Androgen Metabolism and the Maintenance of Spermatogenesis in a Clinical Trial of Hormonal Male Contraception.

Richard Alexander Anderson

Thesis submitted for the Degree of Doctor of Medicine

University of Edinburgh
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All day, the same our postures were,
And wee said nothing, all the day.

But O alas, so long, so farre
Our bodies why do wee forbeare?

This Extasie doth unperplex
(We said) and tell us what we love.

Loves mysteries in soules doe grow,
But yet the body is his booke.

John Donne
I declare that the studies presented in this Thesis are the result of my own independent investigation with the exception of the plasma and urinary steroid determinations described in Chapter 7, which were carried out by Dr AM Wallace at the Institute of Biochemistry, Glasgow Royal Infirmary.

This work has not and is not being currently submitted for candidature for any other degree.
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And to the men who took part in this study, it has been the greatest of privileges to work with you
Abstract of Thesis

The administration to normal men of supraphysiological doses of testosterone causes a profound inhibition of spermatogenesis and is currently being investigated as a method of male contraception. Azoospermia, however, is achieved in only 50-70% of men, the remainder maintaining a very low rate of spermatogenesis. The object of these studies was to investigate the biochemical basis for the maintenance of spermatogenesis in the oligozoospermic group, and in particular to investigate the hypothesis that the activity of the enzyme 5α-reductase, which converts testosterone to the more potent androgen dihydrotestosterone (DHT), is increased in these men either constitutionally or as a result of treatment.

Thirty-three normal men were recruited to a clinical trial of hormonal male contraception. After a baseline period, subjects were administered 200 mg testosterone cestranate i.m. weekly. Semen samples were analysed at 4 weekly intervals. When the sperm density had fallen below 5 million/ml in 3 consecutive samples, the subjects were required to discontinue all other forms of contraception for one year, during which the weekly injections of testosterone were continued. 18 of the subjects became azoospermic within 20 weeks of testosterone treatment, the other 15 remained severely oligozoospermic with a mean sperm density of 2.0 ± 0.8 million/ml at that time.

Multiple blood sampling over the week following the first injection and after 16 weeks of testosterone treatment demonstrated that there were no differences in plasma concentrations of total or bioavailable testosterone or of oestradiol between those men becoming azoospermic and those remaining oligozoospermic. Similarly, the rate and degree of suppression of gonadotrophin secretion was similar in the two groups.

Measurement of the metabolic clearance rate (MCR) of testosterone and of the conversion rate (CR) of testosterone to DHT by infusion of radiolabelled testosterone showed that MCR was similar in the two groups at baseline, and was increased in both after 16 weeks of testosterone treatment. CR was also similar in the two groups at baseline, but increased after 16 weeks of treatment in the oligozoospermic group only.

The concentrations of DHT and androstenediol glucuronide (AdiolG), products of 5α-reduction of testosterone, were measured in blood plasma and seminal plasma. After 16 weeks of testosterone treatment, blood plasma concentrations of both steroids were increased, but the increase was greater in the oligozoospermic group. In seminal plasma, there was an increase in testosterone and AdiolG in both groups. However, the concentration of DHT was only increased in the oligozoospermic group. The urinary excretion of steroid metabolites was measured to investigate the effect of testosterone treatment on hepatic 5αR activity. There was no consistent effect on the ratio of 5α/5β-reductase metabolites of either C19 or C21 steroids. These results suggest that testosterone treatment causes an increase in peripheral but not hepatic 5αR activity in those men who remain oligozoospermic, but not in those who become azoospermic.

The concentration in plasma of the testicular steroid 17OH-progesterone was reduced by 50% in both groups. In contrast, the plasma concentration of dehydroepiandrosterone sulphate, an adrenal steroid, was reduced in the azoospermic group only. The urinary metabolites of these steroids showed parallel changes. These results suggest that there may be an interaction between testicular and adrenal steroidogenesis, and that adrenal steroids may be a source of androgen precursor when testicular steroidogenesis is reduced.

The sebum excretion rate (SER) was investigated as a measure of end-organ response to androgen. Testosterone treatment resulted in an increase in SER of both forehead and back, but there were no differences between the two groups.

An increase in 5αR activity is suggested to be the basis for the maintenance of spermatogenesis in those men who remain oligozoospermic on supraphysiological doses of testosterone, and this suggests new approaches for the development of effective hormonal male contraception.
### Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>5αR</td>
<td>5α-reductase</td>
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<tr>
<td>5βR</td>
<td>5β-reductase</td>
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<tr>
<td>11βOH-A</td>
<td>11β-hydroxyandrostenedione</td>
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<tr>
<td>11βOH-E</td>
<td>11β-hydroxyaetiocholanolone</td>
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<tr>
<td>11βOHSD</td>
<td>11β-hydroxysteroid dehydrogenase</td>
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<tr>
<td>17OH-pregnenolone</td>
<td>17α-hydroxypregnenolone</td>
</tr>
<tr>
<td>17OH-progesterone</td>
<td>17α-hydroxyprogesterone</td>
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<tr>
<td>A</td>
<td>Androsterone</td>
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<tr>
<td>ABP</td>
<td>Androgen binding protein</td>
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<tr>
<td>Adioll</td>
<td>Androstandiol (5α-androstan-3α,17β-diol)</td>
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<tr>
<td>AdiollG</td>
<td>Androstandiol glucuronide</td>
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<tr>
<td>aTHF</td>
<td>Allo-tetrahydrocortisol</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3':5' cyclic monophosphate</td>
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<tr>
<td>CG</td>
<td>Chorionic gonadotrophin</td>
</tr>
<tr>
<td>CR</td>
<td>Conversion ratio</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<tr>
<td>DHA</td>
<td>Dehydroepiandrosterone</td>
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<td>DHAS</td>
<td>Dehydroepiandrosterone sulphate</td>
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<tr>
<td>DHT</td>
<td>Dihydrotestosterone (5α-androstan-17β-ol-3-one)</td>
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<tr>
<td>E</td>
<td>Aetiocholanolone</td>
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<tr>
<td>EDS</td>
<td>Ethane dimethane sulphonate</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<tr>
<td>i.m.</td>
<td>Intramuscular</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<td>LH</td>
<td>Luteinising hormone</td>
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<tr>
<td>LSC</td>
<td>Liquid scintillation counting</td>
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<tr>
<td>MCR</td>
<td>Metabolic clearance rate</td>
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<tr>
<td>PCOS</td>
<td>Polycystic ovarian syndrome</td>
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<tr>
<td>PSU</td>
<td>Pilosebaceous unit</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>SER</td>
<td>Sebum excretion rate</td>
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<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
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<td>THE</td>
<td>Cortisone</td>
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<tr>
<td>THF</td>
<td>Tetrahydrocortisol</td>
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<td>TLC</td>
<td>Thin layer chromatography</td>
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Chapter 1
General Introduction

The importance of the testes in normal male development and fertility was known to Aristotle. Confirmation that the testes are the source of a male factor was provided by the experiments of Berthold, who in 1849 showed that castration of a cockerel was followed by regression of the comb, and that this could be prevented by the transplantation of the testis to a new site. The administration of testicular extracts to men achieved prominence following the experiments of Brown-Séquard in the last century. In his publication of 1889, he described the effect of injection into himself of an aqueous extract of dog and guinea-pig testis. At that time he was aged 72, and reported that the injection gave enhanced strength, heightened intellectual capacity and increased sexual potency, and took 30 years off his age. This dramatic description led to an explosion of “organotherapy”, and although this method of treatment became synonymous with quackery, the scientific stimulus arising from Brown-Séquard’s description resulted in the birth of modern endocrinology (Wilson, 1990).

Testosterone was purified in 1931 (Butenandt, 1931), and synthesised chemically shortly thereafter. The studies in this Thesis concern the administration of testosterone to normal adult men as a hormonal method of contraception, and in this Chapter testicular function and its control are described.

1.1 Testicular steroidogenesis.

1.1.1 The pathways.

It has long been recognised that the principle intratesticular androgen is testosterone, present in the testis in a concentration approximately 100 fold greater than in peripheral plasma, and that steroidogenesis in the testis is largely localised to the Leydig cell (Eik-Nes, 1970). Leydig cells differentiate and start to secrete androgens in the seventh week of intrauterine life, which correlates with the onset of androgen-dependent sexual differentiation (Bloch, 1964; Siiteri and Wilson, 1974). The plasma level of testosterone in the male foetus approaches that of the adult at 12-16 weeks gestation, with a subsequent fall as the Leydig cells involute until puberty (Tapainen, Kellokompu-Lehtinen, Pelliniemi and Huhtaniemi, 1981). This is not dependent on the secretion of gonadotrophins from the foetal pituitary, as it occurs in anencephalic foetuses: however it may be dependent on chorionic gonadotrophin (CG), as CG levels in foetal plasma correlate in time with Leydig cell development and foetal plasma testosterone concentration (Clements, Reyes, Winter, and Faiman, 1976).
Much of the early information regarding the presence of steroid pathways in the testis was obtained from experiments in which testicular tissue (often not fractionated) was incubated with radioactive substrates. From the relative rates of production of steroid products, inferences were made as to the presence of those pathways. As the rate of enzyme activity will be dependent on the presence of cofactors, the pH of the medium etc, the quantification of the contributions of various pathways is difficult using such techniques. Quantification requires the determination of the size of the endogenous pools of the intermediates, and this has become possible with the refinement of radioimmunoassay and mass spectrometric techniques, and, more recently, the advent of techniques of molecular biology has allowed the sequencing and detailed characterisation of some of the enzymes involved.

The testes are able to convert acetate to cholesterol, and most cholesterol is believed to be synthesised de novo in the Leydig cells (Morris and Chaikoff, 1959). This step takes place on the inner mitochondrial membrane rather than in the endoplasmic reticulum, which is the site in most other tissues (Pignatoro, Radicella, Calvo and Charreau, 1983). Subsequent conversion to pregnenolone by side-chain cleavage is the most important step in the pathway (vide infra), and also takes place within the mitochondria (Toren, Menon, Forchielli and Dorfman, 1964; van der Vusse, Kalkman, and van der Molen, 1973). This and subsequent steps are catalysed by members of the cytochrome P-450 superfamily. Pregnenolone can then be further converted by either the Δ5 or Δ4 pathways in the endoplasmic reticulum. In man, the Δ5 pathway is considered to be more important (Yanaihara and Troën, 1972; Weusten, Smals, Hofman, Kloppenborg and Benraad, 1987), in contrast to the rat, in which the Δ4 pathway appears to be of greater importance (Samuels, Bussman, Matsumoto and Huseby, 1975). The intermediates in the Δ5 pathway are 17α-hydroxypregnenolone (17OH-pregnenolone), dehydroepiandrosterone (DHA), and androstenediol. The Δ4 intermediates are progesterone, 17α-hydroxyprogesterone (17OH-progesterone), and androstenedione. These pathways, with the enzymes responsible, are shown in Figure 1.1.

It has been found that pregnenolone and progesterone are converted to DHA and androstenedione respectively by the action of a single enzyme, 17α-hydroxylase/C17,20-lyase (Nakajin, Shively, Yuan and Hall, 1981), although 17OH-pregnenolone and 17OH-progesterone appear to be distinct intermediates. There is a single copy of the gene for this enzyme on chromosome 10 in both testis and adrenal (Matteson, Picardo-Leonard, Chung, Mohandas and Miller, 1986). It therefore appears that the two catalytic functions can be separately regulated, with 17α-hydroxylation being preponderant in the adrenal, resulting in the production of cortisol, a 17-hydroxylated
Figure 1.1
Steroidogenic pathway from cholesterol to testosterone, showing further conversion to DHT, 17-ketosteroids and oestrogens.
Enzymes:
1. Cholesterol side chain cleavage
2. 17α-hydroxylase/17,20-lyase
3. 3β-hydroxysteroid dehydrogenase
4. 17β-hydroxysteroid dehydrogenase
5. Aromatase
6. 5α-reductase
Figure 1.1
Steroidogenic pathways
C₂₁ steroid, while in the testis both activities are present, resulting in the synthesis of testosterone.

The final step in the synthesis of testosterone is mediated by the enzyme 17β-hydroxysteroid dehydrogenase, which reversibly converts androstenedione to testosterone (Rivarola, Podestá, Chemes and Aguilier, 1973). This enzyme also converts DHA to androstenediol and oestrone to oestradiol.

The further metabolism of testosterone to dihydrotestosterone (DHT) is catalysed by the enzyme 5α-reductase (5αR), and DHT can be converted to the androstanediols by 3α-hydroxysteroid dehydrogenase (Dorrington and Fritz, 1975). The activity of 5αR in the testis is discussed further in Section 1.6.3. Testosterone can also be converted to oestradiol by the enzyme aromatase, the activity of which is high in the Sertoli cells of immature rats but decreases thereafter (Dorrington and Armstrong, 1975). The intratesticular functions of these potentially active steroid metabolites of testosterone are unknown.

1.1.2 Regulation.

The importance of the pituitary in the control of testicular steroidogenesis was demonstrated by the finding that hypophysectomy is followed by regression of the testes and a decrease in testosterone synthesis (Smith, 1927; Woods and Simpson, 1961). This can be prevented by the administration of luteinizing hormone (LH) or CG, and the synthesis of testosterone from Leydig cells in vitro can be stimulated by LH (Hall, 1966; Janszen, Cooke, van Driel and van der Molen, 1976). It is now recognised that pituitary secretion of LH is the most important hormonal regulator of testicular steroidogenesis.

Incubation of slices of rabbit testis with LH causes a stimulation of the conversion of cholesterol to testosterone, but not of pregnenolone to testosterone (Hall, 1966). The enzyme which cleaves the side chain of cholesterol is a cytochrome P-450 known as P450<sub>SCC</sub>, located on the inner mitochondrial membrane (Moyle, Jungas and Greep, 1973). This site contains only small amounts of cholesterol: the supply of cholesterol may therefore be the rate-limiting step, and the site of control. This hypothesis is supported by experiments in which mitochondria were isolated from Leydig cells and incubated with LH, and the rate of pregnenolone synthesis determined (Hall, 1983). LH had no effect under these circumstances, demonstrating that LH has no effect on isolated mitochondria. In contrast, when Leydig cells are incubated with aminogluthethimide, an inhibitor of P450<sub>SCC</sub>, LH causes an increase in the amount of cholesterol found in the inner mitochondrial membrane, and mitochondria isolated from
treated cells and washed to remove the aminogluthethimide show an increased capacity to produce pregnenolone. A labile "steroidogenesis activator peptide" has been described, which promotes the passage of cholesterol from the outer to the inner mitochondrial membrane (Mertz and Pederson, 1989).

The effect of LH is mediated by binding to specific receptors on the surface of Leydig cells (Davies, Dufau and Catt, 1978; Loosfelt, Misrahi, Atger, Saless, Thi, Jolivet, Guiochon-Mantel, Sar, Jallal, Garnier and Milgrom, 1989). The binding of LH causes an increase in intracellular cyclic adenosine monophosphate (cAMP), by activation of the enzyme adenylate cyclase via a guanyl nucleotide regulatory subunit (Abramowitz, Iyengar and Birnbaumer, 1979). In turn, cAMP activates protein kinase, although other intracellular transducing systems may also be involved, such as lipoxygenase resulting in the formation of leukotrienes, and direct activation of adenylate cyclase by calmodulin and protein kinase C (Hall, 1988; Cooke, 1990).

The immediate response to LH stimulation is mobilisation of cholesterol stores, as described above, with increased supply of cholesterol to the inner mitochondrial membrane as a substrate for P450scc. This results in increased production of pregnenolone. With more prolonged stimulation, LH enhances transcription of genes encoding steroidogenic enzymes, giving rise to de novo synthesis of enzyme (Waterman and Simpson, 1989). If LH stimulation is more prolonged, an adaptive response is seen, in which there is loss of LH receptors, a reduced effect on cAMP, and also interference with steroidogenesis itself (Saez, Haour and Cathiard, 1978; Dufau, 1988). While the pulsatile nature of LH secretion may result in this effect being of little physiological importance (vide infra, Section 1.4), it may alternatively be involved in the feedback of testosterone on LH secretion, and enhance LH pulsatility.

Leydig cells also have receptors for prolactin, which potentiates the response to LH (Hafiez, Bartke and Lloyd, 1972; Aragona and Friesen, 1975). Gonadotrophin releasing hormone (GnRH) has also been reported to have direct effects on the testis (Hsueh and Erikson, 1979), and thus the precise regulation of Leydig cell function and testicular steroidogenesis may be controlled by the interaction of several hormones. The intratesticular control of steroidogenesis is largely unexplored in humans, although the condition of "testicular toxicosis", in which there is precocious puberty associated with adult plasma levels of testosterone in the absence of gonadotrophin secretion (Rosenthal, Grumbach, and Kaplan, 1983), may demonstrate the potential importance of local control. There are reports of stimulatory substances secreted by human seminiferous tubules in vitro (Verhoeven and Cailleau, 1987). Indeed, many substances have been proposed to be paracrine factors within the testis, but evidence as to a physiological role for any of these factors is lacking (Sharpe, 1990). One such
possible substance is oxytocin: this is present in the testis (Nicholson, Swann, Burford, Wathes, Porter and Pickering, 1984), and may have a role in the paracrine regulation of steroidogenesis (Adashi, Tucker and Hseuh, 1984) or of seminiferous tubule contractility (Worley, Nicholson and Pickering, 1985).

1.2 Spermatogenesis.

1.2.1 The Sertoli cell.

Primordial germ cells migrate from the endoderm of the yolk sac to the genital ridges at 5 to 6 weeks post conception (Witschi, 1951). Sertoli cell precursors then surround the germ cells, forming the sex cords (Jost, Magre, Cressant and Perlman, 1974) and thus the beginnings of the seminiferous tubules of the differentiated testis. This process is controlled by a product of the putative testis-determining "TDF" gene, located on the short arm of the Y chromosome. The product of this gene was held to be the male-specific histocompatibility antigen H-Y, until male mice were found which lack this antigen (McLaren, Simpson, Tomanari, Chandler and Hogg, 1984). An alternative candidate is a recently-cloned gene termed SRY (Sinclair, Berta, Palmer, Hawkins, Griffiths, Smith, Foster, Frischauf, Lovell-Badge and Goodfellow, 1990). This process clearly predate the development of Leydig cells and the onset of androgen synthesis (vide supra, Section 1.1.1).

The Sertoli cells are thus in a position to have full influence over the germ cells, and they further influence embryological development by the secretion of the peptide Müllarian inhibitory hormone (Josso, Picard and Tran, 1977; Cate, Mattaliano, Hession, Tizard, Farber, Cheung, Ninfa, Frey, Gash, Chow, Fisher, Bertoni, Torres, Wallner, Ramuchandra, Ragin, Manzanaro, MacLaughlin and Donahoe, 1986) which suppresses the development of the Müllarian system, the precursor of the female internal genitalia. A further indicator of the influence of Sertoli cells is the development of tight intercellular junctions between adjacent Sertoli cells, which develops at puberty (Nicander, 1967; Dym and Fawcett, 1970). This blood-testis barrier limits the passage of ions as well as larger molecules, and results in the effective production of two compartments: the basal compartment adjacent to the basal membrane containing the spermatogonia, and the adluminal compartment, the location of the spermatocytes and spermatids. The fluid of the seminiferous tubule has a high concentration of potassium and bicarbonate, with low concentrations of sodium and chloride: it thus resembles intracellular rather than extracellular fluid (Waites and Gladwell, 1982). The importance of this ionic environment in the development of spermatozoa is unknown.
Chapter 1

The nature of the tight junctions is such that all nutrients and hormonal signals reaching the adluminal germ cells have to pass through the cytoplasm of the Sertoli cells. This also provides an immunological barrier. The nature of the substances regulating this passage is largely unknown, although a number of secretory products of the Sertoli cell have been identified, including androgen binding protein (APB), inhibin, transferrin and the protease inhibitor plasminogen activator which appear to be controlled by FSH (\textit{vide infra}, Section 1.3.2; Syed, Karpe, Plöen and Ritzén, 1986; Lacroix, Smith and Fritz, 1977).

1.2.2 Organisation.

After the high activity of foetal life, the testis is quiescent during childhood. An increase in testicular volume is the first clinical sign of puberty (Marshall and Tanner, 1970), and reflects the increasing mass of the seminiferous tubules. This can be detected biochemically by measuring the increasing nocturnal pulsatile secretion of LH and an overnight rise in plasma testosterone level (Boyar, Finkelstein, Roffwarg, Kapen, Weitzman and Hellman, 1972; Wu, Butler, Kelnar, Stirling and Huhtaniemi, 1991).

Unlike the situation in the female, male germ cells do not enter meiosis until puberty and the initiation of spermatogenesis. At that stage, the spermatogonia enter a series of mitotic divisions to form primary spermatocytes, which then enter meiosis to produce haploid secondary spermatocytes and, after a second division, spermatids. The anatomical organisation of this sequence was described by Clermont (1963), who found that activity in the human seminiferous epithelium could be divided into 6 stages, each containing a specific constellation of cell types at particular stages of maturation. In cross-section, there are usually several stages visible in each tubule, in an apparently irregular fashion, but reinvestigation of the topographic arrangement by Schulze (1982) has demonstrated that the stages are arranged in a helical pattern. This contrasts with the organisation in many other species, eg the rat and most primates, in which the stages are arranged radially in a longitudinal sequence.

Spermatids undergo the differentiation process of spermiogenesis to become spermatozoa. This involves formation and maturation of the acrosome cap, condensation of the nuclear chromatin and reshaping of the nucleus with loss of residual cytoplasm, and development of the spermatid midpiece containing the mitochondria and tail containing an axial filament complex (the axoneme) flanked by nine pairs of microtubules (Clermont, 1963; de Kretser, 1969). The spermatozoa are then released from the Sertoli cell into the lumen of the tubule (the process of
spermiation), whence they pass through the rete testis to the epididymis and so to the vas deferens. The process of spermatogenesis is estimated to take 74 days in man (Heller and Clermont, 1964).

1.3 The hormonal control of spermatogenesis.

The involvement of the pituitary gland in the control of spermatogenesis was first described by Smith (1927). Using the classic endocrine technique of gland removal followed by replacement of the postulated active substances, he demonstrated the importance of pituitary factors in the stimulation of testicular growth and spermatogenesis in the rat by observing the effect of hypophysectomy and subsequent administration of pituitary extracts. It was subsequently recognised that two pituitary hormones are involved, with separate effects on the Leydig cells and on spermatogenesis (Greep, Fevold and Hishaw, 1936). This finding provides the basis for current understanding of the dual control of the endocrine and spermatogenic functions of the testes, by the glycoproteins LH and follicle-stimulating hormone (FSH) respectively. The control of testicular steroidogenesis has been discussed above, and in this section experimental findings relating to the hormonal control of spermatogenesis are reviewed.

1.3.1 The influence of testosterone.

The precise relative contributions of LH-controlled androgens and of FSH in the control of the onset and maintenance of spermatogenesis remain the subject of debate. After hypophysectomy in rats, elongated spermatids are progressively lost, followed by a reduction in round spermatids and spermatocytes (Bocabella, 1963; Clermont and Morgantheler, 1955), but this could be prevented (in qualitative terms) by the administration of testosterone at or soon after surgery (Walsh, Cuyler and McCullagh, 1934; Steinberger, 1971; Ahmad, Haltmeyer and Eik-Nes, 1975). This effect was also apparent following the administration of other steroids, including DHT and androstenediol (Ahmad et al., 1975; Chowdhury and Steinberger, 1975; Dubé, Pelletier and Labrie, 1988), as well as other precursors of testosterone (Harris and Bartke, 1975). LH itself, or CG, maintained spermatogenesis (Clermont and Harvey, 1965; Russell and Clermont, 1977; Vernon, Go and Fritz, 1975), but the interpretation of these experiments is difficult because of the presence of FSH-like activity in the preparations used. The use of the Leydig cell toxin dimethanesulphonate (EDS) to completely destroy Leydig cells has allowed the demonstration that testosterone alone, if given in sufficient dose to maintain intratesticular testosterone concentrations, will
maintain spermatogenesis quantitatively without loss of fertility (Sharpe, Fraser and Ratnasooriya, 1988).

In man, the administration of testosterone in sufficient dose to inhibit gonadotrophin secretion causes profound inhibition of spermatogenesis, but a low rate of spermatogenesis was maintained in some subjects (Swerdloff, Campfield, Palacios and McClure, 1979; Nieschlag, Behre and Weinbauer, 1989; vide infra, Section 1.7). This partial maintenance of spermatogenesis was also found when a progestogen was used to inhibit gonadotrophin secretion, combined with replacement doses of testosterone (Wu and Aitken, 1989).

Similar results have been obtained using gonadotrophin-releasing hormone (GnRH) analogues to inhibit the secretion of LH and FSH. Treatment with a GnRH antagonist inhibited spermatogenesis in rats (Rivier, Rivier and Vale, 1980). The simultaneous administration of testosterone, however, was reported to maintain spermatogenesis quantitatively (Rea, Marshall, Weinbauer and Nieschlag, 1986), but under those conditions, the administration of testosterone was found to stimulate FSH secretion (Rea et al., 1986; Bhasin Fielder and Swerdloff, 1988), thus making interpretation difficult. Others have used the anti-androgen flutamide in combination with a GnRH antagonist (Chandolia, Weinbauer, Fingscheidt, Bartlett and Nieschlag, 1991). These studies demonstrated that flutamide accelerated testicular involution, and reduced the stimulatory effect of subsequent administration of FSH on spermatogenesis.

The administration of gonadotrophins (either as partially “purified” FSH or as human menopausal gonadotrophin) was shown to partially restore spermatogenesis in men following hypophysectomy (Genzell and Kjessler, 1964; MacLeod, Pazianos and Ray, 1964), and administration of human FSH following suppression of spermatogenesis by supraphysiological doses of testosterone caused some restoration of spermatogenesis (Matsumoto, Karpas, Paulsen and Bremner, 1983). In a non-human primate, daily injection of a GnRH antagonist caused azoospermia, but the addition of androgen replacement (using the long-acting androgen 20-Aet-1) at physiological or supraphysiological doses caused progressive restoration of spermatogenesis (Weinbauer, Göckeler and Nieschlag, 1988). Others have shown more reproducible azoospermia using a GnRH antagonist with testosterone replacement (Bremner, Bagatell and Steiner, 1991). The difference may lie in the use of mini-pumps to administer more constant and effective levels of the antagonist in the latter study, so the influence of the replacement androgen is inconclusive.

In addition to studies using GnRH antagonists, the effect of administration of the GnRH agonist buserelin (as a subcutaneous implant) during treatment with the androgen 19-nortestosterone hexyloxyphenylpropionate has recently been studied in
man (Behre, Nashan, Hubert and Nieschlag, 1992). Those authors found that treatment with the GnRH agonist partially reversed the inhibition of spermatogenesis caused by the androgen, and this was associated with a restoration of FSH secretion to almost normal levels. LH secretion remained suppressed.

It therefore appears that, in rats, testosterone can maintain spermatogenesis in a quantitative manner in the complete absence of gonadotrophins, and that the presence of FSH is not essential. The crucial factor appears to be the maintenance of a sufficient intratesticular concentration of testosterone by exogenous administration after destruction of endogenous synthesis, which is not possible in human experiments. However, the results of both human and animal experiments suggest that the presence of FSH in some way “facilitates” the effect of testosterone, supporting spermatogenesis at lower testosterone concentrations. Many aspects of the mechanisms of initiation and control of spermatogenesis by testosterone and FSH remain unexplored.

1.3.2 The effects of FSH.

The intratesticular injection of FSH was found to specifically stimulate Sertoli cells (Murphy, 1965), and receptors for FSH are found on Sertoli cells (but not on germ cells) (Castro, Alonso and Mancini, 1972). The mechanism of action of FSH is believed to be by a similar method to that of LH (and indeed to most peptide hormones), as discussed above. This involves a guanyl nucleotide regulatory subunit, linked to adenylate cyclase, with subsequent activation of protein kinases (Abramowitz et al., 1979; Jahnsen, Purvis, Birnbaumer and Hansson, 1980).

Several proteins have been identified as Sertoli cell products, most particularly androgen binding protein (ABP) (Hagenäs, Ritzén, Ploén, Hansson, French and Nayfeh, 1975). This substance is very similar to SHBG, differing only in the carbohydrate moieties (Joseph, Hall and French, 1987; Reventos, Hammond, Crozat, Brooks, Gunsalus, Bardin and Musto, 1988). ABP is secreted into tubular fluid and is transported to the epididymis, and in rats (a species without SHBG) is also found in plasma where it has been used as a marker of Sertoli cell activity (Gunsalus, Musto and Bardin, 1978); this has not been possible so far in the human because of the similarities between ABP and SHBG. The production of ABP is stimulated by both FSH and testosterone (Hansson, Reusch, Trygstad, Torgersen, Ritzén and French, 1973; Rommerts, Krüger-Sewnaraïn, van Woerkom-Blik, Grootegoed and van der Molen, 1978), and although a role as a tubular store and buffer of androgen appears likely, its precise role remains obscure.
Other proteins identified as Sertoli cell products whose production is stimulated by FSH and which may be involved in spermatogenesis include transferrin and plasminogen activator (Syed et al., 1986; Lacroix et al., 1977). Again, the precise role of these and other proteins remains to be established. The secretion and function of inhibin by Sertoli cells is discussed below (Section 1.4.2).

1.3.3 Androgen action within the testis.

It is apparent from the findings discussed above that, at least in the rat, testosterone alone can maintain spermatogenesis after hypophysectomy if given in sufficient dose. For this to occur, it must be given before the seminiferous epithelium has regressed. This suggests a facilitatory interaction between testosterone and FSH within the testis, and there is also biochemical evidence for this.

Androgen receptors are found in the testis, and have been localised to both Sertoli and Leydig cells, peritubular cells and testicular arteriole muscle cells (Hansson, McLean, Smith, Tindall, Weddington and Nayfeh, 1974; Mulder, Peters, de Vries and van der Molen; 1975; Bergh and Damber, 1992). Both testosterone and FSH stimulate ABP, acting synergistically (Hansson et al., 1973), and a similar additive effect of testosterone and FSH has been demonstrated on the enhancement of androgen receptor binding by both hormones (Verhoeven and Cailleau, 1988). This also provides a basis for the results of Chandolia et al. (1991) (vide supra, Section 1.3.1).

It is not known whether germ cells are androgen target cells, but an essential role for androgens in their development appears unlikely from the work of Lyon (Lyon, Flenister, and Lamoreux, 1975). Those authors fused normal and androgen-insensitive (i.e. lacking androgen receptors) Tfm mouse blastocysts, making chimaerae. Some resulting males were fertile, and mating with normal females produced female offspring carrying the Tfm gene. This gene is carried on the X chromosome and thus the spermatozoon bearing it had matured despite the absence of androgen receptors. This assumes that androgen effects are always mediated via the androgen receptor, but there is increasing evidence that cell surface actions may be important (Rommerts, 1992).

Testosterone can be metabolised within the Sertoli cell. The activity of the aromatase enzyme is high in the Sertoli cell of young rats and is FSH-dependent, but declines to low levels with maturity, whereas aromatase activity in the Leydig cells increases (Dorrington and Armstrong, 1975; Tsai-Morris, Aquilano and Dufau, 1985). The potential importance of 5αR in the testis is discussed further in Section 1.6.3.
1.4 The control of gonadotrophin secretion.

The gonadotrophins LH and FSH are secreted by the gonadotrophes of the anterior pituitary gland in response to stimulation by GnRH. This decapetide is secreted into hypophyseal portal blood from the terminals of neurones projecting from the medial preoptic area of the hypothalamus to the median eminence (Davidson and Bloch, 1978; Elde and Hökfelt, 1978; Silverman, Krey and Zimmerman, 1979). The pattern of both LH and FSH secretion in the peripheral circulation is pulsatile (Santen and Bardin, 1973; Veldhuis, King, Urban, Rogol, Evans, Kolp and Johnson, 1987), and in experimental animals pulsatile secretion of LH has been demonstrated to directly parallel secretion of GnRH into portal blood (Levine, Pau, Ramirez, and Jackson, 1982; Clarke and Cummins, 1982). The pulsatile secretion of gonadotrophins in man is assumed to have a similar basis, and in many studies the pulse frequency of LH secretion is taken to reflect that of GnRH. In investigations of the feedback control of gonadotrophin secretion, both frequency and amplitude of this pulsatile secretion can therefore be assessed, but interpretation is complicated by the possibility of both hypothalamic and pituitary sites of action of regulatory hormones. The discussion here will concentrate on human data, with evidence from animal studies included to provide details of some effects not subject to investigation in humans because of the limitations of clinical experimental design. In addition, as the secretion of GnRH in humans cannot be detected in vivo, this discussion will refer only to feedback control by testicular hormones.

1.4.1 Sex steroids.

The testes secrete DHT and oestradiol in addition to testosterone (Kelch, Jenner, Weinstein, Kaplan and Grumbach, 1971; Baird, Galbraith, Fraser and Newsam, 1973; Pazzagli, Borrelli, Forti, and Serio, 1974), although most of these two hormones in peripheral plasma is derived from peripheral conversion (Longcope, Kato and Horton, 1969; Ito and Horton, 1971). Aromatisation of testosterone also occurs in nervous tissue (Naftolin, Ryan, Davies, Reddy, Flores, Petro, Kuhn, White, Takaoki and Wolin 1975), but DHT is not subject to this. Thus there are (at least) three interconverting steroid hormones to be considered in the investigation of feedback control.

The administration of testosterone or DHT inhibits gonadotrophin secretion (Sherins and Loriaux, 1973; Santen, 1975), and several studies have reported that this is by a reduction in pulse frequency with no effect on pulse amplitude (Winters, Janick, Loriaux and Sherins, 1979; Santen, 1975; Matsumoto and Bremner, 1984). This was
interpreted to imply a site of action at the hypothalamus, by reducing the frequency of GnRH secretion. Oestradiol also caused an inhibition of gonadotrophin secretion, but this was by a reduction in pulse amplitude with no effect on pulse frequency (Kulin and Reiter, 1972; Sherins and Loriaux, 1973; Santen 1975; Winters et al., 1979; Gooren, 1989).

The non-steroidal anti-oestrogen clomiphene and the anti-androgen flutamide have been used to investigate the effect of endogenous steroids. Clomiphene increased basal LH secretion and prevented the inhibitory effect of testosterone (Winters et al., 1979; Veldhuis and Dufau, 1987), and flutamide increased LH pulse frequency (Balzano, Migliari, Sica, Scarpa, Pintus, Loviselli, Usai and Balestrieri, 1987; Urban, Davis, Rogol, Johnson and Veldhuis, 1988) and the mass of bioactive LH released per pulse (Veldhuis, Urban and Dufau, 1992), although in that study LH pulse frequency was unaffected. Reduction of endogenous DHT concentrations by administration of the 5αR inhibitor finasteride had no effect on basal or GnRH-stimulated gonadotrophin concentrations, although there was a slight but significant increase in plasma testosterone concentrations (Rittmester, Lemay, Zwicker, Capazzi, Winch, Moore and Gormley, 1992). These studies demonstrate the importance of differentiating between the effects of steroids in physiological and supraphysiological concentrations.

Urban, Dahl, Padmanabhan, Beitins and Veldhuis (1991) have also recently reported that DHT or oestradiol infusion did not affect FSH pulse frequency, which finding they interpret in terms of dissociation between LH and FSH secretion. Similarly, earlier studies showed that pulsatile GnRH administration is required for the stimulation of LH but not FSH secretion (Fauser, Dony, Doesberg and Rolland, 1983), and increasing frequency of administered GnRH pulses increased LH but not FSH secretion (Spratt, Finkelstein, Butler, Badger and Crowley, 1987; Sauder, Frager, Case, Kelch and Marshall, 1988).

