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CHARACTERISATION OF FEMALE RAT HEPATIC TESTOSTERONE 5α-Reductase

A thesis submitted to the University of Glasgow in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science.

by

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August, 1987
FOR LINDA
"Where there is much desire to learn, there of necessity will be much arguing, much writing, many opinions; for opinion in good men is but knowledge in the making."

John Milton (1608-1674)
ACKNOWLEDGEMENTS

My gratitude is expressed to Professors R. Mackie and R.M.S. Smellie for making available the resources and facilities of the Departments of Dermatology and Biochemistry at the University of Glasgow, where this work was undertaken.

My thanks are also expressed to Drs. M.B. Hodgins and H.G. Nimmo for their continuous support, advice and encouragement throughout the course of this work.

To Dr. L.M. Fixter for his helpful suggestions and general interest in the work.

To the Medical Research Council for financial support provided.

Finally, to my wife, Linda, not only for her patience and fortitude during the typing of this thesis but also for her unstinting support during the entire course of this work.
Abbreviations

CAII&III Isoenzymes II&III of carbonic anhydrase
Chaps (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate
Diazo-MAPD 21-diazo-4-methyl-4-aza-5α-pregnane-3,20-dione
DMSO di-methyl sulphoxide
DTT dithiothreitol
EDTA ethylene diamine tetra-acetic acid
FPLC fast protein liquid chromatography
GH growth hormone
g.l.c. gas liquid chromatography
HPLC high performance liquid chromatography
MUP major urinary protein
NADPH nicotinamide adenine dinucleotide phosphate (reduced form)
PPO di-phenyl oxazole
SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis
t.l.c. thin layer chromatography
Tris 2-amino-2-(hydroxymethyl)propane-1,3-diol
5α-DHT 5α-dihydrotestosterone
5β-DHT 5β-dihydrotestosterone

The following trivial names of steroids have been used:
androstenedione = 4-androstene-3,17-dione ; 5-androstenediol = 5-androstene-3β,17β-diol ; androstanedione = 5α-androstan-3,17-dione ; epiandrosterone = 3-epiandrosterone ; epiaetiocholanolone = 3-epiaetiocholanolone ; pregnenolone = 3β-hydroxy-5-pregn-20-one ; oestradiol = 1,3,5 (10)-oestratriene-3,17β-diol.
SUMMARY

Following a short discussion of steroid hormone biosynthesis and catabolism and mechanisms of action, the existing literature concerning the biochemistry of $\Delta^4$-3-oxosteroid 5α-oxidoreductase (E.C. 1.3.99.5; 5α-reductase) is reviewed under several main headings—viz. historical background, physiological and clinical significance of testosterone 5α-reduction, properties of hepatic and androgen-dependant tissue 5α-reductase, the mechanism of steroid 5α-reduction, the solubilisation of 5α-reductase and finally the hormonal control of 5α-reductase enzyme activity.

Evidence is discussed concerning the possible relationship between enzymes of steroid hormone and fatty acid metabolism and at the end of the introduction the aims and scope of this investigation are delineated.

The experimental section of this thesis is divided into five sections. In the first section, the microsomal 5α-reductase of female rat liver microsomes is characterised with respect to kinetic properties and pH optimum. In the next section experiments undertaken to solubilise the enzyme from microsomal membranes are described. The behaviour of solubilised enzyme on gel filtration is reported in the third section of results. The fourth part of the experimental work deals with the photo-affinity labelling of microsomal 5α-reductase. The specificity of photo-affinity labelling is described and the behaviour of the solubilised, photo-affinity labelled enzyme on gel filtration is reported and compared...
to the behaviour of solubilised 5α-reductase enzyme activity. Further characterisation of the radio-labelled conjugate by SDS-PAGE and stability of radio-ligand to precipitation and organic solvent extraction is also reported. In the final part of the results section experiments undertaken to elucidate the relationship between 5α-reductase and enoyl CoA reductase (a microsomal enzyme involved in fatty acid metabolism) are described.

The results obtained are discussed in relation to the findings obtained by other workers at the end of each appropriate experimental section.

In the final chapter, some conclusions are derived from the experimental work and some suggestions for future investigations are forwarded.

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4 GENERAL DISCUSSION AND CONCLUSIONS

REFERENCES
1.1 Biochemistry of the steroid hormones

1.1.1 Biosynthesis and catabolism of steroids

The pathways of steroid hormone biosynthesis have been described in detail by several authors (Dorfman and Sharma, 1965; Dorfman and Ungar; 1965; Ryan and Smith, 1965). A simplified scheme of these biosynthetic pathways is outlined in Fig. 1.
Fig. 1. Outline of Steroid Hormone Biosynthesis


Key to compounds
A. Cholesterol  G. Cortisol
B. Pregnenolone  H. Dehydroepiandrosterone
C. Progesterone  I. Androstenedione
D. 17α-hydroxyprogesterone  J. Aldosterone
E. 17α-hydroxyprogrenolone  K. 5α-androstenediol
F. Corticosterone  L. Testosterone
M. Estradiol
Enzyme Activities

1. = 22-hydroxylase, 20-hydroxylase, C_{20}-C_{22} lyase
   [side-chain cleavage enzyme]
2. = 17\alpha-hydroxylase
3. = 3\beta-hydroxysteroid dehydrogenase, \Delta^{4-5}_{\beta}\text{isomerase}
4. = C_{17}-C_{20} lyase
5. = 3\beta-hydroxysteroid dehydrogenase, \Delta^{4-5}_{\beta}\text{isomerase}
6. = 17\alpha-hydroxylase
7. = 21-hydroxylase, 11\beta-hydroxylase
8. = 17\beta-hydroxysteroid dehydrogenase
9. = \Delta^{5}_{\beta}-3\beta-hydroxysteroid dehydrogenase
10. = C_{17}-C_{20} lyase
11. = 21-hydroxylase, 11\beta-hydroxylase
12. = 18-hydroxylase
13. = 3\beta-hydroxysteroid dehydrogenase, \Delta^{4-5}_{\beta}\text{isomerase}
14. = 17\beta-hydroxysteroid dehydrogenase
15. = aromatase

With the exception of the side-chain cleavage enzyme and
the 11\beta and 18-hydroxylases involved in corticosteroid
biosynthesis, which are mitochondrial, the steroid bio-
synthetic enzymes are present in the microsomes. Soluble
17\beta-hydroxysteroid dehydrogenases are known however
(Kautsky and Hagerman, 1970).
The common precursor for the biosynthesis of all the physiologically active steroid hormones is cholesterol. The total biosynthesis of cholesterol from acetyl CoA via isoprenoid intermediates has been reviewed by Clayton, (1965). The biosynthetic routes from cholesterol lead to the four main classes of steroid hormone – the oestrogens (C18), the androgens (C19), progesterone (C21) and the mineralocorticoids and glucocorticoids (C21). The numbers appearing in brackets indicate the number of carbon atoms in the steroid nucleus of each of the classes of hormone.

The main pathway of androgen biosynthesis is indicated by bold arrows in Fig. 1. Thus 17α-hydroxy progesterone undergoes oxidative side-chain cleavage between C17 and C20 to form androstenedione which is further transformed by the action of 17β-hydroxysteroid dehydrogenase to testosterone. A minor route of androgen biosynthesis involves the conversion of 17α-hydroxy pregnenolone to dehydroepiandrosterone and 5-androstene-3β, 17β diol. These serve as precursors for androstenedione and testosterone respectively. The main site of androgen production in the adult male is the testis and in the female is the adrenal cortex. The biosynthetic inter-relationships between the steroid hormones are illustrated in Fig. 2.
As this report is concerned with the characterisation of the enzyme activity testosterone 5α-reductase it is necessary to indicate where in the scheme of androgen metabolism this enzyme belongs. To that end, a simplified scheme of androgen catabolism is outlined in Fig. 3. This topic has been extensively reviewed by Dorfman and Unger (1965).
Fig. 3 Outline of Androgen Catabolism
### Enzyme activities

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<td>2.</td>
<td>5β-reductase</td>
<td>1.3.99.6</td>
</tr>
<tr>
<td>3.</td>
<td>17β-hydroxysteroid dehydrogenase</td>
<td>1.1.1.64</td>
</tr>
<tr>
<td>4.</td>
<td>3α/3β-hydroxysteroid dehydrogenase</td>
<td>1.1.1.50/1.1.1.51</td>
</tr>
</tbody>
</table>
The basic pattern of metabolism of both androstenedione and testosterone is that they are subject to two NADPH-dependent reductions - one at the C4-C5 double bond and one at the 3-keto group. Reduction at these points in the molecule can give products of α or β configuration at C3 and C5. In the case of testosterone this leads to the formation of four isomeric androstandiols and for androstenedione four isomeric 3-hydroxy, 4, 5-reduced 17-ketosteroids. These products are formed by the sequential action of 5α-reductase/5β-reductase and 3α-hydroxysteroid dehydrogenase/3β-hydroxysteroid dehydrogenase as outlined in Fig. 3. The 3α-hydroxysteroids are the predominant isomers present in human urine, and the main metabolites of testosterone are the 17-ketosteroids-androsterone and aetiocholanolone (Slaunwhite and Sandberg, 1957).

