support this hypothesis is the observation that 5 α -reductase deficient males have smaller prostates than normal males and do not develop BPH or prostate cancer (Imperato-McGinley, 1974). Therefore, if a critical level of DHT in the prostate is necessary for the development and maintenance of BPH and prostate cancer then a drug that reduces DHT in prostate tissue should provide an effective treatment alternative to surgery.

The enzyme 5 α -reductase is an ideal target for inhibition of DHT production because its prime function in the prostate is to convert testosterone to DHT and there should be no effect on the production of the other major steroid hormones and also no impairment of sexual potency. The development of effective compounds for the selective inhibition of 5 α -reductase requires appropriate in vitro models in which to evaluate selectivity and potency. For example it is known that the major form of 5 α -reductase in human BPH tissue is 5 α -reductase 2 and

therefore any in vitro model of BPH should also display 5 α -reductase 2 activity. The study of androgen metabolism and the characterisation of 5 α -reductase activity in prostate cell systems such as those investigated in this thesis is fundamental in establishing their use as in vitro models of the human prostate.

7.1 Testosterone Metabolism in Human BPH Tissue: Implications for the Treatment of BPH with 5 α -Reductase Inhibitors

Other investigators have evaluated the potency of 5 α -reductase inhibitors using homogenates of fresh human BPH tissue incubated with non-physiological concentrations of testosterone (1-10 μ M) and an NADPH regeneration system (Liang *et al.*, 1983, 1985; Jenkins *et al.*, 1992). Recently, COS cells expressing 5 α -reductase 1 and 2 isozymes have been used in the same way, under similar conditions to tissue homogenates to evaluate the selectivity of new inhibitors (Andersson *et al.*, 1991; Harris *et al.*, 1992; Jones *et al.*, 1993; Hirsch *et al.*, 1993; Mellin *et al.*, 1993). The expression of 5 α -reductase isozymes in COS cells has the advantage of being able to produce large amounts of relatively concentrated enzyme. Furthermore, the properties of COS cell expressed 5 α -reductase 2 have been found to closely resemble those of 5 α -reductase activity in human BPH homogenates indicating that this is an appropriate system with which to study 5 α -reductase 2. To date, 5 α -reductase 1 activity has not been demonstrated in freshly isolated BPH tissue so is not possible to be certain of the extent to which the properties of COS cell expressed 5 α -reductase 1 resembles those of 5 α -reductase 1 in human prostate tissue. However, 5 α -reductase 1 does resemble the 5 α -reductase activity in homogenates of human scalp and non-genital skin (Harris *et al.*, 1992; Mellin *et al.*, 1993).

The main disadvantage to using 5 α -reductase enzymes expressed in non-prostate cells is that the enzymes are being studied out of their natural environment and away from prostate-specific factors that may serve to regulate their activity. This

study is the first to report the investigation of testosterone metabolism and the evaluation of 5 α -reductase inhibitors using intact BPH cell suspensions incubated in the presence of physiological concentrations of testosterone without added co-factors. A comparison of the profiles of testosterone metabolism in cell suspensions with that formed by BPH tissue slices confirmed that the characteristic pattern of prostate testosterone metabolism was preserved in the BPH cell suspensions. Furthermore the 5 α -reductase activity in BPH cell suspensions has similar properties to COS cell expressed 5 α -reductase 2.

Further study revealed that the cell suspensions provided a reproducible system ideal for comparing inhibitors of 5 α -reductase. In the cell suspensions 5 α -reductase activity was distributed evenly when the cells were aliquoted in an assay system. This contrasts the situation of using tissue slices where the activity can vary between experimental replicates. BPH homogenates provides a reproducible system for evaluating 5 α -reductase inhibitors but, unlike BPH cell suspensions, has the disadvantage that the cells are disrupted and the enzyme is released from its normal in vivo environment allowing the inhibitor free access. In BPH cell suspensions the enzyme remains confined inside the plasma membrane of the cells and as in vivo the inhibitor must first cross the plasma membrane in order to gain access to the enzyme. This feature of BPH cell suspensions offers a significant advantage over using tissue homogenates which have frequently been used to evaluate 5 α -reductase inhibitors.

In addition to providing a suitable system for testing 5 α -reductase inhibitors, the BPH cell suspensions provided a source of raw material for establishing primary cultures. These cultures offer the possibility of studying the distribution and types of enzyme activity, the levels of 5 α -reductase activity, and the effects of endogenous and exogenous molecules on growth of the cells. Furthermore, cell cultures potentially provide the opportunity for evaluating the potency of 5 α -reductase inhibitors to inhibit DHT production and DHT-stimulated cell

proliferation. However, in this study the experiments were limited by the fact that capacity to form DHT declined in the cultured epithelium and also by the low activity of 5 α -reductase in the cultures of fibroblasts.