It therefore appears that androgens reduce gonadotrophin pulse frequency without affecting amplitude, while oestrogens reduce pulse amplitude. Furthermore, it has been concluded that the effect of androgens is mediated at the level of the hypothalamus. Animal experiments have provided clear evidence of an inhibitory effect of androgens on gonadotrophin secretion at the level of the pituitary. This effect is evident both in vivo (Debeljuk, Arimura and Schally, 1972) and in vitro (Kao and Weisz, 1975; Drouin and Labrie, 1976; Speight and Fink, 1981), although stimulatory effects have been reported on FSH secretion in rats in vivo (Rea et al., 1986; Bhasin et al., 1988), and at the level of FSH β mRNA (Wierman and Wang, 1990) after GnRH antagonist treatment. The human data thus requires reinterpretation, especially in the light of recent reports of the effects of sex steroids on gonadotrophin secretion in men with
idiopathic hypogonadotropic hypogonadism (IHH, Kallmann's syndrome) (Finkelstein, Whitcomb, O'Dea, Longcope, Schoenfeld and Crowley, 1991; Finkelstein, O'Dea, Whitcomb and Crowley, 1991). These authors used the model of men with IHH receiving intravenous pulsatile GnRH therapy to circumvent the possibility of hypothalamic site of action of steroids in the negative feedback control of gonadotrophin secretion, as the GnRH pulse frequency and amplitude is controlled. Under these conditions, testosterone markedly suppresses LH pulse amplitude, demonstrating a direct effect on the pituitary of testosterone, or of a metabolite of testosterone. This effect was greater in normal men, which is compatible with an additional effect on the hypothalamus. Administration of testolactone, an aromatase inhibitor, caused an increase in LH secretion when given alone to men with IHH, and prevented the effect of testosterone when given in combination. The inhibitory effect of testosterone on the pituitary is therefore largely due to aromatisation to oestradiol. This was supported by similar experiments in which oestradiol was infused (Finkelstein et al., 1991a): the degree of inhibition of LH and FSH secretion was similar in men with IHH and normal men, indicating that the pituitary is the major site of action of oestradiol in the negative feedback inhibition of gonadotrophin secretion.

In conclusion, it appears that both androgens and oestrogens are involved in the control of gonadotrophin secretion in men, with multiple sites of action and interaction, with a greater control of LH secretion by testosterone than of FSH. A hypothalamic site of action appears most important for the effect of androgens, but the pituitary may be a more important site of action of oestradiol, either from the peripheral circulation or produced by local aromatisation of testosterone.

1.4.2 Inhibin.

Further control of FSH secretion is mediated by the peptide hormone, inhibin. The existence of a protein hormone secreted by the testes and involved in the feedback inhibitory control of FSH secretion was originally proposed by McCullagh (1932). This hormone, which he termed inhibin, has now been extensively characterised, including description of the primary amino acid sequence in the pig and cow (Mason, Hayflick, Ling, Esch, Ueno, Ying, Guillimin, Niall and Seeberg, 1985; Forage, Ring, Brown, McInerney, Cobon, Gregson, Robertson, Morgan, Hearn, Findlay, Wettenhall, Burger and de Kretser, 1986). This established that it is composed of α and β subunits, with two forms of the β subunit (βA and βB), either of which could combine with the common α subunit. The structure of human inhibin has also been described (Mason, Niall and Seeberg, 1986), and shows 85% homology of the α chain
sequence with bovine and porcine inhibin, and an even greater homology of both $\beta$ chains.

Castration results in a rapid fall in plasma inhibin levels in man (Ishida, Tashiro, Watanabe, Fujii, Yoshida, Imamura, Minowada, Shinohara, Fukutani, Aso and de Kretser, 1990) as in the rat (Robertson, Hayward, Irby, Jacobsen, Clarke, McLachlan and de Kretser, 1988), confirming a gonadal source. In the rat testicular inhibin levels are reduced after hypophysectomy and restored by FSH administration but not by testosterone (Au, Robertson and de Kretser, 1985). In a non-human primate, GnRH antagonist treatment caused a decrease in serum inhibin levels (Weinbauer, Khurshid, Fingscheidt and Nieschlag, 1989), which was restored by FSH administration (Weinbauer, Behre, Fingscheidt and Nieschlag, 1991). The physiological importance of an inhibin-like hormone is suggested by the selective elevation of FSH but not LH in men with severe impairment of spermatogenesis (de Kretser, Burger, Fortune, Hudson, Long, Paulsen and Taft, 1972), although decreases in plasma inhibin are not always found (Tsatsoulis, Shalet, Morris and de Kretser, 1990). These results suggest that inhibin may be involved in the control of FSH secretion by a classical negative feedback loop.

Cultured monkey Sertoli cells secrete inhibin (Noguchi, Keeping, Winters, Saito, Oshima and Troën, 1987), and are thought to be the main or only site of inhibin secretion in the testis. Secretion of inhibin is reduced in Sertoli cell preparations from animals after hypophysectomy, and can be restored by gonadotrophin administration (Steinberger, 1981): it thus follows the same pattern as other Sertoli cell products. In man, pulsatile secretion of inhibin into spermatic venous blood has been reported, and the pulses of inhibin secretion coincided with pulses of testosterone secretion (Winters, 1990). This may imply a common mechanism of control involving LH and Leydig cells.

Inhibin was originally characterised on the basis of its ability to inhibit FSH secretion, in in vivo and in vitro bioassays: the secretion of both basal and GnRH stimulated release is inhibited, as is cellular FSH content (de Jong, 1988) and there is a rapid reduction in cellular FSH $\beta$ mRNA levels (Attardi, Keeping, Winters, Kotsuji, Maurer and Troën, 1989). These results support direct studies in which inhibin preparations suppressed FSH secretion in vivo (Hermans, van Leeuwen, Debets and de Jong, 1980; Lorenzen, Dworkin and Schwartz, 1981). A synergistic effect between inhibin and testosterone on FSH secretion has been suggested based on the inhibition of FSH secretion in response to GnRH stimulation of cultured pituitary cells (Kitahara, Kotsuji, Keeping, Oshima, Troën and Winters, 1991). A local, paracrine role for
Inhibin has been also been proposed in the ovary, in the regulation of preovulatory oestrogen synthesis (Hillier, 1991): a similar role in the testis awaits description.

Direct evidence for a role for inhibin in the control of FSH secretion in man is lacking, but several animal studies support this. The administration of the Leydig cell toxicant EDS to rats, which results in a decline in plasma testosterone concentration, caused a rise in LH comparable to that following castration but a lesser rise in FSH (Jackson and Morris, 1977). Cryptorchidism cause a similar rise in FSH secretion with no change in testosterone concentration, but subsequent administration of EDS caused a further rise in FSH secretion, to concentrations found after castration (O'Leary, Jackson, Averill and de Kretser, 1986). These studies suggest that both inhibin and testosterone have a role in the normal control of FSH secretion.

Serum inhibin levels rise during puberty in both boys and girls (Burger, McLachlan, Bangah, Quigg, Findlay, Robertson, de Kretser, Warne, Werther, Hudson, Cook, Fielder, Greco, Yong and Smith, 1988), and fall with advancing age in men (Tenover, McLachlan, de Kretser, Burger and Bremner, 1988). Testosterone-induced suppression of gonadotrophin secretion caused a fall of 60% in inhibin levels, which was only partially restored by gonadotrophin treatment (McLachlan, Matsumoto, Burger, de Kretser and Bremner, 1988). Others have reported that 19-nortestosterone also suppressed inhibin levels in normal men, but subsequent administration of a human FSH preparation had no effect (Jockenhovel, Fingscheidt, Khan, Behre and Nieschlag, 1990).

Although, as mentioned above, FSH is often elevated in infertile men with damaged seminiferous tubules, the expected decrease in inhibin concentrations is not consistently seen in these men. Thus inhibin levels were normal or slightly elevated in men with spontaneous or chemotherapy-induced damage to the seminiferous tubule (de Kretser, McLachlan, Robertson and Burger, 1988; Tsatsoulis et al., 1990), and there was a positive correlation between FSH and inhibin in the men studied following chemotherapy. More recently, a close negative correlation has been reported between serum concentrations of inhibin and FSH or sperm density in normal men, but these relationships were not found in men with varicoceles, whether or not they were fertile (Plymate, Paulsen and McLachlan, 1992).

It therefore appears that inhibin may have a role in the control of FSH secretion, albeit subsidiary to that of testosterone, although further studies are required to define its importance in physiological and pathological states. The existence of the BA:βA dimer, activin, with stimulatory effects on FSH secretion has been described (Ling, Ying, Ueno, Esch, Denoroy, Guillimin, 1985; Vale, Rivier, Vaughan, McClintock, Corrigan, Woo, Karr and Speiss, 1986), and a paracrine role for both these peptides
within the testis is currently being explored. The highly vectorial secretion of inhibin towards the lumen of the seminiferous tubule in vivo and by cultured primate Sertoli cells (Maddocks and Sharpe, 1990; Handelsman, Spaliviero and Phippard, 1990) suggests a negative paracrine role in the control of spermatogenesis. In contrast, activin has been shown to have stimulatory effects on spermatogonial proliferation in vitro (Mather, Attie, Woodruff, Rice and Phillips, 1990).

1.5 Action of Androgens.

1.5.1 The androgen receptor.

Androgens are believed to exert their biological effects after binding to an intracellular protein receptor. This was first demonstrated by Bruchovsky and Wilson (1968) and Anderson and Liao (1968). Bruchovsky and Wilson showed that [3H]testosterone and [3H]DHT are present in rat prostatic cytoplasm within one minute after administration of [3H]testosterone to rats, and that both were found in prostatic nuclei as much as 2 hrs after the injection. Anderson and Liao showed that nuclear chromatin from prostate, but not from liver, retained [3H]DHT with high affinity after incubation of homogenate with [3H]testosterone. Both studies thus demonstrated the rapid conversion of testosterone to DHT within the cytoplasm of the prostatic cell, and nuclear binding of both steroids. The importance of DHT in the action of androgens is discussed further below, and in Section 1.6.

Complementary DNA encoding the androgen receptor has now been cloned, and the gene localised to the X chromasome (Chang, Katoris and Liao, 1988; Lubahn, Joseph, Sullivan, Willard, French and Wilson, 1988). This has confirmed that it belongs to the steroid receptor superfamily, a family of proteins which bind steroids (including glucocorticoids, mineralocorticoids, oestrogens, progestogens, vitamin D derivatives and thyroxine) and subsequently interact with the genome. The structure of the receptor has many characteristics in common with other members of the family, having 4 domains: a hormone binding region, a hinge region, a DNA-binding domain and an N-terminal domain (Wilson, 1992).

Androgen resistance syndromes result when the physiological response to testosterone is reduced or absent. The two most important are disorders of the androgen receptor and of conversion of testosterone to DHT. These result in very different clinical syndromes, respectively testicular feminisation and pseudovaginal perineoscrotal hypospadias or 5α-reductase (5αR) deficiency. These syndromes illustrate the effect of androgens on the development of the normal male phenotype, and
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in particular the importance of the conversion of testosterone to DHT in the development of the external genitalia.

1.5.2 Male sexual development.

There are three stages in normal intra-uterine sexual development.

1. The determination of chromosomal sex, which is completed on fusion of the germ cells.

2. Translation of genetic sex into gonadal sex. The testis develops at 6 weeks of life after migration of the primordial germ cells to the genital ridges, and after 7 weeks Leydig cells appear. As discussed in Section 1.2, the mechanism of translation of genetic into gonadal sex is incompletely understood, but it appears that the development of the testis is dependent on a product of the testis-determining gene, located on the Y chromasome (Simpson, Chandler, Goulmy, Disteche, Ferguson-Smith and Page, 1987; Wartenberg, 1989; vide supra, Section 1.2.1), and the absence of this factor results in the development of an ovary.

3. Gonadal sex is translated into somatic or phenotypic sex. This involves differentiation of the internal and external genital “anlage” into male or female forms. The internal genitalia in both sexes are derived from a dual duct system, the Wolffian (mesonephric) and Müllerian (paramesonephric) ducts. In males, the Wolffian system develops into the epididymis, vas deferens and seminal vesicles, and the Müllerian duct degenerates. The external genitalia and the urethra develop from a common anlagen in both sexes, with the genital tubercle, genital folds and genital swellings forming the glans penis, the scrotum and the shaft of the penis respectively, and the urogenital sinus forming the prostate and prostatic urethra.

The first two of these stages of sexual differentiation occur before the development of the ability of the foetus to produce androgens (Siiteri and Wilson, 1974), but the third stage is critically dependent on the secretion of hormones from the testis. This was demonstrated by Jost (1953), who showed that foetal rabbits castrated in utero at a sufficiently early stage of gestation developed a female phenotype regardless of gonadal sex. Androgens were able to replace the masculinising effect of the testis, but failed to inhibit Müllerian duct development. Müllerian inhibiting hormone is secreted by the Sertoli cells and has a local effect on the ipsilateral Müllerian duct (Josso et al., 1977), and the stimulatory effect of testosterone on the internal ducts is also a local effect (Jost, Vigier, Prépin and Perchelet, 1973). The tissues of the urogenital sinus and external genitalia have been shown to have 5αR activity, and it appears that the development of
these structures is dependent on the conversion of testosterone to DHT (Siiteri and Wilson, 1974). The effect of deficiency of 5αR activity is described in Section 1.6.

1.5.3 Testicular feminisation.

Complete testicular feminisation is at the extreme of a range of conditions of defective virilisation, a spectrum varying from XY individuals with female phenotype to mild undervirilisation in phenotypically normal men, but all have similar X-linked inheritance and hormonal profiles (Griffin, 1992). Testicular feminisation is characterised by a normal male 46,XY karyotype, bilateral testes, absent Müllerian structures and a female phenotype but usually diminished axillary and pubic hair (Morris, 1953). The plasma levels of testosterone and LH are normal or elevated (Wilson and MacDonald, 1978).

It had previously been suggested that the condition was due to unresponsiveness of the target tissues to testosterone. This was confirmed by studies of the testicular feminised mouse, which demonstrated a defect in the binding of androgens to the androgen receptor (Bardin, Bullow, Sherins, Mowszowicz and Blackburn, 1973). Subsequently Keenan, Meyer, Hadjian, Jones and Migeon (1974) reported very low levels of high affinity binding of DHT to skin fibroblasts from patients with this condition.

The androgen receptor has now been extensively characterised in many families with testicular feminisation (Griffin and Durrant, 1982), and the genetic mutation responsible for the condition characterised in several. This approach has allowed the demonstration of a deletion at the carboxy terminal steroid binding domain (Brown, Lubahn, Wilson, Joseph, French and Migeon, 1988), or more often single base deletions (Marcelli, Tilley, Wilson, Wilson, Griffin and McPhaul, 1990) or substitutions (Tincello, Hargreave, Wu, Padayachi and Saunders, 1992). Complete deletion of the androgen receptor gene has also been described (Quigley, Friedman, Johnson, Lafreniere, Silverman, Lubahn, Brown, Wilson, Willard and French, 1992).

The wide variations in the underlying genetic basis for the condition may provide an explanation for the spectrum of clinical manifestation of testicular feminisation, and for the finding that in vitro androgen binding is normal in some patients with complete androgen insensitivity (Griffin, 1992). The clinical manifestations of testicular feminisation thus reveal the effect of defective androgen stimulus throughout the body.
1.6 Dihydrotestosterone and 5α-Reductase.

Testosterone is converted to DHT by the enzyme 5α-reductase (5αR). This conversion is regarded as a key reaction in androgen action, essential for the development of the male phenotype during embryogenesis and subsequently for androgen-mediated growth of tissues such as the prostate. The conversion of testosterone to DHT was first demonstrated by Farnsworth and Brown (1963) using prostatic tissue in vitro, and this conversion was subsequently confirmed in vitro and in vivo following injection of [3H]testosterone and the subsequent isolation of [3H]DHT (Bruchovsky and Wilson, 1968). These authors also reported that 5αR activity was absent from rat skeletal muscle, a finding that has been confirmed in the adult human (Gloyna and Wilson, 1969), although there may be some activity in fetal skeletal muscle (Kelch, Lindholm and Jaffe, 1971). DHT has a greater biological activity than testosterone (Dorfman and Shipley, 1956), binding to the androgen receptor (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968), but with a greater affinity than testosterone (Keenan et al., 1974; Grino, Griffin and Wilson, 1990). It has also been suggested that the DHT-receptor complex has a higher affinity for acceptor sites in the nuclear chromatin than does the testosterone-receptor complex (Kovacs, Griffin, Weaver, Carlson and Wilson, 1984). Both these effects will therefore act as an amplifier of androgen action, and this appears to be required for some but not all androgen-mediated functions.

1.6.1 The effects of 5α-Reductase deficiency and inhibition.

The importance of 5αR and of DHT as essential components of androgen action is demonstrated by the effect of its deficiency (Imperato-McGinley, Guerrero, Gautier and Peterson, 1974; Walsh, Madden, Harrod, Goldstein, MacDonald and Wilson, 1974; Peterson, Imperato-McGinley, Gautier and Sturla, 1977). The condition of male pseudohermaphroditism (previously described as pseudovaginal perineoscrotal hypospadias by Nowakowski and Lenz (1961)), due to autosomal recessive inheritance of 5αR deficiency has been described in the Dominican Republic (Imperato-McGinley et al., 1974), Papua New Guinea (Imperato-McGinley, Miller, Wilson, Peterson, Shackleton and Gajdusek, 1991) and Great Britain (Ng, Taylor, Hughes, Ransley and Grant, 1990). Affected males have ambiguous external genitalia at birth, often resulting in rearing as girls (although rearing in a specific intersex, prepubertal male, role has been described in some tribes in Papua New Guinea), but they have male internal urogenital tracts. At puberty, a male muscular habitus develops, with growth of the phallus and scrotum and some descent of the testes, and most assume the male
gender role (Imperato-McGinley, Peterson, Gautier and Sturla, 1979). These subjects have been extensively investigated, and as many of the results are of relevance to the studies carried out in this Thesis, they will be discussed here, as described by Peterson et al. (1977).

The plasma concentration of testosterone is slightly higher than normal, but the concentration of DHT is markedly reduced, and there is no increase in plasma DHT after injection of hCG despite a normal increase in testosterone. LH and FSH concentrations are higher than normal, despite the mildly elevated concentration of testosterone, suggesting reduced negative feedback at the hypothalamo-pituitary axis. The metabolic clearance rate of both testosterone and DHT is normal, but conversion of testosterone to DHT is reduced. Urinary excretion of androgens is abnormal, with an aetiocholanolone:androsterone ratio between 6 and 22 (normal 1.0 - 2.1). Androsterone is the major 5α-reduced metabolite of testosterone in urine, whereas aetiocholanolone is the major 5β-reduced metabolite. The ratio of the two is therefore taken as an index of 5αR activity. Testicular biopsy has been reported as showing reduced spermatogenesis at less than 10% of normal (Johnson, George, Neaves, Rosenthal, Christensen, Decristoforo, Schweikert, Sauer, Leshin, Griffen and Wilson, 1986), although this result must be interpreted in the light of the concurrent presence of partial maldescent, as the testes are found in the inguinal canal or labio-scrotal folds. These subjects show reduced body hair, female pubic hair distribution, and little acne. The prostate is small.

The urinary excretion of steroids has also been used to diagnose 5αR deficiency in children (Imperato-McGinley, Gautier, Pichardo and Shackleton, 1986). In addition to the androgen β/α pair aetiocholanolone/androsterone, the equivalent cortisol metabolites can be quantified as the same enzyme is responsible for 5α-reduction of C21 and C19 steroids (Fisher, Kogut, Moore, Goebelsmann, Weitzman, Isaacs, Griffin and Wilson, 1978). The C21 metabolites are tetrahydrocortisol (THF) and allo-tetrahydrocortisol (aTHF). In children as in adults with 5αR deficiency, the THF/aTHF ratio is greatly increased, allowing diagnosis at an age when urinary excretion of androgens is too low for accurate quantification.

The isolation of complementary DNA to 5αR from the prostate has recently been described (Andersson and Russell, 1990; Andersson, Berman, Jenkins and Russell, 1991), suggesting the presence of two types of 5αR. One type was absent in two males with 5αR deficiency, and that enzyme was demonstrated to have many of the characteristics of the major isoenzyme in genital tissue, including acidic pH optimum and sensitivity to inhibition by the 4-azasteroid derivative, finasteride. This enzyme has
been designated \(5\alpha R\) 2, in contrast to \(5\alpha R\) 1, which was also cloned from human prostate and has an alkaline pH optimum, is relatively insensitive to finasteride and is present in subjects with \(5\alpha R\) deficiency. The significance of the two isoenzymes is unknown, but it appears that \(5\alpha R\) 1, which is expressed at only a low level, may be sufficient (in the presence of increased concentrations of testosterone) for the partial virilisation that occurs at puberty in boys with \(5\alpha R\) deficiency (Imperato-McGinley et al., 1979), but is not sufficient for the in utero masculinisation of the external genitalia. The gene encoding \(5\alpha R\) 1 (SRD5A1) has now been localised to chromosome 5, with a pseudogene mapped to the X chromosome (Jenkins, Hsieh, Milatovich, Normington, Berman, Francke and Russell, 1991).

The abnormalities described in males with \(5\alpha R\) deficiency illustrate the profound influence of DHT on the development of the male phenotype, with the demonstration of those androgen-mediated effects which are dependent on DHT, despite the elevated level of testosterone. The partial virilisation that occurs in these males at puberty is compatible with the demonstration that there is only one molecular species of androgen receptor (Quarmby et al., 1990), with the relative binding of testosterone and DHT according to the law of mass action (Grino et al., 1990). The study of these subjects does not, however, shed much light on the influence of DHT on spermatogenesis, because of the complicating effect of often-coexisting cryptorchidism (Johnson et al., 1986).

The development of compounds with inhibitory effects on \(5\alpha R\) activity has heralded a new stimulus into the study of the importance of \(5\alpha R\) in human pathophysiology, with particular emphasis on the condition of benign prostatic hyperplasia (Horton, 1992). Inhibitors of \(5\alpha R\) cause regression of the hyperplastic canine prostate, with a reduction in prostatic DHT levels (Brooks, Berman, Glitzer, Gordon, Primka, Reynolds and Rasmusson, 1982), and prevent testosterone-induced growth of the prostate in castrated dogs (Wenderoth, George and Wilson, 1983). These parent drugs, however, are also weak inhibitors of androgen binding to the androgen receptor, and thus part of the effect could be due to a direct antagonistic effect. This is not the case for finasteride, which has very low affinity for the androgen receptor (Liang, Heiss, Cheung, Reynolds and Rasmusson, 1984). When given to new-born male rats, finasteride prevents development of some but not all androgen-dependent organs: thus growth of the penis, prostate, and seminal vesicles was inhibited despite elevated plasma levels of testosterone, but the perineal muscles and preputial glands were not affected (George, Johnson and Wilson, 1989). Exposure in utero was without effect in female rats, but caused abnormal development of the mesenchymal
wedge between the urogenital sinus and rectum in male rats, which resulted in the opening of the urethra as a groove on the ventral surface of the penis, i.e. hypospadias (Anderson and Clark, 1990). These experiments provide valuable evidence as to the importance of 5αR in various organs in animals, but the administration of finasteride to humans is of necessity limited to adults. Several studies have now been described.

Finasteride is a very potent inhibitor of 5αR in men, a single dose of 0.04 mg having effects for several days (Gormley, Stoner, Rittmaster, Gregg, Thompson, Lasseter, Vlasses and Stein, 1990). Plasma DHT and AdiolG levels were reduced by approximately 65% and 40% respectively, testosterone levels were unchanged or increased, and LH and FSH levels were unchanged (Vermuelen, Giagulli, De Schepper, Buntinx and Stoner, 1989; Rittmaster, Stoner, Thomson, Nance and Lasseter, 1989; Gormley et al., 1990). The urinary excretion of the 5αR metabolite of cortisol, αTHF, was also reduced, indicating an inhibition of the hepatic as well as the peripheral enzyme (Vermuelen et al., 1989). Comparison of men with congenital 5αR deficiency with normal men treated with finasteride demonstrated many similarities between the two groups in both plasma and urinary steroid metabolism (Imperato-McGinley, Shackleton, Orlic and Stoner, 1991). Prostatic concentrations of DHT were reduced by finasteride to a greater extent than in plasma, to approximately 10% of controls despite prostatic tissue testosterone concentrations being increased 10-fold (Geller, 1990; McConnell, Wilson, George, Geller, Pappas and Stoner, 1992). Shrinkage of prostatic volume has also been reported (Stoner, 1992).

1.6.2 The control of 5α-Reductase activity.

An important characteristic of 5αR is that its activity is dependent on androgen levels in many tissues, with both stimulatory and inhibitory effects described. In pubic skin, 5αR activity increases at puberty (Mauvais-Jarvis, 1977), and enzyme activity is therefore thought to be androgen-dependent, as it is in the prostate (Moore and Wilson, 1973). Pubic skin fibroblast 5αR activity is increased by DHT (Mowszowicz, Melanitou, Doukani, Wright, Kuttenn and Mauvais-Jarvis, 1983), and in the prostate a similar effect has been demonstrated at the level of both enzyme activity and mRNA (Andersson, Bishop and Russell, 1989; George, Russell and Wilson, 1991). These results suggest that in these two tissues, there is a positive feedback effect of DHT on its rate of production, and this effect may be the basis for the profound inhibition of prostatic DHT levels seen after finasteride administration despite elevated tissue testosterone levels (McConnell et al., 1992).
Further evidence for a positive influence of the androgen receptor on 5αR is provided by studies on patients with testicular feminisation, in whom the androgen receptor is absent (Keenan et al., 1974; Griffin, Punyasthiti and Wilson, 1976). These subjects have normal, high 5αR activity in perineal skin, but it is very low or absent in pubic skin (Northcutt, Island and Liddle, 1969; Kuttenn, Mowszwowicz, Wright, Baudot, Jaffiol, Robin and Mauvais-Jarvis, 1979; Quigley et al., 1992). 5αR activity is also present in foetal urogenital tissue before the onset of testosterone secretion by the testis, and is similar in males and females (Siiteri and Wilson, 1974). 5αR activity in these tissues in the adult is also much higher than in pubic skin (Kuttenn et al., 1979). This suggests that the low 5αR activity in pubic skin is secondary to the absence of the androgen receptor (Imperato-McGinley, Peterson, Gautier, Cooper, Danner, Arthur, Morris, Sweeney and Shackleton, 1982).

It therefore appears that 5αR in tissues exhibiting secondary sexual characteristics is androgen dependent, whereas in urogenital tissue the activity of the enzyme is much greater, but is not androgen-dependent. Thus in the tissues exhibiting secondary sexual characteristics the effect of the increase in testosterone secretion at puberty will be amplified. This is in contrast with 5αR activity in rat adrenal cortex, which is greatly increased after hypophysectomy or castration and is reduced by androgen administration (Trzeciak and Malendowicz, 1981; Andò, Canonaco, Valenti, Aquila, Tavolaro, Maggiolini, Panno and Dessi-Fulgheri, 1989; Lephart, Simpson and Trzeciak, 1991), and in the rat liver, which is increased after orchidectomy (Andersson et al., 1989). This has been demonstrated at the mRNA level in both organs (Andersson et al., 1989; Lephart et al., 1991). In the rat adrenal cortex, Lephart et al. (1991) found that DHT administration reduced 5αR activity to almost undetectable levels.

A further difference between liver and prostatic 5αR lies in its subcellular location: in the liver, 5αR activity was equally distributed between mitochondria and microsomes with negligible amounts in the nuclear fraction, whereas in the prostate the nuclear fraction contained the most activity (Roy, 1971).

1.6.3 5α-Reductase in the testis.

Much of the information available in this field is from work carried out on species other than man for reasons of limitations of availability of tissue. Extrapolation to the human must therefore be cautious. The problem of scarcity of tissue is compounded by the fact that much of the human tissue that has been studied may not be normal.
The role of testosterone in the maintenance of spermatogenesis has been discussed above. Thus testosterone has been shown to maintain spermatogenesis in hypophysectomised rats (Walsh et al., 1934; Steinberger, 1971). The ability of rat interstitial tissue and seminiferous tubules to convert testosterone to DHT and androstanediol (Adiol) in vitro has been demonstrated (Nayfeh, Barefoot and Baggett, 1966; Rivarola and Podestá, 1972), and the activity of 5αR in the rat testis was found to be dependent on the maturity of the animal. Thus the immature rat testis rapidly converted [3H]progesterone to [3H]Adiol, with little accumulation of [3H]testosterone, but in the adult [3H]testosterone became the major product (Nayfeh et al., 1966; Coffey, French and Nayfeh, 1971). Others have confirmed a higher 5αR activity in immature than mature rat testis (Folman, Ahmad, Sowell and Eik-Nes, 1973; Matsumoto and Yamada, 1973; Dorrington and Fritz, 1975). Maximal formation of [3H]Adiol from [3H]testosterone was found in 20-26 day old rats, the time of the first meiotic division, at which time endogenous levels of Adiol are elevated (Rivarola, Podestá and Chemes, 1972; Rivarola, Podestá, Chemes and Calandra, 1975). 5αR activity in the rat testis is also differentially distributed between seminiferous tubules and interstitial cells, with greater activity in the tubules (Rivarola and Podestá, 1972; Nayfeh, Coffey, Hansson and French, 1975), which are also the site of 3α-hydroxysteroid dehydrogenase activity, the enzyme which reversibly converts DHT to the androstanediols (Dorrington and Fritz, 1975). Spermatocytes also appear to have 5αR activity but not 3α-hydroxysteroid dehydrogenase activity (Dorrington and Fritz, 1975).

Following hypophysectomy of immature rats, 5αR activity declined rapidly in both tubules and interstitial tissue (Nayfeh et al., 1975), and this decline was partly restored by either LH or FSH, but not by testosterone. In mature hypophysectomised rats, 5αR activity in seminiferous tubules but not interstitial tissue was increased by LH (Nayfeh et al., 1975). 3α-hydroxysteroid dehydrogenase activity in seminiferous tubules from adult rats, however, was greatly increased following hypophysectomy, to a rate similar to that found in immature rats (Dorrington and Fritz, 1975). These authors also speculated that ABP, secreted by the Sertoli cells in response to FSH stimulation (Hansson et al., 1973), might influence androgen metabolism in the Sertoli cell, by competing with 3α-hydroxysteroid dehydrogenase for DHT, and thus reducing the metabolism of DHT. This is compatible with the finding that the concentration of Adiol is low relative to that of testosterone and DHT in the seminiferous tubules of mature rats (Podestá and Rivarola, 1974).

In important studies, Ahmad, Haltmeyer and Eik-Nes (1973 and 1975) showed that DHT capsules implanted into the testis of hypophysectomised rats maintained
spermatogenesis in the ipsilateral but not the contralateral testis: 5α-reduction of testosterone is not reversible, therefore the maintenance of spermatogenesis in that study cannot be attributed to the effect of testosterone. The development of inhibitors of 5αR have enabled further investigation into the role of DHT in the testis. The administration of one such inhibitor, 4-MA, did not impair fertility in male rats (Brooks et al., 1982), nor did finasteride affect spermatogenesis (George et al., 1989). The testicular concentration of testosterone was 250% of normal in the finasteride-treated rats, however, which complicates interpretation.

Data from the human is scanty. Payne, Kawano and Jaffe (1973) demonstrated the ability of testicular tissue from a 17-year old to reduce testosterone to DHT \textit{in vitro}, and this has been confirmed by others (Rivarola et al., 1973). As in the rat, there is a differential anatomical distribution, and changes in activity at different stages of maturity: activity was higher in the seminiferous tubule preparation than in interstitial tissue, and there appeared to be an increase in activity with increasing maturity, with a maximum in tissue from a 15 year old (Rivarola et al., 1975). A single adult in that study had low activity, but also had reduced spermatogenesis, making interpretation difficult. 5αR activity was undetectable in human foetal testis (Kelch et al., 1971). The level of 5αR in the human testis may therefore show a similar pattern of development to that in the rat, with an increase at puberty and fall subsequently.

Testicular 5αR may also be androgen-controlled, in common with the activity of the enzyme in other tissues, as it is decreased after hypophysectomy in the rat (Nayfeh et al., 1975). No data are available for the human. However, 5αR activity in the rat testis was not restored by testosterone administration, although gonadotrophin administration partially restored activity (Nayfeh et al., 1975). An increase in activity at the time of development of the seminiferous epithelium would be expected, and has been reported in both human and rat (Rivarola et al., 1973, 1975; Matsumoto and Yamada, 1973; Nayfeh et al., 1975). In both species, there appears to be a decrease in 5αR with full maturity. The presence of high intratesticular levels of testosterone may also interfere with the enzyme assays by competing with the \([3H]\)testosterone added as the enzyme substrate, and differences in the level of endogenous testosterone between tissue preparations and experimental models will complicate interpretation. Firm conclusions as to the androgen-dependence of testicular 5αR therefore cannot be drawn, but it is possible that 5αR in the seminiferous tubules, as elsewhere in the body, acts as an amplifier of androgen action. This may be particularly important at puberty, when testosterone production is not at its adult level. The resulting increased androgen stimulus may be important in the initiation of spermatogenesis.
5αR is also present in the rat epididymis, and was decreased after orchidectomy (Robaire, Ewing, Zirkin and Irby, 1977). This effect was partially restored by testosterone administration. It has subsequently been shown in experiments measuring 5αR mRNA that concentrations of testosterone 5-8 fold higher than in peripheral plasma are necessary to restore 5αR mRNA in the caput, where activity is highest, but even then mRNA levels were only 50% of control (Viger and Robaire 1991). Replacement of testosterone in amounts sufficient to restore normal peripheral concentrations restored 5αR mRNA in the corpus and cauda epididymis. These results were interpreted as suggesting that 5αR in the caput is regulated by a factor in addition to testosterone, but as epididymal concentrations of testosterone are 10-12 fold higher than in peripheral plasma (Turner, Ewing, Jones, Howards and Zegeye, 1985), replacement may have been inadequate. Nevertheless, it appears that the epididymis of the rat contains a high level of 5αR, which is to a large extent androgen-dependent. Similar studies at the mRNA level on 5αR in the testis would be of value.

1.6.4 The origin of plasma DHT.

DHT in the plasma could be the result of direct glandular secretion, or the product of peripheral or splanchnic conversion of testosterone. The classical method for investigating glandular secretion of a hormone is to determine the difference in concentration between the venous blood draining the gland under investigation and that in mixed peripheral venous blood. Thus for testosterone, it is accepted that testicular secretion accounts for almost all the circulating hormone. Using this methodology, it has been found that the testes in the adult male secrete DHT (Pazzagli et al., 1974; Hammond, Ruokonen, Kontturi, Koskela and Vihko, 1977), and it has been estimated that the daily testicular production is approximately 100μg per day (Horton, 1978), compared to 6 mg/day for testosterone. The metabolic clearance rate of DHT is 760 l/day, and the blood production rate is therefore 300μg/day (Ito and Horton, 1971). Testicular production therefore accounts for approximately one third of circulating DHT.

Measurement of the conversion rate of testosterone to DHT allows calculation of the contribution of circulating testosterone to DHT, and a value of 75% in men has been suggested (Ito and Horton, 1971). In women, the contribution of testosterone is less than 20%, and androstenedione is the major precursor (Ito and Horton, 1971). The conversion of testosterone to DHT may occur in the splanchnic or the peripheral circulations. This has been investigated in men by infusion of [3H]testosterone with selective cannulation of the splanchnic circulation: the concentration of DHT did not
differ across the splanchnic circulation, nor was $[^{3}\text{H}]$testosterone converted to detectable concentrations of $[^{3}\text{H}]$DHT in the hepatic vein (Ishimaru, Edmiston, Pages and Horton, 1978), despite the presence of 5αR in the liver. It therefore appears that in men the majority of the conversion of testosterone to DHT which is subsequently released into the circulation occurs peripherally, in tissues such as the skin and prostate and that DHT is therefore a paracrine hormone. Experiments to measure the conversion rate do not, however, allow for the subsequent conversion of DHT to metabolites such as Adiol before being released into the circulation, and may thus underestimate this value. This further metabolism has been suggested to be of importance in androgen metabolism in the skin, and to account for the poor discrimination between groups of women with clinical hyperandrogenism by comparison of DHT concentrations in plasma. This is discussed further in Chapter 5.

The effects of finasteride confirms the importance of 5α-reduction of testosterone in determining the concentration of DHT in both plasma and tissues (vide supra, Section 1.6.1), but does not provide information as to the site of reduction of testosterone, as both hepatic and peripheral 5αR are inhibited by this drug.

1.7 The development of hormonal male contraception.

The choice of contraception currently available to men is between the condom and vasectomy. Both have considerable drawbacks, particularly the reliability and acceptability of condoms and the irreversible nature of vasectomy, although the advent of the human immunodeficiency virus has resulted in an increase in the advantages offered by condoms. This paucity of choice of method contrasts with the wide range of methods available for the control of female fertility, most notably in the use of hormonal methods.