Inactivation of physiologically active steroid occurs mainly in the liver, but also to some extent in the kidney and intestine. Following their reductive metabolism in these tissues, steroids are conjugated with glucuronic acid or sulphate and excreted in the urine. Dehydroepiandrosterone sulphate (DHAS), produced by the adrenal gland is excreted in the urine as such or converted in the liver to 5α-androstene-3β, 17β-diol which is converted to a glucuronide or sulphate conjugate and excreted.

Although Fig. 3 outlines the scheme of androgen catabolism, it is important to appreciate that the other classes of Δ^4-3-ketosteroids are metabolised by the same route - i.e. the NADPH-dependent reduction of the 4-5 double bond and the 3-keto group prior to conjugation and excretion.
Fig. 3 therefore serves as a general scheme for $\Delta^4$-3-ketosteroid catabolism. The sequential 4-5 double bond, 3-keto, reduction is also involved in the biosynthesis of the bile acids from cholesterol. In this instance the product has the 3$\alpha$, 5$\beta$ reduced configuration. It can be seen, therefore, that this reductive metabolism of steroids is a biochemical pathway of major significance.
1.1.2. Mechanism of action of androgens

The mechanisms by which steroid hormones produce their effect on target cells has been the subject of great interest and has subsequently produced an enormous literature concerning this topic. Several of the steps between entry of steroid hormone into a target cell and the manifestation of that target cell's response to steroid are the subject of great debate and indeed remain controversial. It is not the intention, in this section of the introduction, to describe in detail the differences in various worker's views of this problem (which in any case is outwith the scope of this thesis). Instead, a brief and simplified scheme of the mechanism of androgen action will be outlined, together with the possible role played by 5α-reductase in the expression of androgen action. The mechanism of androgen action in normal individuals and various defects which can occur in this process were the subject of a recent review (Kovacs et al, 1987). A simplified scheme of androgen action is outlined in Fig. 4.

Fig. 4 Mechanism of action of androgens

![Mechanism of action of androgens diagram](image)

(After Kovacs et al, 1987)
Following entry of testosterone into the target cell, it can be reduced by the enzyme 5α-reductase to 5α-DHT (see Section 1.2.1.). Although testosterone and 5α-DHT appear to bind to the same receptor protein, (Rc) (Wilson et al, 1983) the kinetics of the interaction between these two steroids and the receptor protein appear to differ. For example Kd values of testosterone for the cytosolic fibroblast androgen receptor was reported as 3·01 nmol/l. The affinity of 5α-DHT for receptor was some 7-fold higher with a Kd value of 0·46 nmol/l. Hodgins (1982) had previously demonstrated a 2-fold difference in Kd values between testosterone and 5α-DHT for the androgen receptor in genital skin fibroblasts (T = 0·44 nmol/l; 5α-DHT = 0·20 nmol/l). Furthermore, in the same publication he reported a faster dissociation rate constant for the testosterone-receptor complex than the 5α-DHT-receptor complex (average values T·Rc = 0·045/min; 5αDHT·Rc = 0.010/min). These differences in the kinetic properties of androgen-receptor complexes may be a significant amplification of the androgenic signal as it is known that in some cases prior reduction of testosterone to 5α-DHT is necessary for the proper expression of androgen action. (see Section 1.2.4.).

Transformation of the receptor \((R \rightarrow R^*)\) in Fig. 4 is associated with changes in receptor structure or conformation such that it acquires the ability to bind to DNA. Several aspects of the process of transformation remain unknown. In a recent paper (Kovacs et al, 1983) demonstrated transformation of 5αDHT-androgen receptor complexes from genital skin fibroblast by heating pre-labelled/...
cytosol preparations from 0°C to 25°C or by treatment of cytosol with 0.5 mol/l NaCl. Transformation of receptor was assessed by measuring its binding to DNA-cellulose. The authors reported that the acquisition of this ability to bind DNA-cellulose was associated with the loss of the form of the receptor which sedimented at 7.5S on continuous sucrose density gradients. Later Kovacs et al (1984) described an androgen receptor mutant which displayed an impaired ability to be transformed to the DNA-binding state.

The next step in the mechanism of action of androgens involves the binding of transformed receptor to chromatin of the target cell nucleus. Both the DNA and protein components of the chromatin are believed to be important in providing binding sites. For example Klyzseko-Stefanowicz et al (1976) demonstrated that the DNA-associated non-histone protein (NHP) fraction contained tissue-specific binding sites for 5α-DHT-receptor complexes. Thus the NHP fraction from prostate or testis contained three times the amount of receptor binding sites than the same fractions of liver or pancreas chromatin. Buttyan et al (1983) demonstrated the importance of DNA in providing binding sites for receptor when he showed that digestion of nuclei with DNAase I (which preferentially removes active DNA sequences) led to a loss of tissue-specific acceptor sites. These two views were synthesised in a report which demonstrated specific, high affinity, saturable binding of pre-labelled androgen receptors to preparations of prostatic nuclear matrix - a structure /.
known to contain both actively transcribed sequences of DNA and non-histone proteins (Barrack, 1983). This binding was shown to be dependent on steroid and on receptor activation.

As indicated in Fig. 4, the final stage in androgen action involves the stimulation of mRNA transcription and subsequent protein synthesis. Several proteins have been shown to be under androgenic control. These include a prostatic cholesterol binding protein (Chen et al., 1982) and spermine binding protein (Hiipakka et al., 1984), hepatic glycolithocholate sulphotransferase (Kirkpatrick et al., 1985), mouse kidneyβ-glucuronidase (Bullock et al., 1985), testicular and epididymal angiotensin converting enzyme (Jaiswal et al., 1985) and at least five different proteins in the epididymal cytosol of the adult rhesus monkey (Arslan et al., 1986). Section 1.2.7 - "Hormonal control of 5α-reductase" discusses the different levels of proteins found in the livers of male and female rats and describes a possible mechanism underlying this sexual differentiation.
1.2 Biochemical properties of 5α-reductase

1.2.1 Historical background

\[ \Delta^4 \text{-}3\text{-}\text{oxosteroid} \ 5\alpha\text{-oxidoreductase} \ (E.C.1.3.99.5; 5\alpha\text{-reductase}) \] is a membrane-bound enzyme which catalyses the NADPH-dependant reduction of the 4-5 double bond of \[ \Delta^4 \text{-}3\text{-}\text{ketosteroids} \] to give the corresponding 5α-dihydro reduced steroid (see Fig.5)

Fig. 5 NADPH-dependant reduction of \[ \Delta^4 \text{-}3\text{-}\text{ketosteroids} \]

\[ \Delta^4 \text{-}3\text{-}\text{oxosteroid} \ 5\alpha\text{-oxidoreductase} \ (E.C.1.3.99.5; 5\alpha\text{-reductase}) \] is a membrane-bound enzyme which catalyses the NADPH-dependant reduction of the 4-5 double bond of \[ \Delta^4 \text{-}3\text{-}\text{ketosteroids} \] to give the corresponding 5α-dihydro reduced steroid (see Fig.5)

The reduced steroid has a trans configuration at the junction of the steroid A and B rings and these rings are essentially co-planar. (Liao et al, 1973).

Schneider and Horstmann (1951) demonstrated the reduction of the 4-5 double bond of desoxycorticosterone by rat liver slices, a finding confirmed by Schneider (1952). Later it was demonstrated that the supernatant obtained following the centrifugation of rat liver homogenate at 6000g for 30min. catalysed the 5α-reduction of /...
11-deoxycortisol. (Forchielli et al, 1955). Subsequently, $5\alpha$- and $5\beta$-reductases were separated by differential centrifugation (Forchielli and Dorfman, 1956). Following centrifugation of a rat liver homogenate at 78000g for 90min, $5\beta$-reductase was found exclusively in the supernatant fraction and $5\alpha$-reductase in the particulate fraction. The soluble cytosolic $5\beta$-reductase (Tomkins, 1957) and particulate $5\alpha$-reductase enzymes (McGuire et al, 1960) were both partially characterised. Since these early studies, $5\alpha$-reductase has been shown to have a wide tissue distribution in the rat being found in tissues as diverse as the ventral prostate (Farnsworth and Brown, 1963; Bruchovsky and Wilson, 1968(a)), seminal vesicles (Gloyna and Wilson, 1969), epididymis (Inano et al, 1969) hypothalamus (Cheng and Karavolas, 1975(a)) anterior pituitary (Cheng and Karavolas, 1975(b)) and kidney (Verhoeven and De Moore, 1971).

The enzyme has also been partially characterised in human skin (Gomez and Hsia, 1968; Wilson and Walker, 1969; Voigt et al, 1970) and prostate (Habib et al, 1981; Houston et al 1984). In addition the presence of $5\alpha$-reductase has been demonstrated in the prostate and seminal vesicles of the dog, lion, mouse, guinea pig, cat and bobcat (Gloyna and Wilson, 1969). The presence of $5\alpha$-reductase has also been demonstrated in the skin and skeletal muscle of the fish (Hay et al, 1976) and a procaryotic $5\alpha$-reductase has been described in Mycobacterium smegmatis by Lestrovaja et al, (1977). Studies in these tissues have used mainly androgens as substrates for $5\alpha$-reductase, although the $5\alpha$-reduction of progesterone by neuro-endocrine organs has also been investigated (Bertics and Karavolas, 1984; Bertics and Karavolas, 1985).
1.2.2. Properties of hepatic microsomal 5\(^{-}\)-reductase

McGuire and Tomkins (1960a) demonstrated that in the presence of NADPH rat liver microsomes catalysed the 5\(^{-}\)-reduction of five different \(\Delta^4\)-3-ketosteroids - cortisol, cortisone, 11-deoxycortisol, 11-deoxycorticosterone and androstenedione. The same laboratory (McGuire et al., 1960) went on to demonstrate that in fact 21 different \(\Delta^4\)-3-ketosteroids were substrates for hepatic microsomal 5\(^{-}\)-reductase. They also showed that 13 other \(\Delta^4\)-3-ketosteroids could not be reduced by hepatic microsomes. A common feature of these non-reducible steroids was C1-C2 unsaturation of the steroid A ring or \(\|\) methyl substitution at C2 or C6 (see Fig. 6).