The finding that SKF 105,657, a potent inhibitor of 5 α -reductase activity in freshly isolated BPH cells, was less active against the 5 α -reductase activity in cultured BPH epithelium supports the interpretation that the 5 α -reductase activity expressed by cultured BPH epithelium is different to that expressed by freshly isolated cells. The results, supported by the recent report of Hirsch *et al.* (1993), indicate that BPH cells may express 5 α -reductase 1 but it is only detected in primary cultures when the level of 5 α -reductase 2 activity drops.

Growth of BPH cells on a solid surface, immersed in a nutrient medium does not appear to be an environment conducive to maintaining differentiation and sustaining normal cell function (in particular 5 α -reductase 2 activity) even though the cells continue to proliferate. In vivo the typical prostate epithelial cell is arranged in a complex histological structure, separated from stromal elements by a basement membrane. Nutrients and chemical signals from the blood must pass through the stroma and basement membrane to reach the cells. Luminal cells are bathed in secretory products and absorption of various factors through their apical surface may play a role in regulating the functioning cell. Therefore, cell culture conditions need to be designed so that the specialised functions of the prostate epithelial cells are retained. This will lead to the development of more relevant in vitro models of prostate cells in vivo.

Finasteride is the first 5 α -reductase inhibitor licensed for the treatment of BPH. Although treatment with finasteride reduces the concentration of DHT in the prostate by up to 80% i.e. to a level equivalent to that following castration, this is accompanied by only a modest reduction (approximately 20%) in the volume of the prostate and small improvements in urine flow rate (The MK906

(Finasteride) Study Group, 1991; Gormley *et al.*, 1992). Agents designed to treat BPH have to reduce the enlarged gland in addition to preventing further growth. The ideal goal is that 5 α -reductase inhibitors will be able to shrink the enlarged gland so that urine outflow from the bladder is improved to an extent approaching that achieved by surgery. Finasteride is a selective inhibitor of 5 α -reductase 2 and perhaps the development of potent dual inhibitors of 5 α -reductase 1 and 2 may prove more efficacious in the treatment of BPH. A potent inhibitor of both isozymes potentially could completely inhibit DHT production and may produce a greater reduction in prostate volume, providing more effective relief of the symptoms caused by BPH.

7.2 Testosterone Metabolism in Human Prostate Cancer Cell Lines: Implications for the Treatment of Prostate Cancer with 5 α -Reductase Inhibitors

This is the first study to compare, in detail, the pathways of testosterone metabolism in the human prostate cancer cell lines, and also the first to report characterisation of 5 α -reductase activity in HPC-36M and DU145. The cell lines differed in their rate of testosterone metabolism and in the profile of metabolites formed even though they have been established from tissue taken from prostatic tumours. The only overall similarity between the four cell lines was the low amount of DHT produced.

Two of the cell lines, PC-3/MA2 and LNCaP, had extremely high capacity for removal of the potent androgen testosterone. This was by the conversion to androstenedione in PC-3/MA2 and by the formation of testosterone-glucuronide in LNCaP. The two cell lines, HPC-36M and DU145, which did form DHT as the major metabolite had the lowest rates of testosterone metabolism. The formation of testosterone glucuronide by LNCaP is an interesting phenomenon and may relate to its greater sensitivity to testosterone and DHT observed in

growth assays. The fact that the other cell lines could tolerate much higher concentrations of testosterone and DHT is probably unrelated to their ability to metabolise testosterone. Some investigators have postulated that the growth inhibitory effect of androgens on LNCaP cells is mediated by the androgen receptor (de Launoit *et al.*, 1991). However, the androgen receptor negative cell lines did not form testosterone glucuronide in large amounts, like LNCaP, and therefore it may be speculated that accumulation of testosterone-glucuronide may be toxic to LNCaP cells.

The 5 α -reductase activity in HPC-36M and DU145, the only lines to form predominantly DHT, differed in its sensitivity to 5 α -reductase inhibitors compared with BPH cell suspensions. The sensitivity of the 5 α -reductase activity in HPC-36M and DU145 to inhibitors resembled that of 5 α -reductase 1. Based on the findings with epithelial primary cultures it is reasonable to postulate that during the culture of the prostate tumour cells the expression of 5 α -reductase 2 has been lost leaving only 5 α -reductase 1 activity. To date the characterisation of 5 α -reductase activity, using selective inhibitors, in fresh human prostate cancer tissue has not been reported. This is necessary to determine how closely human prostate cancer cell lines resemble the *in vivo* situation. If the 5 α -reductase activity in the prostate cancer cell lines does resemble 5 α -reductase in prostate cancer tissue *in vivo* then potent inhibitors of 5 α -reductase 2, such as finasteride, would not be expected to be as efficacious as in the treatment of BPH.