Hormonal methods of contraception in the male have been investigated for over 50 years, following the demonstration that administration of testosterone (as the propionate ester) to normal men caused a reversible reduction in spermatogenesis without affecting libido or potency (McCullagh and McGurl, 1939; Heller, Nelson, Hill, Henderson, Maddock and Jungck, 1950). This has subsequently been confirmed and enlarged upon in many other studies using a variety of testosterone derivatives, alone or in combination with oestrogens or progestogens (de Kretser, 1974), but the goal of complete suppression of spermatogenesis to provide the required degree of contraceptive efficacy with the maintenance of libido, without side effects, and in an acceptable method of delivery, remains elusive. This combination of effects is thought to require:
1. Complete suppression of LH and FSH secretion.
2. Consequent complete (but fully reversible) arrest of spermatogenesis.
3. Normal circulating concentrations of sex steroids, in particular testosterone and oestradiol.

Several approaches have been used to achieve this end, each with different advantages and disadvantages.

1. **Androgens alone.**

Several studies have shown a profound inhibition of spermatogenesis when androgen is given alone (*vide supra*, Section 1.3.1; Mauss, Börsch, Richter and Bormacher, 1974; Steinberger and Smith, 1977; Swerdloff, Palacios, McClure, Campfield and Brosman, 1978), and most recently in a multicentre trial organised by the World Health Organisation (World Health Organisation, 1990). In that study, 200mg testosterone oenanthate was given weekly i.m. to 271 men in a protocol very similar to that of the study described in this Thesis. Those men who became azoospermic continued on the testosterone treatment and used it as their only method of contraception for one year. Plasma levels of gonadotrophins were undetectable in all men, but only 65% became azoospermic within 6 months of testosterone treatment. Side-effects largely reflected the supraphysiological dose of testosterone, and included acne, hypertension and polycythaemia. Behavioural disturbances were noted in 3 men. Only one pregnancy was reported in a total of 1486 months of exposure, giving a Pearl Index of 0.8.

While this large trial provides much important information, a major finding is the confirmation of incomplete suppression of spermatogenesis in a large proportion of men, as reported in the earlier studies referred to above. Incomplete suppression of spermatogenesis has been a consistent finding in all studies of hormonal male contraception, whether using androgens alone or in combination with progestogens or GnRH analogues (*vide infra*), with azoospermia being achieved in 50-70% of men in most studies. The proportion of men achieving azoospermia varied greatly between centres in the WHO study, with those with a predominantly Caucasian population reporting much lower rates of azoospermia than those with Chinese populations, in which azoospermia was achieved in over 90% of men studied. The basis for this difference is unknown, and is discussed further in Chapter 5.

Whether androgens are used alone or in combination, the ideal is replacement of physiological concentrations of testosterone or equivalent, and elimination of the high peak concentrations which are the result of administration of currently used
preparations. To this end, various compounds with longer half-lives have been tested. They include 19-nortestosterone (Schürmeyer, Knuth, Belkien and Nieschlag, 1984) and testosterone buiclate (20-Aet-1). The latter maintained effective plasma concentrations of testosterone for up to 4 months following single injection in monkeys (Weinbauer, Marshall and Nieschlag, 1986) and is currently undergoing clinical testing. Different pharmaceutical preparations of testosterone such as incorporation into pellets (Handelsman, Conway and Boylan, 1990) or microspheres (Bhasin, Swerdloff, Steiner, Peterson, Meridues, Galmirini, Pandian, Goldberg and Berman, 1992), also cause constant concentrations of testosterone over several months but have only been used as replacement therapy in hypogonadal men.

Potential fertilising capacity of residual spermatozoa of men on testosterone alone or in combination with MPA has been found to be severely impaired but still present as determined by the in vitro hamster oocyte penetration test (Matsumoto, 1988; Wu and Aitken, 1989; Wallace, Aitken and Wu, 1992), emphasising the need for azoospermia to ensure complete contraceptive efficacy.

2. Androgen-Progestogen combinations.

Progestogens inhibit gonadotrophin secretion in man, with subsequent suppression of spermatogenesis (Bremner and de Kretser, 1976). This also causes a suppression of testicular testosterone synthesis and secretion, and replacement androgen therapy is therefore required for the maintenance of libido and potency in particular.

The most widely studied combination has been depot medroxyprogesterone acetate (MPA) with testosterone oenanthate (Brenner, Mishell, Bernstein and Ortiz, 1977; Melo and Coutinho, 1977), but as with testosterone administration alone, azoospermia was not uniformly achieved. Pregnancies have been reported in the partners of men who had become oligozoospermic on this regime (Barfield, Melo, Coutinho, Alvarez-Sanchez, Faundes, Brache, Leon, Frich, Bartsch, Weiske, Brenner, Mishell, Bernstein and Ortiz, 1979). The combination of 19-nortestosterone with MPA caused azoospermia in 9 out of 12 men (Knuth, Yeung and Nieschlag, 1989).

3. Analogues of GnRH.

Analogues of GnRH with both agonist and more recently antagonist properties have been developed, and investigated as male contraceptive agents alone or in combination with androgens. GnRH agonists initially stimulate then inhibit gonadotrophin secretion through a complex process involving depletion of cell surface receptors and uncoupling of signal transduction, a process known as “downregulation” (Bhasin and Swerdloff,
The decline in gonadotropin secretion results in a fall in testosterone secretion and spermatogenesis (Schürmeyer, Knuth, Freischem, Sandow, Akhtar and Nieschlag, 1984), but again azoospermia was not consistently achieved. This finding is perhaps related to the incomplete suppression of FSH secretion in that study. Similar findings have been reported using an implant of the long-acting GnRH agonist (buserelin), in which the combination of buserelin with a long-acting androgen caused less consistent suppression of spermatogenesis than when the androgen was given alone. Treatment with buserelin resulted in FSH concentrations returning towards baseline after 9-15 weeks treatment despite continuing inhibition of LH secretion (Behre et al., 1992).

The more recent development of GnRH antagonists, which competitively inhibit the action of endogenous GnRH at the pituitary gland (Perrin, Haas, Rivier and Vale, 1983; Wierman, Rivier and Wang, 1989), has led to a resurgence in interest in this method of male fertility regulation (Jockenhovel, Bhasin, Steiner, Rivier, Vale and Swerdloff, 1988; Pavlou, Wakefield, Schlechter, Linder, Souza, Kamilaris, Konidaris, Rivier, Vale and Toglia, 1989) despite initial problems with histamine-like side-effects (Pavlou, Interlandi, Wakefield, Island, Rivier, Vale and Kovacs, 1987). As with other non-androgen methods, replacement androgen is required. This has the theoretical advantage of having an inhibitory effect on GnRH secretion, which would otherwise increase and thus tend to overcome the effect of the antagonist.

As with the GnRH agonists, FSH secretion is relatively resistant to suppression (Jockenhovel et al., 1988; Pavlou et al., 1989) but azoospermia was achieved in 7 out of 8 subjects using a protocol in which the antagonist was administered followed by a low dose of testosterone enanthate after 2 weeks (Pavlou, Brewer, Farley, Lindner, Bastias, Rogers, Swift, Rivier, Vale, Conn and Herbert, 1991). The plasma concentration of testosterone was only approximately 20% of baseline with the replacement dose used, but this appeared sufficient to prevent complaints of reduced libido or sexual activity. This finding has recently been confirmed with a further 7 out of 8 subjects achieving azoospermia following treatment with the same GnRH antagonist but a more physiological replacement dose of testosterone (Tom, Bhasin, Salameh, Steiner, Peterson, Sokol, Rivier, Vale and Swerdloff, 1992).

It therefore appears that inhibition of gonadotrophin secretion by progestogen or GnRH antagonists with replacement testosterone administration results in azoospermia in the majority of men, and the use of these agents rather than androgen alone avoids or reduces the incidence of side-effects related to the supraphysiological doses of androgen required. This is associated with a reduction in risk (Bardin, Swerdloff and Santen, 1991). The use of GnRH antagonists may result in a greater rate of
azoospermia with presumably increased contraceptive efficacy, but this has yet to be established in a large trial. There also remains the possibility of allergic reaction, and the current necessity for daily injection of the antagonist. A hormonal method of male contraception for widespread use therefore remains elusive for the present, although the most recent results outlined above provide some grounds for continuing development.

1.8 Aims of this Thesis.

One major factor which remains to be established is the degree of fertility of those men who become oligozoospermic as a result of one of the treatment regimes discussed above. There is \textit{in vitro} evidence that the fertilising capacity of such residual spermatozoa is reduced, but there are no estimates available for the clinical risk of pregnancy based on large-scale prospective trials. The results reported in this Thesis are part of such a trial, the background, protocol, and preliminary results of which are presented in Chapter 2.

The other studies in this Thesis were carried out to investigate the basis for the maintenance of spermatogenesis in those men who remain oligozoospermic on this regime by comparison with those who become azoospermic. The hypothesis tested is that the maintenance of spermatogenesis in the oligozoospermic group of men is related to an increased activity of the enzyme 5αR. This will result in increased conversion of testosterone to the more potent androgen DHT in the testis as elsewhere in the body, and such amplification of the androgen stimulus may be sufficient to maintain a low rate of spermatogenesis in the presence of low concentrations of testosterone. The possible contribution of continuing endogenous production of androgens in the testis and adrenal cortex was also investigated.
Chapter 2
Outline of Contraceptive Trial.

2.1 Introduction.
The studies reported in this Thesis are based on a trial conducted by the World Health Organisation Task Force on Methods for the Regulation of Male Fertility, entitled “Evaluation of the requirement for severe oligospermia (< 5 x 10^6/ml) in an antifertility method based on the suppression of sperm production with testosterone enanthate in normal men: multicentre study”. The Medical Research Council Reproductive Biology Unit in Edinburgh is one of the participating centres, and the men described here form the Edinburgh cohort of the trial.

The background to this trial was described in Chapter 1, in which previous studies in the development of a hormonal method of male contraception were discussed. The rationale for this trial is that while administration of supraphysiological doses of testosterone, in the form of testosterone enanthate, is known to induce reversible azoospermia and infertility in about 2/3 of normal men for a period of one year (World Health Organisation, 1990), the fertility of the remaining third of men who maintain non-zero sperm counts despite continuing to receive testosterone is unknown. The major object of this trial is to determine the fertility of this group of men.

In this Chapter, an outline of the Protocol of the trial is described as a background to the results presented in subsequent Chapters. The results presented here are the demographic characteristics of the men and their partners recruited to the trial, and the results of semen analyses. The semen analysis results are the part of the main trial most relevant to the studies described in subsequent Chapters of this Thesis, as they form the basis for the discrimination of the azoospermic and oligozoospermic groups of men.

2.2 Protocol of Trial.

2.2.1 Outline of design.
This was a prospective study to evaluate the contraceptive efficacy of severe oligozoospermia (sperm density < 5 x 10^6/ml) and azoospermia. There were 4 phases in the trial:
1. Pretreatment phase. Duration of 1 month, in which pretreatment semen analyses and blood tests were carried out.
2. Suppression phase. Variable duration of up to a maximum of 6 months from the date of first injection. Couples entered the Efficacy phase when they had submitted three consecutive semen samples with sperm densities of < 5 x 10^6/ml.

3. Efficacy phase. One year duration, throughout which no other form of contraception is used. Sperm density was required to remain < 5 x 10^6/ml.

4. Recovery phase. Variable duration, from the date of last injection, to when mean pretreatment sperm density was regained.

2.2.2 Requirement characteristics of subjects.

Couples recruited were in a stable relationship, requiring contraception, and with no history of infertility. The male partner was aged 21 to 45, in good health without cardiac, renal, prostatic or hepatic disease or active genitourinary infection. Two pretreatment semen samples both had sperm density of ≥ 20 x 10^6/ml, with ≥ 50% normal morphology and ≥ 50% forward motility. Two pretreatment blood samples gave normal values for full blood count, clinical chemistry (including cholesterol, high density lipoprotein and low density lipoprotein) and hormone concentrations (LH, FSH, testosterone). The female partner was not be older than 35 years, with a regular menstrual cycle and no pelvic inflammatory disease in the last year. Before testosterone treatment was started and before entering the Efficacy phase of the trial, she had a negative pregnancy test.

Full informed consent was obtained from both partners, in writing and witnessed. Ethical approval was given by the Lothian Region Division of Reproductive Medicine Ethical Committee. Subjects had the right to withdraw at any stage and for any reason.

2.2.2 Drugs administered.

Suppression of spermatogenesis was achieved by weekly administration of 200 mg Testosterone Oenanthate ("Testoviron Depot", Schering AG, Germany) by deep intramuscular (i.m.) injection, in a volume of 0.8 ml.

2.2.3 Semen analyses.

Two semen samples were submitted during the Pretreatment Phase at an interval of at least 2 weeks. During the Suppression Phase, samples were submitted after 4 and 8 weeks treatment, and then at fortnightly intervals until 3 consecutive samples with sperm densities of < 5 x 10^6/ml had been submitted thus enabling the subject to enter the Efficacy Phase. Samples were then submitted every 4 weeks, during both the
Efficacy and Recovery Phases. Analysis was according to the World Health Organisation *Manual for the examination of human semen and sperm-cervical mucus interaction* (1987). Azoospermia was confirmed by examination of the pellet after centrifugation of the semen sample at 2000 g for 5 min.

### 2.2.4 Other procedures.

Subjects had a general Medical examination during the Pretreatment Phase, and at 4 week intervals during testosterone treatment and during the Recovery Phase. The blood tests performed during the Pretreatment Phase were repeated at 12 week intervals.

### 2.2.5 Statistical analysis.

Data was analysed using Students “t” test, for paired samples where appropriate, or by Analysis of Variance with Neuman-Keuls test for post-hoc analysis for multiple results.

### 2.3 Results.

#### 2.3.1 Recruitment.

Couples were recruited following articles in the newspapers and features on radio and television. All General Practitioner practices in Lothian Region received a letter informing them of the trial, and a poster for display. Posters were also displayed in Family Planning Clinics and Public Libraries.

A total of 33 couples were recruited to the trial and started testosterone treatment. Their ages, marital status, contraceptive method at the time of recruitment, and fertility are shown in Table 2.1, together with source of recruitment and stated reason for involvement in the trial. These results show that approximately 75% of the subjects were of proven fertility, as were 60% of their partners. Approximately 2/3 of couples were married. Problems with oral contraception accounted for 55% of couple’s primary reasons for interest in the trial, and 33% gave interest in the development of a hormonal method of male contraception as their primary reason for involvement in the trial.

The age, weight, and height of the men in the trial are given in Table 2.1. Further analysis demonstrated that there were no differences in these parameters between those becoming azoospermic and those remaining oligozoospermic: mean ages were $29 \pm 1$ and $32 \pm 1$ years, weights were $73.1 \pm 2.3$ and $80.3 \pm 3.7$ kg, and heights were $173 \pm 2$ and $177 \pm 2$ cm in the two groups respectively.
Table 2.1.

Demographic characteristics of couples at recruitment, and age, height and weight of the male partners.

<table>
<thead>
<tr>
<th>Marital Status</th>
<th>59% married*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertility:</td>
<td></td>
</tr>
<tr>
<td>Proven (as couple)</td>
<td>58% (19)</td>
</tr>
<tr>
<td>Male only</td>
<td>15% (5)</td>
</tr>
<tr>
<td>Female only</td>
<td>3% (1)</td>
</tr>
<tr>
<td>Neither partner</td>
<td>24% (8)</td>
</tr>
<tr>
<td>Current contraception:</td>
<td></td>
</tr>
<tr>
<td>Condom</td>
<td>42% (14)</td>
</tr>
<tr>
<td>Oral</td>
<td>39% (13)</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>3% (1)</td>
</tr>
<tr>
<td>Depo Provera</td>
<td>3% (1)</td>
</tr>
<tr>
<td>Withdrawal</td>
<td>3% (1)</td>
</tr>
<tr>
<td>Current pregnancy</td>
<td>9% (3)</td>
</tr>
</tbody>
</table>

Reason for involvement:

- Problems with oral contraception 55% (18)
- General interest in development of male method 33% (11)
- Other contraceptive problems 12% (4)

Male Partner

<table>
<thead>
<tr>
<th>Age</th>
<th>mean 31 years (range 21 - 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>175 ± 8 cm</td>
</tr>
<tr>
<td>Weight</td>
<td>76.4 ± 12.4 kg</td>
</tr>
</tbody>
</table>

* 3 couples got married during the course of the trial, increasing this value to 67%.
2.3.2 Suppression of spermatogenesis.

Testosterone treatment caused a profound inhibition of spermatogenesis in all men, with 18 becoming azoospermic by 20 weeks of treatment. The rate of suppression of spermatogenesis was very variable, with one man becoming azoospermic after 4 weeks of treatment while others showed no change in sperm density at that time although subsequently achieving azoospermia. The cumulative number of men becoming azoospermic is shown in Figure 2.1. From this it can be seen that there was a rapidly rising incidence of azoospermia up to 20 weeks of testosterone treatment, but no men became azoospermic between 20 and 40 weeks of treatment. This group of men, who rapidly achieved azoospermia which was confirmed on centrifugation of the semen sample, are referred to as the “azoospermic” group throughout this Thesis, and the remaining group of men are referred to as the “oligozoospermic” group.

Division into these two groups revealed that there was no difference in baseline sperm density (mean of two pretreatment samples, $62 \pm 8 \times 10^6$/ml in the azoospermic group, $76 \pm 13 \times 10^6$/ml in the oligozoospermic group), but that a difference was apparent in the first treatment sample, submitted after 4 weeks. At this time the azoospermic group had a mean sperm density of $15 \pm 8 \times 10^6$/ml, compared to $39 \pm 11 \times 10^6$/ml in the oligozoospermic group ($p<0.05$). The mean sperm density of the two groups at up to 24 weeks of treatment is shown in Figure 2.2, using a log scale for clarity.

The oligozoospermic group had a mean sperm density of $2.0 \pm 0.8 \times 10^6$/ml after 20 weeks testosterone treatment, and this continued to decline. In a subgroup of oligozoospermic men ($n=6$), sperm density of $<0.1 \times 10^6$/ml was achieved at 16 weeks, with sperm usually only detectable in the pellet after centrifugation of the sample. 5 of these men became azoospermic after prolonged testosterone treatment, as indicated by the gradual secondary rise in the incidence of azoospermia after 40 weeks of testosterone treatment shown in Figure 2.1. This subdivision of the oligozoospermic group is discussed further in Chapter 5.

2.3.3 Discontinuation from the trial.

Five men discontinued the trial before completing one year in the Efficacy Phase. One man found the injections too painful, and left the trial after 4 weeks of the Suppression Phase. Results from this man are not included in any analyses in this Thesis. One man discontinued after 8 months of testosterone treatment as he emigrated, and two men discontinued because of separation from their partners, in both cases after more than a
Figure 2.1
Cumulative percentage of men achieving azoospermia during testosterone treatment.
Figure 2.2
The effect of testosterone treatment on sperm density in the ejaculate in those men becoming azoospermic and those remaining oligozoospermic.

Sperm density at week 0 is the mean of two baseline samples.

Open symbols, Azoospermic group, n=18
Filled symbols, Oligozoospermic group, n=15

Note the logarithmic ordinate
year of testosterone treatment. There was one pregnancy during the Efficacy Phase, 2.3.4 Contraceptive Efficacy

There was one pregnancy during the Efficacy Phase. Semen analysis carried out within 7 days of the estimated date of conception showed a sperm density of 4.1 x 10⁶/ml with 25% forward motility and 35% normal morphology. This pregnancy resulted in spontaneous abortion at 6 weeks gestation. This outcome may be related to a functional deficit of the spermatozoa, but previous studies using similar regimes have reported successful pregnancy outcomes (Wallace et al., 1992). It therefore appears unlikely that the outcome was related to TE treatment.

A mean and median age of 31 years. A little over half of the couples were married, and a similar proportion were of proven fertility. While the combined oral contraceptive pill was used by 39% of couples at recruitment, problems with that method were the single most common reason given for interest in this study. This small population thus illustrates the main reasons for requirement for a hormonal method of male contraception and the breadth of interest in such a method.

The results shown in Figures 2.1 and 2.2 illustrate the effect of testosterone treatment on sperm density in the ejaculate in these men. There was a pronounced fall in sperm density in all men, but there was a clear separation of response into those men who rapidly became azoospermic and those who remained oligozoospermic. This difference was apparent in the rate of inhibition of sperm density, with the group who were to become azoospermic having a significantly lower sperm density after only 4 weeks of testosterone treatment, i.e. the first treatment sample submitted. The resistance of spermatogenesis to suppression in those men who remained oligozoospermic was therefore evident in the rate of decline of sperm density as well as in the degree of inhibition achieved.

Figure 2.1 shows the percentage of men achieving azoospermia during testosterone treatment. One man was azoospermic by 4 weeks, and the number of azoospermic men increased rapidly over the first 20 weeks of testosterone treatment. At that time, 18 men (55%) were azoospermic, a similar proportion to that previously reported (World Health Organisation, 1990). No further men became azoospermic up to 40 weeks of treatment, although there was then a gradual secondary increase. The shape of the curve thus obtained suggests that the two groups form distinct populations, and illustrates the validity of the definition of the oligozoospermic group used in subsequent studies in this Thesis.

Testosterone administration therefore causes profound inhibition of spermatogenesis in all men investigated, but there appear to be two clearly distinct
patterns of response. This cannot be simply explained on the basis of the age, height or weight of the subjects, which did not differ between the two groups. The object of the further studies in this Thesis was to investigate the biochemical basis for this difference, and in particular to investigate the involvement of the enzyme 5αR in the maintenance of spermatogenesis under these conditions.
3.1 Introduction.

The evidence that testosterone is required for spermatogenesis was discussed in Chapter 1. The concentration of testosterone in the testis is estimated to be approximately 100-fold higher than that in peripheral plasma, but is the subject of continuing debate (Sharpe, 1987; Rommerts, 1988). By administering testosterone to hypophysectomised rats or stalk-sectioned monkeys to give conditions in which the intratesticular concentration of testosterone is estimated to be 10%-20% of normal, spermatogenesis is maintained qualitatively but not quantitatively (Bocabella, 1963; Buhl, Cornette, Kirkton and Yuan, 1982; Marshall, Wickings and Nieschlag, 1984), and replacement of physiological intratesticular concentrations of testosterone following the administration of EDS results in normal spermatogenesis (Sharpe et al., 1988).

The administration of supraphysiological doses of testosterone to normal men reduces intratesticular testosterone concentrations to approximately 5% of normal, at which time the intratesticular testosterone concentrations were only 2 fold higher than in plasma (Morse, Horike, Rowley and Heller, 1973): whether this alone (ie in the absence of gonadotrophins) is sufficient to maintain a degree of spermatogenesis is unclear. Indirect evidence as to the intratesticular concentration of testosterone in the current study is provided by studies involving the administration of GnRH antagonist with replacement testosterone to non-human primates. This caused a reduction in intratesticular testosterone to 38% of pretreatment concentrations (Weinbauer et al., 1988), under which conditions there was some maintenance of spermatogenesis. However, the administration of GnRH antagonist with replacement testosterone to normal men results in a high rate of azoospermia (vide supra, Chapter 1.7), which suggests that there may be a significant species difference.

Thus the simplest explanation for some men remaining oligozoospermic on supraphysiological doses of testosterone is that the intratesticular concentrations of testosterone remain sufficient to support a very low rate of spermatogenesis, whereas in the men becoming azoospermic, that concentration is below a threshold for spermatogenesis. Previous trials using this experimental design have reported that the concentration of testosterone in plasma does not differ between the two groups of men, although the data were not presented (World Health Organisation, 1990).
Following administration of testosterone oenanthate, plasma concentrations of testosterone increase rapidly (Schulte-Beerbühl and Nieschlag, 1980), with peak concentrations approximately 3 times baseline within 24 hours, followed by a decline over 10 days. With repeated administration, trough concentrations will increase to a steady state, when an equilibrium is established between absorption and metabolism. Similarly, peak concentrations after the first injection are likely to be lower than peak concentrations achieved after the new equilibrium is established, but the precise pharmacokinetics and pharmacodynamics with repeated testosterone injections in normal men are unknown. It is thus possible that there are differences between men becoming azoospermic and those remaining oligozoospermic that are apparent only on close examination of the profile of testosterone absorption which are not seen when trough concentrations alone are measured. With the suppression of endogenous secretion of testosterone by the testes, the germinal epithelium may be exposed to concentrations of testosterone similar to that those present in the peripheral circulation. Thus the measurement of that circulating testosterone profile may provide information relevant to the maintenance of spermatogenesis in some men under these conditions.

Testosterone is converted to oestradiol by the action of the enzyme aromatase both within the testis and in the periphery (Longcope et al., 1969; Baird, Horton, Longcope and Tait, 1969; Dorrington and Armstrong, 1975), and the administration of the dose of testosterone oenanthate used in these studies causes an elevation of plasma oestradiol concentration of approximately 90% (Swerdloff et al., 1979; Nieschlag, Waites and Farley, 1991). A difference in plasma oestradiol concentrations between men becoming azoospermic and those remaining oligozoospermic has been reported (Wallace, Seth and Wu, 1993), but this difference was only detected at one time point during the 12 month course of testosterone treatment. Changes in plasma concentration of oestradiol have therefore been investigated in greater detail here, as such a difference between azoospermic and oligospermic responders may reflect differences in androgen metabolism both within the testis and in the periphery.

The concurrent presence of FSH appears to be of great importance in determining the spermatogenic response to the presence of subphysiological intratesticular concentrations of testosterone. In the experiments quoted above in which hypophysectomised rats were used and there was therefore no FSH secretion, spermatogenesis was greatly reduced. In an experimental protocol in which a GnRH antagonist was used to inhibit gonadotrophin secretion in rats with a subcutaneous testosterone implant, resulting in intratesticular testosterone concentrations of 15% of normal, spermatogenesis was maintained at a normal rate (Rea et al., 1986). In that model there was detectable FSH secretion. Similar results have been obtained in
humans: following the suppression of gonadotrophin secretion by testosterone, the subsequent administration of FSH partially restored spermatogenesis (Matsumoto et al., 1983).

The administration of a GnRH antagonist to men resulted in azoospermia in 7 out of 8 men (Pavlou et al., 1991). This treatment, in which androgen replacement was delayed, resulted in a very rapid decline in gonadotrophin secretion which may be related to the high rate of azoospermia. Furthermore, men who became azoospermic during testosterone treatment show a rebound enhanced secretion of gonadotrophins at the beginning of the recovery period compared to those who became oligozoospermic, despite levels being undetectable in both groups during treatment (Wallace et al., 1993).

In this Chapter, the possible importance of the rate of suppression of gonadotrophin secretion in determining whether spermatogenesis is maintained has been investigated, and whether the apparent difference in responsiveness of the hypothalamo-pituitary axis is detectable at the beginning of testosterone treatment by closely monitoring the rate of decline of LH and FSH in plasma. Previous studies of the importance of FSH in determining the rate of spermatogenesis have been hampered by the relative insensitivity of RIAs for FSH, with levels of sensitivity at approximately 20% of normal male concentrations. Thus FSH secretion at a rate of 10% of normal would be undetectable, making interpretation of experiments relying on complete suppression of FSH (eg by supraphysiological doses of testosterone) difficult. A novel method for the measurement of hormones (including the gonadotrophins) has recently been developed (DELFIA, Pharmacia Ltd), using antibodies labelled with a fluorescent marker, europium. This method has greatly enhanced sensitivity, and has been used to detect the nocturnal secretion of gonadotrophins in early puberty (Wu et al., 1991).

Testosterone binds to proteins in plasma, in particular SHBG and albumin, such that only approximately 2% is free in solution, with approximately 60% being bound to SHBG. The SHBG molecule has a single steroid binding site (Rosner and Smith, 1975). The function of this high-affinity binding to SHBG is generally believed to be to reduce the hepatic clearance of the hormone, so acting as a reservoir of testosterone (Mendel, 1989; Rosner, 1990), and it has been shown that infusion of SHBG in monkeys reduces the metabolic clearance rate of testosterone (MCRT) (Pétrá, Stanczyk, Namkung, Fritz and Nový, 1985). A better correlation between MCRT and free + non-SHBG bound testosterone than with the free fraction alone has been found (Siiteri, Murai, Hammond, Nisker, Raymoure and Kuhn, 1982). The free fraction of plasma testosterone is regarded as being bio-available while that bound to SHBG is not ("the free hormone hypothesis"): whether that fraction bound to albumin is bio-
available or not is the subject of active debate. Pardridge has proposed that the dissociation of testosterone from albumin is sufficiently rapid that an equilibrium will develop within the timespan of capillary blood flow, i.e., approximately 1 second (Pardridge, 1981). This has been supported by direct measurement of the rate of dissociation of testosterone from SHBG and albumin (Mendel, 1990; Mendel, Miller, Siiteri and Murai, 1990). These experiments showed that testosterone dissociated from SHBG with a half-life of 12 sec, compared to 0.5 sec from albumin. Further evidence is provided by experiments in which $[^3]H$testosterone in albumin-containing buffer or in plasma is injected into the rat carotid artery, followed by measurement $[^3]H$ in brain tissue 15 seconds later (the Oldendorf technique). Uptake of $[^3]H$testosterone is presumed to reflect the bioavailability of testosterone, and these experiments showed a good correlation between "bioactive" and albumin-bound testosterone (Manni, Pardridge, Cefalu, Nisula, Bardin, Santner and Santen, 1985).

The presence of high affinity binding of SHBG to prostate membrane preparations has been reported (Hryb, Khan and Rosner, 1985), with a $K_D=0.15$ nM. Binding of $[^3]H$SHBG is reduced in the presence of steroids in proportion to their affinity for SHBG (Hryb, Khan, Romas and Rosner, 1990). It therefore appears that SHBG is an allosteric protein, with binding sites for both steroids and membranes arranged in such a way that steroid binding masks the membrane binding site. Membrane bound SHBG, however, retained the ability to bind steroids (Hryb et al., 1990), and the membrane-SHBG complex was found to enhance the stimulation of cAMP accumulation caused by steroids (Rosner, 1990). The function of SHBG may therefore be more complex than the regulation of steady state circulating concentrations of steroids, and may be involved in the transfer of steroids across cell membranes. Such a function may underlie the finding that the presence of SHBG appears to affect the uptake of testosterone by the prostate, as testosterone was taken up equally by epithelium and stroma when perfused in buffer, but was preferentially sequestered by the stroma in the presence of SHBG (Ellison and Pardridge, 1990). Indeed, SHBG-like immunoreactivity has been demonstrated in a variety of tissues including the testis and prostate (Bordin and Pétra, 1980). These results suggest parallels with the receptor-mediated uptake of cholesterol bound to low density lipoprotein (Goldstein and Brown, 1977).

The concentration of SHBG is altered in a variety of hormonal states. The plasma concentration of SHBG falls at puberty in boys (Blank, Attanasio, Rager and Gupta, 1978) and is reduced in hyperandrogenic states (Plymate, Leonard, Paulson, Fariss and Karpas, 1983). However plasma SHBG concentrations also fell in two boys with complete androgen insensitivity at the time of puberty (Cunningham, Loughlin,
Culliton and McKenna, 1985). Conversely, oestrogen administration to men causes a increase in SHBG concentrations (Anderson, 1974), and changes in plasma SHBG concentration have been detected during the menstrual cycle, with a 20% rise during the luteal phase (Plymate, Moore, Cheng, Bardin, Southworth and Levinsky, 1985). SHBG concentrations are also elevated in thyrotoxicosis, with an associated increase in total testosterone concentration and fall in MCR (Gordon, Southren, Tochimoto, Rand and Olivo, 1969). In that condition however, evidence of hyperandrogenicity is not usually found although there may be abnormalities of the hypothalamo-pituitary-testicular axis (Kidd, Glass and Vigersky, 1979; Hudson and Edwards, 1992). This may be explained by a small fall in non-SHBG bound testosterone, and concentrations of free testosterone may be normal or reduced (Chopra and Tulchinsky, 1974; Loric, Duron, Guechot, Aubert and Giboudeau, 1989; Ford, Cook, Keightley and Feek, 1992; Hudson and Edwards, 1992). SHBG is reduced in obese men in proportion to the degree of obesity, and there is also a reduced total testosterone concentration: from this some have predicted that the two changes compensate for each other resulting in a eugonadal state (Schneider, Kirschner, Berkowitz and Ertel, 1989), but others have found that free and non-SHBG bound testosterone are also reduced, indicating a hypogonadal state (Zumoff, Strain, Miller, Rosner, Senie, Seres and Rosenfield, 1990).

Determination of total testosterone alone therefore provides a very incomplete analysis of the biologically relevant concentration of testosterone in plasma, and changes in SHBG and the free and non-SHBG bound components should also be considered. The examples of thyrotoxicosis and obesity demonstrate that measurement of SHBG and total testosterone do not always allow accurate prediction of “bioavailable” testosterone, and the free and non-SHBG bound components require to be measured directly. In the experiments described in this Chapter, free and non-SHBG bound testosterone were measured in addition to total testosterone and SHBG itself, to further characterise the pharmacokinetics of testosterone administered using this regime, and to investigate whether there might be differences between men becoming azoospermic and those remaining oligozoospermic.

3.2 Materials and Methods.

The methods of radioimmunoassay, immunoradiometric assay and fluoroimmunoassay were used here, and the major differences between these techniques are illustrated in Figure 3.1.
Figure 3.1
Schematic drawing illustrating the differences between the three immunoassay methods used in this Chapter.

(a) Radioimmunoassay (testosterone, oestradiol)
(b) Immunoradiometric assay (SHBG)
(c) Fluoroimmunoassay (LH and FSH).
To investigate the pharmacokinetics of absorption of testosterone, total, free, and non-SHBG bound testosterone were measured in samples taken before the first injection, and 1, 2, 4, and 7 days later, the last being immediately before the next injection was given. This schedule was repeated after 16 weeks. Additional samples were assayed for total testosterone at 2-4 week intervals between these two injections to demonstrate that the steady state had been reached with weekly administration.

3.2.1 Testosterone RIA

Testosterone in plasma was measured by RIA using the method of Corker and Davidson, (1978).

Plasma aliquots (50µL in duplicate) were extracted with 3ml hexane:ether (4:1) by vortexing for 7 min. The organic phase was separated after freezing the plasma in methylated spirit/solid CO₂, dried down under nitrogen and reconstituted in 200µl of phosphate buffered gelatine-saline (PBGS) buffer. 100µl of this was taken for assay. Recoveries were determined by adding 50µl of PBGS containing 2500cpm [³H]testosterone to the plasma sample before extraction, and measuring [³H] by liquid scintillation counting (LSC) in 50µl of the reconstituted sample.

Standards over the range 5 to 640 pg/tube were used. 100µl of buffer containing 12000 cpm ¹²⁵I-Testosterone and 100µl of anti-testosterone antibody at a dilution of 1:35000 were added to each tube. The assay was incubated at room temperature for 3 hrs, then 100µl of normal sheep serum (1:1000 dilution) and 100µl of donkey anti-goat/sheep serum (1:25 dilution) were added. After incubation overnight at 4°C, 1ml of 0.9% NaCl containing 0.2% Triton X-100 was added, and the tubes centrifuged at 2500 rpm for 30 min. The supernatant was decanted, and ¹²⁵I in the pellet was counted using a Wallac 1261 “Multigamma” counter. Results were calculated for this and other immunoassays (DELFIA assays for LH and FSH, oestradiol, DHT and AdiolG RIAs and SHBG IRMA) using the “AssayZap” computer program (Biosoft Ltd, Cambridge). Quality control samples were included in all assays, allowing calculation of an intraassay coefficient of variation (CV) of 6.4%, and an interassay CV of 9.4%. Recovery values were 70-82%.

3.2.2 Free testosterone assay.

Testosterone is bound to SHBG and albumin in plasma, with only about 2% free in men, and 1% in women. The method used in this Thesis involves the incubation of plasma with a tracer concentration of [³H]testosterone, which equilibrates with the various fractions of endogenous hormone. This is followed by ultrafiltration, which is
carried out by centrifugation across a protein-retaining membrane. The concentration of
free hormone is unaffected by this and is therefore the same in the ultrafiltrate as in
plasma (Ekins, 1990). The membranes used here have a molecular weight cut-off of
10,000 (molecular weight of albumin is 67,000).