Fig. 6 Non-reducible \(\Delta^4\)-3-ketosteroid derivatives

The structure of the steroid molecule at these points is therefore important in respect of the steroid being a substrate for 5\(^{-}\)-reductase. The rat liver enzyme showed a very broad pH optimum at pH 5.5-7.5. The enzyme was inhibited by p-chloromercuribenzoate (PCMB). This inhibition could be prevented by the simultaneous addition of reduced glutathione (GSH), suggesting that free sulphydryl groups are necessary /...
for maintenance of enzyme activity.

The observation that sodium dithionite could not replace NADPH as an electron donor for 5α-reduction argued against the involvement of flavin in the mechanism of 5α-reduction (Mahler, 1954). Similarly antimycin A, an inhibitor of electron transport (Chance 1956) only caused slight inhibition of cortisone and 11-deoxycortisol 5α-reduction (McGuire et al, 1960).

Rat liver microsomes will also catalyse (in the presence of NADPH) the reduction of the 4-5 double bond of the C27 steroid cholest-4-en-3-one (Shefer et al, 1966). This enzyme activity resembled those described previously (McGuire and Tomkins, 1959; McGuire and Tomkins, 1960a; McGuire et al, 1960) in that it was NADPH-dependant, displayed a pH optimum of around pH7 and was inhibited by PCMB. However the observation that cholest-4-en-3-one did not inhibit the reduction of C19 Δ4-3-ketosteroids led the authors to suggest that cholestnone 5α-reductase was in fact a separate enzyme from those already described (Shefer et al, 1966).

Roy (1971) demonstrated the NADPH-dependant reduction of [7α-3H] testosterone by female rat liver microsomes. In contrast to previous reports he found an acidic pH optimum for the enzyme of pH 5.5. He also reported that atebrin - an inhibitor of flavin enzymes (McGuire et al, 1960) - and carbon monoxide (CO) - an inhibitor of cytochrome P-450 dependant steroid hydroxylations (Omura and Sato, 1964) - were without effect on testosterone 5α-reduction. This /.
suggested that these intermediates were not involved in electron transport between NADPH and steroid substrate. However, evidence for intermediate electron carriers in the mechanism of 5\(\Delta^4\)-reductase has been provided by Golf and Graef, (1978) (see Section 4). These same workers also claimed to have partially separated testosterone 5\(\Delta^4\)-reductase from a progesterone/cortisone 5\(\Delta^4\)-reductase by iso-electric focussing although these preparations retained the ability to reduce all three steroids. This has been the only demonstration of the separation of \(\Delta^4\)-3-ketosteroid 5\(\Delta^4\)-reductase activities.

The importance of membrane structure in the activity of 5\(\Delta^4\)-reductase was evidenced by the fact that solubilisation of microsomal membranes by certain agents led to a loss of 5\(\Delta^4\)-reductase activity. Even when solubilisation has been achieved it has always been associated with a loss of enzyme activity (see Sections 1.2.6., 3.2.2. and 3.2.3.2). Similarly modulation of the enzyme activity in situ in the membrane by the use of phospholipases has demonstrated the importance of the lipid micro-environment in relation to enzyme activity. Thus Lumper et al (1969) demonstrated that treatment of rat liver microsomes with phospholipase D resulted in a complete loss of 5\(\Delta^4\)-reductase activity. In a similar vein, Golf and Graef (1978) have shown that addition of phosphatidyl choline to solubilised hepatic microsomal 5\(\Delta^4\)-reductase resulted in a 3-fold stimulation of enzyme activity.
Although the evidence of the early studies by McGuire and Tomkins, (McGuire and Tomkins, 1959; McGuire and Tomkins, 1960(a); 1960(b) McGuire et al, 1960) suggested the presence of several 5α-reductase enzymes in liver microsomes, each acting on different Δ⁴-3-ketosteroid substrates, more recent evidence suggests this may not in fact be the case. Thus humans with 5α-reductase deficiency (see Section 1.2.4) have decreased levels of both 5α-reduced androgens and 5α-reduced glucocorticoids in their urine (Peterson et al, 1977). This suggested that in the liver the same enzyme acted on these two classes of steroid hormone prior to their excretion. It has also been demonstrated that epididymal microsomes isolated from a patient with 5α-reductase deficiency were unable to catalyse the 5α-reduction of ten different physiological Δ⁴-3-ketosteroids - including progesterone, testosterone and cortisol (Fisher et al, 1978). If one assumes that a single mutation is involved in the aetiology of 5α-reductase deficiency then these findings suggest that a single gene product was involved in the 5α-reduction of these steroids. However, the data are also consistent with the hypothesis that these steroid 5α-reductases are not identical but do in fact share a common sub-unit and that a structural mutation in this sub-unit leads to a loss of enzyme activity.
1.2.3 Properties of 5α-reductase in androgen-dependent tissues.

Because of the importance of 5α-reductase in the development and maintenance of some androgen-dependent tissues and the association of the enzyme with some pathological conditions in these tissues (see Section 1.2.4), an extensive literature exists on this topic. Indeed as the importance of 5α-reductase in androgen action is one of the reasons for undertaking this work it is appropriate that this subject should be reviewed at this stage.

(a) Prostatic 5α-reductase.

The first report of 5α-reductase activity in the prostate was by Farnsworth and Brown (1963). Using minces or slices of human benign hyperplastic prostate tissue, they identified 5α-DHT as representing 35% of the total metabolites of testosterone. Later Anderson and Liao (1968) demonstrated that following incubation of minced rat prostate with 7α-[3H] testosterone, 75-90% of the radioactivity recovered from purified nuclear preparations was identified as 5α-DHT. In the same year, Bruchovsky and Wilson (1968a) showed that following intravenous administration of 1,2-[3H] testosterone to normal and functionally hepatectomised rats, only [3H] testosterone and [3H] dihydrotestosterone were recovered from prostatic nuclei. It was also shown that in the presence of an NADPH generating system, prostatic /...
nuclei were, in vitro, able to metabolise testosterone to 5α-DHT. In a subsequent publication, Bruchovsky and Wilson (1968b) demonstrated the binding of 5α-DHT, formed following intravenous administration of 1,2-[3H] testosterone to eviscerated, castrated and functionally hepatectomised rats, to isolated preparations of rat prostatic nuclei.

Frederiksen and Wilson (1971) then went on to describe some of the properties of rat prostatic testosterone 5α-reductase. The enzyme displayed an absolute requirement for NADPH and had a pH optimum of pH 6.6. In contrast to the liver, prostatic 5α-reductase was present in both the nuclear and microsomal membranes with each of these intracellular compartments containing approximately 50% of the total enzyme activity. The enzyme was sensitive to divalent cations, with the nuclear 5α-reductase being almost totally inhibited by Hg²⁺, Cu²⁺ and Zn²⁺ at concentrations of 1mmol/l. The inhibition by Zn²⁺ was competitive with respect to NADPH. The authors reported a Km(app.) for testosterone of 1umol/l and their data indicated that the ability of eight different Δ⁴-3-ketosteroids (including progesterone and deoxycorticosterone) to serve as substrates for 5α-reductase was paralleled by their potency as inhibitors of the conversion of [3H] testosterone to 5α-DHT. Thus the measured Km(app.) values for the steroids were in close agreement with their Ki values for inhibition of testosterone 5α-reduction. Inhibition was competitive with respect to testosterone and the authors concluded that in the prostate there was a single 5α-reductase activity with a broad /...
specificity for \( \Delta^4 \)-3-ketosteroids.

Later, Moore and Wilson (1972), working with purified prostatic nuclei, demonstrated that when sonicated extracts of these nuclei were subjected to density gradient centrifugation (in continuous caesium chloride density gradients), all the 5\( \pi \)-reductase activity was associated with a visible band of turbidity at d.1·23-1·27. They reported a 90-fold purification of enzyme activity in this fraction.

Nozu and Tamaoki (1974a) initially confirmed the findings of Frederiksen and Wilson in respect of the pH optimum, Km(app.) testosterone and sub-cellular distribution of prostatic 5\( \pi \)-reductase. However in a later publication (Nozu and Tamaoki, 1974b), the authors reported that when purified, sonicated prostate nuclear extracts were subjected to discontinuous sucrose density gradient centrifugation, 5\( \pi \)-reductase activity was recovered at the d.1·16-1·18 and d. 1·18-1·20 interfaces. The authors suggested that the lighter density of the 5\( \pi \)-reductase they obtained compared to the findings of Moore and Wilson (1972) may have been due to the absorption of Cs\(^+\) ions to the nuclear membrane in the continuous CsCl gradients used by the latter authors.