The precise role of DHT in the pathogenesis of prostate cancer has not been established. Epidemiological studies have shown that men castrated prior to puberty do not develop prostate cancer and males with 5 α -reductase deficiency are also reported not to develop prostate cancer. Thus, if DHT has a role in prostate cancer then androgen ablative therapy should be directed towards the elimination of DHT while retaining circulating testosterone. Potent inhibitors of 5 α -reductase have the potential to achieve complete inhibition of DHT within the

prostate and may also have the potential for prevention of prostate cancer.

There is no known cure for metastatic prostate cancer. Endocrine therapy, based on total androgen withdrawal, is the best palliative treatment available for patients with prostate carcinoma. Such treatment in the short-term, is effective in reducing symptoms due to bone pain and urinary obstruction, and in prolonging time to death. However, androgen ablation remains effective only as long as the cells remain hormone-dependent. Endocrine therapy usually fails in the long-term because the cancer progresses to androgen-independent cell growth. More than 50% of patients who initially respond to androgen ablation therapies show a reappearance of the tumour within one year. Once a tumour has reached this stage medical treatment has little to offer and approximately 50% of the men who relapse die within six months (Labrie *et al.*, 1990).

A potentially promising use of 5 α -reductase inhibitors in the treatment of prostate cancer is in the combination with an androgen receptor antagonist such as flutamide. Such a combination combines the inhibition of DHT production with blockade of the androgen receptor and has the advantage of preserving sexual potency and only minimal side-effects. However, this regimen would only be effective in tumours which are still androgen-dependent and this requires early detection. Using a combination of a 5 α -reductase inhibitor and androgen receptor antagonist it is possible to envisage that prostate cancers detected early could be effectively treated, with minimal side-effects, by starving the tumour cells of androgens before they progress to androgen-independent growth.

7.3 Summary

This thesis has shown that suspensions of BPH cells provide a useful and relevant in vitro model for the evaluation of 5 α -reductase inhibitors, in particular for the development of selective inhibitors of 5 α -reductase 2. The human prostate cancer cells HPC-36M and DU145, provide in vitro models for the evaluation of 5 α -reductase 1 selective inhibitors. Thus a compound may be profiled by comparing its ability to inhibit 5 α -reductase 2 (in BPH cells) with that against 5 α -reductase 1 (in HPC-36M and DU145). The studies presented in this thesis have also shown that cultured human BPH cells differed in their sensitivity to 5 α -reductase inhibitors compared to freshly isolated BPH cells and supports the hypothesis that there is an alteration in the expression of 5 α -reductase 1 and 2 during long term culture.

7.4 Areas of Further Investigation

- i) Characterisation of the 5 α -reductase activity in freshly prepared cell suspensions of prostate cancer tissue using selective inhibitors of 5 α -reductase 1 and 2.
- ii) Investigation of the effects of the potent 5 α -reductase 1 inhibitor LY 191704 in primary cultures of BPH epithelial cells to confirm the presence of 5 α -reductase 1 activity and time-dependence of its activity.
- iii) Investigation of culture techniques to identify factors that modulate the 5 α -reductase 1 and 2 activity in primary cultures.
- iv) Immortalisation by transformation of young primary culture cells to determine if this a way of preserving 5 α -reductase activity.

APPENDIX ONE

**Rf Values for TLC Separation of Testosterone Metabolites in
Dichloromethane : Acetone (12.3:1, v/v) Using Silica Gel Plates**

<u>Metabolite</u>	<u>Rf Value</u>
3 α -Androstanediol	0.155
3 β -Androstanediol	0.167
Testosterone	0.217
Epiandrosterone	0.28
Androsterone	0.29
DHT	0.33
Androstenedione	0.40
Androstanedione	0.52

APPENDIX TWO

**Rf Values for TLC Separation of Testosterone Metabolites in
Dichloromethane : Diethylether (9:1, v/v) using Aluminium Oxide Plates**

<u>Metabolite</u>	<u>Rf Value</u>
3 α -Androstanediol	0.089
3 β -Androstanediol	0.077
Androsterone	0.15
Epiandrosterone	0.18
Testosterone	0.23
DHT	0.31
Androstenedione	0.48
Androstanedione	0.56

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