A 12.5 cm column of hydroxyalkoxypropyl-dextran (H83586, Sigma) was prepared
in a glass pipette, and equilibrated with hexane:chloroform (95:5). 20 µl
$[^3]H$-testosterone ([1,2,6,7-$^3$H]-testosterone, 70 µCi/mmol, Amersham) was dried
down under nitrogen, and redissolved in 1 ml hexane:chloroform. This was added to
the column, which was eluted with the same solvent. The first 15 ml was discarded,
and the next 6 aliquots of 5 ml collected. The purified tracer eluted in fractions 2 and 3,
which were then combined and used in the assay.

100 000 cpm of purified $[^3]H$-testosterone was dried down in each glass assay tube.
500 µl of plasma was added, the sample vortexed, and incubated at 37°C for 30 min.
200 µl aliquots in duplicate were then added to prewarmed Centrifree ultrafiltration
devices (Amicon Ltd), with YMT membranes, and incubated for a further 10 min. The
devices were then centrifuged at 1000 g/4000 rpm at 37°C for 5 min in a fixed angle head
in a 50 µl aliquot of the incubate was also counted. Results were calculated according to
the formula:

$$\text{% Free} = \frac{\text{cpm in ultrafiltrate - background}}{\text{cpm in incubate - background}} \times 100$$

This gives a result as percentage free testosterone, which can be converted to
concentration of free testosterone by multiplying by the total testosterone concentration
as determined by RIA (vide supra, Section 3.2.1). Quality control plasma samples
were routinely included in all assays; intraassay CV was 7.1% and interassay CV was
11.0% at 2.1% free testosterone.

3.2.3 Non-SHBG bound testosterone assay.

The method used in this Thesis is derived from that of O'Connor, Baker, Dulmanis and
Hudson (1973), as modified by Tremblay and Dube (1974). The principle is similar to
that of the free testosterone assay described above, but instead of ultrafiltration to
separate free from protein-bound tracer $[^3]H$-testosterone, ammonium sulphate is used
to precipitate the SHBG. At the concentration of ammonium sulphate used, albumin is
not affected and the concentration of $[^3]H$-testosterone remaining in the supernatant
represents that bound to albumin (as well as a small fraction bound to other globulins) with that free in solution.

$[^3\text{H}]$testosterone was purified as described above. 15000 cpm of $[^3\text{H}]$testosterone was added to each assay tube, and evaporated to dryness. 200μl of 0.9% NaCl was added to each tube, followed by 200μl of plasma in duplicate, or 200μl of 1% BSA to total counts tubes. The tubes were vortexed, and incubated at 37°C for 60 min. The tubes were then cooled in ice water for 10 min, and 400μl of saturated ammonium sulphate added, giving a final concentration of 50%. Tubes were immediately vortexed, and after a further 10 min in the ice water, were centrifuged at 2000g for 20 min at 4°C. 200μl aliquots of the supernatant in duplicate were then taken for determination of $[^3\text{H}]$ by LSC. Results were calculated using the formula

$$\text{% non-SHBG bound} = \frac{\text{cpm in sample} - \text{background}}{\text{cpm in total counts} - \text{background}} \times 100$$

Multiplication of this value by total testosterone determined by RIA gives non-SHBG bound testosterone, in nmol/l. Quality control plasma samples were routinely included in all assays; intraassay CV was 7.7% and interassay CV was 9.2% at 21% non-SHBG bound testosterone.

3.2.4 Oestradiol RIA.

Oestradiol concentrations in plasma was measured by RIA (Glasier, Irvine, Wickins, Hillier and Baird, 1989). 200μl aliquots in duplicate were extracted into 1ml of ethyl ether by vortexing for 1 min. After freezing the aqueous layer, the organic layer was decanted into glass tubes and dried down under nitrogen. The residue was reconstituted in 100μl PBS buffer, and used directly for assay. Extraction efficiency was determined in representative tubes using $[^3\text{H}]$oestradiol, and was found to be greater than 92%. Results were therefore not corrected for extraction.

Standards were used over the range 30-10000 pmol/ml. 200μl $^{125}\text{I}$-oestradiol (approximately 10000cpm) and 100μl of primary antiserum at a dilution of 1:1500000 were added to each tube, mixed, and incubated at room temperature for 3 hrs. Non-specific binding was determined by omitting the primary antiserum, and $B_0$ by using buffer in place of standard.

Donkey anti-sheep/goat antiserum (1:8 dilution) and 100μl of non-immune sheep serum (1:400 dilution) were then added to all tubes. After incubation overnight at 4°C, 1ml of 0.9% NaCl containing 0.4% Triton X-100 and 4% polyethylene glycol was added, tubes were centrifuged at 3000g for 30 min, the supernatant decanted and $^{125}\text{I}$
in the pellet counted. Results were calculated using “AssayZap”. Quality control samples were included in all assays, the interassay CV was 14.3%, and interassay CV was 7.1% at 120 pmol/l. The limit of detection of the assay was 50 pmol/l. Samples giving values below the limit of detection were assigned this value. This occurred in 30% of baseline samples, but no others.

3.2.5 SHBG assay.

SHBG concentration in serum was measured using an immunoradiometric assay (IRMA) system supplied by Farmos Diagnostica Ltd, Finland. This circumvents the instability of pure SHBG which reduces the accuracy of $^{125}\text{I}-\text{SHBG}$ radioimmunoassays by the use of a monoclonal anti-SHBG antibody which recognises a specific determinant on the SHBG molecule. $^{125}\text{I}$-labelling of this antibody with subsequent use in a “sandwich”-type IRMA allows very high specificity and reduces variation in tracer quality.

Reagents were reconstituted as instructed. Standards, serum samples and quality control samples were diluted 1 in 100 in assay buffer. 100µl of diluted standard or sample was then assayed. Equal volumes of anti-SHBG antiserum and $^{125}\text{I}$-anti-SHBG antibody were mixed, and 200µl of this was then added to all tubes. Total counts tubes contained only label, non-specific binding tubes contained the label and 100µl of buffer. Tubes were vortexed and incubated at room temperature for 60 min. 500µl of solid-phase separation agent was then added, and after a further 15 min incubation, tubes were centrifuged at 2000g for 15 min. The supernatant was discarded, and radioactivity in the pellet was counted. Results were calculated using “Assayzap”, and expressed as nmol/l. Quality control sera were provided in the kit by the manufacturer, and gave values within the expected range.

3.2.6 LH and FSH assays.

The gonadotrophins were measured using a time-resolved fluoroimmunoassay (DELFIA, Pharmacia, Finland). This is a solid-phase, two site assay based on the sandwich principle, in which two monoclonal antibodies are directed against antigenic determinants on the α and gonadotrophin-specific β subunits. Standards and samples bind to the first antibody, immobilised on the microtitre plate well, and a second antibody labelled with europium is added. After washing off unbound second antibody, the europium is dissociated by an enhancement solution. This forms highly fluorescent chelates, and the level of fluorescence is proportional to the concentration of hormone.
Both hormones were measured using an identical technique, the only difference being in the specificity of the antibodies to the β-subunit of LH or FSH. The plates were rinsed, then 25μl of standard (0 - 256 IU/l) or plasma sample was added in duplicate followed by 200μl of buffer. Plates were mixed by rotatory shaking, then incubated overnight at 4°C. Plates were then washed, 200μl of tracer-antibody added, and incubated at room temperature for 30 min. Plates were again washed, and 200μl of enhancement solution added. Time-resolved fluorescence was then measured (Wallac model 1234 fluorimeter), and results calculated using Assayzap. A time lapse between excitation and counting allows nonspecific fluorescence to decay, giving increased signal to noise ratio and thus low background values, and the excitation pulse length allows 1000 cycles to be counted per sample well in 1 second. All samples were assayed in one assay, and quality control samples were consistent with results previously obtained in the laboratory. Assay sensitivity was 0.05 IU/l for LH, and 0.06 IU/l for FSH. Samples which gave results below the limit of sensitivity were assigned that value.

3.2.7 Statistical analysis.

For repeated measures, Analysis of Variance with Neuman-Keuls test for post-hoc analysis was used to determine levels of significance. For single results (eg SHBG results), Students t test was used, on paired data where appropriate.

Pharmacokinetic modelling of plasma testosterone results was carried out using the "RSTRIP" computer program (MicroMath Scientific Software, Salt Lake City, Utah, USA), which uses least squares analysis of polyexponential functions (Behre, Oberpenning and Nieschlag, 1990). A biexponential model was used in this analysis.

3.3 Results.

3.3.1 Testosterone results.

Following the first injection, plasma concentrations of total testosterone rose from baseline values of 15.6 ± 1.2 nmol/l to 45.0 ± 4.1 nmol/l on day 1 in the azoospermic group, and from 15.8 ± 2.0 nmol/l to 56.0 ± 2.0 nmol/l in the oligozoospermic group. These concentrations then declined to 21.6 ±2.2 nmol/l in the azoospermic group on day 7, and to 26.7 ± 2.4 nmol/l in the oligozoospermic group. These results, with those of the intervening time points, are shown in Figure 3.2a. The concentrations on days 1, 2, and 4 were significantly different from baseline values in both groups.
Figure 3.2  Plasma concentrations of testosterone in those men becoming azoospermic and those remaining oligozoospermic following injection of 200 mg testosterone enanthate.

(a) Following 1st injection.  
(b) Following 16th injection.

* p<0.01 vs day 0.

Open symbols, Azoospermic group, n=18
Filled symbols, Oligozoospermic group, n=15

Mean ± sem.
(p<0.01), but on day 7 the concentrations were not significantly different from baseline. At no time point were the values for the two groups significantly different.

The profiles of concentrations of plasma testosterone after 16 weeks of testosterone treatment are shown in Figure 3.2b. At this time, day zero concentrations are trough testosterone concentrations, taken immediately before the next injection is given. In the azoospermic group, the trough testosterone concentration was 35.2 ± 4.2 nmol/l, and was 36.8 ± 3.4 nmol/l in the oligozoospermic group. These concentrations rose to 67.0 ± 5.5 nmol/l and 65.6 ± 8.0 nmol/l respectively on day 1 after that injection. As after the first injection, the concentrations on days 1, 2, and 4 were significantly different from baseline values in both groups (p<0.01), but on day 7 the concentrations were not significantly different from trough testosterone concentrations. At no time point were the values for the two groups significantly different.

There therefore appeared to have been an accumulation of testosterone in the plasma of the subjects, as concentrations 7 days after the first injection were significantly lower than trough concentrations taken after 16 weeks of testosterone treatment (p<0.01 in both groups). Determinations of plasma concentrations of testosterone at intermediate time points are shown in Table 3.1, illustrating this accumulation. All samples were taken immediately before the next injection was given, 7 days after the previous one. Trough testosterone concentrations were significantly greater than pretreatment baseline concentrations after 2 weeks of treatment (p<0.05) and at the time of subsequent injections (p<0.01) in both groups, but were not significantly elevated after only one week. Samples taken after 8, 12 and 16 weeks of testosterone treatment also had significantly greater testosterone concentrations than those taken after 2 weeks of treatment. There was therefore a significant rise in trough concentrations of testosterone after 2 weeks of testosterone treatment, with further significant increases following repeated administration but appearing to plateau after 8-12 weeks suggesting that steady state had been reached by 8 weeks of treatment.

Computer pharmacokinetic modelling allowed calculation of area under the curve (AUC), terminal half-life (t1/2), maximum concentration of testosterone (C_max), and time after injection of maximum concentration (t_max), for the two weeks analysed in detail. Results are shown in Table 3.2. There were no differences between the azoospermic and oligozoospermic groups in any of these parameters, but in both groups there were significant increases in AUC and C_max after 16 weeks of treatment as expected, as the baseline after 16 weeks is a trough concentration and as shown in Table 3.1, is considerably higher than the pre-treatment baseline concentration. There were no changes in the apparent half life of the administered testosterone (C_max) or the
time interval between injection and time at which the concentration of testosterone was maximal ($t_{\text{max}}$).

**Table 3.1**

<table>
<thead>
<tr>
<th>Weeks of treatment</th>
<th>Baseline</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoospermic</td>
<td>15.6</td>
<td>21.6</td>
<td>27.7*</td>
<td>31.3**</td>
<td>34.4**</td>
<td>34.8**</td>
<td>35.2**</td>
</tr>
<tr>
<td></td>
<td>(1.2)</td>
<td>(2.2)</td>
<td>(3.2)</td>
<td>(3.2)</td>
<td>(3.0)</td>
<td>(3.6)</td>
<td>(4.2)</td>
</tr>
<tr>
<td>Oligozoospermic</td>
<td>15.8</td>
<td>26.7</td>
<td>29.9*</td>
<td>30.8**</td>
<td>34.0**</td>
<td>40.2**</td>
<td>36.8**</td>
</tr>
<tr>
<td></td>
<td>(2.0)</td>
<td>(2.4)</td>
<td>(2.9)</td>
<td>(3.0)</td>
<td>(3.0)</td>
<td>(3.1)</td>
<td>(3.4)</td>
</tr>
</tbody>
</table>

Plasma concentrations of total testosterone at baseline and following repeated administration of 200 mg testosterone enanthate in men becoming azoospermic and those remaining oligozoospermic. Values are mean (sem)

* $p<0.05$ vs baseline, ** $p<0.01$ vs baseline.

**Table 3.2** Computer-modelled analysis of testosterone concentrations

<table>
<thead>
<tr>
<th>First injection</th>
<th>AUC (nmol.hrs/l)</th>
<th>$t_{1/2}$ (hrs)</th>
<th>$C_{\text{max}}$ (nmol/l)</th>
<th>$t_{\text{max}}$ (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoospermic</td>
<td>6160 ± 440</td>
<td>145 ± 24</td>
<td>55.2 ± 4.9</td>
<td>15.2 ± 2.0</td>
</tr>
<tr>
<td>Oligozoospermic</td>
<td>8720 ± 590</td>
<td>177 ± 27</td>
<td>64.2 ± 6.9</td>
<td>11.7 ± 4.6</td>
</tr>
</tbody>
</table>

After 16 weeks

<table>
<thead>
<tr>
<th></th>
<th>AUC (nmol.hrs/l)</th>
<th>$t_{1/2}$ (hrs)</th>
<th>$C_{\text{max}}$ (nmol/l)</th>
<th>$t_{\text{max}}$ (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoospermic</td>
<td>8720 ± 590(a)</td>
<td>163 ± 18</td>
<td>74.1 ± 5.5(c)</td>
<td>12.8 ± 3.7</td>
</tr>
<tr>
<td>Oligozoospermic</td>
<td>9580 ± 670(b)</td>
<td>155 ± 60</td>
<td>77.5 ± 6.5(d)</td>
<td>8.4 ± 2.2</td>
</tr>
</tbody>
</table>

(a) $p<0.002$, (b) $p<0.005$, (c) $p<0.02$, (d) $p<0.05$, all vs first injection.

3.3.2 Free and non-SHBG bound testosterone.

The results obtained for these two parameters were similar in many respects, and are described here together. The first injection of testosterone caused an increase in free testosterone concentrations from 270 ± 20 pmol/l to 1120 ± 150 pmol/l in the azoospermic group on day 1, and from 260 ± 30 pmol/l to 1370 ± 180 pmol/l in the oligozoospermic group ($p<0.01$ in both groups, Figure 3.3a). This then declined to 470 ± 50 pmol/l in the azoospermic group on day 7, and to 560 ± 50 pmol/l in the oligozoospermic group. In the oligozoospermic group, the concentration on day 7 was significantly greater than the baseline concentration ($p<0.05$), but the concentrations
**Figure 3.3**
Plasma concentrations of free testosterone in those men becoming azoospermic and those remaining oligozoospermic following injection of 200 mg testosterone enanthate.

(a) Following 1st injection.
(b) Following 16th injection.

* p<0.05, ** p<0.01 vs day 0.

Open symbols, Azoospermic group, n=18.
Filled symbols, Oligozoospermic group, n=15
Mean ± sem.
Figure 3.4
Plasma concentrations of non-SHBG bound testosterone in those men becoming azoospermic and those remaining oligozoospermic following injection of 200 mg testosterone oenanthate.

(a) Following 1st injection
(b) Following 16th injection

* p<0.01 vs day 0

Open symbols, Azoospermic group, n=18
Filled symbols, Oligozoospermic group, n=15
Mean ± sem.
were not significantly different in the azoospermic group. There were, however, no significant differences between the two groups.

Plasma concentrations of non-SHBG bound testosterone showed a similar profile after the first injection, rising from 5.5 ± 0.3 nmol/l to 24.6 ± 3.5 nmol/l in the azoospermic group on day 1, and from 4.8 ± 0.5 nmol/l to 30.0 ± 4.4 nmol/l in the oligozoospermic group (p<0.01 in both groups, Figure 3.4a). The concentrations then declined to 9.8 ± 1.1 nmol/l and 11.3 ± 1.1 nmol/l in the two groups respectively. These concentrations were not significantly different from baseline concentrations in either group, and there were no significant differences between the two groups.

Profiles of free and non-SHBG bound testosterone after 16 weeks treatment are shown in Figure 3.3b and 3.4b. At this time, the distribution of testosterone between the bound and free fractions had altered considerably. Thus trough concentrations of free testosterone were 1080 ± 130 pmol/l in the azoospermic group and 1150 ± 160 pmol/l in the oligozoospermic group, similar to the peak concentrations after the first injection and nearly 5 fold greater than baseline concentrations. Trough concentrations of non-SHBG bound testosterone were increased to 17.5 ± 1.7 nmol/l in the azoospermic group, and to 19.3 ± 2.6 nmol/l in the oligozoospermic group (p<0.001 vs day 7 concentration after first injection in both groups). There was still a marked increase in concentrations of both free and non-SHBG bound testosterone after 16 weeks of testosterone treatment, and although the magnitude of the increase was not as great as after the first injection, concentrations of both parameters were more than doubled on day one after that injection. Again, there were no differences between the two groups.

The changes in plasma concentrations of free and non-SHBG bound testosterone expressed as a percentage of total testosterone are shown in Figures 3.5 and 3.6 respectively. Comparison of the values before testosterone treatment started, and over the weeks following the first injection and after 16 weeks of testosterone treatment illustrate the changes in distribution of testosterone between its binding proteins and that free in solution with continuing administration of testosterone at supraphysiological doses. These results differed from those expressed as concentrations, in that both the percentage free and non-SHBG bound testosterone on day 7 following the 1st injection were significantly greater than at baseline in both groups.

3.3.3 Oestradiol results.

The plasma concentration of oestradiol was measured in the same samples as testosterone over the 7 days following the 1st injection and after 16 weeks of
Figure 3.6
Percentage non-SHBG bound testosterone in plasma in those men becoming azoospermic and those remaining oligozoospermic following injection of 200 mg testosterone oenanthate.

(a) Following 1st injection
(b) Following 16th injection

* p<0.01 vs day 0

Open symbols, Azoospermic group, n=18
Filled symbols, Oligozoospermic group, n=15
Mean ± sem.
Figure 3.5
Percentage free testosterone in plasma in those men becoming azoospermic and those remaining oligozoospermic following injection of 200 mg testosterone enanthate.

(a) Following 1st injection
(b) Following 16th injection

* p<0.01 vs day 0

Open symbols, Azoospermic group, n=18
Filled symbols, Oligozoospermic group, n=15
Mean ± sem.
testosterone treatment. In the azoospermic group, oestradiol rose from a baseline of 96.6 ± 9.9 pmol/l to 184 ± 20 pmol/l on day 1 (p<0.01), and then fell to 110.6 ± 10.8 pmol/l on day 7. In the oligozoospermic group, oestradiol rose from 76.9 ± 7.5 pmol/l to 155 ± 20 pmol/l (p<0.01), then fell to 82.4 ± 15.3 pmol/l at the same time points. Values on days 2 and 4 were also significantly greater than baseline in both groups (p<0.01). After 16 weeks of testosterone treatment, oestradiol rose from a trough concentration of 194 ± 27 pmol/l (p<0.001 vs baseline) to 252 ± 20 pmol/l on day 1 (p<0.01 vs trough concentration) in the azoospermic group, and from 173 ± 23 pmol/l (p<0.002 vs baseline) to 220 ± 23 pmol/l (p<0.05 vs trough concentration) in the oligozoospermic group. The concentrations on days 2, 4 and 7 after 16 weeks of testosterone treatment were not significantly different to trough concentrations in either group. These results, and those of the other time points analysed, are shown in Figure 3.7. At no point were there significant differences in plasma concentration of oestradiol between those men who became azoospermic and those remaining oligozoospermic.

3.3.4 SHBG results.

Plasma concentrations of SHBG were determined at baseline before treatment started, and after 16 weeks (Figure 3.8). Baseline concentrations were 31.8 ± 3.6 nmol/l in the azoospermic group, and 34.5 ± 3.6 nmol/l in the oligozoospermic group. After 16 weeks of testosterone treatment, these concentrations had fallen to 19.2 ± 2.3 nmol/l in the azoospermic group (p<0.001), and to 24.0 ± 2.6 in the oligozoospermic group (p<0.01). There were no significant differences between the groups.

3.3.5 Gonadotrophin concentrations.

Plasma concentrations of both LH and FSH showed a rapid decline following the first injection of testosterone (Figures 3.9 and 3.10). Plasma concentrations of LH were significantly inhibited on day 1 following injection compared to baseline concentrations in both groups. In the azoospermic group, LH concentration at the time of first injection was 3.57 ± 0.20 IU/l, and 2.63 ± 0.31 IU/l on day 1; in the oligozoospermic group, LH concentrations were 4.24 ± 0.46 IU/l at baseline and 2.78 ± 0.38 IU/l on day 1 (p<0.01 in both groups). In contrast, the inhibition of FSH secretion was slightly delayed: thus concentrations of FSH on day 1 were not significantly different from those at the time of first injection (3.05 ± 0.39 IU/l, baseline; 2.86 ± 0.35 IU/l, day 1 in the azoospermic group and 3.19 ± 0.24 IU/l, baseline; 2.96 ± 0.28 IU/l, day 1 in the oligozoospermic group). By day 2, concentrations of FSH were significantly
Figure 3.7
Plasma concentration of oestradiol in those men becoming azoospermic and those remaining oligozoospermic following injection of 200 mg testosterone enanthate.

(a) Following 1st injection.
  ** p<0.01 vs day 0

(b) Following 16th injection
  * p<0.05, **p<0.01 vs day 0

Open symbols, Azoospermic group, n=18
Closed symbols, Oligozoospermic group, n=15
Mean ± sem.
Figure 3.8
Effect of testosterone treatment on plasma SHBG concentration in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* \ p<0.01 \ vs \ baseline
** \ p<0.001 \ vs \ baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Days after 1st injection

Figure 3.9
Plasma concentration of LH following injection of 200 mg testosterone enanthate in those men becoming azoospermic and those remaining oligozoospermic.

B1 and B2 are baseline samples, B2 being taken at the time of the first injection of testosterone.

Open symbols, Azoospermic group, n=18
Filled symbols, Oligozoospermic group, n=15

Note non-linear abscissa

Mean ± sem.
Figure 3.10
Plasma concentration of FSH following injection of 200 mg testosterone oenanthate in those men becoming azoospermic and those remaining oligozoospermic.

B1 and B2 are baseline samples, B2 being taken at the time of the first injection of testosterone.

Note non-linear abscissa

Open symbols, Azoospermic group n=18
Filled symbols, Oligozoospermic group n=15

Mean ± sem.
Figure 3.11
Decline in plasma concentration of LH (open symbols) and FSH (filled symbols) following injection of 200 mg testosterone enanthate.

Data are means of results of all subjects, expressed as percentage of baseline concentration.
inhibited in both groups (2.08 ± 0.21 IU/l in the azoospermic group, and 2.09 ± 0.22 IU/l in the oligozoospermic group, p<0.01 in both groups).

Figures 3.9 and 3.10 show that plasma concentrations of both gonadotrophins declined rapidly to the limit of detection in both groups, with no significant differences between groups at any of the time points investigated. None of the azoospermic group and only one of the oligozoospermic group had detectable immunoreactive FSH after 12 weeks of TE treatment (the FSH concentration in that subject was 0.3 IU/l). Immunoreactive LH was not detectable in any subjects after 12 weeks of TE treatment.

To investigate the rate of decline of secretion of the gonadotrophins, results were recalculated as percentages of baseline concentration for each man over the week following the first injection. By 14 days concentrations were less than 10% of baseline. The means of these results, for all subjects, are shown in Figure 3.11, with computer-fitted logarithmic curves. Correlation coefficients were 0.99 for both gonadotrophins. Substitution into the formulae for the curves allows calculation of the apparent half-lives of the gonadotrophins: values of 50.0 hrs for LH and 78.2 hrs for FSH were obtained.

3.4 Discussion.

The results presented here show the changes in plasma concentrations of testosterone following injection of 200mg of testosterone enanthate in normal men, and the accompanying changes in free and non-SHBG bound testosterone. This profile was determined at the time of the first injection, and repeated after 16 weeks of treatment. The main purpose of these experiments was to investigate whether the maintenance of spermatogenesis in some men under these conditions of supraphysiological concentrations of testosterone could be explained by differences in pharmacokinetics or pharmacodynamics of the administered testosterone.

The administration of testosterone caused a rapid increase in the plasma concentration of testosterone, with concentrations after 24 hours which were 3 fold higher than pre-injection levels (Figure 3.2). The plasma concentration then fell over the following 6 days, and had returned to near baseline at the time of the second injection. This trough concentration of testosterone was measured at intervals over the following 16 weeks, demonstrating a rise in trough concentration up to 8 weeks of treatment, with steady state achieved thereafter (Table 3.1). This indicates a degree of accumulation of testosterone over that period.

The profile of testosterone following injection after 16 weeks of treatment showed a similar pattern to that after the first injection (Figure 3.2). At no time point studied
were there differences between the azoospermic group and those men remaining oligozoospermic, either in measured concentration or calculated pharmacokinetic parameters.

The pharmacokinetic analysis shown in Table 3.2 demonstrates that the peak testosterone concentration achieved following the injection of 200 mg testosterone oenanthate was 55-64 nmol/l following the first injection, and 74-78 nmol/l after 16 weeks of testosterone treatment. The terminal half-life was 145-177 hrs. These results are similar to those reported by Behre et al. (1990), who investigated the pharmacokinetics of repeated administration of 250 mg testosterone oenanthate in hypogonadal men.

Plasma free testosterone was measured by ultrafiltration, and pre-treatment concentrations of 200 - 300 nmol/l, i.e. approximately 2% of total testosterone, were obtained. This is in good agreement with previous reports. Other methods for the determination of free testosterone, and indeed of other hormones which are predominantly bound to plasma proteins (for example thyroxine as well as other steroids), have major drawbacks, including the need for a large volume of sample, dilution effects, and prolonged time course for the determination (Hammond, Nisker, Jones and Siiteri, 1980; Ekins, 1990). Centrifugal ultrafiltration was developed by Hammond et al. (1980), using a dialysis membrane across which the free steroid equilibrates, but that method involved the use of a second isotope ([14C]glucose) with subsequent calculation of the [3H]testosterone / [14C]glucose ratio as the glucose will equilibrate across the membrane unimpeded, acting as a marker for the movement of the labelled steroid. This method was refined following the development of protein-retaining membranes such as that used here, which allowed a rapid determination to be carried out at physiological temperatures without dilution of the sample (and thus disturbance of binding equilibriums), although both methods give very similar results (Green and Yucis, 1982; Vlahos, MacMahon, Sgoutas, Bowers, Thompson and Trawick, 1982). This method is currently thought to provide the best available approximation to the physiological concentration of the free steroid (Ekins, 1990). There is general agreement as to the methodology to determine the concentration of non-SHBG bound testosterone, the ammonium sulphate method used here was developed by O'Connor et al. (1973), who found that after precipitation of plasma proteins with 50% ammonium sulphate, albumin was the only protein remaining in solution. The addition of tracer concentrations of [3H]testosterone to plasma in both methods gives a result as a percentage of total testosterone present in the sample: direct assay of total testosterone by RIA in the original plasma sample then allows the calculation of the plasma concentration of free and non-SHBG bound testosterone. It is
also possible to measure testosterone in the ultrafiltrate by RIA, thus avoiding the use of a radiolabelled tracer, but the small volume of ultrafiltrate obtained and the low concentration of testosterone present requires an assay of increased sensitivity to that normally used for the measurement of total testosterone in plasma.

The use of these methods in this study has allowed the description of the pharmacokinetic profile of plasma bioavailable testosterone following the administration of testosterone in normal men. Comparison with the profiles obtained for total plasma testosterone shows that the rises in free and non-SHBG bound testosterone were greater than those of total testosterone, with concentrations approximately 5 fold higher than baseline 24 hours after the first injection (Figures 3.3 and 3.4). This compares to 3 fold rises in total testosterone. This difference is more marked after 16 weeks of treatment: trough concentrations of total testosterone were approximately double baseline values, but concentrations of free and non-SHBG bound testosterone were nearly 5 times those at baseline (and similar to those found 24 hours after the first injection), and there was a further doubling 24 hours after injection. At that time concentrations were therefore approximately 10 times those at baseline.

Therefore measurement of total testosterone alone considerably underestimates changes in plasma concentrations of bioactive testosterone under the conditions of these studies, and thus the increase in androgen load and stimulus to the tissues. This applies to both free and non-SHBG bound testosterone as these two parameters showed very similar changes.

The changes in plasma concentrations of free and non-SHBG bound testosterone expressed as a percentage of total testosterone (Figures 3.5 and 3.6) differ from when expressed as true concentrations, in that both the percentage free and non-SHBG bound testosterone on day 7 following the 1st injection are significantly greater than at baseline in both groups. One explanation for this discrepancy is that the results when expressed as concentrations depend on the values obtained from two different assays, i.e. the total testosterone RIA and the free or non-SHBG bound testosterone assay. Both of the latter two give results as percentages, and thus conversion to concentrations will compound assay variability. Indeed, inspection of the results illustrated in Figures 3.3 and 3.4 show that free and non-SHBG bound testosterone concentrations are higher on day 7, but that this only reached statistical significance in the oligozoospermic group free testosterone results. These results therefore show that there are increases in bioavailable testosterone at a time when total plasma testosterone concentration is similar to baseline, and may be a result of the rapid fall in SHBG concentration following testosterone administration, which is maximal at 7 days (Hampl, Stárka, Lachman, Snajdrová and Kalvachová, 1992).
At no point were differences found in plasma concentration of free or non-SHBG bound testosterone between men becoming azoospermic and those remaining oligozoospermic, in support of the finding that there were no differences in total testosterone between the two groups. Further confirmation of this result was provided by the direct measurement of SHBG in plasma. These results also confirmed the findings of previous studies that androgen administration causes a fall in plasma concentrations of SHBG (Plymate et al., 1983), but the SHBG concentrations of the two groups of men were similar both at baseline and after 16 weeks of testosterone treatment (Figure 3.7). It therefore appears that these two groups of men do not differ in the pharmacokinetics or pharmacodynamics of administered testosterone, and that the maintenance of spermatogenesis in some men cannot be explained by a higher concentration of bioavailable testosterone.

The demonstration that the administration of this dose of testosterone causes a 5 fold elevation in trough concentrations and 10 fold elevation in peak concentrations of bioavailable testosterone is of interest in the light of the finding by Morse et al. (1973) that a similar dose of testosterone propionate caused a fall in intratesticular testosterone concentrations to only double that found in plasma, which was itself approximately double the physiological concentration. If the determination of intratesticular concentration of testosterone in that study is accurate, it suggests that the magnitude of the increase in bioavailable testosterone found here may be sufficient to influence the intratetsticular environment.

Testosterone is converted to oestradiol by the enzyme aromatase (Longcope et al., 1969). The results in Figure 3.7 illustrate the time-course of changes in plasma oestradiol concentration following injection of TE, and show that the changes in plasma oestradiol concentration closely parallel those of testosterone. Thus there was a rapid rise in plasma concentration of oestradiol following TE injection, and an accumulation of oestradiol following repeated administration. There were no differences in plasma oestradiol concentration between men becoming azoospermic and those remaining oligozoospermic at any time-point investigated. This suggests that there is no constitutive difference in aromatase activity between the two groups of men which might underlie the maintenance of spermatogenesis in those remaining oligozoospermic.

The evidence that FSH is involved in the maintenance of spermatogenesis was discussed in Chapter 1: although it appears that FSH is required for initiation and quantitative maintenance of spermatogenesis in primates and man, the absolute requirement of FSH for spermatogenesis to proceed after puberty is uncertain. The
results presented here show a rapid fall in the secretion of both LH and FSH to undetectable concentrations in the first 4 weeks of testosterone treatment, with similar rates of inhibition in men becoming azoospermic and those remaining oligozoospermic (Figures 3.9 and 3.10). This contrasts with the dramatic discrepancy in rate of inhibition of spermatogenesis between the two groups (Figure 2.2). There therefore appears to be no evidence for a difference in the sensitivity of the hypothalamo-pituitary axis to steroid negative feedback between the two groups of men which could underlie the maintenance of spermatogenesis in the oligozoospermic group. Furthermore, these results suggest that the greater incidence of azoospermia reported when a GnRH antagonist was used to inhibit gonadotrophin secretion (Pavlou et al., 1991) is not the result of the more rapid rate of decline of gonadotrophin secretion under those conditions. The sensitivity of the assay method used in the present studies also allows greater confidence that suppression of gonadotrophin secretion was complete.

A bioassay has not been used in these studies to further validate this finding. Although in some situations changes in immunoactive FSH have been found without concomitant changes in bioactive FSH, and vice versa, these changes in ratio have been relatively slight (Urban et al., 1991; Beitins and Padmanabhan, 1991). The extreme sensitivity of the immunoassay used here (0.06 IU/l) would require inordinate dissociation of immuno-active from bioactive FSH, and it is therefore highly unlikely that there is persistent FSH bioactivity during testosterone treatment in this study. This is supported by the finding that 19-nortestosterone administration suppressed the secretion of both immunoactive and bioactive FSH to below the limit of detection (Jockenhovel et al., 1990).

The plasma concentration of LH was significantly reduced 24 hours after the first injection of testosterone, whereas FSH was not significantly reduced until 48 hours post-injection. Several factors will be of importance in determining the plasma concentration of both gonadotrophins after administration of testosterone, in particular the clearance rates of the gonadotrophins and continuing secretion. LH is cleared from the circulation faster than FSH (Yen, Llerena, Little and Pearson, 1968; Yen, Llerena, Pearson and Little, 1970; Santen and Bardin, 1973; Urban et al., 1988; 1991), but the apparent half-lives calculated from the data here (50 hrs for LH and 78 hrs for FSH, Figure 3.11) are much greater than those in the literature derived from the injection of the hormone or following hypophysectomy. It therefore appears that there is continuing secretion of both gonadotrophins from the pituitary after the injection of the first dose of testosterone. The calculated apparent half life of FSH was longer than that of LH, but the shape of the decline in FSH secretion was very similar to that of LH, with the major difference being the delay before a decline in secretion is detected. If
clearance of the gonadotrophins was the major determinant of the decline seen here, the curve for FSH would be much flatter that that for LH: this is not seen, suggesting that suppression of secretion is the major factor. Alternatively, a combination of the two factors, i.e. greater secretion of LH compensating for its more rapid clearance, could result in similarly shaped curves. This interpretation, however, is at variance with observations on the greater sensitivity of LH than FSH to androgen feedback (Urban et al., 1991), thus suggesting that continuing secretion is the more likely explanation. A further factor is the effect of steroids on the rate of clearance of the gonadotrophins. A decrease in FSH half-life during oestradiol infusion has been suggested based on deconvolutional analysis (Urban et al., 1991).

In conclusion, these results demonstrate that there are no differences in total or bioavailable testosterone concentration in plasma between men becoming azoospermic and those remaining oligozoospermic on supraphysiological doses of testosterone, nor is there a difference in hypothalamo-pituitary sensitivity to negative feedback inhibition of gonadotrophin secretion. These results also confirm that androgen administration causes a decrease in plasma SHBG concentration, and support the finding that the clearance of FSH is slower than that of LH. The maintenance of spermatogenesis in the group of men remaining oligozoospermic is therefore not due to a greater plasma testosterone concentration stimulating spermatogenesis directly nor to continuing gonadotrophin secretion, and in the subsequent Chapters of this Thesis the possibility of increased $5\alpha$R activity resulting in an amplification of the androgen stimulus in men remaining oligozoospermic is investigated.
Chapter 4

The Metabolic Clearance Rate of Testosterone, and the Conversion Rate of Testosterone to Dihydrotestosterone

4.1 Introduction.

The steady state plasma concentration of steroids, as with all other biological molecules, is determined by the balance between the rate of synthesis and secretion and that of metabolism. Thus changes in the rate of metabolism are as important as changes in synthesis in determining the plasma concentration of the steroid. A difference in rate of metabolism might account for the difference between men becoming azoospermic and those remaining oligozoospermic, and this hypothesis has been investigated here. There are several possibilities for the metabolism of testosterone: as described in Chapter 1, it can be reduced to DHT, or aromatised to oestradiol. These reactions however constitute only a very small fraction of the metabolism of testosterone, and the great majority is rapidly cleared from the blood by the liver. As a result, it has a half life of only 12 minutes.