Roy (1971) confirmed previous findings on the enzymology of prostatic 5\( \pi \)-reductase. He also reported, however, that as with the hepatic microsomal 5\( \pi \)-reductase (see Section 1.2.2), PCMB at a concentration of 0·1 mmol/l completely inhibited nuclear 5\( \pi \)-reductase activity. Also, as in the case of the hepatic enzyme, carbon monoxide had no effect on enzyme activity, suggesting that cytochrome P-450 played no part in the reaction mechanism.
A recent paper on the comparative biochemistry of prostatic 5\(\alpha\)-reductase indicated that species differences exist in the enzyme obtained from different mammalian sources (Liang et al, 1985(a)). It was shown that prostate particulates of the rat, dog and human had a similar Km(app) for testosterone - at 2.4, 2.2 and 3.3 umol/l respectively. However, only the rat enzyme was sensitive to inhibition by PCMB - a finding previously reported by Roy (1971). Also, the enzyme from the three different species displayed different pH optima, the values being rat (pH7), dog (pH6) and human (pH5). These findings together with the observation that the rank order of potencies of 24 5\(\alpha\)-reductase inhibitors was different in the three species suggested that the enzymes from the prostates of these animals showed significant structural variations. As well as the rat, dog and human prostate, the conversion of testosterone to 5\(\alpha\)-DHT has been demonstrated in the prostates from eight other species (Gloyna and Wilson, 1969; Wilson and Gloyna, 1970). The specific activities of the prostatic 5\(\alpha\)-reductase in these species was (in descending order) - rat, man, baboon, lion, dog, mouse, guinea pig, cat, bobcat, bull and rabbit. (In the bull and rabbit 5\(\alpha\)-DHT formation was practically zero).

(b) Epididymal 5\(\alpha\)-reductase
Scheer and Robaire (1983) undertook a study of the rat epididymal 5\(\alpha\)-reductase. As was the case with the prostate, the enzyme was present in both the nuclear and microsomal membranes. The Km(app) testosterone for the /...
nuclear and microsomal enzymes was in the range 0.17 - 0.45 umol/l. Unlike the prostatic enzyme, the epididymal enzyme had a much broader pH optimum at pH 5.8 - 7.5. As was noted in the case of the hepatic enzyme (see Section 1.2.2), the lipid micro-environment of the membrane was important in the control of 5α-reductase activity. Thus Cooke and Robaire (1985) reported the effects of treating epididymal nuclear and microsomal preparations with phospholipases A₂ and C. (PIₐ₂ and PI C). PIₐ₂ increased the Km (app) testosterone of the microsomal enzyme by a factor of two and decreased the Vmax by 20 - 30%. The Km(app) testosterone of the nuclear enzyme was unaffected by PIₐ₂ but the Vmax was reduced by 50%. In contrast PI C increased the Km(app) testosterone two-fold for both the nuclear and microsomal enzymes and decreased the Vmax of both enzymes by 50%. As was the case with the hepatic enzyme (Golf and Graef, 1978), the authors reported a stimulation of the Lubrol WX - solubilised enzyme activity by the addition of phosphatidyl choline to the assay solution.

(c) Skin 5α-reductase

Since the original demonstration of the transformation of testosterone to 5α-DHT by specimens of human skin (Gomez and Hsia, 1968), several workers have described the properties of 5α-reductase in skin from various anatomical sites. Wilson and Walker (1969) demonstrated that the 5α-reductase activity in skin slices from some perineal areas (labia majora, prepuce, scrotum and clitoris) is higher than in skin from other anatomical areas (trunk and limbs). Voigt et al (1970) published an extensive study of the 5α-reductase enzyme in microsomal preparations /...
of human neonatal foreskin. The authors tested a total of twenty three steroids as inhibitors of the conversion of testosterone to $5\alpha$-DHT. They reported that the necessary structural requirements for a steroid to inhibit the $5\alpha$-reduction of testosterone were a $\Delta^4$-3-ketosteroid structure and $17\beta$ (but not $17\alpha$) substitution. They also reported that substitutions on the steroid nucleus (eg. 11-hydroxylation) also reduced the efficacy of the steroid as an inhibitor of testosterone $5\alpha$-reduction. $5\alpha$-DHT was a very poor inhibitor of $5\alpha$-reductase activity. It was demonstrated, in fact, that progesterone was the best substrate for the $5\alpha$-reductase enzyme - $K_m = 0.7$ umol/l (cf testosterone - $K_m = 1.1$ umol/l). By the mixed substrate method (Dixon and Webb, 1964) it was shown that in the human neonatal foreskin, the same enzyme reduced progesterone and testosterone. The same lab (Voigt and Hsia, 1973) reported the conversion of testosterone to $5\alpha$-DHT by homogenates of the hamster flank organ. This is a sebaceous structure and is androgen-dependant being larger and more pigmented in the male (Voigt and Hsia, 1973). However, topical application of testosterone propionate or $5\alpha$-DHT to the female hamster organ caused it to enlarge. The testosterone propionate (but not the $5\alpha$-DHT) - induced enlargement of the organ could be prevented by the simultaneous addition of 4-androsten-3-one-$17\beta$-carboxylic acid ($17\beta$C) or its methyl ester. It was suggested therefore that the effects of testosterone on the hamster flank organ were mediated by its conversion to $5\alpha$-DHT and that $17\beta$C inhibited $5\alpha$-reductase. This inhibition was shown to occur when $[4-^{14}\text{C}]$ testosterone was incubated with homogenates of flank organ, ...
with 5α-DHT formation being reduced from 75 pmol/100mg tissue/hr to approx. 25 pmol/100mg tissue/hr. In a study of the metabolism of androgens by slices of human skin (Hay and Hodgins, 1973) the authors reported high levels of 5α-reductase activity in three samples of forehead skin from three men (ages 20, 58 and 83), in the axillary skin from a 20 year old woman and a 43 year old man and in cheek skin taken from a 78 year old woman. The authors used dehydroepiandrosterone (DHA), DHA-sulphate, androstene dione and testosterone as substrates in their incubations and as well as 5α-reductase they reported the presence of eight other steroid-metabolising enzymes.

As well as investigating the properties of 5α-reductase in skin samples and skin homogenates or microsomal preparations the 5α-reductase activity of fibroblasts derived from human skin from various anatomical sites has also been investigated (Moore and Wilson, 1976; Moore et al, 1975; Mowszowicz et al, 1980; Leshin et al, 1978; Saenger et al, 1978; Pinsky et al, 1978). In a study of the 5α-reductase enzyme in cell-free extracts of fibroblasts derived from genital skin (foreskin, labia majora and scrotum), Moore et al (1975) confirmed some of the properties previously described by Voigt et al (1970) for the 5α-reductase activity in microsomes from human foreskin. Thus genital skin fibroblast 5α-reductase had a pH optimum at 5.5 with a shoulder of activity at pH 7-9. As was demonstrated in preparations of human foreskin microsomes, (Voigt et al, 1970) in cell-free extracts of fibroblasts derived from human foreskin, …
progesterone is a better substrate for 5α-reductase than testosterone. Six steroids were tested as substrates and their affinities for 5α-reductase were - progesterone > testosterone > androstenedione > cortexolone > 4-androstene-3, 11, 17-trione > cortisol. Cortisol was 5α-reduced at barely perceptable rates. Therefore as was previously reported by Voigt et al (1970), substitution of the steroid nucleus at position 11 led to a decrease in the affinity of steroid for enzyme.

Variation in enzyme activity of fibroblast preparations has been reported. Using homogenates of fibroblast derived from genital skin, (Moore et al., 1975) found activities ranging from 2.6 - 175 pmol/h/mg protein in twelve individuals. Similarly in whole sonicates of fibroblasts derived from genital skin, 5α-reductase activities ranging from 5 - 300 pmol/h/mg protein were reported (Moore and Wilson, 1976). This variation in 5α-reductase activity of fibroblast derived from genital skin was confirmed by other workers (Leshin et al., 1978; Pinsky et al., 1978; Mowszowicz et al., 1980). It had previously been found that there was wide variation in the 5α-reductase activity of slices of whole foreskin (Wilson and Walker, 1969). This variation was in part explained by donor age with younger foreskins containing higher levels of enzyme activity. In fibroblast studies 5α-reductase activity was shown to vary according to the passage number of the cultured cells. Moore et al., (1975) found that 20th passage cells had a two-fold higher activity that 10th passage fibroblasts. Similarly Mowszowicz et al. (1980) demonstrated a four to five-fold increase in /...
12th passage fibroblasts (cultured from foreskin and pubic skin) compared to 2nd passage cells.

Different properties have been described for the 5α-reductase enzyme of fibroblasts cultured from non-genital skin. The total enzyme activity was lower than that of genital skin fibroblasts (Pinsky et al, 1978). Moore and Wilson (1976) compared the properties of 5α-reductase from genital skin (foreskin) - derived fibroblasts and non-genital (inguinal) fibroblasts. The non-genital fibroblast enzyme did not show a peak of activity at pH 5.5 but demonstrated a broad slightly alkaline pH optimum at pH 7-9. As mentioned previously, genital skin fibroblasts display 5α-reductase activity both as a peak at pH optimum of 5.5 and over a broader range at pH 7-9. The observation that the 5α-reductase activity at pH 5.5 had a different subcellular distribution from the activity at pH 9.0 and also that the activity had a different Km(app) for testosterone at the two pH values, led Moore and Wilson (1976) to suggest that these two activities were in fact separate entities.

Before leaving this review of the literature concerned with the properties of the 5α-reductase enzyme from different regions of skin and the fibroblast derived from these sites it must be appreciated that there remain several areas of controversy. For example, Moore and Wilson (1976) reported the results of an experiment which demonstrated that in homogenates of non-genital (inguinal) skin, there was indeed a peak of 5α-reductase activity at pH 5.0 - 6.0 (as is the case in genital skin). The authors suggested that this activity /...
may be due to the presence of hair follicles or sebaceous glands in the skin. Also Pinsky et al (1978) did not demonstrate any difference in 5α-reductase activity in fibroblasts derived from male pubic skin compared to those derived from female pubic skin. However Kuttenn and Mauvais-Jarvis (1975) and Mauvais-Jarvis (1977) demonstrated higher 5α-reductase activity in the male pubic skin fibroblasts than the female. In view of the findings reported by Mowszowicz et al (1980), it is important that 5α-reductase enzyme activity in fibroblasts grown from different skin samples should be compared only when cells have been through the same number of passages.

(d) Neuroendocrine structures

The presence of 5α-reductase in the brain and other nervous tissues of the rat and mouse has been well documented (Massa et al, 1972; Motta et al, 1973; Rommerts and Van der Molen, 1971; Celotti et al, 1987) and has been the subject of a recent review (Martini, 1982). The pituitary, hypothalamus, amygdala and cerebral cortex of the rat were able to 5α-reduce both testosterone and progesterone (Massa et al, 1972). Enzyme activity was highest in prepubertal rats and decreased with age. Castration or incubation in vitro with FSH increased anterior pituitary 5α-reductase activity (by 3,50%). Celotti et al (1987) demonstrated a differential distribution of 5α-reductase in central nervous system structures of the rat and mouse. Enzyme activity was c. threefold higher in white matter structures (sub-cortex, corpus callosum, optic chiasm) than in the cortex or hypothalamus. Further discussion of the significance of the 5α-reduction of steroids in the central nervous system will be presented/...
in the next section - Section 1.2.4 - "Physiological and clinical significance of 5α-reductase."

(e) 5α-reductase in other tissues

Suzuki and Tamaoki (1974) described some of the properties of the testosterone 5α-reductase of seminal vesicles. This enzyme displayed an acidic pH optimum and as with the prostate enzyme (Frederiksen and Wilson, 1971), the seminal vesicle enzyme was also inhibited by Hg$^{2+}$ and Zn$^{2+}$. Similarly to the epididymal enzyme, the seminal vesicle enzyme was inhibited by low concentrations of PCMB. Finally Wilson and Gloyna (1970) published a study demonstrating the conversion of testosterone to 5α-DHT in seventeen different rat tissues - prostate, epididymis, seminal vesicles, penis, preputial gland, scrotum, kidney, skin, liver, ovary, vas, uterus, adrenal, fallopian tube, lung, testis and muscle (almost undetectable in muscle).

Several workers have suggested that the microsomal and nuclear enzymes in these androgen-dependent tissues are in fact different species. Scheer and Robaire (1983) demonstrated that the Lubrol WX - solubilised microsomal and nuclear enzymes displayed different sedimentation co-efficients. They also reported that the microsomal enzyme had a 30-fold higher Km(app) for NADPH that the nuclear enzyme (37umol/l versus 1.2 umol/l). These observations together with the demonstration of the differential effects of phospholipase A$_2$ on the kinetic parameters of the nuclear and microsomal enzymes led the authors to suggest that different enzyme species exist in these different sub-cellular compartments./...
(Cooke and Robaire, 1985). However, it may be that these differences did not reflect dissimilarities in the enzyme protein itself but rather differences in the lipid microenvironment and associated proteins surrounding the enzyme as it existed in the nuclear and microsomal membranes.

Frederiksen and Wilson (1971) demonstrated that the prostatic nuclear and microsomal enzymes were similar in respect of \( K_m(\text{app}) \) testosterone, pH optimum and specific activity per unit protein and concluded that the microsomal and nuclear enzymes were derived from a common source.

Finally, it is interesting to notice some differences in the properties of the enzyme from androgen-dependent tissues and that of liver (see Section 1.2.2).

(i) in the liver, \( 5\alpha \)-reductase is confined to the endoplasmic reticulum. In androgen-dependent tissues the enzyme is equally distributed between the nuclear and microsomal membranes. (Note however recent evidence suggests that in the human prostate the enzyme is confined to the nuclear membrane (Houston et al., 1985).

(ii) withdrawal of androgen support (e.g. by castration) has opposite effects on the levels of \( 5\alpha \)-reductase activity in hepatic and androgen-dependent tissues (see Section 1.2.7)

(iii) a considerable difference exists in the specific activity of the hepatic and androgen dependent tissue \( 5\alpha \)-reductases.
Thus for the rat prostate, epididymal and seminal vesicle enzymes values of 100, 40 and 26 pmol 5α-DHT formed/h/mg of protein have been reported (Frederiksen and Wilson, 1971; Scheer and Robaire, 1983; Suzuki and Tamaoki, 1974). The specific activity of the rat hepatic microsomal enzyme is of the order of 1000-fold higher (See Section 3.1.9).
1.2.4. Physiological and clinical significance of testosterone 5α-reduction

The importance of 5α-DHT in the growth and development of some androgen-dependent tissues was first suggested by the studies of Bruchovsky and Wilson (1968a,b). These workers injected [3H] testosterone into normal rats and then 5 min. later examined portions of various rat tissues to determine the amount of radioactivity present and the % of that radioactivity which was present as [3H] 5α-DHT. Of the eleven tissues examined (plasma, gut, liver, heart, lungs, levatorani muscle, testis, kidney, prostate, seminal vesicles and preputial gland) significant amounts of [3H] 5α-DHT were recovered only in the latter three tissues - 29.5%, 29.1% and 27.8% of the total radioactivity present in the prostate, seminal vesicles and preputial gland respectively (Bruchovsky and Wilson, 1968a). Furthermore only [3H] 5α-DHT was recovered as a metabolite from prostatic nuclei following the intravenous injection of [3H] testosterone in normal or functionally hepatectomized rats. Also the observation that, following the intravenous administration of [3H] testosterone to castrated, eviscerated and functionally hepatectomised rats, over 80% of the radioactivity bound to a protein component of prostatic nuclei was in fact [3H] 5α-DHT suggested that the prostatic nuclear reduction of testosterone to 5α-DHT may be physiologically relevant.

Support for this view came with studies which investigated the metabolism of androgens by hyperplastic prostate tissue.
Siiteri et al (1970), measured the testosterone, 5α-DHT and androstenedione levels of normal and hypertrophic human prostate. Whereas the levels of testosterone and androstenedione were the same in both types of gland, the level of 5α-DHT was five-fold higher in the hyperplastic prostate compared to the normal gland (0.60ug/100g v. 0.13ug/100g). In the same study it was reported that the levels of 5α-DHT in the peri-urethral area of the prostate (the area where hypertrophy usually begins) was two to threefold higher than the outer regions of early hyperplastic prostate. The authors forwarded these findings as being suggestive of a role for 5α-DHT in the aetiology of benign prostatic hyperplasia. It should be noted however that they were unable to demonstrate increased 5α-reductase activity in slices of prostate tissue. A recent study by Habib et al (1983) has demonstrated that 5α-reductase activity is unevenly distributed in different parts of the prostate gland. It remains possible therefore that a local increase in 5α-reductase activity and subsequent 5α-DHT accumulation may be important events in the development of prostatic hyperplasia.

Studies reporting the anabolic effect of 5α-DHT on the prostate were confirmed and extended by other workers. Gloyna et al (1970) demonstrated that the administration of 5α-DHT to castrated dogs over a nine month period caused an acceleration in the rate of prostatic growth. Hammond (1978) reported a five-fold increase in the 5α-DHT content of human hyperplastic prostate and Moore et al (1979) /...
demonstrated that the administration of 2mg 5\alpha-DHT/kg body weight/day induced the development of prostatic hyperplasia and also that 5\alpha-DHT accumulated in naturally occurring prostatic hyperplasia in dogs.

Evidence of a role for 5\alpha-reductase activity in the development of certain structures of the male genitalia was provided by studies which examined testosterone metabolism in the urogenital structures of rat, rabbit and human embryos (Wilson and Lasnitzki, 1971; Wilson, 1973; Siiteri and Wilson, 1974). In the rabbit and rat embryos (at 17 and 15 days gestation respectively) the rate of conversion of testosterone to 5\alpha-DHT was measured in several tissues. In these embryos, substantial 5\alpha-DHT formation was observed only in the urogenital sinus and tubercle. Further studies in the rabbit embryo (Wilson, 1973) demonstrated that when \[^{3}H\] testosterone was incubated with portions of urogenital sinus and tubercle, then the intracellular concentration of \[^{3}H\] 5\alpha-DHT was substantially higher than the \[^{3}H\] testosterone concentration. On the contrary when \[^{3}H\] testosterone was incubated with Wolffian duct tissue fragments no 5\alpha-DHT was formed from the tissue. These findings were confirmed and extended in the human embryo where once again high rates of 5\alpha-DHT formation were demonstrated only in the urogenital sinus and tubercle (Siiteri and Wilson, 1974). Furthermore rates of formation of 5\alpha-DHT in these tissues were maximal just prior to the time at which these tissues differentiate into the adult structures (the prostate and external genitalia). In contrast 5\alpha-DHT formation could not be /...
detected in the Wolffian ducts at this stage of development and was detectable only when these structures had differentiated into the adult male counterparts (the epididymis, vas deferens and seminal vesicles). On the basis of these findings, it was hypothesised that whereas testosterone, secreted by the foetal testis, induced differentiation of the Wolffian duct structures, in the urogenital sinus and tubercle testosterone was in fact a pro-hormone and that its prior reduction to 5α-DHT was necessary to induce differentiation of these tissues (Siiteri and Wilson, 1974).