The metabolic clearance rate (MCR) of a substance is that volume of blood from which the substance is completely and irreversibly removed per unit of time (Tait, 1963), and is expressed in units of litres per day. This can be determined by two methods: in one, a bolus of radiolabelled hormone in tracer quantities is injected, and its elimination is followed by multiple blood sampling thereafter. This allows the calculation of the various exponentials representing the compartments in which the hormone is distributed. The second method involves the constant infusion of the tracer until a steady state is reached, at which time comparison of the rate of infusion and the steady state concentration of the tracer allows calculation of the MCR (Baird et al., 1969; Horton 1978). These techniques were of particular importance before the development of assay methods able to quantify the nanogram quantities of steroids in biological fluids, but still have not been superceded as methods to determine MCR values.

In the previous Chapter it was demonstrated that the plasma concentrations of total and bioavailable testosterone (as both free and non-SHBG bound fractions) do not differ between the groups of men becoming azoospermic and those remaining oligozoospermic as a result of supraphysiological concentrations of testosterone. Nor was there a difference between the two groups of men in the plasma concentration of SHBG either before or after 16 weeks of treatment, although a fall in SHBG
concentration was seen with testosterone treatment in both groups. In the present experimental protocol, the exogenous administration of testosterone replaces endogenous synthesis, but the effect of this on the MCR of testosterone (MCRT) in normal men is unknown. MCRT was therefore determined before and after 16 weeks of testosterone treatment, and both the effect of prolonged supraphysiological concentrations of testosterone and differences between the two groups of men were investigated.

The conversion rate of testosterone to DHT (CRT-DHT) is the most direct measure of in vivo 5αR activity, and can be determined using the same methodology as that used for the measurement of MCRT. It has thus been demonstrated that conversion of testosterone accounts for at least 70% of plasma DHT in the male (Ito and Horton, 1971; Mahoudeau, Bardin and Lipsett, 1971). The conversion of testosterone to DHT is reduced in men with 5αR deficiency (Peterson et al., 1977), and occurs exclusively in the extrasplanchnic circulation (Ishimaru et al., 1978). In the experiments described in this Chapter, CRT-DHT was determined and compared between men becoming azoospermic and those remaining oligozoospermic on supraphysiological doses of testosterone, both before treatment was started and after 16 weeks of treatment.

4.2 Materials and methods.

4.2.1 [3H]Testosterone infusion.

[3H]Testosterone ([1,2,6,7-3H]testosterone, 70 μCi/mmol, Amersham), was purified as described in Chapter 3. 90μCi was dried down, redissolved in 4ml ethanol, and made up to 50 ml with sterile normal saline in a 50ml silanised glass syringe. A 20 gauge iv cannula (Venlon, Viggio, Sweden) was inserted into a forearm vein, and a 10 ml blood sample taken. The cannula was then connected to the syringe using polyolefin-coated tubing (106 cm length, 270μl volume; MiniMed Technologies, Sylmar, California): this is designed to minimise adsorption of drugs to the wall of the tubing. A Harvard infusion pump was used, previously calibrated to deliver 20 ml per hour when connected to the syringes and tubing used.

At time zero, 10ml was injected as a bolus, and the infusion was started after 30 min. Subjects remained semiprone during the infusion, and were given light refreshments. Blood was collected from the contralateral antecubital fossa at 150 min, and the infusion stopped. Plasma was separated and stored at -20°C. Radioactivity in duplicate 50μl aliquots of infusate was determined by LSC to allow calculation of total infused [3H]. In preliminary experiments, plasma was taken at time 120 min and
extracted as below to establish that these conditions were sufficient to allow equilibrium of the infused [3H]testosterone.

4.2.2 Extraction, separation and quantification.
Plasma (5 ml) was extracted with 5 volumes of diethyl ether (AnalaR, BDH) for 7 min by continuous vortexing, and the organic phase decanted after freezing the aqueous layer in methylated spirits / solid CO2. After drying down, the residue was redissolved in 50μl of 50% acetonitrile containing 100 ng/ml testosterone and 1 mg/ml DHT, and injected onto a high performance liquid chromatography (HPLC) column (5 ODS-2, Spherisorb) using a 20μl injection loop and controlled by a BioRad HPLC-minicomputer interface. The column was eluted with isocratic 50% aqueous acetonitrile at 1ml/min. 1 min fractions were collected, and the radioactivity determined by LSC with 10min counting as described in Chapter 2. UV absorption at a wavelength of 254 nM was also recorded to monitor the elution of the unlabelled standards.

Recovery was determined by the addition of 2500cpm of [4-14C]testosterone (Amersham, 50 mCi/mmol) and 1500cpm of [4-14C]DHT (New England Nuclear, 50 mCi/mmol) to the tubes in which the extraction was carried out. These tracers were dried down, and plasma was equilibrated in the tubes for 20 min before extraction. [14C] in the HPLC fractions was then used to calculate recovery (45-60%).

Results were calculated from the LSC results using the formulae:

\[
MCRT = \frac{[^3H] \text{testosterone}/ \text{day infused}}{[^3H] \text{testosterone}/ \text{1 plasma}}
\]

\[
CRT\text{DHT} = \frac{[^3H] \text{DHT}/ \text{1 plasma}}{[^3H] \text{testosterone}/ \text{1 plasma}} \times 100
\]

where MCRT represents the metabolic clearance rate of testosterone, and CRT\text{DHT} represents the conversion rate of testosterone to DHT. This convention is also used below to describe the conversion ratio of precursor to product of other steroids.

4.2.3 Thin layer chromatography.
To compare the results obtained with the HPLC method described above with those obtainable by thin layer chromatography (TLC), the methodology used widely in the
past for separation of steroids, some samples were randomly selected for reanalysis by this method.

Samples were extracted as above, including the addition of $[^{14}\text{C}]$labelled tracers. The extracts were then redissolved in 100μl diethyl ether, and spotted onto silicagel 60 TLC plates (Merck Ltd). The plates were then run in chloroform:diethyl ether (95:5), dried, and cut into areas after visualisation of standards run in parallel by UV absorption (testosterone and androstenedione) or after spraying with 10% sulphuric acid in methanol and heating (DHT and androstanediol). Radioactivity was determined by LSC, and results calculated as above.

4.2.4 Statistical analysis.

All results in this Chapter were analysed using Student's t test, on paired and unpaired results as appropriate.

4.3 Results.

Steroids in low concentration can be absorbed to plastic and glass, and therefore the infusion system used silanised glassware, tubing coated with polyolefin and teflon-coated intravenous tubing. The tubing used has a coating designed to minimise absorption of drugs. That dose of radioactivity actually being infused into the subject was confirmed by comparing the radioactivity in a sample of infusate with that in the syringe, samples being taken from the syringe before the infusion was started and at the end. This data was obtained from trial experiments carried out before any infusions into the subjects were carried out. The radioactivity in the syringe after 2 hours was 96.6 ± 1.1% of that in the syringe at the beginning of the experiment, and that in fluid from the end of the tubing was 98.1 ± 1.4% of that in the syringe initially, and 100.5 ± 2.3% after 2 hours (mean ± sem of 8 determinations). There were no significant differences between the amounts of radioactivity in the syringe and tubing at the beginning of the experiment and in either the syringe or in the tubing after 2 hours, and there was therefore no evidence for any significant absorption of $[^{3}\text{H}]$testosterone by either syringe or tubing.

The HPLC system used was similar to that used by Bonsall, Rees and Michael (1989) to investigate the metabolism of $[^{3}\text{H}]$testosterone in monkey brain after injection of the radioisotope. The retention times of testosterone and dihydrotestosterone were 8.6 and 15.8 minutes respectively, demonstrating good separation. Other steroids tested were oestradiol (retention time 7.5 min), androstenedione (10.8 min) and androstanediol (11.8 min). An example of the HPLC profiles of the $[^{3}\text{H}]$testosterone
and of the [14C]labelled standards is shown in Figure 4.1, to illustrate the degree of separation obtained.

MCR\textsuperscript{T} was calculated as described in Methods, and the results are shown in Figure 4.2. At baseline, MCR\textsuperscript{T} in the azoospermic group was 1120 ± 60 l/day, and 1020 ± 90 l/day in the oligozoospermic group. After 16 weeks of testosterone treatment, MCR\textsuperscript{T} had significantly increased to 1300 ± 100 l/day in the azoospermic group (p<0.02), and to 1280 ± 100 l/day in the oligozoospermic group (p<0.02). In 5 men blood samples were taken at 100 minutes (ie after 70 minutes of infusion) to establish that equilibrium conditions had been reached. In this group MCR\textsuperscript{T} was 1120 ± 120 l/day at 120 minutes, and 1090 ± 160 l/day after 150 minutes, ie at the end of the infusion.

The blood production rate (P\textsubscript{B}) of testosterone is shown in Figure 4.3, calculated using values for plasma testosterone concentration taken from Chapter 3. Before treatment, P\textsubscript{B} was 6.6 ± 0.3 mg/day in the azoospermic group, and 6.9 ± 0.6 mg/day in the oligozoospermic group. These values were not significantly different. During testosterone treatment, production of endogenous testosterone is assumed to be greatly reduced secondary to inhibition of LH secretion (as shown in Chapter 3), and the plasma concentration of testosterone reflects absorption of the administered dose. The value for P\textsubscript{B} calculated is therefore an “apparent” rather than a real value. Figure 4.3 shows that apparent P\textsubscript{B} increased in both groups of men, to 13.5 ± 0.7 mg/day in the azoospermic group and to 13.7 ± 0.9 mg/day in the oligozoospermic group (p<0.001 for both groups). Data from Chapter 3 was used to calculate the mean plasma concentration of testosterone over a week, by extrapolation between the data points shown in Figure 3.2 and this value was then used to recalculate mean P\textsubscript{B} over a week. By this method, mean plasma testosterone concentrations were 49.5 ± 3.4 nmol/l in the azoospermic group and 55.2 ± 4.0 nmol/l in the oligozoospermic group. Calculation of average P\textsubscript{B} thus gives a result of 17.6 ± 1.2 mg/day in the azoospermic group and of 20.9 ± 2.5 mg/day in the oligozoospermic group (values not significantly different), compared to the administered dose of 140mg/week of free unesterified testosterone, equivalent to 20 mg/day.

Results of the determination of CRT\textsuperscript{DHT} are shown in Figure 4.4. CRT\textsuperscript{DHT} was 3.39 ± 0.20% in the azoospermic group and 3.18 ± 0.25% in the oligozoospermic group pretreatment. After 16 weeks of testosterone treatment, CRT\textsuperscript{DHT} in the azoospermic group was unchanged (3.12 ± 0.25%), but in the oligozoospermic group CRT\textsuperscript{DHT} was increased to 4.00 ± 0.35% (p<0.05). CRT\textsuperscript{DHT} of the oligozoospermic group was significantly greater than that of the azoospermic group after 16 weeks of treatment (p<0.05).
Figure 4.1
(b) HPLC profile of $[^{14}$C]testosterone and $[^{14}$C]DHT standards, used for determination of recovery.
Figure 4.2.
Effect of testosterone on MCR\textsuperscript{T} in those men who became azoospermic and those remaining oligozoospermic.

Open columns, baseline
Hatched columns, after 16 weeks testosterone treatment.

* p<0.05 vs baseline in both cases.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 4.3
Blood production rate of testosterone at baseline in those men who became azoospermic and those who remained oligozoospermic.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 4.4
The effect of testosterone administration on CR$^T\text{-DHT}$ in men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks of testosterone treatment.

* $p<0.05$ vs baseline

Mean ± sem, $n=18$, azoospermic group
$n=15$, oligozoospermic group.
To validate the method further, 5 samples were analysed by TLC using the method of Mowszowicz and Bardin (1977) to estimate $MCRT^T$ and $CRT^T$-DHT, and in addition the conversion of testosterone to androstenedione and androstanediol. The steroids were well separated, with $R_f$ values of 0.18 for androstanediol, 0.28 for testosterone, 0.34 for DHT, and 0.49 for androstenedione. Thus testosterone and DHT were not as well separated as by HPLC. This method gave values for $MCRT^T$ of 1240 ± 90 l/day, compared to 1050 ± 90 l/day for the same samples by HPLC. $CRT^T$-DHT was 4.80 ± 0.38% by TLC, and 4.20 ± 0.56% by HPLC. In both cases the values obtained by TLC were not significantly different from those obtained by HPLC. $CRT^T$-Androstenedione was 5.4 ± 1.2%, and $CRT^T$-Androstanediol was 1.9 ± 0.4% in these samples.

### 4.4 Discussion.

The metabolic clearance rate of steroids is determined by one of two methods. The most widely used, and that used here, is the constant infusion method in which a constant intravenous infusion of the radiolabelled steroid is given (sometimes after an initial loading dose to reduce the duration of infusion required) until a steady state is reached. The second is the bolus method, in which a single bolus of labelled steroid is given, and the amount remaining in the blood at times thereafter is determined (Baird et al., 1969; Horton, 1978).

The principle of the infusion method is that the infused steroid is in tracer quantity, and therefore equilibrates with the endogenous steroid in the circulation. It is then metabolised at the same rate as the endogenous steroid, and comparison of the quantity of steroid infused with that measured in the blood after equilibrium is reached allows calculation of MCR. This method does not distinguish between hepatic and extra-hepatic metabolism: these components can be separated by determining the gradient of steroid across the splanchnic circulation but this requires invasive methodology, including the catheterisation of the central veins, and cannot therefore be performed on healthy volunteers whereas the standard method requires only peripheral venous access.

Two further values can be determined from these experiments: the conversion ratio (CR) of the infused steroid (ie the precursor) to a metabolite, and the blood production rate ($P_B$). CR is usually expressed as a percentage of radioactivity converted from the precursor to the metabolite, and $P_B$ is the product of the MCR and the plasma concentration of the steroid, giving the production in mg/day.
The values for MCRT obtained from plasma samples taken at 120 min were similar to those at 150 min. This suggests that the infusion had reached equilibrium, an essential prerequisite for the validity of the method (Baird et al., 1969). The results for MCRT are similar to those reported in the literature (Lipsett, Wilson, Kirschner, Korenman, Fischman, Sarfaty and Bardin, 1966; Southren, Gordon, Tochimoto, Pinzon, Lane and Stypulkowski, 1967; Ito and Horton, 1971). These results were further validated by comparison to those obtained using TLC. The retention times of androstenedione and androstanediol under these conditions for HPLC were 10.8 and 11.4 min respectively. Thus, with the 1 min fractions collected in these experiments, androstenedione and androstanediol were not sufficiently clearly separated from testosterone to allow CRT-Androstenedione and CRT-Androstanediol to be accurately determined. Determinations of CRT-Androstenedione and CRT-Androstanediol were obtained in a few experiments by TLC, and the results (5.4% and 1.9% respectively) were in good agreement with those previously reported (3.4% for CRT-Androstenedione, and 1.3% for CRT-Androstanediol; Longcope et al., 1969; Mahoudeau et al., 1971). This suggests that any error in the calculation of MCRT from incorporation of the other androgens would be very small.

Testosterone administration increased MCRT by approximately 20% (Figure 4.2). Testosterone has been previously reported to increase MCRT in women (Southren et al., 1967), but this effect has not been previously reported in normal men. MCRT is thought to be dependent on the non-SHBG bound testosterone in the blood. Thus MCRT is decreased in conditions such as thyrotoxicosis and hepatic cirrhosis when the plasma concentration of SHBG is increased (Gordon et al., 1969; Baker, Burger, de Kretser, Dulmanis, Hudson, O'Connor, Paulson, Purcell and Seah, 1976), and increased in obesity, in which SHBG is reduced (Kirschner, Samojlik and Silber, 1983). Under the conditions of this study, plasma testosterone concentrations were increased, but the non-SHBG bound testosterone was increased by a greater amount as a result of the reduction in SHBG (Chapter 3). The increase in MCRT was similar to the fall in SHBG (approximately 30%), in contrast to the rise in total testosterone of approximately 100% and of 400% in free and non-SHBG bound testosterone, which supports the hypothesis that MCRT is determined largely by the concentration of SHBG (Vermuelen, Verdonck, van der Straeten and Orić, 1969).

There were no differences in MCRT between the azoospermic and oligozoospermic groups, before or during testosterone administration. This result, together with the results presented in Chapter 3 of the measurement of concentrations of free and non-SHBG bound testosterone and of SHBG itself, suggest that the basis of the differences
between these two groups is not in the pharmacokinetics or pharmacodynamics of testosterone.

Calculation of values for \( P_B \) (Figure 4.3) confirmed that 6-7 mg of testosterone is produced by the adult male per day (Horton 1978). There was no difference between the azoospermic and oligozoospermic groups. During testosterone administration, endogenous production of testosterone is reduced, and absorption from the site of injection replaces it. A value for "apparent" \( P_B \) can be calculated, representing this. The results shown in Figure 4.3 show that apparent \( P_B \) is increased to 13.5 ± 0.7 mg/day in the azoospermic group, and to 13.7 ± 0.9 mg/day in the oligozoospermic group. Over one week the apparent production of testosterone is therefore approximately 50 mg, compared to 140 mg of free testosterone administered. \( P_B \), however, is dependent on the concentration of testosterone for its calculation, and the infusion experiments were carried out 7 days after the last testosterone injection had been given, when the next was due. The concentration of circulating testosterone was therefore at its nadir, and the data in Chapter 3 show that at this time plasma testosterone concentrations are approximately half those found on the two days following injection. If it is assumed that \( MCRT \) does not vary over the week (which may not be the case as the concentration of testosterone varies so greatly), then \( P_B \) can be recalculated using the values for plasma testosterone shown in Chapter 3 with extrapolation between the data points to give mean values for plasma testosterone. Calculation of average \( P_B \) by this method gives a result of 17.6 ± 1.2 mg/day in the azoospermic group and of 20.9 ± 2.5 mg/day in the oligozoospermic group. The apparent production per week is therefore approximately 123 mg in the azoospermic group, and 146 mg in the oligozoospermic group, in much closer agreement to the administered dose of 140 mg. These results do not allow any speculation as to whether there is any continuing secretion of testosterone under these experimental conditions, despite the good agreement between calculated apparent \( P_B \) and known administered dose, particularly as it is not known whether \( MCRT \) varies as the plasma concentration of testosterone varies but do support the contention that endogenous secretion of testosterone is greatly suppressed.

\( CRT-DHT \) was 3.39% in the azoospermic group and 3.18% in the oligozoospermic group at baseline, values which were not significantly different (Figure 4.4). Values obtained by HPLC were similar to those obtained by TLC in a trial experiment, and are similar to those previously reported (Ito and Horton 1971; Saez, Forest, Morera and Bertrand, 1972; Horton, 1978). This is a measure of the peripheral conversion of testosterone to DHT, as this is not thought to occur in the splanchnic circulation despite
the presence of the enzyme 5αR in the liver (Ishimaru et al., 1978). It only measures the conversion of testosterone to DHT which is then released back into the circulation, and does not reflect any further metabolism of DHT at the site of action, for example conversion to androstanediol or glucuronidation (Moghissi, Ablan and Horton, 1984). If further metabolism at the site of action is important, then the value for CRT-DHT as estimated by this method will give an underestimate of the true 5αR activity of the body. Despite the direct nature of this method, it can therefore only be regarded as an index of peripheral 5αR activity.

After 16 weeks of testosterone administration, CRT-DHT had increased significantly in the oligozoospermic group but was unchanged in the azoospermic group (Figure 4.4). This implies that the activity of 5αR was increased by testosterone treatment in the oligozoospermic group but not in the azoospermic group. This increased activity would tend to increase tissue concentrations of DHT and thus amplify the androgen stimulus. In the skin, it has been shown that 5αR activity is androgen dependent: this has been demonstrated in vivo (Mauvais-Jarvis, Bercovici, Crepy and Gauthier, 1970; Kuttenn, Mowszowicz and Mauvais-Jarvis, 1980), and in vitro (Kuttenn and Mauvais-Jarvis, 1975; Mowszowicz, Melanitou, Kirchhoffer and Mauvais-Jarvis, 1983), and individuals with androgen insensitivity have a secondary deficiency of 5αR (Kuttenn et al., 1979; Imperato-McGinley et al., 1982). Thus men remaining oligozoospermic may have a greater androgen stimulus at the level of the tissues than those becoming azoospermic, without any differences in plasma testosterone, as was shown in Chapter 3. The finding that the two groups of men had the same CRT-DHT in the pretreatment experiments implies that under normal physiological conditions, peripheral 5αR activity is similar in the two groups, and thus any screening method to try to identify which men would become azoospermic and which would remain oligozoospermic would fail to distinguish between them.

In conclusion, the results presented so far demonstrate that there is no difference in the pharmacokinetics or pharmacodynamics of testosterone in these two groups of men. There is, however, evidence that the conversion of testosterone to the more potent androgen DHT is increased in the men remaining oligozoospermic, reflecting enhanced activity of the enzyme 5αR in that group. In the next Chapter, plasma concentrations of DHT and its metabolite androstanediol glucuronide have been investigated to substantiate this finding.
Chapter 5
Plasma concentrations of
Dihydrotestosterone and Androstanediol Glucuronide

5.1 Introduction.

In this Chapter androgen metabolism in acne and hirsutism, the common disorders involving the pilosebaceous unit (PSU), will be reviewed, as the study of these conditions has provided the majority of our knowledge of the metabolism of androgens in the skin. The skin is the largest androgen-dependent organ in the body, and the object of this discussion is to determine which androgens in plasma most accurately reflect the concentration of 5αR activity in the PSU and would therefore be most appropriate index to investigate possible differences in peripheral androgen metabolism in men receiving supraphysiological doses of testosterone. While the prostate is also an important androgen-dependent organ, there is little data available regarding the contribution of prostatic metabolism to plasma androgen concentrations.

Although hirsutism and acne are both conditions of the PSU, they affect different populations: thus hirsutism is a condition exclusively affecting women, whereas acne affect both sexes. Although males are more commonly affected, it has been suggested that if the study is rigorous enough, some degree of acne can be detected in almost all Caucasians at some point during puberty (Rosenfield, 1986). These sex differences may therefore introduce discrepancies in comparing the two conditions relating more to gender rather than to the condition itself. However it will become apparent that despite this possible cause for confusion, there is considerable similarity in androgen metabolism in the two conditions.

5.1.1 Androgen metabolism in hirsutism.

The bulk of the endocrinological literature on androgen metabolism by the skin concerns hirsutism. Early studies to investigate whether hirsutism was a hyperandrogenic condition yielded largely negative results. Thus plasma testosterone concentrations were only mildly elevated, as were urinary androgens (Lloyd, Lobotsky, Segre, Kobayashi, Taymor and Bath, 1966) and plasma androstenedione (Kuttenn, Mowszowicz, Schaison and Mauvais-Jarvis, 1977). Androstenedione is the major source of DHT in women (Ito and Horton, 1971, Mahoudeau et al., 1971) and this has also been demonstrated in the skin in vitro (Silva, Gentzschein and
Androstenedione is converted to DHT via 5α–androstanedione rather than testosterone (Stanczyk, Matteri, Kaufman, Gentzschein and Lobo, 1990). The origin of plasma DHT is therefore different in the two sexes. These minor increases in circulating concentrations of androgens were more marked in women with polycystic ovarian syndrome (PCOS), and it is now thought that PCOS is the underlying condition in over 90% of hirsute women (Kirschner, Zucker and Jespersen, 1976, Conway and Jacobs, 1990). There was, however, a greater increase in free testosterone concentration (Rosenfield, 1971), and this population of women have lower SHBG concentrations (Vermuelen et al., 1969, Cunningham and McKenna, 1988). In all these parameters, however there is considerable overlap with normal non-hirsute women, and these results therefore suggest that if there is an increased supply of androgen to the skin, it is relatively minor.

An alternative possibility is that there is an increased utilisation of androgen. In support of this, an increase in both MCRT (Bardin and Lipsett, 1967) and CRT-DHT (Mahoudeau et al., 1971) have been reported in hirsute women, as has an increased plasma concentration of DHT. This slight elevation of plasma DHT (also reported by Kuttenn et al., 1977) has not been widely confirmed (Lobo, Goebersmann and Horton, 1983). In vitro studies, however, have demonstrated a dramatic increase in 5αR activity of skin homogenates from hirsute women (Thomas and Oake, 1974; Kuttenn et al., 1977; Mowszowicz, et al., 1983; Serafini, Ablan and Lobo, 1985), and there is evidence that the rate of hair growth correlates with plasma androstenedione and DHT concentrations (Ebling, Randall and Sawers, 1984).

DHT is further metabolised to androstanediol (Adiol), which can be measured in the urine. Although hirsute women were found to have slightly elevated plasma concentrations of DHT in some studies but not others, the urinary excretion of Adiol was greatly increased (Kuttenn et al., 1977), as was the plasma concentration of androstanediol glucuronide (AdiolG) (Horton, Hawks and Lobo, 1982; Paulson, Serafini, Catalino and Lobo, 1986). Women with PCOS and hirsutism have been reported to have markedly elevated concentrations of AdiolG, whereas those with PCOS but not hirsutism did not (Lobo et al., 1983). Again, others have been unable to confirm these findings (Scanlon, Whorwood, Franks, Reed and James, 1988), but in that study there was a very wide variation in AdiolG concentrations in the hirsute women studied. A comparison of AdiolG concentrations with the plasma concentrations of androgen precursors in mildly hirsute women found evidence of increased conversion of precursors to AdiolG, but there was considerable overlap of AdiolG concentrations with normal controls (Giagulli, Giorgino and Vermuelen,
Furthermore, clinical cure (following treatment with cyproterone acetate and ethinyl oestradiol) did not correlate with changes in AdiolG concentration; a reduction in AdiolG concentration occurred in all women treated, but only half showed a clinical improvement.

Androsterone glucuronide is also a product of DHT metabolism, and its plasma concentration is increased in hirsutism (Scanlon, Whorwood, Reed, Franks and James, 1987; Thomson, Horton and Rittmaster, 1990) but has been subject to less investigation. This metabolite has recently been suggested to be preferentially increased in women with acne but not hirsutism, in contrast to AdiolG which was elevated in both conditions (Carmina, Stanczyk, Matteri and Lobo, 1991).

The increased concentrations of these metabolites may reflect both the increased supply of androstenedione and the increased conversion rate in the tissues. One possible conclusion that can be drawn from these studies is that the increased local conversion of precursor androgens to DHT is of major importance in the pathogenesis of hirsutism. Indeed, the ratio of α/β steroid metabolites in urine, interpreted as reflecting hepatic 5αR activity, was increased in women with PCOS (Stewart, Shackleton Beastall and Edwards, 1990), and these authors concluded that increased 5αR activity in liver and skin is the major underlying abnormality in this condition.

The androgen receptor has not been widely studied in hirsutism, but in one study no abnormality was detected (Eil, Cutler and Loriaux, 1985).

### 5.1.2 Androgen metabolism in acne

Similar hormonal processes have been implicated in the development of acne, although there is less data, and the other pathological factors involved in the aetiology of acne complicate interpretation. Thus bacterial infection of the blocked duct of the sebaceous gland is of major importance (Pochi, 1990), in addition to the increased secretion of sebum (Pochi and Strauss, 1964; Cunliffe and Shuster, 1969).

There are reports of testosterone concentrations being normal or elevated in patients with acne, and of SHBG being reduced (Marynick, Chakmakjian, McCaffree and Herndon, 1983; Lawrence, Katz, Robinson, Newman, McGarrigle, Shaw and Lachelin, 1981). 5αR activity in skin has been shown to be raised (Sansone and Reisner, 1971, Hay and Hodgkins, 1974), and in facial skin the enzyme is concentrated in the sebaceous glands (Hay and Hodgkins, 1978). An important study (Lookingbill, Horton, Demers, Egan, Marks and Santen, 1985) showed that in a group of young women with mild to moderate acne (only one of whom had any degree of hirsutism), plasma total and free testosterone and androstenedione were normal, whilst
plasma DHT was elevated to a mean of double that of controls and plasma AdiolG was nearly 3 times that of controls. The conclusion drawn was that precursor androgens were normal, but that "tissue" androgens, the result of local 5α-reduction, were elevated. In a similar study, the same authors investigated the correlation between AdiolG and acne and chest hair in men (Lookingbill, Egan, Santen and Demers, 1988), and found that there were correlations between the serum concentration of the metabolite and both skin markers. There was also a good correlation between AdiolG and non-SHBG bound testosterone but not with total testosterone. One important caveat to the interpretation of these data suggesting that increased 5αR is involved in the pathophysiology of acne is that the increase in enzyme activity may be because of proliferation or hyperplasia of the sebaceous glands rather than be causal.

5.1.3 Ethnic differences in clinical and biochemical markers of 5α-reductase.

Lookingbill's group have more recently investigated differences in androgen metabolism between caucasian and Chinese subjects (Lookingbill, Demers, Wang, Leung, Rittmaster and Santen, 1991). They found that caucasian men had hairier chests and more acne than Chinese men, and thus clinical evidence for a greater androgen stimulus to the PSU. Biochemical studies showed that plasma concentrations of total and bioavailable (non-SHBG bound) testosterone were the same in the two groups, but that concentrations of dehydroepiandrosterone sulphate (DHAS) and androstenedione were higher in caucasian men. Concentrations of the 5α-reduced androgens AdiolG and androstenedione glucuronide were, however markedly higher in the caucasians, both in men and women, to approximately double the concentrations found in Chinese subjects. The plasma concentration of DHT was similar in the two groups of men, but higher in the caucasian than in the Chinese women. These biochemical results provide indirect evidence that there is a racial difference in 5αR activity in both sexes, and provide a possible explanation for the differences in hair growth and the incidence of acne. It may be said that the higher 5α-reduced androgens in caucasian than Chinese men is because of the increased mass of hair follicles, but because 5α-reduced androgens are also increased in caucasian women compared to Chinese women despite similar Ferriman Galway scores (very low in both groups), it is unlikely that the markedly higher 5α-reduced metabolite concentrations in Caucasian women are due to a greater mass of hair follicles. This make genetic and ethnic differences in androgen metabolism more likely. As yet, there are no studies comparing androgen metabolic clearance rate and CRT-DHT or 5αR activity in skin biopsies in caucasian versus
Chapter 5

Chinese men, which might provide more direct evidence for a lower activity of this enzyme in Chinese men and women.

A recent study has investigated serum AdiolG and androsterone glucuronide concentrations (as indices of 5αR activity) in Japanese men compared to caucasian and black US men (Ross, Bernstein, Lobo, Shimizu, Stanczyk, Pike and Henderson, 1992). Concentrations of both 5αR markers were higher in the two US populations, but there were no differences serum testosterone concentrations. These results were discussed with respect to the much lower incidence of prostatic carcinoma in Japanese than American men. However, although the two US groups had similar AdiolG concentrations, the incidence of prostatic carcinoma is considerably higher in blacks.

These studies are of particular interest to the present investigation, in view of the striking differences in the incidence of azoospermia between Chinese and caucasian men with testosterone treatment (World Health Organisation, 1990), with over 90% azoospermia being achieved in the Chinese centres in this study, compared to 50-70% in the caucasian centres. An incidence of 100% azoospermia has also been reported in a group of Indonesian men receiving a combination of progestogen and testosterone as a contraceptive (Pangkahila, 1991).

5.1.4 The skin as a source of circulating androgens

There is therefore a body of evidence showing that there is increased 5αR activity in both hirsutism and acne, in caucasians compared to Chinese and Japanese, and that AdiolG may be a good plasma marker for this. What is the evidence for the skin being the origin of this metabolite, and that its concentration reflects 5αR activity? This was first suggested by Mauvais-Jarvis et al. (1970), who found that there was greater conversion of radiolabeled testosterone to AdiolG when it was applied to the skin rather than given intravenously. An extrasplanchnic source of AdiolG was supported by infusion studies in men (Morimoto, Edmiston, Hawks and Horton, 1981; Moghissi et al., 1984), and the results were compatible with the major pathway of metabolism being testosterone to DHT to AdiolG, with DHT glucuronide being a possible intermediate. These studies also revealed that plasma Adiol was derived from a different pool from its glucuronide (Moghissi et al., 1984). Some 75% of plasma Adiol is derived from DHT (Kinouchi and Horton, 1974), but the reason for the apparently different metabolic involvements of Adiol and AdiolG are unknown. There may also be a contribution by adrenal androgens to AdiolG, as there is a correlation between plasma DHA and AdiolG concentrations in castrated men (Bélanger, Brochu and Cliche, 1986), and AdiolG concentrations rise at adrenarche in boys (Brochu and
Bélanger, 1987). Urinary excretion of AdiolG was found to rise to very high levels after prolonged adrenal stimulation in normal men, and men having undergone adrenalectomy for Cushing’s disease were found to have lower urinary AdiolG excretion than normal men (Deslypere, Sayed, Punjabi, Verdonck and Vermeulen, 1982). It therefore appears that urinary AdiolG has a combined splanchnic and extrasplanchnic origin.

Direct production of AdiolG in rat prostate but not liver was reported by Chung and Coffey (1977), but it was not until 1987 that human skin was shown to be capable of converting testosterone and DHT to AdiolG (Lobo, Paul, Gentzschein, Serafini, Catalino, Paulson and Horton, 1987). The rate of conversion was higher in men than women, paralleling differences in plasma concentrations (Mauvais-Jarvis et al., 1970).

Mauvais-Jarvis et al. (1970) also reported that AdiolG was found in reduced concentration in the plasma of subjects with testicular feminisation. Similar findings have been reported in subjects with 5αR deficiency (Horton, Imperato-McGinley and Peterson, 1984). While the latter result provides further evidence that AdiolG reflects 5αR activity, the former is less easy to explain as the major defect in that condition is believed to lie with the androgen receptor rather than the enzyme. However the activity of the enzyme in homogenates of pubic skin from subjects with testicular feminisation was undetectable, whereas it was normal in genital skin (Northcutt et al., 1969; Kuttenn et al., 1979). This may reflect the importance of activation of the androgen receptor in determining the concentration of activity of 5αR (vide supra, Section 1.6.2), with the very low or absent 5αR activity in skin from subjects with testicular feminisation being a secondary effect.

Further evidence for the validity of serum AdiolG as a marker of 5αR activity in skin was provided by the demonstration of a very good correlation between the two using genital skin biopsies (Paulson et al., 1986), in a study of 4 groups of subject: premenopausal and postmenopausal women, hirsute premenopausal women, and men.

On the basis of these results it has been proposed that in skin, 5αR converts testosterone to DHT, which is then converted by the enzyme 3α-hydroxysteroid dehydrogenase to Adiol, an inactive metabolite, which is further conjugated to the polar compound AdiolG and released into the circulation (Lookingbill et al., 1985). The circulating concentration of AdiolG therefore appears to be a useful marker of 5αR activity in peripheral tissues, to which the skin and in particular the PSU may be a major contributor. Infusion of testosterone into normal men causes a prompt rise in serum AdiolG concentrations, of a similar magnitude to the rise in testosterone
concentrations (Reed, Whorwood, Scanlon, Beranek, Polson, Franks and James, 1986).

The importance of the plasma concentration of DHT is less clear. Whilst being the primary product of 5αR in skin as in other androgen-dependant tissues, the above discussion demonstrates that elevated plasma concentrations are only demonstrated in some studies of disorders of the PSU, and the elevations are usually slight when detected. One possibility is that DHT is rapidly metabolised to Adiol at the site of action, and indeed plasma concentrations of AdiolG are considerably higher than those of DHT and are similar to those of testosterone (Moghissi et al., 1984; Lookingbill et al., 1991). DHT may therefore be predominantly a local paracrine amplifier of androgen action. However it is clear that the bulk of plasma DHT derives from 5α-reduction of testosterone in men, and in view of the differences in origin of DHT in men and women, it is unclear to what extent the studies on androgen metabolism in women discussed above are relevant to the male. In the current study the plasma concentrations of both DHT and AdiolG were therefore determined to investigate whether this would provide further evidence as to whether men remaining oligozoospermic when administered supraphysiological doses of testosterone have a higher level of peripheral 5αR activity than those becoming azoospermic.