This hypothesis was confirmed by the description, in the same year, of a syndrome of 5α-reductase deficiency (Imperato-McGinley et al, 1974). This defect was reported among a family in the Dominican Republic. It was clearly an autosomal recessive trait and was diagnosed biochemically by elevated plasma testosterone: 5α-DHT ratios (normal = 14; affected = 40) and by an increased ratio of 5β/5α urinary 17-keto steroids (8.5 v. 1.2) and 5β/5α androstaniols (normal range = 0.8 - 3.0; affected = 6.0 - 11.8). Infusion of radio active testosterone and analysis of the urine for radio active metabolites also demonstrated an increased 5β/5α metabolite ratio in the urine. Phenotypically the principle defect in 5α-reductase deficient individuals was limited to an incomplete differentiation of the male external genitalia and prostate. As predicted by the work of Siiteri and Wilson, (1974) differentiation of Wolffian duct structures was normal in these individuals. Other testosterone-depantent events of puberty such as the increase in muscle...
mass, growth of phallus and scrotum, the voice change and the acquisition of the male psychosexual orientation also occurred normally. However these individuals had a scant beard and no temporal recession of the hairline or acne and it was suggested that these processes may be 5\&-DHT dependant. Again in the same year (Walsh et al, 1974) used the term "familial incomplete pseudohermaphroditism Type II" (FIMPII) to describe the lesion of 5\&-reductase deficiency. Because of the appearance of the genitalia in these individuals the term pseudovaginal perineoscrotal hypospadias (PPSH) has also been used to describe this condition. However it was subsequently noted that there was considerable phenotypic heterogeneity in individuals with 5\&-reductase deficiency (Peterson et al, 1977). This together with the observation of a case of PPSH due to a deficiency of the enzyme 17\-hydroxy steroid dehydrogenase led to the term FIMPII being adopted to describe pathological cases of 5\&-reductase deficiency. Walsh et al (1974) were also the first to describe deficient conversion of testosterone to 5\&-DHT in foreskin, labia majora, epididymis and corpus cavernosum in an individual with FIMPII. However some years earlier the studies of Mauvais-Jarvis and co-workers (Mauvais-Jarvis et al, 1969; Mauvais-Jarvis et al, 1970) demonstrated an increased ratio in 5\-/5\& metabolites in the urine when patients with testicular feminisation syndrome were treated percutaneously with \[^{3}H\] testosterone. They concluded that there appeared to be deficient conversion of testosterone to 5\&-DHT in the skin of these patients.
Several workers have gone on to examine the properties of this mutant enzyme in fibroblasts grown from various anatomical sites of 5α-reductase deficient individuals (Moore et al, 1975; Pinsky et al, 1978; Leshin et al, 1978; Saenger et al, 1978; Hodgins et al, 1977,1979), in whole skin homogenates (Kutten et al, 1979; Savage et al, 1980) and in epididymis (Fisher et al, 1978). Thus Moore et al (1975) reported low 5α-reductase activity in homogenates of foreskin-derived fibroblasts from two FIMPII patients <0.2 pmol/h/mg protein. Note however as mentioned earlier (see Section 1.2.3) there was considerable variation in 5α-reductase activity among the seven control strains used in this experiment (2.6 - 175 pmol/h/mg protein). Using fibroblast monolayers grown from foreskin, Pinsky et al, (1978) also reported low levels of 5α-reductase activity in two siblings with FIMPII. Again it was noted that there was considerable variation in 5α-reductase activity among 18 control strains (over 40-fold). However the values for the two FIMPII siblings were 5-fold lower than the lowest control value. In a study of the kinetic properties of the 5α-reductase in fibroblasts cultured from normal foreskin and fibroblasts derived from the genital skin of an FIMPII patient, Hodgins et al (1977) reported a 100-fold difference in the Km(app) testosterone for the two enzymes from these sources (normal = 50-200 nmol/l; FIMPII = 20-50 umol/l). These findings were confirmed in a subsequent study (Hodgins, 1979) and led the author to postulate the existence of two forms of the enzyme - a "high Km" and "low Km" species, with 5α-reductase deficiency due either to a decrease or mutation of the /..
"low Km" species. Indeed in one patient with 5α-reductase deficiency evidence was obtained of two forms of the enzyme in genital skin fibroblasts (Hodgins, 1979).

In 1978, Leshin et al. published a study of the properties of the mutant 5α-reductase enzymes in five different FIMPII patients. These patients belonged to three families - 2 Los Angeles siblings, 2 Dallas siblings and 1 patient from the Dominican Republic. In the Dallas siblings and the Dominican patient they demonstrated a 20-fold increase in the Km(app) testosterone value in cell-free extracts of foreskin fibroblasts. (1.5 - 3.4 umol/l versus 80 nmol/l). In the Los Angeles sibship the foreskin fibroblast 5α-reductase had only a slightly elevated Km(app) testosterone. However the Km(app) NADPH was elevated 40-fold (0.9 and 1.76 mmol/l versus 40 umol/l). This enzyme was also thermolabile being denatured rapidly at 45°C in the presence of concentrations of NADPH which stabilised the normal enzyme. Further evidence for the instability of this enzyme (Los Angeles) was obtained when it was shown that following addition of cyclohexamide to intact fibroblasts enzyme activity was undetectable after 24h. In the normal controls and the Dallas and Dominican patients no effects were seen on enzyme levels 24h after the addition of cycloheximide. The authors concluded that different mutations were present in the Los Angeles sibship and the Dallas/Dominican families. This paper also demonstrated that caution was required when the 5α-reductase activity of genital-skin fibroblasts is used diagnostically as a test of 5α-reductase deficiency. Thus while the activity in the two Dallas patients was below the/...
normal range - 0.9 and 1.3 pmol/h/mg protein (normal range = 2.3 - 110 pmol/h/mg) in the Dominican patient and two Los Angeles patients activity was in the lower normal range (5, 4 and 3.9 pmol/h/mg protein).

In a study of two patients with 5α-reductase deficiency Fisher et al (1978) demonstrated that epididymal microsomes were unable to catalyse the 5α-reduction of ten different Δ4-3-keto steroids which were normally metabolised by control epididymis. This of course provided further proof that 5α-DHT was not involved in the differentiation of Wolffian duct structures.

In humans, skin is a major site of testosterone 5α-reduction (Gomez and Hsia, 1968; Wilson and Walker, 1969; Voigt et al 1970), and the properties of 5α-reductase in fibroblasts derived from skin from various anatomical sites were described in some detail in the previous section. In this section the involvement of 5α-reductase in the development of some pathological conditions involving skin (or skin derived structures) will be discussed. This is a controversial area and conflicting data have been presented.

It has been suggested that increased 5α-reductase activity is involved in the aetiology of idiopathic hirsutism (Kuttenn and Mauvais-Jarvis, 1975) male-pattern baldness (Bingham and Shaw, 1973) and acne vulgaris (Sansone and Reisner, 1971). These topics have also been the subject of a review by Price (1975).
Kuttenn and Mauvais-Jarvis (1975) measured the % conversion of testosterone to 5α-DHT (+ 3α/3β androstandiols) in pubic skin samples from hirsute women and compared them to values obtained for normal men and women. The value for hirsute women (13.5% conversion of testosterone to 5α-reduced products) was much higher than the value obtained in normal women (3.6%) and was in fact close to the value obtained for male pubic skin (14.6%). However, in a subsequent publication the same group (Kuttenn et al, 1977) reported elevated levels of 5α-reductase activity in pubic skin homogenates from women with idiopathic hirsutism. But in women whose hirsutism was established to be of ovarian or adrenal origin levels of 5α-reductase activity in pubic skin homogenates were only slightly above normal. Furthermore in a study of forty hirsute women it was demonstrated that the plasma levels of androgens (testosterone, 5α-DHT and androstenedione) were significantly elevated above normal control values. This led the authors to suggest that there were two factors involved in the development of hirsutism: increased levels of androgens circulating in the plasma and also increased local conversion of testosterone to 5α-DHT by target cells in the skin. Further ambiguity concerning the role of 5α-DHT in the development of female hirsutism was noted in a study by Jenkins and Ash (1973). In a study of six hirsute women they reported elevated levels of 5α-reductase in supra-pubic skin samples of only four of these subjects. Note however the endocrinological status of these patients was not described in detail and it is possible that the hirsutism observed in the two patients with /.../
normal levels of 5\(\alpha\)-reductase may have been of adrenal or ovarian origin.

Sansone and Reisner (1971) demonstrated differential rates of 5\(\alpha\)-reductase activity in normal and in acne-bearing skin of both men and women. In skin from the chin of women suffering from acne they found the level of 5\(\alpha\)-DHT formation to be 49.6 pmol/mg tissue/3h (cf. normal female chin = 3.03 pmol/mg tissue/3h. Hay and Hodgins (1974) however did not detect any difference in the metabolism of testosterone, androstenedione or dehydroepiandrosterone between the skin of male controls and acne patients. The observation that human (and rat) skin was able to convert 5\(\alpha\)-androstene-3\(\beta\), 17\(\beta\)-dihol into testosterone and 5\(\alpha\)-DHT and that 5\-androstene-3\(\beta\), 17\(\beta\)-dihol stimulated the growth of sebaceous glands (Hodgins and Hay, 1974) has led to the suggestion that the enzyme which converts 5\-androstene-3\(\beta\), 17\(\beta\)-dihol into testosterone (3\(\beta\)-hydroxy steroid dehydrogenase -\(\Delta^{4}\)-5 isomerase -\(\Delta^{5}\)-3\(\beta\)-HSD) may be involved in the aetiology of acne vulgaris. (Baillie et al, 1966)

Local excess production of 5\(\alpha\)-DHT has also been implicated in the development of male pattern alopecia in genetically susceptible men (Adachi and Kano, 1970; Bingham and Shaw, 1973; Schweikert and Wilson, 1974). Bingham and Shaw (1973) demonstrated that although male pattern baldness was not associated with any grossly abnormal changes in testosterone metabolism, nevertheless testosterone uptake and metabolism to 5\(\alpha\)-reduced products was greater in bald than in hairy /...
skin. Schweikert and Wilson (1974) also reported elevated levels of 5α-reductase in the frontal scalp of balding individuals. Note however that the situation is complicated by the fact that different types of hair follicles metabolise testosterone differently. For example in growing hair follicles (anagen follicles) androstenedione is the main metabolite of testosterone, whereas in resting (telogen) follicles 5α-DHT formation is higher than in anagen follicles. Therefore the pattern of testosterone metabolism observed in a skin sample will depend to a large extent on the relative distribution of different types of hair follicle.

It must be recognised however that skin is not a homogeneous tissue but does in fact contain several different structures. Using micro-dissection and collagenase-digestion, Hay and Hodgins (1978) separated samples of forehead and axillary skin into five different components viz. epidermis, sweat glands, sebaceous glands, hair follicles and dermis. 5α-reductase activity was found in each of these tissue compartments. However uneven distribution of some steroid-metabolising enzymes was observed (eg. 90% of Δ5-3β-HSD activity of forehead was located in the sebaceous gland). It was suggested therefore that some of the changes seen in androgen metabolism by skin affected by pathological changes described above may occur secondarily to changes in the anatomical structure of the skin (eg. the hypertrophy of the sebaceous gland which occurs in acne vulgaris — Plewig (1974)). Furthermore, it is also appropriate to take /...
into account the temporal pattern of $5\alpha$-DHT production by skin. Thus $5\alpha$-reductase activity may increase at a specific time in specific tissues of the skin when the effect of $5\alpha$-DHT is produced. When this is over enzyme activity may decline (Price, 1975). This phenomenon was reported in skin slices from foreskin, where $5\alpha$-reductase activity was highest in foreskins from young donors (Wilson and Walker, 1969).

Finally in this section on the physiological significance of testosterone $5\alpha$-reduction, some discussion of the $5\alpha$-reductase activity in the brain and central nervous system is appropriate.

The presence of $\Delta^4$-3-ketosteroid $5\alpha$-reductase in several tissues of the central nervous system is well-documented (Bertics et al, 1984; Bertics and Karavolas, 1984; Bertics and Karavolas, 1985; Cheng and Karavolas 1975a; Cheng and Karavolas 1975b; Stupnicka et al, 1972) and the biochemical and physiological significance of $5\alpha$-reduction in neuroendocrine structures has been the subject of a recent review (Martini, 1982). The levels of $5\alpha$-reductase in neuroendocrine structures are known to be sensitive to hormonal manipulations. For example castration increased the activity of rat anterior pituitary $5\alpha$-reductase in vitro (Kniewald and Milkovic, 1973) and testosterone administration returned activity levels back to normal. An interesting finding was that exposure of male rats to constant darkness decreased the levels of $5\alpha$-reductase in the hypothalamus, suggesting that the pineal gland (a photo-sensitive structure) may control hypothalamic $5\alpha$-reductase activity (Shapiro et al, 1976). Indeed melatonin (a pineal hormone)
increased the activity of hypothalamic $5\alpha$-reductase in male rats (Frehn et al, 1974).

It was demonstrated that systemically administered $5\alpha$-DHT was more effective than testosterone at inhibiting LH release both in the male rat (Naftolin and Feder, 1973) and in men (Stewart-Bently et al, 1974). Confirmation of this was provided by the observation of elevated levels of LH in the plasma of individuals with $5\alpha$-reductase deficiency (Imperato-McGinley et al, 1974). These observations together with the finding that the amount of $5\alpha$-DHT formed in the anterior pituitary, hypothalamus and cerebral cortex of pre-pubertal rats was higher than in pubertal or post-pubertal rats led to the hypothesis that the LH surge seen at puberty is due to a decrease in $5\alpha$-reductase activity of these neuroendocrine structures and the subsequent decrease in inhibition of LH release by $5\alpha$-DHT (Massa et al, 1975).

Reports in the literature have suggested a role for $5\alpha$-DHT in the maintenance and development of male sexual behaviour. Administration of testosterone to castrated male rats was shown to restore male sexual behaviour (Beyer, 1976). As this could be blocked by inhibitors of the aromatase enzyme complex or by anti-oestrogens (Beyer et al, 1976; Christensen and Semens, 1975) the effects of testosterone were presumed to be mediated via its conversion to oestrogens. Indeed administration of oestrogens to neonatal female rats caused a masculinisation of behaviour (Doughty et al, 1975).
However prolonged systemic treatment with sufficiently high doses of 5α-DHT (which is not an aromatisable androgen) can restore sexual behaviour in the castrated male rat. (Sodersten, 1975; Paup et al, 1975). It was demonstrated also that the effects of 5α-DHT on sexual behaviour were greatly enhanced when oestradiol was simultaneously administered (Baum and Vreeberg, 1973). It is possible therefore that both 5α-reduced and oestrogenic steroids are involved in the establishment and maintenance of male sexual behaviour. Evidence from studies of 5α-reductase deficient patients showed that, at puberty, these individuals developed a normal male psycho-sexual habitus (Peterson et al, 1977). However for obvious technical reasons it was not possible to directly demonstrate a lack of 5α-reductase in the neuro-endocrine structures in these patients and it is possible that local accumulation of 5α-DHT could still have occurred in tissues of the central nervous system.
1.2.5. Mechanism of steroid 5α-reduction

There have been several reports by investigators on the catalytic mechanism of steroid 5α-reductase. It was shown that incubation of 4-androstenedione with NADPH and rat liver microsomes in a medium containing $^3$H$_2$O resulted in incorporation of $^3$H at the 4-position of the steroid (McGuire and Tomkins, 1960b). This provided the first evidence for a simple ionic mechanism being involved in the reduction of the 4-5 double bond with transfer of a hydride ion from NADPH to C5 followed by the abstraction of a proton from the reaction medium (see Fig. 7).

Fig. 7 Proposed reaction mechanism of steroid 5α-reductase

![Proposed reaction mechanism of steroid 5α-reductase](image)

The putative enolate intermediate present during the reaction is shown in brackets in Fig. 7.

Support for this theory was obtained later when the incorporation of $^3$H into the 5α position was demonstrated following the 5α-reduction of 7α-hydroxycholest-4-en-3-one by rat liver microsomes in the presence of NADP$^3$H (Bjorkhem,/)..
(Bjorkhem, 1969a). The same author reported that $^3$H incorporation was five times greater with $[4B-^3H]$ NADPH than with $[4A-^3H]$ NADPH*. It was also noted that as at least half of the hydrogen incorporated at the 4-position is in the configuration the reaction mechanism does not proceed as a straightforward trans addition of hydrogen across the 4-5 double bond.

Subsequently the specificity of steroid 5α-reductase for the 4B-hydrogen of NADPH was shown in the reduction of testosterone by rat liver microsomes (Abul-Hajj, 1972; Bjorkhem, 1969b), by seminal vesicle microsomes (Suzuki and Tamaoki, 1974) and by both the nuclear and microsomal enzymes of rat prostate (Nozu and Tamaoki, 1974b). This specificity for the 4B-hydrogen of NADPH may be a general feature of membrane-bound steroid metabolising enzymes as the hepatic microsomal 3β-hydroxysteroid dehydrogenase also abstracts this hydrogen from NADPH (Bjorkhem and Danielsson, 1970). Soluble steroid-metabolising enzymes, for example the steroid 5β-reductase (Bjorkhem 1969(b)) and 3α-hydroxysteroid dehydrogenase (Bjorkhem and Danielsson, 1970) of rat liver cytosol show the opposite stereo-specificity in that they preferentially remove the 4A-hydrogen from NADPH.