5.2 Methods.

5.2.1 DHT assay.

The concentration of DHT in plasma was determined by RIA, using [3H]testosterone / dihydrotestosterone assay system kits supplied by Amersham International plc. The technique used enables the separate measurement of DHT from testosterone in this assay by the chemical oxidation of the double bond in testosterone, which causes it to lose immunoreactivity without affecting DHT. DHT can then be determined, despite the antibody used showing 50% crossreactivity for DHT compared to testosterone.

Reagents supplied were [3H]DHT (5α-dihydro[1,2,4,5,6,7-3H]testosterone), 4μCi (148kBq) in ethanol; oxidation reagent (KMnO4 in water); anti-testosterone antiserum and charcoal precipitating reagent. Assay buffer consisted of 6.06g AnalR Tris, 1.49g AnalR EDTA, disodium salt, and 1g gelatin per litre, pH 8.0.

500μl of plasma made up to 1ml with assay buffer was extracted twice into 3ml of ether. The extracts were combined and dried under nitrogen, and reconstituted in 1ml of buffer. 500μl of water was added, followed by 50μl of oxidation reagent. The
samples were vortexed and reacted for 20 min., before being extracted twice as before with 3ml ether. The extracts were dried down, and reconstituted in 1ml assay buffer. To 200µl duplicate aliquots of sample or standard (range 25-800 pg/tube) 200µl of [3H]DHT was added, then 200µl of antiserum. Non-specific binding determinations consisted of 400µl buffer and tracer, and Bo of 200µl of buffer, tracer, and antiserum. The tubes were vortexed, and incubated at room temperature for 1 hr. 200µl of charcoal reagent was then added, the tubes vortexed and allowed to stand for 10 min before being centrifuged at 3000 rpm for 30 min. [3H] in 500µl aliquots of supernatant was then determined by LSC, and results calculated using the AssayZap. Quality control samples were included, and recoveries were determined in parallel samples. Assay sensitivity (lowest standard concentration, corrected for recovery) was approximately 1.1 nmol/l, when 500µl of sample was extracted. Intraassay CV was 7.5%, interassay CV 9.9%, and mean recovery was 74%. The efficiency of the oxidation stage was checked in view of the high concentrations of testosterone in the plasma of the men in this study, and found to be complete in plasma spiked with a concentration of 100 nmol/l testosterone.

Recovery was also confirmed by spiking plasma samples with known quantities of DHT: mean recovery was 103 ± 6% over the range 50-800 pg DHT added.

5.2.2 Androstanediol Glucuronide assay.

The concentration of AdiolG in serum was determined by RIA using a kit supplied by DSL Inc (Texas, USA) which uses an antibody directed against 3α-androstanediol 17-glucuronide-7-hemisuccinate and which has been shown to give values in good agreement with previous laborious chromatographic methods (Samojlik and Kirschner, 1990). This antibody has 10.7% cross reactivity with unconjugated Adiol, 5.9% with Adiol-3-glucuronide (which contributes approximately 20% to total serum AdiolG; Thomson, Rittmaster, Rodriguez, Moore and Rao, 1991) and <2% with testosterone glucuronide and DHT glucuronide.

Standards were supplied in over the range 0.5-100 ng/ml plus a zero standard. Non-specific binding was estimated using 200µl of zero standard in the absence of antiserum. 100µl of standard or sample were assayed in duplicate. A high (10.5 ng/ml) and low (2.5 ng/ml) quality control sample were included with the kit. 500µl of 125I-AdiolG was added to each tube, followed by 100µl of AdiolG antiserum. Tubes were vortexed, and incubated at room temperature for 3 hrs. 1ml of goat anti-rabbit globulin with polyethylene glycol precipitating reagent was then added, the tubes vortexed, and incubated at room temperature for a further 15 min before being
centrifuged at 1500g for 20 min. The supernatant was decanted, and radioactivity in the pellet counted in a gamma counter. Results were calculated using AssayZap. Quality control samples gave results within the range quoted by the manufacturer, and over 3 assays the interassay CV was 6.4% and the intraassay CV was 6.1% at 10 ng/ml. The sensitivity of the assay was 0.5 nmol/l.

The assay was validated by assessing linearity of serial dilution of serum samples and seminal plasma samples (for Chapter 7 results), and recovery was investigated by spiking serum and seminal plasma with AdiolG standards in the range 0.5 to 30 ng/ml. The results of these checks are shown in Figure 5.1, showing linearity of dilution and complete recovery of standard with both sample types. Mean recovery was 103% in serum and 105% in seminal plasma (n=8 in both cases).

5.2.3 Statistical analysis.

Results in this Chapter were analysed using Student's t test, on paired and unpaired results as appropriate, or the Mann-Whitney U test on non-parametric data.

5.3 Results.

5.3.1 Dihydrotestosterone.

Plasma concentrations of DHT were determined at baseline before testosterone treatment was started, and after 16 weeks of treatment at the time the next injection was due. Baseline concentrations of DHT were 2.51 ± 0.19 nmol/l in the azoospermic group, and 2.64 ± 0.30 nmol/l in the oligozoospermic group (Figure 5.2a). The concentrations of DHT were significantly increased in both groups after 16 weeks of testosterone treatment, to 3.08 ± 0.20 nmol/l in the azoospermic group (p<0.01), and to 4.08 ± 0.35 nmol/l in the oligozoospermic group (p<0.001). The concentrations of DHT were similar at baseline, but after 16 weeks, the concentrations in the oligozoospermic group were significantly greater than in the azoospermic group (p<0.02). The plasma concentrations of DHT in the two groups after 16 weeks of testosterone treatment expressed as a percentage of baseline are shown in Figure 5.2b, illustrating the greater increase in the oligozoospermic group at that time, to 166 ± 16% of baseline compared to 133 ± 11% in the azoospermic group (p<0.05).

Plasma DHT concentrations were compared to values for CRT-DHT reported in Chapter 4 (Figure 5.3). There was no significant correlation between plasma DHT concentrations and CRT-DHT before treatment (r=0.27, for all men; r=0.24, azoospermic group; r=0.30, oligozoospermic group; p>0.05 in each case). After 16
Figure 5.1
(a) Recovery of AdiolG from spiked samples of serum and seminal plasma. Known concentrations of AdiolG were added to 100µl of serum or seminal plasma.

(b) Linearity of dilution of serum and seminal plasma.

Open symbols, serum.
Closed symbols, seminal plasma.
Figure 5.2
Effect of testosterone treatment on plasma DHT concentration in those men becoming azoospermic and those remaining oligozoospermic.

(a) Open columns, baseline. Hatched columns, after 16 weeks testosterone treatment.

(b) Increase in DHT after testosterone treatment, results expressed as percentage of baseline concentration.

a: p<0.05 vs baseline
b: p<0.01 vs baseline
c: p<0.05 vs Azoospermic group

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 5.3
Correlation between plasma concentration of DHT, and CR T-DHT in those men becoming azoospermic and those remaining oligozoospermic.

(a) Baseline
(b) After 16 weeks of testosterone treatment.

Open symbols, Azoospermic group.
Filled symbols, Oligozoospermic group.

Correlation coefficients:
(a) All men, r= 0.27, p>0.05
(b) All men, r=0.64, p<0.001
weeks treatment, the correlation coefficient for the whole group was 0.64, p<0.001, and there was a significant correlation between these two parameters at that time in the oligozoospermic group (r=0.67, p<0.01) but not in the azoospermic group (r=0.40, p>0.05), although the correlation in that group was greater than at baseline. The treatment-induced changes in plasma DHT concentration and CRT-DHT were also compared (Figure 5.4), after calculating the values after 16 weeks testosterone treatment as a percentage of baseline values for each set of data. The correlation coefficient for the whole group of men was 0.47 (p<0.01), and Figure 5.4 also shows the distribution of the results according to the azoospermic/oligozoospermic criterion. This Figure demonstrates that there is a significant correlation between increases in CRT-DHT and plasma DHT with testosterone treatment, and also illustrates that there is a degree of separation between the two groups of men. It thus amplifies the results presented in Figures 4.4 and 5.2, showing the greater increase in both CRT-DHT and DHT in the oligozoospermic group of men.

The correlation between plasma testosterone and DHT was also determined using plasma testosterone concentrations measures in Chapter 3. The correlation was significant in both groups at baseline (r=0.54 in the azoospermic group, p<0.05; r=0.77 in the oligozoospermic group, p<0.01), but not after 16 weeks of treatment (r=0.09 in the azoospermic group; r=0.08 in the oligozoospermic group).

### 5.3.2 Androstanediol Glucuronide.

AdiolG concentrations in serum were measured at the same time points as DHT, i.e. before testosterone treatment was started, and after 16 weeks of treatment. There was no difference in baseline AdiolG concentrations between men who subsequently became azoospermic (20.1 ± 3.0 nmol/l), and those who remained oligozoospermic (18.2 ± 2.3 nmol/l). Testosterone treatment caused a significant increase in AdiolG concentrations in both groups (Figure 5.5), to 31.0 ± 4.8 nmol/l (p<0.01) in the azoospermic group and to 40.3 ± 7.1 nmol/l in the oligozoospermic group (p<0.001). The difference between the values of the oligozoospermic group and the azoospermic group after 16 weeks of testosterone treatment did not reach statistical significance, but when the results were recalculated to give the concentration after 16 weeks as a percentage of baseline, this revealed that testosterone treatment caused a greater increase in AdiolG in the oligozoospermic group than in the azoospermic group (111 ± 16% increase in the oligozoospermic group vs. 69 ± 15% in the azoospermic group, p<0.05; Figure 5.5b).
Correlation between increase in plasma concentration of DHT and increase in CRT-DHT after 16 weeks treatment, in those men becoming azoospermic and those remaining oligozoospermic. In both cases, data points are values after 16 weeks testosterone treatment as a percentage of value at baseline.

Open symbols, Azoospermic group.
Filled symbols, Oligozoospermic group.

Correlation coefficient:
All men, $r = 0.47$, $p<0.01$
Figure 5.5
Effect of testosterone on serum AdiolG concentrations in those men becoming azoospermic and those remaining oligozoospermic.

(a) Open columns, baseline
Hatched columns, after 16 weeks testosterone treatment.

(b) Increase in AdiolG after testosterone treatment, results expressed as percentage of baseline concentration.

* $p<0.05$ vs Azoospermic group
** $p<0.01$ vs baseline
*** $p<0.001$ vs baseline.

Mean ± sem, $n=18$, azoospermic group;
$n=15$, oligozoospermic group.
There was no significant correlation between serum AdiolG and plasma DHT concentrations in either group, either at baseline or after 16 weeks testosterone treatment \((r=0.34\) and \(0.05\) in the azoospermic group, \(r=0.16\) and \(0.25\) in the oligozoospermic group, baseline and after 16 weeks testosterone treatment respectively). However when the results after 16 weeks treatment were recalculated as percentages of baseline concentrations, there was found to be a significant correlation between the increases in DHT and AdiolG concentrations in the oligozoospermic group \((r=0.58, p<0.05)\) but not in the azoospermic group \((r=0.1, p>0.05)\). This is shown in Figure 5.6, with the correlation line for the whole group of men shown \((r=0.33, p>0.05)\).

### 3.4 Discussion.

#### 5.4.1 Dihydrotestosterone.

The results in Figure 5.2 show that administration of supraphysiological doses of testosterone causes an increase in plasma DHT at 16 weeks of approximately 40%. This increase is thus considerably lower than the change in plasma testosterone concentrations, which are increased by approximately 100% at this time (Table 3.1). Schulte-Beerbühl and Nieschlag (1980) reported similar increases in plasma DHT following a single injection of testosterone oenanthate, but their study did not involve repeated administration. An increase in plasma DHT was also reported following infusion of testosterone (Reed et al., 1986), with an increase within 40 min of starting the infusion. There are no previous data on the plasma concentrations of DHT following repeated administration of testosterone to normal men.

It is accepted that in men most of the DHT in the circulation results from the peripheral 5α-reduction of testosterone (Ito and Horton, 1971, Ishimara et al., 1978). The smaller increase in plasma DHT than testosterone therefore suggests a low rate of conversion of testosterone to DHT in the periphery, ie a low 5αR activity. In Chapter 1, the literature reporting changes in 5αR activity after hypophysectomy or testosterone treatment was discussed. 5αR activity is increased by androgens and decreased by hypophysectomy in the androgen-dependent organs (skin, prostate, and epididymis), but the opposite is true of 5αR in the liver and adrenal cortex. It is not always clear from those studies whether the effect of hypophysectomy is mediated by a decrease in testosterone or other hormones (including a direct effect of the gonadotrophins). Under the conditions of the current study, testosterone treatment causes an increase in plasma testosterone, and a decline in gonadotrophin secretion to undetectable concentrations.
Figure 5.6
Correlation between increases in AdiolG and DHT concentrations after 16 weeks of testosterone treatment, results expressed as percentage of baseline concentrations.

Open symbols, Azoospermic group.
Filled symbols, Oligozoospermic group.

Correlation coefficient:
All men $r=0.33$, $p>0.05$. 
5αR in the skin (and prostate) might therefore be expected to be increased, with an increased production of DHT relative to the concentration of testosterone, and thus a greater increase in DHT than testosterone. The small increase in plasma DHT observed therefore requires an explanation.

One possibility is that DHT in plasma does not reflect 5αR activity directly, but is the result of a "leak" of the hormone from within the cell in which it is produced, while most of the DHT thus produced is further metabolised, eg to Adiol; DHT thus has a paracrine rather than an endocrine role. This is consistent with the finding that finasteride treatment causes a much greater inhibition of prostatic tissue DHT concentrations than plasma DHT (McConnell et al., 1992), as well as the results discussed in the Introduction to this Chapter, which suggest that AdiolG is a better index of hyperandrogenic status (eg in hirsutism) than is DHT. If increased 5αR activity is coupled with increased metabolism of DHT, then increasing the concentration of the substrate, testosterone, will preponderantly increase the concentration of AdiolG in the circulation, even though tissue concentrations of DHT may also be raised. Any leak of DHT might also be increased, but to a smaller extent. Thus the circulating concentration of DHT is a poor index of 5αR activity.

After 16 weeks of testosterone treatment, the plasma concentration of DHT in the oligozoospermic group was significantly greater than in the azoospermic group (Figure 5.2). As the plasma concentration of testosterone was not different between the two groups (Chapter 3), this result suggests that the 5αR activity of the oligozoospermic group was greater than that of the azoospermic group, and supports the finding of a selective increase in CRT-DHT in the oligozoospermic group after 16 weeks of testosterone treatment (Chapter 4). An alternate explanation is that the metabolism of DHT was increased to a greater extent in the azoospermic group: no data are available on this point, but the demonstration in Chapter 4 that MCRT was the same in the two groups, and increased to the same extent with testosterone treatment argues against it.

There was also a significant correlation between plasma DHT and CRT-DHT results (Figure 5.3), but only after 16 weeks of treatment. This was confirmed by comparing changes in CRT-DHT and DHT concentration as a result of testosterone treatment (Figure 5.4), showing a significant correlation between the two measures of 5αR activity. The experimental protocol for the determination of CRT-DHT requires the release of [3H]DHT into the circulation after reduction of infused [3H]testosterone, and as discussed in Chapter 4, any further metabolism of the [3H]DHT produced will tend to reduce the value for CRT-DHT. It is thus at best only an index of tissue 5αR activity, but the correlation with plasma DHT is consistent with the suggestion that under conditions of (exogenously-administered) supraphysiological testosterone
concentrations, plasma DHT is more closely related to 5αR than under physiological conditions.

Information on the relationship between plasma testosterone and DHT is also obtained by determining correlations between them directly. The correlation between plasma testosterone and DHT was significant in both groups at baseline as reported by others (Rittmaster et al., 1989), but not after 16 weeks of treatment. This result suggests that under physiological conditions, the amount of DHT in the circulation is related to the amount of testosterone, i.e. that the concentration of product is related to the concentration of precursor. During testosterone treatment, this relationship no longer appears to be present: the large increase in plasma testosterone has perhaps "swamped" the 5αR. This would also explain the smaller increase in plasma DHT than testosterone.

5.4.2 Androstanediol Glucuronide.

Baseline concentrations of AdiolG were similar to those reported by others using chromatographic/hydrolytic methods (Morimoto et al., 1981; Lookingbill et al., 1988), although the results reported by Lookingbill et al. (1991) are considerably higher (mean AdiolG in normal caucasian men 35 nmol/l in that study, compared to 11 nmol/l in Lookingbill et al. 1988). The results presented here are very similar to those reported in a study comparing the assay kit used here with a chromatographic method (Samojlik and Kirschner, 1990).

Testosterone treatment caused a significant increase in AdiolG concentrations in both groups of men after 16 weeks of treatment. Although the concentration of AdiolG in the oligozoospermic group was higher than in the azoospermic group after 16 weeks of treatment (40.3 ± 7.1 nmol/l vs 31.0 ± 4.8 nmol/l), this did not reach statistical significance. The increase in AdiolG with treatment was however significantly greater in the oligozoospermic group when the treatment concentration is expressed as a percentage increase (111% vs 69%, p<0.05, Figure 5.5). These results therefore support the hypothesis that there is an increased 5αR activity in men remaining oligozoospermic.

Comparison of Figures 5.2 and 5.5 shows that the increase in AdiolG is greater than the increase in DHT, and is similar in magnitude to the increase in plasma testosterone described in Chapter 3. This is consistent with DHT being further metabolised to AdiolG before being released into the circulation, and both supports the hypothesis that AdiolG is a better marker of hyperandrogenism than DHT and provides a further explanation for the lesser rise in plasma DHT concentrations observed. While
the lack of direct correlation between AdiolG and DHT concentrations found here confirms the previous report of a lack of correlation between DHT and AdiolG (or androsterone glucuronide) concentrations (Rittmaster et al., 1989), further analysis of the data with recalculation to show changes after testosterone treatment showed that there was a significant correlation between increases in DHT and AdiolG concentrations with testosterone treatment in the oligozoospermic group (Figure 5.6). This demonstrates the importance of measuring dynamic changes in steroid concentrations in the investigation of relationships between them, and provides further support for the hypothesis that there is a difference in 5αR activity between those men who became azoospermic and those remaining oligozoospermic, but that this difference is only unmasked during testosterone treatment.

The regulation of 5αR was discussed in Chapter 1.6, where the evidence which suggests that 5αR in the skin and prostate is androgen-dependent was presented. The hypothesis of this Thesis is that men remaining oligozoospermic on supraphysiological doses of testosterone have a higher 5αR than those remaining azoospermic, resulting in more testosterone being converted to DHT thus providing a greater androgen stimulus systemically and also presumably in the testis. This may be sufficient to maintain a low rate of spermatogenesis. If this is correct, then the rate of decline of sperm density might inversely correlate with measures of 5αR. This was investigated by subdivision of the oligozoospermic group into those who exhibited a rapid decline in sperm density to less than 0.1 x 10^6/ml but remained oligozoospermic ("rapid" group), and those who showed a slower decline and maintained a greater sperm density ("slow" group). Examination of the data revealed 6 men from the oligozoospermic group who all had a sperm density of <0.1 x 10^6/ml after 16 weeks of testosterone treatment. The mean sperm density of the remaining "slow" oligozoospermic group (n=9) at 16 weeks was 4.2 ± 1.7 x 10^6/ml, and that of the azoospermic group was <0.1 x 10^6/ml (13 of that group had already become azoospermic at that time). The sperm densities of these 3 groups during testosterone treatment are shown in Figure 5.7. DHT and AdiolG results were presented in the same groups, and these results (normalised to baseline) are shown in Figure 5.8. This shows a progressive rise in the testosterone-induced increase in the circulating concentrations of both hormones with the degree of maintenance of spermatogenesis, such that the increases in concentration of both hormones was greatest in the more resistant oligozoospermic group and lowest in those who rapidly achieved azoospermia. Those men whose sperm density declined rapidly but in whose ejaculates spermatozoa remained detectable formed an intermediate
Figure 5.7
Effect of testosterone treatment on sperm density, subjects divided into 3 groups.

- □ Azoospermic group, n=18
- ▲ "Rapid" oligozoospermic group, n=6
- ■ "Slow" oligozoospermic group, n=9.
Figure 5.8
Plasma concentrations of DHT and AdiolG after 16 weeks of testosterone treatment expressed as a percentage of baseline concentration, subjects divided into 3 groups.

Open columns, azoospermic group.
Hatched columns, "rapid" oligozoospermic group.
Filled columns, "slow" oligozoospermic group.
group. These results did not reach statistical significance due to the small numbers in each group.

The results presented in this Chapter thus demonstrate increases in circulating DHT and AdiolG concentrations with testosterone administration. The increases in both hormones were greater in the group of men remaining oligozoospermic than in those becoming azoospermic, and there also appeared to be a correlation between degree of maintenance of spermatogenesis and increases in DHT and AdiolG. There were also correlations between testosterone-induced changes in the three measures of 5αR activity (CRT-DHT, DHT and AdiolG), particularly in the oligozoospermic group of men. These results therefore support the results presented in Chapter 4, suggesting that there is increased activity of the enzyme 5αR in those men who remain oligozoospermic, and that this may underlie the maintenance of spermatogenesis in these men. This is further investigated in the following two Chapters, in which androgen concentrations in seminal plasma, and steroid precursors of testosterone metabolism in urine and plasma are measured. In Chapter 8, the effect of testosterone administration on sebum secretion is reported, to investigate whether increased 5αR activity in those men remaining oligozoospermic can be demonstrated at the level of an end-organ response.
Chapter 6
Androgens in Seminal Plasma.

Introduction.

In the preceding Chapters, results have been presented of measurements of the concentration of testosterone, DHT and AdiolG in blood before and after 16 weeks of testosterone treatment, and these results have been interpreted in terms of the activity of the enzyme $5\alpha$R. The concentrations of DHT and AdiolG are believed to reflect peripheral conversion of testosterone. Interpretation of these data in relation to the degree of inhibition of spermatogenesis by supraphysiological concentrations of testosterone is difficult, as testicular and peripheral androgen metabolism may differ considerably. An alternate, and perhaps closer, approach to the investigation of testicular steroidogenesis may be by the measurement of androgens in seminal plasma.

The development of specific and sensitive RIAs has allowed the analysis of steroids in seminal plasma in addition to their study in blood plasma, with improved accuracy compared to previously available methods such as bioassay (Raboch, Gregorová and Rezábek, 1963) and fluorimetry (Diczfalusy, 1954). Such earlier methods are now recognised to have often given very erroneous values. However, Purvis, Landgren, Cekan and Diczfalusy (1975) demonstrated the presence and concentration of several steroids in seminal plasma by RIA, including testosterone, DHT, pregnenolone, androsterone and oestradiol, in both normal and infertile men. These results showed that the concentration of testosterone in seminal plasma is much lower than in blood, whereas the concentration of DHT in seminal plasma is approximately double that of testosterone (compared to being 10% of the concentration of testosterone in blood plasma). These results have since been confirmed by others (Biswas, Ferguson, Stedronska, Baffoe, Mansfield and Kosbab, 1978; Purvis, Calandra, Sander and Hansson, 1978; Le Lannou, Massart, Chambon, Nicol and Allannic, 1980; Hudson, Hayes, Crawford and McKay, 1983).

Seminal plasma is composed of the secretions of the male sex accessory glands, the prostate and seminal vesicles, as well as fluid from the testis and epididymis. Evidence for the relative contributions of these tissues to the concentrations of steroids in seminal plasma has been obtained from the study of men after vasectomy, and from direct assay of steroids in the tissues. DHT is found in much greater concentration in the epididymis than in the testis, but whereas testosterone is found predominantly in the testis, the concentration of testosterone in the epididymis is still 3 fold greater than that
of DHT (Purvis et al., 1978). These authors also demonstrated the absence of a concentration gradient for either androgen along the human epididymis, in contrast to the gradient (decreasing from the Caput towards the Cauda) found in several species including the rat, rabbit and bull, a gradient which parallels that of ABP (Aafjes and Vreeburg, 1972; Guerrero, Ritzén, Hansson, Purvis and French, 1975; Pujol, Bayard, Louvet and Boulard, 1976).

The epididymis contains 5αR, with an activity approximately half that of the prostate in the human (Gloyna and Wilson, 1969), and in the epididymis as in the prostate, the activity of the enzyme is androgen-dependent (Viger and Robaire, 1991). Epididymal DHT is therefore likely to be produced locally from testosterone, and the concentration of DHT in the epididymis is 10 fold higher than in seminal plasma (Purvis et al., 1975; 1978).

The operation of vasectomy physically prevents the passage of spermatozoa and fluid from the testis and epididymis, and thus provides a useful model for the investigation of the relative contributions of the testis/epididymis and the accessory glands to the components of seminal plasma. The concentration of DHT in seminal plasma after vasectomy is reduced to 20-40% of normal, but that of testosterone is unchanged or only slightly reduced (Le Lannou et al., 1980; Ying, Hedman, de la Torre, Jensen, Pedersen and Diczfalussy, 1983; Hudson et al., 1983). Measurement of steroids in split ejaculates also supports a differential contribution, as DHT is found predominantly in the first fraction (mostly consisting of prostatic and testicular/epididymal fluid), whereas testosterone is found equally in that fraction and the second, mostly from the seminal vesicles (Cohen, Delafontaine and Grenier, 1978). There have also been similar reports of selectively reduced concentrations of DHT but not testosterone in seminal plasma of men who are azoospermic, of both obstructive and non-obstructive aetiologies (Purvis et al., 1975; Schoenfeld, Amelar, Dubin and Numeroff, 1978; Biswas et al., 1978; Le Lannou et al., 1980).

These findings therefore suggest that most DHT in seminal plasma derives from the testis/epididymis rather than the accessory glands, and in particular from the conversion of testosterone to DHT in the epididymis, whereas testosterone in seminal plasma is derived in more equal measure from the testis/epididymis and accessory glands. The relative concentrations of testosterone in the epididymis and seminal plasma suggest that testicular/epididymal fluid makes up only a small proportion (no more that 1/50th) of seminal plasma, which is supported by the lack of effect of vasectomy on the volume of the ejaculate. The estimation of DHT in seminal plasma may thus be a marker for epididymal 5αR activity.
This has been investigated in this Chapter. If the analysis of the results of the preceding Chapters are correct, the concentration of DHT in seminal plasma would be expected to be increased in men remaining oligozoospermic on supraphysiological doses of testosterone to a greater extent than in those men becoming azoospermic. A complication of this is that the testosterone substrate for epididymal 5αR may be derived mostly from direct testicular secretion, rather than via the peripheral circulation. It may therefore be reduced under the conditions of these studies. Comparison with changes in seminal plasma testosterone concentrations, which may predominantly reflect accessory gland concentrations, may provide some insight to this.

Adiol has been detected in the human epididymis (Purvis et al., 1978), although at concentrations close to the sensitivity of the assay used (approximately 5 ng/ml, compared to 20 ng/ml in serum). AdiolG has been reported in seminal plasma (Paulson, Bernstein, Marrs and Lobo, 1986), at a concentration 25% that in serum, i.e. several fold greater than the concentration of testosterone in seminal plasma. The concentrations of conjugated steroids are very high in seminal plasma, with for example the concentrations of sulphated testosterone and dihydrotestosterone being approximately 5 times that of the free steroid (Purvis et al., 1975). Serum concentrations of AdiolG are elevated by supraphysiological testosterone treatment (Chapter 5), and the effect of supraphysiological testosterone treatment on concentrations in seminal plasma are reported here.

6.2 Methods.

Semen samples were centrifuged at 2000g for 5 min to give a preparation of seminal plasma, which was stored at -20°C until assay. Subjects were asked to abstain from ejaculation for at least 2 days previously.

Testosterone, DHT and AdiolG were determined in seminal plasma samples at baseline and after 16 weeks of treatment. Radioimmunoassays were as described in Chapter 4 for testosterone, and in Chapter 5 for DHT and AdiolG.

For the testosterone assay, 100μl of sample in duplicate was assayed, compared to 50μl for plasma. This was because of the lower concentration of testosterone in seminal plasma. The antibody used shows 30% cross-reactivity with DHT (Corker and Davidson, 1978). Results presented here are not corrected for this.

Validation of the AdiolG assay for use with seminal plasma samples was carried out by testing for linearity of dilution and for recovery after spiking of seminal plasma with authentic AdiolG. Results of these experiments are shown together with results of similar experiments using serum in Figure 5.1.
6.3 Results.

The concentrations of testosterone in seminal plasma at baseline and after 16 weeks of testosterone treatment are shown in Figure 6.1. Testosterone treatment increased seminal plasma testosterone concentrations in both groups of men, from 1.31 ± 0.13 nmol/l to 2.55 ± 0.47 nmol/l in the azoospermic group (p<0.02), and from 1.25 ± 0.21 nmol/l to 2.58 ± 0.46 nmol/l in the oligozoospermic group (p<0.01). The mean increase was thus 94% in the azoospermic group and 106% in the oligozoospermic group, and similar to the increase in peripheral plasma concentrations reported in Chapter 3.

There was no significant effect of testosterone administration on seminal plasma DHT concentrations in the azoospermic group, which were 2.18 ± 0.31 nmol/l at baseline, and 2.54 ± 0.27 nmol/l after 16 weeks of testosterone treatment. In the oligozoospermic group, however, DHT was increased from 2.12 ± 0.29 to 2.94 ± 0.33 nmol/l (p<0.05). These results are shown in Figure 6.2.

The effect of testosterone administration on seminal plasma concentrations of AdiolG was similar to the effect on testosterone. In the azoospermic group, AdiolG concentrations were increased from 5.1 ± 1.5 to 9.3 ± 2.3 nmol/l (p<0.005), and in the oligozoospermic group from 6.0 ± 3.0 to 11.1 ± 3.1 nmol/l (p<0.001) (Figure 6.3).

6.4 Discussion.

Baseline seminal concentrations of testosterone (=1.3 nmol/l) are much lower than concentrations in peripheral plasma, as previously described, although the concentrations reported here may be an overestimate due to cross-reactivity of the antiserum with DHT (present in much greater concentration relative to testosterone in seminal plasma than in peripheral plasma). The concentration of testosterone in saliva is also much lower than in plasma, but in that case it closely approximates to (and has been claimed to be equivalent to) the concentration of free testosterone in plasma (Fahmy, Read, Walker and Griffiths, 1982). The concentration of testosterone in seminal plasma is intermediate to these two values, and also contrasts with the concentrations of LH and FSH in seminal plasma, which are similar to those found in plasma (Schoenfeld et al., 1978).

Testosterone administration caused an increase in seminal plasma concentrations of testosterone in both groups of men, and the magnitude of the increase was similar to that of testosterone in peripheral plasma. After 16 weeks of testosterone treatment, testicular production of testosterone is assumed to be greatly reduced secondary to the suppression of gonadotrophin secretion, and it therefore appears that the concentration
Figure 6.1
The effect of testosterone treatment on seminal plasma testosterone concentrations in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* p<0.02
** p<0.01 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 6.2
The effect of testosterone treatment on the concentration of DHT in seminal plasma in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* p<0.05 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 6.3
The effect of testosterone treatment on the concentration of AdiolG in seminal plasma in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* p<0.005
** p<0.001 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
of testosterone in seminal plasma reflects the systemic rather than the intratesticular concentration of testosterone. This is supported by the only small fall in seminal plasma testosterone concentration following vasectomy (Ying et al., 1983; Hudson et al., 1983).

The concentration of DHT in seminal plasma is reported to be similar to that in peripheral plasma (Purvis et al., 1975), and appears to be largely of testicular/epididymal origin. Thus it is reduced after vasectomy and in obstructive azoospermia to approximately 25% of normal (Le Lannou, 1980; Ying et al., 1983; Hudson et al., 1983). Baseline concentrations of DHT reported here are similar to those in previous reports, and are similar to the values obtained in peripheral plasma in Chapter 5. After 16 weeks of testosterone treatment, the concentration of DHT in seminal plasma was not significantly altered in the azoospermic group, but was increased in the oligozoospermic group (Figure 6.2). The lack of effect in the azoospermic group may be due to the combination of two opposing influences: an increased peripheral concentration tending to increase seminal plasma DHT concentrations, and a reduced supply from the testis/epididymis secondary to reduced testicular production of testosterone and thus less conversion to DHT within the epididymis. In contrast, the increase in seminal plasma DHT concentrations in the oligozoospermic group may reflect both the higher peripheral plasma concentration than in the azoospermic group, and increased 5αR activity in the epididymis, converting a greater proportion of the available testosterone. This result is therefore consistent with the hypothesis that the maintenance of spermatogenesis is associated with increased 5αR activity. This conclusion must, however, be cautious in view of the mixed origin of DHT in seminal plasma.

The baseline concentration of AdiolG in seminal plasma was 5.5 nmol/l, which is approximately 25% of that in serum (Chapter 5). There is only one previous report of AdiolG in seminal plasma, and the concentration found in that study was 161 ng/dl, i.e. 3.4 nmol/l. (Paulson et al., 1986). Testosterone treatment caused an increase in the concentration of AdiolG in seminal plasma in both groups of men, and to a similar degree (Figure 6.3). In both groups, the concentration of AdiolG was increased by approximately 80%. The magnitude of this increase is therefore similar to that of testosterone, and greater than the change in concentration of DHT. It therefore appears that both testosterone and AdiolG are found in seminal plasma in considerably lower concentrations than in blood, and in both fluids show large increases with testosterone treatment. In contrast DHT is present in similar amounts in seminal plasma and the increases in DHT are much smaller, and in fact there was no change in seminal plasma DHT concentration in the azoospermic group.
The origin of AdiolG in seminal plasma is unknown. In the only previous report of its presence, in a study of the steroid content of seminal plasma of men with idiopathic oligospermia (Paulson et al., 1986), there was no difference in seminal plasma AdiolG concentration between normal men and those with idiopathic oligospermia, although the latter had lower seminal plasma testosterone and DHT concentrations. The results presented here suggest that seminal plasma AdiolG concentration is related to testosterone concentration, but not DHT concentration. Pathways of androgen metabolism in the accessory glands appear to be similar to those elsewhere in the body, and have been extensively investigated in the prostate in particular (Krieg and Tunn, 1990). The small or non-existent changes in DHT observed here are therefore compatible with the DHT in seminal plasma being mostly from a different source to the testosterone and AdiolG, as suggested above. Any influence of the selective increase in seminal plasma DHT concentrations on AdiolG concentrations in the oligozoospermic group would be difficult to detect under such circumstances.

In conclusion, the selective increase in DHT concentration in seminal plasma in those men remaining oligozoospermic during testosterone treatment is compatible with the hypothesis that there is increased 5αR activity in this group, but the lack of clear evidence as to the relative contributions of various organs to androgens in seminal plasma complicates interpretation.
Chapter 7

7.1 Introduction.

In previous Chapters of this Thesis, results of the investigation of the possible role of the enzyme 5αR in the maintenance of spermatogenesis in those men who remain oligozoospermic on supraphysiological doses of testosterone have been presented. In particular, this has involved the determination of the conversion rate of testosterone to DHT, and plasma concentrations of DHT and AdiolG, i.e. products of 5α-reduction of testosterone. In this Chapter, the plasma concentrations of precursor steroids and urinary excretion of steroid metabolites are investigated.

The liver contains the enzymes 5αR and 5βR, which result in the formation of the 17-ketosteroids androsterone (A) and aetiocholanolone (E) respectively, which can be measured in the urine. In congenital 5αR deficiency, the urinary excretion of A is reduced relative to that of E, such that the E/A ratio is greatly elevated, from approximately unity in normal men to a mean of 5 in men with 5αR deficiency, whereas it is normal in men with testicular feminisation (Peterson et al., 1977). This enzyme pair also converts cortisol to the 5αR / 5βR metabolites allo-tetrahydrocortisol (aTHF) and tetrahydrocortisol (THF), and the determination of these corticosteroid metabolites in urine has proved useful in the early diagnosis of 5αR deficiency in infancy when A and E are excreted in insufficient quantity for detection (Imperato-McGinley et al., 1986). In particular, the ratios of these metabolites (i.e. E/A and THF/aTHF) provides an index of hepatic 5αR activity.

Treatment of men with the 5αR inhibitor, finasteride, alters urinary excretion of androgens, resulting in an E/A ratio similar to that of men with 5αR deficiency (Vermuelen et al., 1989; Imperato-McGinley et al., 1990). Finasteride therefore appears to inhibit hepatic as well as peripheral 5αR. If men who remain oligozoospermic on supraphysiological doses of testosterone as used in this study have a greater 5αR activity than those who become azoospermic, then this may be reflected in the pattern of urinary androgen excretion as well as in plasma androgen concentrations, with increased excretion of A relative to that of E, and with increased excretion of aTHF relative to that of THF. Such differences, however, may not be nearly as clear-cut as in men with congenital 5αR deficiency.
Andersson, Bishop and Russell (1989), however, found that orchidectomy increased 5αR activity and mRNA in the liver of rats, and similarly androgens (in particular DHT) have been reported to inhibit 5αR in the rat adrenal cortex (Andò et al., 1989; Canonaco, Andò, Valenti, Tavolaro, Panno, Maggiolini and Dessl-Fulgheri, 1989; Lephart et al., 1991). Administration of testosterone may therefore decrease hepatic and adrenal 5αR activity in men, resulting in increased E/A and THF/aTHF ratios.

The measurement of THF, aTHF and of tetrahydrocortisone (THE) allows the estimation of the activity of the enzyme 11β-hydroxysteroid dehydrogenase (11βOHSD). This enzyme converts cortisol to the inactive metabolite cortisone, which is excreted as THE in the urine, and the ratio of THE to the sum of THF and aTHF is an index of its activity (Walker and Edwards, 1991). This enzyme is of particular importance in the distal tubule of the kidney, “protecting” the mineralocorticoid receptor from cortisol (Fraser, 1990), but is also found in the testis (Phillips, Lakshmi and Monder 1991) where its function is unknown.

The results of two further series of investigations are also presented here: the plasma concentrations of precursors of testosterone synthesis, and the possible contribution of adrenal secretion of androgens to the maintenance of spermatogenesis. Androstenedione is the proximate precursor of testosterone in steroidogenesis and is reversibly converted to testosterone by the enzyme 17β-hydroxysteroid dehydrogenase, both in the testis and peripherally, eg in the skin (Mowszowicz et al., 1983). 17OH-progesterone is a crucial intermediate, as it is the metabolite at which the synthetic pathways for C19 and C21 steroids diverge, and its urinary metabolite is pregnanetriol. Dehydroepiandrosterone-sulphate (DHAS) is the major adrenal androgen, and 11β-hydroxy steroids have been proposed to be specifically of adrenal origin (Goldzieher, Pena and Aivaliotis, 1978; Polson, Reed, Franks, Scanlon and James, 1988). These steroids and their urinary metabolites were measured to investigate the possible contribution of continuing androgen synthesis in the testis and adrenal to the maintenance of spermatogenesis in those men remaining oligozoospermic on supraphysiological doses of testosterone.

### 7.2 Methods.

Subjects collected 24 hr urine samples before testosterone treatment started and after 16 weeks of treatment. The second collection was made during the 24 hrs before the next testosterone injection was due, and therefore reflects steroid excretion at a time when
testosterone concentrations were at a nadir. The volume of each collection was recorded, and aliquots were stored at -20°C until assay.

7.2.1 Urinary steroid measurements.

Urinary steroid profile analysis was performed by capillary column gas chromatography after derivatisation of unconjugated steroids. This method allows the determination of up to 30 steroid metabolites excreted as either free steroids or conjugated to glucuronides or sulphates.

Steroid metabolites were extracted from 10 ml of urine by passage through a Sep-Pak C18 column and elution with methanol. The extract was dried down under nitrogen, reconstituted in acetate buffer to which Helix Pomatia juice was added for hydrolysis of steroid conjugates. Samples were incubated at 37°C for 36 hrs, steroids extracted using Sep-Pak columns, and internal standards (5α-androstane-3α,17α-diol, cholesterol butyrate and Stigmasterol) were then added. After washing with 8% NaHCO₃, the samples were derivitised by incubation overnight with methoxyoxime trimethylsilyl ether at 100°C. Excess derivatisation agent was removed by passage down a Lipidex 5000 column. Steroid derivatives were separated using a model 438 gas chromatograph (Packer-Becker BV, Netherlands), fitted with a “Flexsyl” 25m x 0.32mm capillary column coated with OV-1 (Phase-Sep, Queensferry, Clywd) and quantified by comparison with peak height of the internal standards.

7.2.2 Radioimmunoassays of androstenedione, 17OH-Progesterone, and dehydroepiandrosterone.

Androstenedione and 17OH-progesterone were measured by established “in-house” RIAs (Wallace and Wood, 1984; Thomson, Wallace and Cook, 1989). The 17OH-progesterone assay involved the use of microencapsulated antibody. DHAS was measured directly by RIA using a commercial kit (Diagnostic Products Corporation).

7.3 Results.

7.3.1. Urinary excretion of androsterone and aetiocholanolone.

The excretion of both A and E was significantly increased by testosterone administration in all men (Figure 7.1). Excretion of A was increased from 2.12 ± 0.35 mg/24 hrs to 3.49 ± 0.38 mg/24 hrs in the azoospermic group (p<0.005), and from 2.08 ± 0.32 mg/24 hrs to 3.43 ± 0.57 mg/24 hrs in the oligozoospermic group
Figure 7.1.
The effect of testosterone treatment on urinary excretion of androsterone and aetiocholanolone in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks of testosterone treatment.

* $p<0.02$
** $p<0.005$
*** $p<0.001$ all vs baseline.

Mean ± sem, $n=18$, azoospermic group
$n=15$, oligozoospermic group.
Excretion of E was increased from 2.00 ± 0.29 mg/24 hrs to 3.15 ± 0.39 mg/24 hrs in the azoospermic group (p<0.02), and from 1.86 ± 0.27 mg/24 hrs to 3.07 ± 0.40 mg/24 hrs in the oligozoospermic group (p<0.001). Calculation of the E/A ratio (Figure 7.2) showed that there was a small but statistically significant decrease in the ratio in the azoospermic group from 1.02 ± 0.08 to 0.93 ± 0.07 (p<0.01), but there was no change in the ratio in the oligozoospermic group (0.97 ± 0.08 to 0.99 ± 0.08).

There was a close correlation between A and E excretion both at baseline and after 16 weeks of testosterone treatment (Figure 7.3, a and b). The correlation coefficient was 0.90 at baseline, and 0.84 after 16 weeks of testosterone treatment (p<0.001 in both cases), using results for all subjects.

### 7.3.2 Urinary excretion of cortisol metabolites.

Three major urinary metabolites of cortisol were measured: the 5αR and 5βR pair aTHF and THF, and THE. The excretion of all three metabolites was not significantly affected in either group after 16 weeks of testosterone treatment (nor were there any significant effects when the results were analysed as one group). Results are shown in Table 7.1 and Figure 7.4. This shows that although the 24 hr excretion of the metabolites appeared to be unchanged in both groups, there was a statistically significant increase in the THF/aTHF ratio in the oligozoospermic group from 1.13 ± 0.14 to 1.30 ± 0.15 (p<0.005), but no change in that ratio in the azoospermic group (1.27 ± 0.14 to 1.28 ± 0.12) (Figure 7.5).

<table>
<thead>
<tr>
<th>Group</th>
<th>aTHF Baseline</th>
<th>aTHF Treatment</th>
<th>THF Baseline</th>
<th>THF Treatment</th>
<th>THE Baseline</th>
<th>THE Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoospermic</td>
<td>2.01 ± 0.30</td>
<td>2.02 ± 0.25</td>
<td>2.20 ± 0.27</td>
<td>2.41 ± 0.29</td>
<td>3.82 ± 0.46</td>
<td>3.95 ± 0.53</td>
</tr>
<tr>
<td>Oligozoospermic</td>
<td>2.70 ± 0.51</td>
<td>2.41 ± 0.53</td>
<td>2.49 ± 0.29</td>
<td>2.46 ± 0.28</td>
<td>4.45 ± 0.47</td>
<td>3.94 ± 0.56</td>
</tr>
</tbody>
</table>

24 hr urinary excretion of aTHF, THF and THE. All values mg/24 hrs, mean ± sem.

The THE/(THF+aTHF) ratio was also significantly reduced in the oligozoospermic group after 16 weeks of testosterone treatment, from 0.94 ± 0.10 to 0.84 ± 0.06
Figure 7.2.
Effect of testosterone on urinary E/A ratio in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* p<0.01 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 7.3.
Correlation between 24 hr urinary excretion of Androsterone and Aetiocholanolone.

(a) Baseline.
(b) After 16 weeks testosterone treatment.

Correlation coefficients:
(a) r=0.90, p<0.001
(b) r=0.84, p<0.001.
Figure 7.4
The effect of 16 weeks testosterone treatment on 24 hr urinary excretion of the major metabolites of cortisol, aTHF, THF and THE, in those men becoming azoospermic and those remaining oligozoospermic.

(a) aTHF.
(b) THF.
(c) THE

Open columns, baseline
Hatched columns, after 16 weeks testosterone treatment.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 7.5
The effect of testosterone treatment on THF/aTHF ratio in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* $p<0.005$ vs baseline

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
(p<0.05), but was unchanged in the azoospermic group (0.93 ± 0.05 to 0.90 ± 0.05) (Figure 7.6).

The correlation between urinary excretion of αTHF and THF was determined. The correlation coefficient was 0.82 (p<0.001) both at baseline and after 16 weeks of testosterone treatment, using results for all subjects (Figure 7.7). Correlations were also determined between C₁₉ and C₂₁ β/α ratios, i.e. between E/A and THF/αTHF ratios (Figure 7.8). The correlation coefficient was 0.78 at baseline and 0.90 after 16 weeks of testosterone treatment.

7.3.3 Plasma concentration of 17OH-progesterone and urinary excretion of pregnanetriol.

Plasma concentrations of 17OH-progesterone were reduced in both groups, from 4.6 ± 0.4 nmol/l to 2.5 ± 0.2 nmol/l in the azoospermic group (p<0.001), and from 5.4 ± 0.3 nmol/l to 2.4 ± 0.2 nmol/l in the oligozoospermic group (p<0.005) (Figure 7.9a) after 16 weeks of testosterone treatment.

Pregnanetriol excretion was also significantly reduced in both groups after 16 weeks of testosterone treatment. In the azoospermic group, pregnanetriol excretion was reduced from 0.95 ± 0.14 mg/24 hrs to 0.59 ± 0.11 mg/24 hrs (p<0.005), and in the oligozoospermic group from 0.99 ±0.13 mg/24 hrs to 0.55 ± 0.15 mg/24 hrs (p<0.01) (Figure 7.9b).

7.3.4 Plasma concentration of androstenedione.

Plasma concentrations of androstenedione were significantly increased in both groups after 16 weeks of testosterone treatment (Figure 7.10). In the azoospermic group, plasma androstenedione was increased from 6.8 ± 0.5 nmol/l to 9.3 ± 0.5 nmol/l (p<0.001), and from 6.9 ± 0.5 nmol/l to 8.5 ± 0.7 nmol/l in the oligozoospermic group (p<0.005).

7.3.5 Plasma concentration and urinary excretion of DHAS.

The plasma concentration of DHAS was reduced by testosterone treatment, but only in those men who became azoospermic (Figure 7.11a). Thus after 16 weeks of testosterone treatment, plasma concentrations of DHAS were reduced from 11.5 ± 1.6 μmol/l to 9.4 ± 1.0 μmol/l (p<0.05) in the azoospermic group, whereas there was no change in the plasma concentration of DHAS in the oligozoospermic group (9.1 ± 1.3 μmol/l to 9.1 ± 1.4 μmol/l).
Figure 7.6
The effect of testosterone treatment on the urinary ratio of \( \text{THE}/(\text{aTHF} + \text{THF}) \) in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline
Hatched columns, after 16 weeks testosterone treatment.

* \( p<0.05 \) vs baseline

Mean ± sem, \( n=18 \), azoospermic group
\( n=15 \), oligozoospermic group.
Figure 7.7
Correlation between the urinary excretion of aTHF and THF, the 5αR and 5βR metabolites of cortisol. Data from all men.

(a) At baseline
(b) After 16 weeks testosterone treatment.

Correlation coefficient r=0.82, p<0.001 in both cases.
Figure 7.8
Correlation between β/α ratios of C19 and C21 steroids. Results from all men.

(a) Baseline
(b) After 16 weeks testosterone treatment.

Correlation coefficients
r= 0.78 (p<0.001) baseline
r= 0.90 (p<0.001) after 16 weeks.
Figure 7.9
The effect of testosterone treatment on plasma concentrations of 17αOH-progesterone and the urinary excretion of pregnanetriol in those men becoming azoospermic and those remaining oligozoospermic.

(a) 17αOH-progesterone.
(b) Pregnenetriol.

Open columns, baseline
Hatched columns, after 16 weeks testosterone treatment.

* p<0.01
** p<0.005
*** p<0.001 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 7.10
Effect of testosterone on plasma concentrations of androstenedione in those men becoming azoospermic and those remaining oligozoospermic.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* p<0.005
** p<0.001 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
DHAS in urine is hydrolysed to DHA prior to analysis by this methodology, and the result thus obtained is the sum of DHAS and DHA. To distinguish between plasma and urinary results, the convention of referring to the urinary results as DHA will be followed.

The urinary excretion of DHA showed parallel changes. Thus it was significantly decreased by testosterone treatment in the azoospermic group, from 0.91 ± 0.19 mg/24 hrs to 0.37 ± 0.10 mg/24 hrs (p<0.002), but excretion of DHA in the oligozoospermic group was unchanged (0.85 ± 0.26 mg/24 hrs to 0.72 ± 0.30 mg/24 hrs). These results are shown in Figure 7.11b. Urinary excretion of 16OH-DHA was also measured, and similar results were obtained. Excretion of 16OH-DHA was 0.91 ± 0.14 mg/24 hrs in the azoospermic group at baseline, and 0.79 ± 0.15 mg/24 hrs after 16 weeks of testosterone treatment, and were 0.93 ± 0.20 mg/24 hrs in the oligozoospermic group at baseline and 0.92 ± 0.30 mg/24 hrs after 16 weeks of testosterone treatment. The decrease in the azoospermic group did not reach statistical significance due to the wide individual variation, but the sum of DHA + 16OH-DHA was significantly reduced in this group, from 1.82 ± 0.31 mg/24 hrs to 1.15 ± 0.25 mg/24 hrs (p<0.05), while there was no change in the oligozoospermic group (1.78 ± 0.45 mg/24 hrs to 1.65 ± 0.53 mg/24 hrs).

Urinary excretion of 11β-hydroxy androsterone (11β-OHA) and 11β-hydroxy aetiocholanolone (11β-OHE) was also measured (Figure 7.12). In the azoospermic group, excretion of both metabolites was reduced, although that of 11β-OHA did not reach statistical significance. Excretion of 11β-OHA was reduced from 0.92 ± 0.15 mg/24 hrs to 0.72 ± 0.11 mg/24 hrs and of 11β-OHE from 0.37 ± 0.06 mg/24 hrs to 0.29 ± 0.04 mg/24 hrs (p=0.05). In the oligozoospermic group, neither metabolite was significantly affected. 11β-OHA excretion was 1.04 ± 0.12 mg/24 hrs at baseline and 0.91 ± 0.17 mg/24 hrs after 16 weeks of testosterone treatment, 11β-OHE excretion was 0.35 ± 0.05 mg/24 hrs and 0.34 ± 0.04 mg/24 hrs respectively.

7.4 Discussion.

There were no differences in the plasma concentration or urinary excretion of any of the steroid metabolites reported in this Chapter between those men who became azoospermic and those remaining oligozoospermic, either at baseline or after 16 weeks of testosterone treatment. The absolute concentrations of these parameters cannot therefore be used to distinguish between the groups, either before or during treatment. There were, however, several differences between the two groups of men in the pattern of response to supraphysiological doses of testosterone. These will be discussed under
Figure 7.11
The effect of testosterone on the plasma concentrations of DHAS and urinary excretion of DHA in those men becoming azoospermic and those remaining oligozoospermic.

(a) Plasma concentration of DHAS.
(b) 24 hr urinary excretion of DHA.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* p<0.005
** p<0.002 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
Figure 7.12
The effect of testosterone treatment on 24 hr urinary excretion of 11β-hydroxyandrosterone and 11β-hydroxyaetiocholanolone.

(a) 11β-hydroxyandrosterone.
(b) 11β-hydroxyaetiocholanolone.

Open columns, baseline.
Hatched columns, after 16 weeks testosterone treatment.

* p<0.05 vs baseline.

Mean ± sem, n=18, azoospermic group
n=15, oligozoospermic group.
the headings of effects on 5α/β reductase metabolites, cortisol metabolites, testosterone precursors, and adrenal androgens.

7.4.1 5α/β reductase metabolites

Androsterone and aetiocholanolone are 17-ketosteroids excreted by the liver after the metabolism of testosterone by 5αR and 5βR respectively. The increase in urinary excretion of these two steroids (Figure 7.1) therefore reflects the increase in plasma testosterone concentrations during testosterone treatment (Chapter 3), although excretion of both A and E was increased by approximately 60% compared to increases of over 100% in plasma testosterone. In contrast there was, as expected, no evidence for an effect on the concentrations of cortisol metabolites in urine (Figure 7.4), either in the excretion of aTHF, THF or THE.

These results were further examined to investigate whether there was any evidence for an effect of testosterone administration on the activity of hepatic 5αR. The activity of this enzyme in vivo is often expressed as a ratio of the products of 5βR to 5αR, i.e. E/A or THF/aTHF, as it has been shown that the same enzyme converts testosterone to A as cortisol to aTHF (Fisher et al., 1978). This assumes that the two enzymes are independently controlled, which is without experimental basis, but has provided useful in the investigation of 5αR deficiency, the effects of finasteride on 5αR activity, and steroid metabolism in PCOS (Peterson et al., 1977; Imperato-McGinley et al., 1990; Stewart et al., 1990). In these studies, β/α ratios were found to be greatly elevated in 5αR deficiency or following finasteride administration, from approximately unity to between 2 and 12 in 5αR deficiency (E/A) and to 25 (E/A) or as much as 150 (THF/aTHF) following finasteride administration (Imperato-McGinley et al., 1990). In PCOS, the E/A ratio was 0.75 compared to 1.35 in controls, and the THF/aTHF ratio was 1.10 compared to 1.64 in controls, and this increase in activity of 5αR has been suggested to be the aetiological abnormality in this condition (Stewart et al., 1990). Plasma androgen concentrations (androstenedione and testosterone) were 75% higher in the PCOS group than in the control group (Stewart et al., 1990), whereas plasma testosterone was unaffected by finasteride treatment but was approximately doubled in the subjects with 5αR deficiency (Peterson et al., 1977; Imperato-McGinley et al., 1990).

These comparisons suggest that finasteride causes a much greater inhibition of hepatic 5αR than is present in subjects with autosomal recessive 5αR deficiency, and may be a consequence of the normal expression of 5αR 1 in that condition (Andersson et al., 1991). Finasteride has a much lower IC₅₀ for 5αR 2 than 5αR 1 (Andersson et
al., 1991), but this degree of selectivity in vitro may not be so clear in vivo, especially at the higher doses used in the study of Imperato-McGinley et al. (1990).

In the present study, testosterone administration was found to cause a small but statistically significant decrease in the E/A ratio in the azoospermic group, with no effect in the oligozoospermic group (Figure 7.2). In contrast, there was a significant increase in the THF/aTHF ratio in the oligozoospermic group but not in the azoospermic group (Figure 7.5). The E/A ratio result therefore suggests increased 5αR or decreased 5βR activity in the azoospermic group during testosterone treatment, while the THF/aTHF result suggests decreased 5αR activity in the oligozoospermic group. It therefore appears that testosterone administration has little overall effect on hepatic 5αR activity in either group, particularly compared to the effect of 5αR deficiency or finasteride administration.

Close correlations were found between the urinary excretion of 5αR and 5βR pairs, both before and after 16 weeks of testosterone treatment (Figure 7.3). Similar correlations were found for both androgens and cortisol metabolites (Figure 7.7), and between β/α ratios of both classes of metabolite (Figure 7.8). These results suggest that the same enzymes convert C_{19} and C_{21} steroids, as previously suggested (Fisher et al., 1978), and that the same pool of precursor is metabolized by the two enzymes.

Orchidectomy has been reported to increase 5αR activity and mRNA in the liver and adrenal of rats, and androgens have been shown to inhibit 5αR activity and reduce the concentration of 5αR mRNA in the adrenal cortex (Andersson et al., 1989; Andò et al., 1989; Canonaco et al., 1989; Lephart et al., 1991). These effects contrast with the effects of androgens in the skin, prostate and epididymis (using human tissue), in which an enhancing effect of DHT on 5αR activity and mRNA concentrations has been demonstrated (Chapter 1.6). A further contrast is evident in reports of urinary excretion of androgens by men with testicular feminisation: this group of men were found to have a normal urinary E/A ratio, in contrast to the very low concentration of 5αR activity in pubic skin in this condition (Peterson et al., 1977; Kuttenn et al., 1979). It therefore appears that hepatic and adrenal 5αR may have different control mechanisms to the enzyme elsewhere in the body, but extrapolation between species may be misleading.

### 7.4.2 Other effects on cortisol metabolites

The conversion of cortisol to cortisone is a major route of inactivation of the biologically active hormone, and is carried out by the enzyme 11β-hydroxysteroid dehydrogenase (11βOHSD). This enzyme has a striking similarity of function to 5αR
as a modulator of potency of steroid effect, in that it is believed to protect the distal tubule of the kidney from the effects of cortisol (Fraser, 1990; Walker and Edwards, 1991). The mineralocorticoid receptor has a similar affinity for aldosterone and cortisol, but as the latter is produced in 1000 times the amount and does not have the biological effect of the mineralocorticoid, the kidney appears to be protected from it. This is afforded by the conversion of cortisol to cortisone (which has low affinity for the mineralocorticoid receptor) by the enzyme 11βOHSD, which occurs in a paracrine manner within the tubule. Deficiency of this enzyme results in hypokalaemia and hypertension but without mineralocorticoid excess. Cortisone is predominantly excreted in the urine as tetrahydrocortisone (THE), and the activity of this enzyme can be expressed as the ratio of THE to THF and αTHF, i.e. THE/(αTHF+THF). The results presented here suggest that 11βOHSD activity is reduced by testosterone administration, but only in the oligozoospermic group, with no effect on the ratio in the azoospermic group (Figure 7.6). As with the β/α ratios discussed above, this effect was only small, but was statistically significant.

Physiological modulation of 11βOHSD activity is not established, although several steroid hormones have been reported to affect it and it is inhibited by liquorice (Walker and Edwards, 1991). Thus hepatic 11βOHSD activity is higher in male than female rats, it is reduced by orchidectomy, and is restored by testosterone administration (Lax, Ghraf and Schriefers, 1978). 11βOHSD is present in the rat testis; immunofluorescent staining was undetectable in the foetal testis and before sexual maturation, but in the adult the entire interstitial region showed dense staining (Phillips et al., 1989). These authors proposed that 11βOHSD serves to protect the adult testis from the effects of corticosterone, as corticosteroids are known to inhibit testosterone synthesis (Bambino and Hsueh, 1981). Men with Cushing’s syndrome have reduced plasma concentrations of testosterone and pathological changes in the testes (McKenna, Lorber, Lacroix and Rabin, 1979).

No clear conclusion can be drawn from these preliminary findings, but the demonstration of a difference between men remaining oligozoospermic and those becoming azoospermic on supraphysiological doses of testosterone in the activity of an enzyme which may be of importance in intratesticular steroidogenesis and which shows some functional parallels with 5αR is noteworthy. The results presented here do not provide any evidence as to the site of action of testosterone on 11βOHSD activity.
7.4.3 Effects on testosterone precursors

Plasma and urinary concentrations of two intermediates in steroid biosynthesis were also determined, to investigate the effect of testosterone administration for a prolonged period on steroid synthesis and whether there were detectable differences between men becoming azoospermic and those remaining oligozoospermic.

17β-progesterone is a key intermediate in steroid biosynthesis, as it is the product of 17α-hydroxylase on progesterone and the site at which corticosteroid and sex steroid biosynthetic pathways diverge (Figure 1.1). As an intermediate of the Δ4 pathway in the testis, it may not be of as great importance as the Δ5 intermediate 17β-pregnenolone, but it is found in spermatic vein blood in concentrations approximately 40 fold greater than in peripheral plasma (Laatikainen, Laitinen and Vihko, 1971; Hammond et al., 1977) and parallel increases in plasma testosterone and 17β-progesterone concentrations are seen after hCG injection and LH infusion in men (Laatikainen et al., 1971; Forest, Lecog and Saez, 1979; Nankin, Lin, Murono, Osterman and Troën, 1980). The blood production rate has been estimated to be 1.8 mg/24 hrs, with 90% secreted by the testes (Strott, Yoshimi and Lipsett, 1969). The major urinary metabolite of 17β-progesterone is pregnanetriol.

Plasma concentrations of 17β-progesterone and 24 hr urinary excretion of pregnanetriol were reduced after 16 weeks of testosterone treatment in both groups of men (Figure 7.9), with a fall of approximately 45% in both plasma and urinary steroids. These results suggest that the Δ4 pathway is suppressed by testosterone treatment in all men, and confirms the result of Strott et al. (1969), who found that androgen administration (40 mg fluoxymesterone for 3 days) reduced the plasma concentration of 17β-progesterone by 90% in normal men. As the great majority of plasma 17β-progesterone is derived from the testis, it is likely that this is secondary to the suppression of LH secretion by testosterone (Chapter 3), although the rapidity of effect reported by Strott et al. is difficult to reconcile with the time-course of the fall in LH secretion reported here. It is not possible to make deductions as to the degree of ongoing activity of testicular steroidogenesis on the basis of this result, as even on the most simple assumption that the rate of secretion of intermediates reflects the intracellular concentration, this will be affected by changes in both the rate of synthesis of the intermediate and the rate of further metabolism. Thus if the rate of synthesis is drastically reduced, an equal fall in the rate of metabolism will approximately maintain the intracellular concentration. LH enhances the transcription of genes encoding enzymes in the steroidogenic pathway, and a fall in the concentration of these enzymes results from the absence of LH (Waterman and Simpson, 1989).
Androstenedione is the proximate precursor of testosterone (Chapter 1.2), and is secreted by the testis (Hammond et al., 1977) and adrenal cortex. In some patients, plasma androstenedione concentrations were increased following orchidectomy, and in this group (who also showed a lesser fall in plasma testosterone), the remaining plasma concentrations of testosterone and androstenedione were suppressed by dexamethasone (Sciarrta, Sorcini, di Silverio and Gagliardi, 1973). In a second group of men whose plasma testosterone concentrations fell more after orchidectomy, there was only a small, transient increase in plasma androstenedione concentrations. Others have reported a decrease in plasma androstenedione concentrations after orchidectomy (Bélanger, Dupont and Labrie, 1984). These studies were carried out on older men being treated for prostatic carcinoma, and may thus not be comparable with healthy, younger men. Nevertheless, these results show a smaller effect of orchidectomy on plasma androstenedione concentrations than on plasma testosterone or 17 OH-progesterone concentrations, and are therefore consistent with the adrenal being an important source of androstenedione in men.

The fall in plasma 17OH-progesterone and urinary pregnanetriol is thought to reflect reduced testicular steroidogenesis, and it would appear reasonable to assume that testicular androstenedione secretion would be similarly reduced. However, after 16 weeks of testosterone administration, plasma androstenedione concentrations were increased in both groups of men, by approximately 35% (Figure 7.10). This increase may reflect an increase in secretion from the adrenal, but perhaps a more likely explanation is an increased conversion of administered testosterone to androstenedione in the skin. The skin has 17β-hydroxysteroid dehydrogenase activity (Sansone-Bezzano, Seeler, Cummings and Reisner, 1979) and contains a high concentration of androstenedione (approximately 3 fold greater than that of testosterone, Toth and Faredin, 1985). Perhaps clearer evidence for an effect of testosterone administration on the secretion of androgens by the adrenal is discussed below, in the light of the effect of testosterone treatment on the plasma concentration of DHAS and urinary excretion of DHA and 11β-OHA and 11β-OHE.

The plasma concentration of androstenedione is also elevated by the administration of finasteride in doses which caused an increase in plasma testosterone without changes in plasma LH concentrations (Gormley et al., 1990). In rats, finasteride causes an increase in intratesticular testosterone concentration (George et al., 1989). The increase in plasma androstenedione with finasteride treatment may therefore reflect increased back-conversion from testosterone both in the skin and testis.
7.4.4 Effects on adrenal androgens

DHAS and DHA are secreted by the human testis, but only in small quantities (Gandy and Peterson, 1968; Laatikainen et al., 1971; Nieschlag, Loriaux, Ruder, Zucker, Kirschner and Lipsett, 1973; de la Torre, Hedman, Norën and Sjöberg, 1986). This is supported by the finding that orchidectomy does not affect plasma concentrations (Gandy and Peterson, 1968, Parker, Lai, Wolk, Lifrak, Kim, Epstein, Hadley and Miller, 1984; Bélanger et al., 1984). In contrast, the gradient of DHA across the adrenal is large (Nieschlag et al., 1973). DHAS is also secreted by the adrenal (Nieschlag et al., 1973), but in small quantities; the very low metabolic clearance rate (20 l/day) compensates for this, resulting in a large plasma pool (Baulieu, Corpéchot, Dray, Emiliozzi, Lebeau, Mauvais-Jarvis and Robel, 1965). Thus most plasma DHAS is thought to be secreted directly, with only approximately 15% arising from peripheral conversion of DHA, whereas DHA has a very high rate of secretion and metabolic clearance rate (1800 l/day, Horton and Tait, 1967), and the majority of circulating DHA is thought to arise from conversion of DHAS.

Infusion of ACTH increases the secretion of DHA (Nieschlag et al., 1973; Vermuelen and Andö, 1978), and dexamethasone caused approximately 65% suppression of plasma DHA concentrations (Nishida, Matsumara, Masaharu, Oyama and Tenku, 1977). Synchronous secretion of DHA and cortisol has been reported (Rosenfield, Hellman, Roffwarg, Weitzman, Fukushima and Gallagher, 1971), presumably in response to ACTH, but others have proposed that ACTH may have a more long-term permissive effect on adrenal androgen secretion in physiological conditions (Parker and Odell, 1980).

The existence of one or more additional adrenal androgen stimulatory factors has been proposed to account for situations in which cortisol secretion appears to be dissociated from that of adrenal androgens (Grumbach, Richards, Conte and Kaplan, 1978; Parker and Odell, 1980), and Parker, Lifrak and Odell (1983) reported the isolation of a glycoprotein of 60,000 daltons molecular weight from human pituitary which stimulated the production of DHA but not cortisol from dog adrenal cells. The situations in which adrenal androgen secretion is dissociated from that of cortisol include adrenarche, during fasting, and during recovery from adrenal suppression. The term adrenarche refers to the rise in adrenal androgens which occurs before puberty, and contributes to the development of pubic and axillary hair (Albright, 1947). At this time there is increased secretion of DHA without increased secretion of cortisol (Hopper and Yen, 1975), and it is associated with the development of the zona reticularis of the adrenal cortex (Dhom, 1973).
Steroidogenesis and the response to ACTH are similar in cells from the zona fasciculata and the zona reticularis in culture (Neville and O’Hare, 1979), which implies that there is no fundamental difference in the properties of the two cell types. During steroidogenesis, pregnenolone leaves the mitochondrion, and is then converted by the enzyme 17α-hydroxylase to 17α-hydroxy pregnenolone and then by C17,20-lyase to DHA. Alternatively, 3β-hydroxysteroid dehydrogenase can convert 17α-hydroxy pregnenolone to 17α-hydroxy progesterone, which is then converted to cortisol. ACTH induces increases in the activity of all these enzymes, but in the human adrenal, 3β-hydroxysteroid dehydrogenase activity is low (Hornsby and Aldern, 1984). It has therefore been suggested that discrepancies between adrenal androgen and cortisol secretion may result from changes in the activity of 3β-hydroxysteroid dehydrogenase, with a decrease in activity reducing the conversion of 17α-hydroxy pregnenolone to 17α-hydroxy progesterone and thus promoting the synthesis of DHA relative to cortisol (Adams, 1985).

An alternative possibility is that the regulatory site is the enzyme C17,20-lyase. This is suggested by the finding that, as the adrenal matures, there is increased supply of 17α-hydroxy pregnenolone in response to ACTH stimulation, the percentage converted to DHA increases, and there is less converted to the A4 pathway (Rich, Rosenfield, Lucky, Helke and Otto, 1981). These findings suggest that there is an increase in activity of C17,20-lyase with maturation, with reduced activity of 3β-hydroxysteroid dehydrogenase. A reduction in activity of C17,20-lyase has been suggested to underlie the attenuation of ultradian and circadian rhythms of DHA secretion in postmenopausal women, who showed a reduced pulse amplitude of DHA secretion without any change in pulse frequency, and a decrease in the acrophase amplitude compared to younger cycling women, without any effect on cortisol secretion (Liu, Laughlin, Fischer and Yen, 1990).

These findings account for the dissociation between adrenal androgen and cortisol metabolism without the need for an adrenal androgen-stimulating hormone, although Rich et al. (1981) suggest the existence of an “adrenarche factor”, to bring about the development of an ACTH-responsive zona reticularis.

Urinary excretion of DHA was reduced from 910 ± 140 μg/24 hrs to 370 ± 101 μg/24 hrs in the azoospermic group after 16 weeks of testosterone treatment, but there was no effect in the oligozoospermic group (Figure 7.11). Urinary excretion of 16OH-DHA was also selectively reduced in the azoospermic group, although individual concentrations were very variable and the result did not reach statistical significance. These changes were paralleled by changes in plasma concentrations of DHAS, also selectively reduced in the azoospermic group, although the change was smaller. There
was no change in the plasma concentration of DHAS in the oligozoospermic group (Figure 7.11).

11β-OHA and 11β-OHE are products of the enzyme 11β-hydroxylase. This enzyme is thought to be selectively present in the adrenals: thus activity was undetectable in the ovary, and castrated men had normal levels of plasma 11β-hydroxyandrostenedione (Goldzieher et al., 1978). Urinary excretion of both these metabolites were reduced in the azoospermic group, although the reduction in excretion of 11β-OHA did not reach statistical significance (Figure 7.12). There was no effect of testosterone treatment on either metabolite in the oligozoospermic group. These results therefore support the DHA and DHA results, and provide further evidence for a selective effect of testosterone treatment on adrenal androgens.

The physiological importance of adrenal androgens is uncertain, particularly in men. DHA is a weak androgen, but both DHA and DHAS can be converted to DHT within the prostate in vivo and in vitro (Farnsworth, 1973; Harper, Pike, Peeling and Griffiths, 1974). In castrated rats, infusion of DHA increases prostatic DHT concentrations and prostatic size (Labrie, Bélanger and Labrie, 1988). A stimulatory effect on androgen-dependent prostatic gene expression has also been reported in response to DHA infusion in the castrated rat, and this effect was antagonised by flutamide (Labrie, Simard, Zhao, Bélanger, Pelletier and Labrie, 1989). Castration has a limited effect on prostatic DHT in man, causing only 70% reduction in tissue concentrations (Geller, Albert, Loza, Geller, Stultzing and Vega, 1978), which supports an important role for adrenal androgens as precursors. This is also supported by the recent demonstration of a much more complete suppression of prostatic DHT concentrations by finasteride (Geller, 1990, McConnell et al., 1992). Conversion of androstenedione, DHA and DHAS to testosterone and DHT has also been demonstrated in skin (Cameron, Baillie, Grant, Milnes and Thompson, 1966; Gomez and Hsia, 1968; Kaufman, Stanczyk, Matteri, Gentszchein, Delgado and Lobo, 1990), by which these compounds may contribute to hair growth and sebum secretion (Pochi, Strauss and Mescon, 1963). The adrenal androgens may therefore contribute to the androgenic load throughout the body, and the two sites discussed here suggest the importance of 5αR in their action.

Under the conditions of this study, intratesticular testosterone synthesis is presumed to be suppressed secondary to the suppression of gonadotrophin secretion. The maintained supply of adrenal androgens may therefore contribute to the maintenance of spermatogenesis in the oligozoospermic group, whereas the fall in adrenal androgens in the azoospermic group may contribute to the complete
suppression of spermatogenesis in these men. However, the high circulating concentrations of administered testosterone is likely to be of greater importance as a substrate for 5αR than the weak adrenal androgens.

The selective effect of testosterone on adrenal androgen secretion is also of interest in the light of the finding that the concentration of DHAS in plasma is lower in Chinese men than in Caucasians (Lookingbill et al., 1991). The difference between plasma concentrations reported in that study (9.5 μmol/l in Caucasians vs. 6.5 μmol/l in Chinese) is very similar to the magnitude of the effect of testosterone in the azoospermic group reported here, although Lookingbill et al. (1991) did not feel that the differences in adrenal androgens could account for the larger differences in the concentrations of 5α-reduced androgens which they found.

In the present study, no effects on the excretion of THF, aTHF or THE, the major urinary metabolites of cortisol, were found, suggesting a selective effect of testosterone on adrenal androgen but not glucocorticoid secretion.

In summary, the results presented in this Chapter demonstrate that during testosterone administration:
1. There is no significant change in hepatic 5αR activity, as reflected in the metabolism of either C₁₉ or C₂₁ steroids
2. There is a selective reduction in the plasma concentration of DHAS and urinary excretion of its major metabolite DHA in the azoospermic group, suggesting decreased adrenal production. These weak androgens may contribute to the maintenance of spermatogenesis in the oligozoospermic group.
3. Excretion of 17OH-progesterone, an intermediate in testicular steroidogenesis, was inhibited, whereas the plasma concentration of androstenedione was increased, probably reflecting increased conversion of administered testosterone.

The administration of supraphysiological doses of testosterone therefore appears to demonstrate an interaction between testosterone and adrenal androgen metabolism, and to result in a condition in which there is dissociation of adrenal androgen and cortisol secretion. Whether this reflects alteration of enzyme activity in the adrenal, or the secretion of an adrenal androgen-stimulating hormone from the pituitary remains to be established, but the lack of differential sensitivity of the hypothalamo-pituitary axis to the negative feedback effects of testosterone on gonadotrophin secretion between the men becoming azoospermic and those remaining oligozoospermic may indicate that the site of action lies in the adrenal.
Chapter 8
The Effect of Testosterone on Sebum Excretion Rate

8.1 Introduction.

Sebum is the holocrine secretion of the sebaceous gland. These glands are widely situated in the body but are absent from the palms and soles, with up to 900 glands/cm² in areas such as the face (Benfenati and Brillanti, 1939). They are usually associated with hair follicles (thus making up the pilosebaceous unit, PSU), but can be found alone at sites of transition between skin and mucous membrane, e.g., the Meibomian glands of the eyelids.

Sebaceous glands throughout the body are similar in structure. There is a single or collection of acini opening into the piliary duct, surrounded by a connective tissue capsule, which also acts as a fibrous support. The lipid-producing cells are arranged according to their degree of differentiation, with small cells which are relatively undifferentiated peripherally. As the cells divide and mature, they accumulate lipid which is produced by the smooth endoplasmic reticulum, and are progressively found more centrally. These cells may be 100-fold larger than the peripheral cells (Tosti, 1974). Finally, the cells become necrotic, releasing the sebum into the duct and thence to the skin approximately 7 days later. This mixture of lipids consists of triglycerides, squalene, wax esters and a relatively low concentration of cholesterol (Greene, Downing, Pochi and Strauss, 1970; Lewis and Hayward, 1971).

The pilosebaceous units are formed in the foetal skin at 2-4 months of age and are active in utero, with a similar rate of sebum secretion in the first week of life as in the adult (Agache, Blanc, Barrand and Laurent, 1980). The glands then involute and remain quiescent until puberty, when there is an increase in size and activity, with sebum secretion increasing approximately 5 fold in men (Pochi, Strauss and Downing, 1979). The rate of secretion remains constant through adult male life, only decreasing in old age (Plewig and Kligman, 1978). In women, the adult rate is 75% of that of men, and declines after the age of 50 (Pochi and Strauss, 1974). This sex difference (and post menopausal decline) clearly suggests a hormonal influence in the control of sebum secretion.

Early studies suggested a stimulatory effect of testosterone on sebum secretion (Hamilton, 1941; Jarrett, 1959), and the role of androgens was clearly demonstrated by Strauss and Pochi (1961). These authors showed that the oral administration of
methyltestosterone to prepubertal boys stimulated sebum secretion and increased the size of the sebaceous glands, and they obtained similar results in eunuchs. However, they failed to find any effect of testosterone administration in normal adult men (Strauss, Kligman and Pochi, 1962). This was attributed to sebum secretion being already maximally stimulated in adult men. An alternative explanation is that the methodology used was insufficiently sensitive, as they later found that a dose of 300mg testosterone propionate weekly produced a small increase in sebum secretion (Pochi and Strauss, 1974). 200mg testosterone cypionate i.m. by weekly injection for 10 weeks also increased sebum secretion slightly in 2 out of 3 elderly men (Smith and Brunot, 1961).

More recently, it has been shown that anabolic steroids increase sebum secretion and the size of sebaceous glands in athletes (Király, Alén, Rahkila and Horsmanheimo, 1987; Király, Collan and Alén, 1987) although the mixture of steroids taken and the variable self-administered doses makes quantification of the androgenic stimulus difficult. There is also a considerable body of data from animal experiments showing similar effects of androgen administration on sebum production (Ebling, 1974; Shuster and Thody, 1974).

There are two main processes in sebaceous glands which may be the site of action of androgens. One is cell division, the other lipid synthesis. Both these appear to be stimulated by testosterone (in animal experiments), but by separate mechanisms (Ebling, 1957; Ebling and Skinner, 1967). Thus testosterone stimulates lipid synthesis and the mitotic activity of the gland, and both effects are inhibited by the antiandrogen cyproterone acetate. This contrasts with the inhibitory effect of oestradiol on lipid production; this effect was evident at doses which did not affect mitotic activity, either basally or after simulation by testosterone (Ebling, 1973).

The mechanism of action of testosterone in the PSU appears to be similar to that at other androgen target organs. Thus androgen receptors are found in skin, and pubic and axillary hair are deficient in subjects with testicular feminisation (Keenan et al., 1974). The skin is capable of metabolising testosterone, and DHT is a major product (Gomez and Hsia, 1968). The skin therefore contains the enzyme 5αR (Wilson and Walker, 1969; Northcutt et al., 1969; Voigt, Fernandez and Hsia, 1970). 5αR activity is very high in genital skin, which is capable of converting up to 80% of incubated testosterone to 5α-reduced metabolites (Gomez and Hsia, 1968). Activity in non-genital areas is low, and in sexual but not genital skin, eg the pubic area, is intermediate but higher in men than women (Wilson and Walker, 1969). Similar regional differences have been reported in experiments using cultured monolayers of fibroblasts
(Wilson, 1975), which technique has allowed further characterisation of the enzyme in skin.

5αR activity is therefore sex-dependent, and in both sexes is high in skin derived from the urogenital ridge. Indeed, 5αR activity has been measured in human foetal tissue, and was found to be high in the urogenital tubercle, fold and swelling, but very low in non-genital skin (Siiteri and Wilson, 1974). This may explain the differences in pubic hair growth at puberty between the sexes, and why males with a congenital deficiency of the enzyme show female-type pubic hair growth (Peterson et al., 1977).

Micro-dissection techniques have allowed the demonstration that the majority of 5αR activity in the skin is found in the sweat and sebaceous glands, with minimal activity in the epidermis (Hay and Hodgins, 1978; Takayashu, Wakimoto, Itami and Sano, 1980), and the observation that adolescents with 5αR deficiency do not suffer from acne suggests that DHT may be the major active androgen, although there are no reports of sebum secretion rate in these individuals (Peterson et al., 1977).

Puberty in males is accompanied by an increase in 5αR activity in pubic skin (Kuttenn and Mauvais-Jarvis, 1975), having been very low before puberty. In contrast, activity in genital skin is high throughout childhood, and is the same as at birth and after puberty. Similar results were obtained when males with hypogonadotrophic hypogonadism were treated with hCG; in these subjects there was an increase in 5αR activity in pubic skin before the clinical onset of puberty (Kuttenn and Mauvais-Jarvis, 1975). In vitro studies using cultured fibroblasts have shown that testosterone or DHT itself can increase the activity of 5αR, an effect that is mediated by the androgen receptor (Mowszowicz et al., 1983). These results suggest that the 5αR activity associated with the appearance of secondary sexual characteristics, including the increase in sebum secretion, is androgen-dependent, and acts as an androgen amplifier at and after puberty.

The rate of secretion of sebum is therefore androgen-dependent, and the PSU contains the necessary metabolic pathways for the conversion of testosterone to 5α-reduced metabolites. There is evidence that DHT is, as elsewhere, the active androgen, and that sebaceous gland 5αR activity is androgen-dependent. The rate of secretion of sebum is therefore a possible marker of skin 5αR activity, and one that has not been previously explored: there are for example no studies on sebum secretion rate in women with hirsutism. This study was therefore carried out to investigate whether there are differences in the rate of secretion of sebum between those men becoming azoospermic on supraphysiological doses of testosterone, and those remaining oligozoospermic. Comparison with results presented elsewhere in this Thesis allows a discussion of the usefulness of this approach as a marker of tissue 5αR activity.
8.2 Method.

The method used was the gravimetric technique of Strauss and Pochi (1961), with minor modifications. The basis of the method is that sebum is absorbed onto paper from a delimited area of the skin over a defined period of time, extracted into ether, dried and weighed.

8.2.1 Preparation.

Pads of 4 cigarette papers (Rizla brand, standard weight) were prepared, stapled at both ends and the adhesive strip cut off. All pads were then washed in ether and dried before use. After washing the pads were handled using clean tweezers only. 3 pads for each of the 4 skin sites sampled were required per subject for each determination.

The mask used was made from Micropore tape, 4 cm wide. A 5 cm² hole from a strip of tape 10 cm long was cut using a graph paper template. This was conveniently done by first sticking the tape onto a thin sheet of clear plastic.

8.2.2 Method.

Measurements were carried out starting at 9 am, and the temperature of the room was maintained at 21-23°C. The areas measured were the forehead above the eye and the upper back over the blade of the scapula. Both sides of the body were sampled to give duplicate determinations. The skin was first wiped with a gauze swab, and the mask applied. The first pad of papers was then attached, and held in place with thin strips of Micropore tape at each end. A folded gauze swab was then applied as a pressure pad, and held in place with a gauze bandage around the head or strips of wide Micropore tape on the back.

After 15 min the first pad of papers was removed and discarded. A second pad was applied, again for 15 min, and discarded. The final pad was then applied, and left in place for 3 hours. At the end of the collection period, the sebum-loaded pads were removed and the central area bearing the sebum was cut out. This was facilitated by holding the pad up to the light, when the sebum area could be clearly seen.

The paper was transferred to foil gallipots which had been pre-weighed on a 5-figure balance, and the sebum extracted into 20 ml ether for 10 min. The papers were then transferred into glass beakers, and rewashed twice more in 10 ml ether. All washings were combined in the foil gallipots, dried, and the gallipots reweighed. Subtraction gave the quantity of sebum per sample, and determinations on the two sides of the body were averaged. In each run, blanks were included consisting of pads
prewashed as in the preparation stage but not applied to the skin, and the blank value was subtracted from the sample value. Extraction blanks gave values of less than 100μg. Results are expressed as mg/3hrs/10cm². This investigation was carried out on 32 of the subjects: the other man declined to attend for such a relatively time-consuming procedure.

The incidence of acne was assessed on both occasions, and was graded using an adaptation of the scoring described by Lookingbill et al. (1978) into none, mild (comedones with no or only a few inflammatory papules or pustules), and moderate / severe.

Statistical significance was calculated using Student's t test for paired or unpaired values except when results are expressed as percentage increase when the Wilcoxin signed rank test was used.

8.3 Results.

To assess the reproducibility of the method, samples from the right and left of the body were compared, before being combined to give the final value. The correlation between left and right for forehead and upper back is shown in Figure 8.1. This gave a correlation coefficient of r=0.91, p<0.001. These results are from the baseline measurements: very similar results were obtained when the measurements were repeated after 16 weeks of treatment.

The results of SER determinations are shown in Figure 8.2. Mean baseline SER from forehead was 2.02 ± 0.02 mg/3 hrs/10cm² in the men who became azoospermic, and was 2.06 ± 0.22 mg/3 hrs/10cm² in those who remained oligozoospermic. SER was consistently less from upper back skin, being 0.78 ± 0.04 mg/3 hrs/10cm² in the azoospermic group and 0.77 ± 0.08 mg/3 hrs/10cm² in the oligozoospermic group. This difference between forehead and back was highly significant for both groups (p<0.001). There was thus no difference in SER at either anatomical site between the two groups at baseline.

There was a significant correlation between SER measurements from forehead and upper back at baseline (r=0.47, p<0.02, Figure 8.3). Further analysis revealed that a statistically significant correlation was only found in those men who became oligozoospermic on testosterone treatment (r=0.67, p<0.001 in the oligozoospermic group; r=0.17 in the azoospermic group). During testosterone treatment there was no significant correlation in either group, or when all subjects were analysed together.

After 16 weeks of testosterone treatment, many of the subjects reported a subjective increase in greasiness of the skin. This was confirmed by measurement of the SER,
Figure 8.1
Correlation between SER from left and right sides at baseline, data from both forehead and upper back.
Figure 8.2
The effect of testosterone on SER in those men becoming azoospermic and those remaining oligozoospermic.

(a) Forehead
(b) Upper back

Open columns, baseline
Hatched columns, after 16 weeks testosterone treatment.

* p<0.05
** p<0.01 vs baseline.

Mean ± sem, n=17, azoospermic group
n=15, oligozoospermic group.
Figure 8.3
Correlation between SER of forehead and upper back at baseline.

(a) All men.
(b) Oligozoospermic group only.
Figure 8.4
Increase in SER after 16 weeks of testosterone treatment in those men becoming azoospermic and those remaining oligozoospermic, expressed as a percentage of baseline value.

Open columns, forehead
Hatched columns, upper back.

* p<0.02
** p<0.005, both vs forehead.

Mean ± sem, n=17, azoospermic group
n=15, oligozoospermic group.
which showed a significant increase in both groups of men on both forehead and back (Figure 8.2). In the azoospermic group, forehead SER had increased to 2.90 ± 0.31 mg/3 hrs/10cm² (p<0.01 vs baseline), and from the upper back to 1.51 ± 0.15 mg/3 hrs/10cm² (p<0.001 vs baseline). In the oligoospermic group, forehead SER had increased to 2.77 ± 0.15 mg/3 hrs/10cm² (p<0.02 vs baseline), and from the upper back to 1.58 ± 0.29 mg/3 hrs/10cm² (p<0.01 vs baseline). There were again no differences in SER between the two groups after 16 weeks of testosterone treatment.

Results as percentage increase in each area for the two groups are shown in Figure 8.4. In the azoospermic group, SER was increased to 150±13% of baseline rate from the forehead, and to 203±31% from the back. In the oligoospermic group, SER was increased to 156±11% and 206±20% respectively. This demonstrates the greater increase in SER from the upper back than forehead in both groups (p<0.005, azoospermic group; p<0.02, oligoospermic group).

The incidence and severity of acne at the time of the SER determinations is shown in Table 8.1., together with the sebum excretion results recalculated according to acne grading.

Table 8.1

<table>
<thead>
<tr>
<th>Acne Grade</th>
<th>None</th>
<th>Mild</th>
<th>Moderate/Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline n=</td>
<td>29</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Treatment n=</td>
<td>5</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>SER (+testosterone)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>3.50 ± 0.59</td>
<td>2.81 ± 0.19</td>
<td>2.31 ± 0.37</td>
</tr>
<tr>
<td>Back</td>
<td>1.18 ± 0.08</td>
<td>1.63 ± 0.23</td>
<td>1.55 ± 0.12</td>
</tr>
<tr>
<td>SER (% of baseline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forehead</td>
<td>195 ± 22%</td>
<td>140 ± 9%</td>
<td>161 ± 24%</td>
</tr>
<tr>
<td>Back</td>
<td>214 ± 27%</td>
<td>195 ± 19%</td>
<td>237 ± 34%</td>
</tr>
</tbody>
</table>

Effect of testosterone on acne scoring; values are numbers of men with each grade of acne, before and after 16 weeks of testosterone treatment. The centre section of the table gives SER results after 16 weeks testosterone treatment for the men grouped according to acne score, in mg/3 hrs/10 cm², and the lower section gives results recalculated as SER after 16 weeks of testosterone treatment as a percentage of baseline rate.
These results revealed that men who developed acne tended to have a higher SER from the back than those who remained free of acne, but this did not reach statistical significance, nor did it reach significance when the two acne groups were combined. The percentage increase in SER from the back was similar in the three groups. There was no such trend in SER from the forehead.

8.4 Discussion.

The "Gravimetric" technique originally described by Strauss and Pochi (1961) has been widely used in human studies, and most of what is known about the endocrine control of the gland is based on studies in which it was used. More recent developments include the Lipometre, which measures lipid secretion after absorption onto a ground glass plate (Cunliffe, Kearney and Simpson, 1980), but this has been reported to give erroneous results at high rates of sebum excretion (Chivot, Zeziola and Saurat, 1981).

Other developments include depletion of the follicular reservoir by application of bentonite clay on polyester cloth over a period of 14 hours, followed by collection of sebum onto clay discs for 3 hours and analysis of the lipid by thin layer chromatography (Harris, Downing, Stewart and Strauss, 1983; Hughes and Cunliffe, 1989), and the analysis of the secretion of individual follicles (Nordstrom, Schmus, McGinley and Leyden, 1986). The gravimetric method was therefore used because of the body of information previously obtained using it, its relative ease, and reproducibility.

Sebum secretion measured by this method is linear for at least 5 hours (Strauss and Pochi, 1961) but is not sustained over a more prolonged period. This implies that a pool of sebum which has already been secreted is being sampled: this pool is thought to represent the sebum on the skin and in the duct of the gland (Downing, Stranieri and Strauss, 1982). This method thus measures sebum excretion rate (SER), not sebum production rate, and therefore reflects the activity of the sebaceous gland indirectly (Shuster and Thody, 1974).

There is a diurnal variation in SER which parallels that of testosterone, being higher in the morning, but there is no seasonal variation (Burton, Cunliffe and Shuster, 1970). SER is also temperature-dependent, which may reflect either increased liquefaction of sebum and therefore greater flow from the duct or improved absorption by the papers at higher temperatures rather than increased activity of the gland (Cunliffe, Burton and Shuster, 1970). In this study all measurements were therefore carried out in the morning starting at 9 am, and the temperature of the room was controlled at 21-23°C.
Baseline values for SER of the forehead and back are in good agreement with those in the literature determined by this and other methods (Shuster and Thody, 1974; Cunliffe et al., 1980), and the lower SER of the skin of the back is as previously described (Greene et al., 1970). The degree of correlation between determinations from left and right side of the body is also similar to that previously reported (Simpson, 1987), although that study used the photometric method, and the subjects (including both men and women) had acne. Simpson (1987) also found a high correlation between SER of forehead and back. The results reported here suggest that in men without acne the correlation is lower, and was only significant at baseline in those men who subsequently became oligozoospermic on testosterone treatment. There was no correlation in either group after 16 weeks of testosterone treatment. The significance of this observation is unclear. It may reflect the wider range of SER found by Simpson (1987) as that population was less homogeneous than the population of normal men without acne studied here, or may be a function of Simpson’s population all having acne. However, the lack of correlation found here suggests that in normal skin local factors might be important in determining the SER at a particular anatomical site, rather than a more systemic influence such as the concentration of a hormone such as testosterone in the blood.

Testosterone administration caused a significant increase in SER from both forehead and upper back. The magnitude of the increase is similar to that reported with anabolic steroid abuse in athletes (Király et al., 1987a), who found an increase in SER of forehead skin to approximately 150% of baseline after 2 weeks of self-medication, with no further change up to 12 weeks. The subjects in that group had all abused steroids in the past, but had remained free of all hormonal preparations for at least 12 weeks before the baseline measurement.

The increase in SER of upper back was significantly greater than that of forehead, with an increase to 200% of baseline for back vs. 150% for forehead (Figures 8.2 and 8.4). Using surface microscopy, it has been revealed that there are approximately 4 times as many follicular openings in the forehead as in the skin of the back, but only 2-3 times the sebum production (Cunliffe, Perera, Thackray, Williams, Forster and Williams, 1976). This suggests that under basal conditions the SER per gland is higher in the back, which might reflect an increased sensitivity to stimulatory factors of sebaceous glands in that region. Such a greater sensitivity might underlie the larger effect of testosterone treatment on the SER of the back.

The increase in SER in response to testosterone treatment shown in Figure 8.2 is considerably greater than that reported by Pochi and Strauss (1974). In their initial
studies, they were unable to detect any effect of testosterone administration to adult men, although an effect on adolescents was clear (Strauss et al., 1962). They only studied a small group of subjects, however, and used low doses of testosterone. It therefore appears that failure to demonstrate the effect of testosterone on SER in adult men in previous studies is due to experimental design rather than a true lack of effect. The present results suggest that the sebaceous glands are not under maximal stimulation under physiological conditions and their activity can be considerably increased, by as much as a factor of two in some areas.

These results therefore suggest either that the rate of sebum secretion is not primarily dependent on 5αR activity, or that this method is not sufficiently sensitive to detect small differences in the activity of that enzyme between groups. The evidence presented in the Introduction to this Chapter and to Chapter 5 strongly suggests that DHT is the active androgen in the skin, and that the activity of 5αR is therefore crucial to the degree of androgenic stimulation of the PSU. If the interpretation of the results presented in Chapters 4 and 5 is correct, namely that those men remaining oligozoospermic have a higher 5αR activity during testosterone treatment than those becoming azoospermic, then this suggests that sebum secretion is not closely related to 5αR activity under these conditions. Other more important effects may include the concentration of testosterone itself (and it is noteworthy that the increases in SER are similar to the increases in plasma testosterone concentration, and greater than the increases in plasma DHT concentration), and the increase in plasma oestradiol. It may be that an interaction of stimulatory androgenic and inhibitory oestrogenic effects has prevented the clear expression of any difference in 5αR activity. Local controlling mechanisms may also have influenced this result: as discussed above, it appears that PSU activity is partially dependent on anatomical location.

The second major possibility is that the method is insufficiently accurate or precise to detect small differences in SER. While repeated measurement of SER from the same individual was not possible with the design of this trial, measurements were taken from both left and right sides of the forehead and back. The results in Figure 8.1 show the degree of correlation obtained from left and right sides. These values are not true duplicates, as different sites are being sampled, and the correlation obtained reflects the precision of the method. Determination of the accuracy of the method would require validation against an external “gold standard”. This was not possible, and indeed there is no definitive method of measuring SER. A further point is that SER is an indirect measure of the true rate of sebum secretion by the gland, although there is a correlation between surface lipid and both gland size and mitotic activity (Ebling, 1973).
There are therefore a number of possible reasons why differences in SER were not detected between those men becoming azoospermic and those remaining oligozoospermic on testosterone treatment, and it appears that SER may not be a marker of PSU 5αR activity under these conditions. In situations in which there are greater differences in 5αR activity, for example in men with congenital 5αR deficiency or after finasteride administration, SER may reflect the biochemical abnormality. These experiments have not been carried out.

Patients with acne have seborrhoea which persists even after resolution of the condition, and the degree of acne correlates with SER (Cunliffe and Shuster, 1969): a rate of sebum excretion of double normal is associated with moderate to severe acne. None of the subjects in this study had a history of acne requiring medical treatment, and the incidence and severity of acne at the time of the SER determinations is shown in Table 8.1. These results suggest that those men who developed acne had an increased SER from the back after 16 weeks testosterone treatment, although this did not reach statistical significance. Acne is a recognised side effect of androgen administration, and in a previous study of testosterone as a male contraceptive 9 out of 271 men withdrew from the trial because of it (World Health Organisation, 1990). In an earlier trial, 69% of the subjects reported acne or increased oiliness of the skin (Swerdloff et al., 1978). These results underline the importance of increased sebum secretion in the pathogenesis of acne (Cunliffe and Shuster, 1969).
Chapter 9
Conclusions and Hypothesis

The administration of supraphysiological doses of testosterone to normal men, sufficient to cause more than a 100% increase in trough plasma concentrations of testosterone, was shown to cause a fall in sperm density in all men in this study. 55% of these men achieved azoospermia within 20 weeks of testosterone treatment. The remainder maintained a low rate of spermatogenesis, with sperm densities of less than $5 \times 10^6$/ml, and although 5 achieved azoospermia after more prolonged treatment, this took more than one year in some cases.

The primary objective of the studies described in this Thesis was to investigate the basis for this observed heterogeneity of spermatogenic response to testosterone administration, and in particular to test the hypothesis that the maintenance of spermatogenesis under these conditions is caused by an increased activity of the enzyme $5\alpha$-reductase, $5\alpha$R, which converts testosterone to the more potent androgen dihydrotestosterone.

The administration of this dose of testosterone caused a rapid and complete suppression of gonadotrophin secretion in all men. The removal of LH stimulation of the Leydig cells is presumed to cause a fall in testicular steroidogenesis, and this is supported by the demonstration of parallel falls in both the plasma concentration of 17OH-progesterone and the urinary excretion of pregnanetriol. Further evidence for the degree of suppression of steroidogenesis under similar conditions in humans is provided by the work of Strott et al. (1969), who showed a fall of 90% in the plasma concentration of 17OH-progesterone in men treated with a synthetic androgen, and by the demonstration that intratesticular testosterone concentrations were reduced to 5% of pretreatment values (such that the intratesticular concentrations of testosterone were only double those in peripheral plasma) by treatment with testosterone propionate (Morse et al., 1973).

It therefore appears that under the conditions of the present trial, the major differences in the intratesticular environment from the physiological state are that:
1. There is no gonadotrophin stimulation of Leydig and Sertoli cells.
2. Steroidogenesis is reduced, but the intratesticular concentration of testosterone is still higher than that in peripheral plasma. The intratesticular concentration is therefore probably little influenced by the wide fluctuations in plasma concentration of testosterone with the weekly injection schedule used.
3. The rate of spermatogenesis falls rapidly to zero in 55% of men, with the remaining men having a rate of spermatogenesis of less than 5% of normal (on the basis of sperm density in the ejaculate).

The main hypothesis of this Thesis is that the maintenance of spermatogenesis in the oligozoospermic group of testosterone-treated men is the result of higher 5αR activity in these men. What evidence has been produced to substantiate this?

This conclusion can be considered in two parts:

I. What is the evidence that 5αR may be of importance in the regulation of spermatogenesis, in the human or any other species?

II. What is the evidence for a difference in 5αR activity between men becoming azoospermic and those remaining oligozoospermic?

Subsidiary points include whether such a difference is present under physiological conditions, or only under the conditions of this trial, and the mechanism of such differences.

Evidence for the presence of 5αR in the rat and human testis is presented in Chapter 1. In brief, 5αR activity is higher in immature than adult rat testicular tissue, and the onset of meiosis within the seminiferous tubules is associated with an increase in 5αR activity with a decline subsequently (Rivarola et al., 1975; Nayfeh et al., 1975). A similar pattern is present in the human. Although the level of activity is lower than in the rat (Payne et al., 1973; Rivarola et al., 1973, 1975; Nayfeh et al., 1975), this is also true for the rate of testosterone synthesis. In both species, 5αR activity is predominantly localised to the seminiferous tubules rather than the interstitial tissues, in itself circumstantial evidence for a paracrine role in the regulation of spermatogenesis rather than an endocrine role related to the secretion of steroids from the testis. This maturational change in enzyme activity suggests that 5αR may act as an amplifier of androgen action at the time of puberty, as indeed it acts in other androgen-dependent organs throughout life, from the in utero development of the external genitalia to the prostatic hyperplasia of old age. In the mature adult testis, it appears that the high local concentration of testosterone produced by the Leydig cells is sufficient to maintain spermatogenesis, and this is associated with a fall in 5αR activity. The period of elevated 5αR activity at the onset of spermatogenesis also corresponds to the period during which the secretion of both LH and FSH has yet to achieve the adult pattern, and therefore amplification of the effect of the lower concentrations of testosterone may be required: this is supported by the observation that, in the rat, hypophysectomy
caused a rapid fall in testicular 5αR activity, which could then be increased by gonadotrophin administration (Nayfeh et al., 1975).

There are two pieces of experimental evidence which suggest that 5αR may be of functional importance in the regulation of spermatogenesis under conditions in which the intratesticular concentration of testosterone is low. The first is provided by the demonstration by Ahmad et al. (1973) that intratesticular implants containing DHT could maintain spermatogenesis in the ipsilateral but not the contralateral testis in hypophysectomized rats. There therefore appears to be no absolute requirement for testosterone itself, as indeed would be expected from the finding that there is only one androgen receptor which binds both testosterone and DHT. The second, perhaps more important but not so clearly interpretable piece of evidence is the study by Nicholson, Guldenaar, Boer and Pickering (1991), who inserted oxytocin implants into the testes of normal rats. This caused a marked fall in both intratesticular and peripheral concentrations of testosterone after 21 days, but spermatogenesis was maintained. The plasma concentration of LH was not affected, whereas the plasma concentration of FSH was reduced. Measurement of intratesticular and peripheral concentrations of DHT, however, showed that concentrations of that steroid were elevated. This result is compatible with an inhibition of steroidogenesis by oxytocin (Adashi et al., 1984), but with increased 5α-reduction of testosterone to DHT resulting in sufficient androgen stimulus for the maintenance of spermatogenesis. A direct effect of oxytocin on 5αR is also possible, and these results require confirmation and elaboration to elucidate the mechanisms involved.

These studies therefore provide preliminary evidence that 5αR may be of importance in the maintenance of spermatogenesis in the presence of low intratesticular testosterone concentrations. The demonstration that 5αR inhibition by finasteride does not affect spermatogenesis (George et al., 1989) does not contradict this hypothesis, as finasteride administration is associated with an increase in intratesticular testosterone concentrations, in the presence of normal plasma gonadotrophin concentrations. Indeed, this supports the hypothesis that 5αR is only important when concentrations of testosterone are low. The increase in intratesticular testosterone concentration with finasteride administration is compatible with a negative feedback pathway in which DHT, or some factor dependent on DHT, is involved in the local regulation of testosterone synthesis.

Evidence for Part II above is provided by the results presented in this Thesis. The results in Chapter 3 demonstrate that there are no differences in plasma concentrations
of both total and bio-available testosterone, or in the sensitivity of the hypothalamic-pituitary axis to the rate or degree of steroid suppression of gonadotrophin secretion between men becoming azoospermic and those remaining oligospermic. Thus the rate of decline of gonadotrophin stimulation of the testes does not appear to be of importance in determining whether azoospermia is achieved. Furthermore, plasma oestradiol concentrations did not differ between the two groups of men, nor did MCRT.

In contrast, it was found that testosterone administration caused an increase in CRT-DHT in the oligozoospermic group, without any change in the azoospermic group. This increase was associated with a greater elevation of the plasma concentrations of the 5α-reduced androgens DHT and AdiolG during testosterone treatment. These results suggest that there is increased 5αR activity in men remaining oligozoospermic. This difference is not apparent under physiological conditions, but is induced by administration of supraphysiological doses of testosterone.

The activity of 5αR in the liver was measured by quantification of the urinary excretion of C19 and C21 steroid metabolites. No consistent effect of testosterone treatment was demonstrated on the ratio of α:β metabolites. This may reflect the results of animal experiments, which have shown that hepatic 5αR activity is increased following hypophysectomy in male rats, but testosterone administration has little inhibitory effect. The mechanisms of control of hepatic 5αR therefore appear to differ from those in androgen-dependent tissues such as the prostate and skin, where 5αR is increased by androgens.

Sebum excretion rate was measured as an index of tissue 5αR activity, and although a clear increase was found in both groups of men, there were no differences in SER between those men becoming azoospermic and those remaining oligozoospermic either at baseline or after 16 weeks of testosterone treatment. SER therefore appears to reflect the hyperandrogenaemia caused by the administration of supraphysiological doses of testosterone but no differentiation on the basis of 5αR activity was possible. This may reflect the limitations of the method, or may imply that the increase in plasma testosterone concentrations is of greater importance in terms of tissue response in the PSU than any difference in 5αR activity. As with the lack of effect of finasteride on spermatogenesis, this is compatible with 5αR activity being of greater importance under circumstances in which tissue testosterone concentrations are low.

These results therefore support the hypothesis that an increase in 5αR activity is associated with the maintenance of spermatogenesis during treatment with supraphysiological doses of testosterone. A causal relationship can only be postulated, and further proof must await the direct measurement of 5αR in the testis or of
intratesticular concentrations of DHT or other 5α-reduced steroids under these conditions.

An interaction between testicular and adrenal steroidogenesis was suggested by the results presented in Chapter 7. Testosterone treatment caused a selective fall in DHAS production in those men who became azoospermic (with a trend towards a fall in the plasma concentrations of other adrenal steroids), without any effect in those remaining oligozoospermic. There was no effect on urinary excretion of cortisol metabolites in either group. Plasma androstenedione concentrations were elevated in both groups, and the production of 17OH-progesterone was reduced in both.

The results presented here are not easily explicable in terms of current understanding of the mechanisms of control of adrenal androgen secretion, but they do demonstrate a state in which there is dissociation between adrenal secretion of androgens and cortisol. They also allow speculation as to the possible role of these weak androgens (in particular DHA and its sulphate) in the maintenance of spermatogenesis in those men remaining oligozoospermic. Thus the maintained plasma concentration of DHAS may contribute to the supply of androgens to the seminiferous tubules. These steroids are thought to provide an important supply of tissue androgens in the prostate, as castration for the treatment of carcinoma of the prostate does not sufficiently lower prostatic androgens, and current therapies involve the concurrent administration of an anti-androgen. In this respect, it is of great interest that finasteride causes a more pronounced fall in prostatic DHT than does castration (McConnell et al., 1992). This implies that adrenal androgens are a physiological source of precursor for conversion to DHT by 5αR within the prostate, as originally suggested by Harper et al. (1974), and that the tissue concentrations of DHT (and thus the magnitude of the androgen stimulus to the genome) is more directly determined by 5αR activity than by the concentration of the precursor, testosterone, in plasma.

The recent studies by Lookingbill et al. (1991) on ethnic variation in androgen metabolism reveal close parallels with the results presented here. These authors investigated differences in androgen metabolism between caucasian and Chinese men and women, as the former show clinical evidence of higher 5αR activity: thus caucasian men have more body hair and a higher incidence of acne, and the incidence of prostatic hyperplasia is much greater in caucasian populations. Biochemical evidence was provided by the demonstration that caucasian men had higher plasma concentrations of AdiolG (but not DHT), and caucasian women had higher plasma
concentrations of both $5\alpha$-reduced steroids. Higher plasma concentrations of AdiolG and androsterone glucuronide have also been reported in both black and caucasian Americans compared to Japanese men (Ross et al., 1992). These ethnic differences were interpreted as suggesting greater $5\alpha$R activity in caucasians than Chinese, and it is possible that this biochemical difference underlies the higher incidence of azoospermia in Chinese men than caucasians treated with the same dose of testosterone as used in this study (World Health Organisation, 1990). Of more fundamental importance is the suggestion by Ross et al., (1992) that the higher $5\alpha$R activity in both caucasian and black American men, suggested by the increased plasma concentrations of these two steroids, may have a role in producing the 3 fold higher incidence of prostatic carcinoma in these populations.

Caucasian men also had higher plasma concentrations of DHAS than Chinese men in Lookingbill’s study, which also parallels the finding of a suppression of plasma DHAS concentrations in those men becoming azoospermic in the present study. While the significance of this result is not clear, it underlines the possible role of adrenal androgens as precursors for tissue $5\alpha$R.

In summary, these results suggest that the maintenance of spermatogenesis in a subgroup of men during treatment with supraphysiological doses of testosterone is associated with an increased activity of the enzyme $5\alpha$R. This is proposed to result in the production of sufficient DHT and other $5\alpha$-reduced androgens for the maintenance of a low rate of spermatogenesis in the seminiferous tubules, despite the greatly reduced local concentration of testosterone and in the absence of gonadotrophin stimulus. Adrenal androgens may have a supplementary role in this process.

The relative importance of the two isoenzymes of $5\alpha$R, in the testis as elsewhere in the body, remains to be determined. Although there will be great toxicological problems to be overcome (especially the teratogenic effect on the developing male foetus of such drugs, which may be absorbed by the female genital tract from seminal plasma) the recent clinical availability of potent inhibitors of $5\alpha$R such as finasteride ("Proscar", Merck) provides a new therapeutic option to be explored in the development of safe, effective, reversible and convenient hormonal methods of male contraception.
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