*Absolute configurations for differentially labelled reduced co-factors are $[4B-^3H] = [4S-^3H]$ NADPH and $[4A-^3H]$ NADPH = $[4R-^3H]$ NADPH (Cornforth et al, 1966).
This model of steroid $5\alpha$-reduction involving the direct transfer of reducing equivalents from NADPH to the steroid was challenged when it was observed that reduced co-enzyme Q ($QH_2$) could replace NADPH as hydrogen donor in the reduction of testosterone by rat liver microsomes (Golf and Graef, 1978). This topic will be discussed in detail in Section 4 "General discussion and conclusions".
1.2.6. Solubilisation of 5α-reductase

The first successful solubilisation of 5α-reductase was achieved with the enzyme from rat prostate (Moore and Wilson, 1974). Using digitonin at a final *D/P ratio of 2 and in a buffer containing 3 mol/1 KCl the enzyme was removed from the nuclear membrane in a form which did not sediment after centrifugation at 105000 g for 1 h and which was included on a gel filtration column. The Mr was estimated at 250000 - 300000 and the enzyme displayed a sedimentation co-efficient of 13.5 - 15S. The hypothalamic microsomal progesterone 5α-reductase has also been solubilised by a combination of high salt and digitonin (Bertics and Karavolas, 1984). The authors reported that 39% of the total progesterone 5α-reductase was solubilised by this procedure.

The solubilisation of the rat hepatic microsomal 5α-reductase by the non-ionic detergent Lubrol WX has also been achieved (Golf and Graef, 1978).

[*D/P ratio = detergent/protein ratio (w/w)]
Solubilisation was again performed in the presence of high concentrations of salt (0.1 mol/l KCl and 0.1 mol/l sodium citrate). The criterion of solubility was that the enzyme remained in the supernatant of a solubilisation mixture which contained 40% (v/v) glycerol, following centrifugation at 105000g for 1h. Lubrol WX has also proved successful in the solubilisation of the microsomal and nuclear enzymes from rat epididymis (Scheer and Robaire, 1983). 66% of the total microsomal 5\(\Delta\)-reductase activity was released from the membrane. The sedimentation co-efficient for the nuclear enzyme was 10.1S, somewhat less than that previously reported for the nuclear enzyme of rat prostate (Moore and Wilson, 1974). The epididymal microsomal enzyme sedimented at 11.6S. It must be recognised however, that the different detergents used for solubilisation of the prostatic and epididymal enzymes could bind to 5\(\Delta\)-reductase to different degrees and that the difference seen in the sedimentation co-efficients of the prostatic and epididymal enzymes may reflect this. Alternatively differing sedimentation co-efficients may reflect different vesicle sizes between the digitonin and Lubrol WX solubilised species.

Finally, the successful solubilisation of 5\(\Delta\)-reductase from human tissues has also been described. Thus repeated (x3) extraction of human prostatic microsomal fraction with 0.08% (w/v) Lubrol PX, in the presence of 0.1 mol/l KCl and 0.1 mol/l sodium citrate solubilised 70% of the total 5\(\Delta\)-reductase (Houston et al, 1985).
In most of these solubilisation experiments, the generation of solubilised enzyme species has necessitated the inclusion of NADPH in the solubilisation buffers (0.5 - 5 mmol/l).

As, in most cases, the criterion of solubilisation of enzyme activity was simply that activity was recoverable in the supernatant of the solubilising solution following treatment of the enzyme-containing membrane with detergent and high-speed centrifugation (usually 1h at 105,000g), it is important to appreciate that the density of the solubilisation mixture will have an effect on the sedimentation behaviour of the enzyme. In the case of the rat epididymis, pituitary and liver and the human prostate, where solubilisation was achieved by the use of the non-ionic detergents Lubrol WX, Lubrol PX or n-octyl glucoside, the solubilisation solution contained 20% (v/v) glycerol [40% (v/v) glycerol in the case of the rat liver enzyme]. Therefore in these instances the density of the solubilisation mixture is clearly > 1. When digitonin was used to solubilise the rat prostate and hypothalamic enzymes no glycerol was present during the solubilisation procedure.

It is probably wise therefore to distinguish between large, slowly-sedimenting enzyme complexes and truly solubilised species by using the partitioning of enzyme activity between the void and included volumes of a gel filtration column as an additional criterion of solubility. This was only shown for the digitonin/KCl solubilisation of rat prostatic nuclear 5α-reductase (Moore and Wilson, 1974).
1.2.7. Hormonal control of 5α-reductase

As early as 1958 Yates et al demonstrated a sex-dependance in the rate of ring A reduction of corticosteroids by hepatic 5α-reductase. 5α-reductase activity was found to be higher in the female than the male. Later it was demonstrated that steroid hydroxylating activity also showed sex-dependance (Leybold and Staudinger, 1959). In contrast to 5α-reductase the hydroxylation activity in the male was higher than the female. The finding that castration increased 5α-reductase activity and testosterone treatment returned activity levels to normal male values was the first indication that the sex steroids were involved in the control of these enzyme activities (Yates et al, 1958). This was later confirmed by the demonstration that oestrogen-treatment of intact male rats caused "feminisation" of hepatic steroid-metabolising enzymes (Einarsson et al, 1974).

The involvement of the pituitary gland in this mechanism was suggested by the studies of Denef (1974) and independently by Gustafsson and Stenberg in the same year. They both demonstrated that hypophysectomy of female animals led to hepatic steroid metabolism reverting to the male-type. Furthermore the fact that androgens or oestrogens have no effect on the hepatic steroid metabolising enzymes of hypophysectomised rats was a further indication of the necessity of an intact pituitary for control of these enzyme activities. (Gustafsson and /...
Stenberg, 1976). The authors suggested that these findings were compatible with the hypothesis that the pituitary secreted a substance (feminising factor) which was necessary for the female-type pattern of hepatic steroid metabolism and that gonadal steroids acted to influence the secretion of this substance.

The first indication as to the nature of this pituitary feminising factor came with the observation that administration of growth hormone to intact male rats resulted in the feminisation of hepatic steroid metabolising enzymes (Kramer and Colby, 1976). The situation was complicated however by the finding that administration of GH alone twice daily to hypophysectomised rats had no effect on enzyme activities (Rumbough and Colby, 1980) whereas continuous infusion of the hormone did indeed produce feminisation of the enzyme activities (Mode et al., 1981).

It was already known that there were sex-related differences in the temporal pattern of growth hormone secretion (Eden, 1979). In the male, plasma levels of GH peak every 3-4h. and fall to very low levels in the troughs between peaks. In the female there are fewer peaks of GH secretion and there is a much narrower range of GH levels in the plasma. It was therefore suggested that it was not GH per se which caused feminisation of the enzyme activities, but the different temporal pattern of plasma GH levels in the female compared to the male (Mode et al., 1981).

This hypothesis was confirmed when it was observed that in hypophysectomised female rats, where hepatic /..
steroid metabolising enzymes have reverted to the male-
type pattern of activity, administration of GH to the
animals would feminise enzyme activities only if it was
infused continuously or administered in several divided
doses daily (Mode et al, 1982). Thus human GH (120ug/day)
given as 8 x 15 ug doses at 3h intervals, as 4 x 30 ug
doses at 6h intervals or infused continuously at 5 ug/h
all had the effect of feminising enzyme activities.
However, if the hormone was administered as 2 x 60 ug doses
at 12 h intervals, no effect was seen on the enzyme
activities - i.e. they remained at the male levels. The
same workers also demonstrated that castration or oestrogen
treatment of male rats (which caused feminisation of
enzyme activities) caused the plasma pattern of GH levels
to change from the male-type pattern to the female type.
Administration of testosterone reversed these changes in
the plasma pattern of GH levels. It thus seemed that
gonadal sex steroids influence the temporal pattern of
GH secretion differentially and that this was responsible
for the different levels of hepatic steroid metabolising
enzymes in the two sexes.

As is the case with other pituitary trophic
hormones the hypothalamus exerts an effect on the release
of GH by the secretion of release/inhibiting factors. In
this respect it was found that de-afferentation of the
hypothalamus led to the feminisation of hepatic steroid
metabolising enzymes (Mode et al, 1980). Independently
it was reported that castration led to a decrease in the / .
levels of somatostatin (GH-inhibiting factor) in the median eminence of the hypothalamus and that these levels could be restored by testosterone administration (Gross, 1980). These findings led to the suggestion by Mode et al (1982) that androgen deprivation may cause a decrease in somatostatin secretion which would lead to an incomplete inhibition in secretion of GH, leading in turn to a female-type plasma pattern of GH levels and a feminisation of hepatic steroid metabolising enzyme activities.

Hepatic steroid metabolising enzymes are not the only liver proteins to display sexual dimorphism. There are different levels of synthesis of various proteins in male and female liver (Roy and Chatterjee, 1983). Interestingly, two of these proteins—carbonic anhydrase isoenzymes II and III (CAII & CAIII) exhibited what has been called a "reversed sexual dimorphism" (Jeffery et al, 1986). Thus the level of CAIII is 10–20 times higher in the male than the female, whereas CAII is 2–3 times higher in female than male liver. Furthermore, the control of the levels of CAII and CAIII in the liver appeared to be identical to that described above for the steroid metabolising enzymes. Thus, hypophysectomy led to a masculinisation of enzyme activities in the female, whereas continuous infusion of growth hormone into male rats led to a feminisation of both CAII and CAIII levels. It may be therefore that this mechanism was responsible for controlling the levels of several proteins in the livers of the /..
different sexes. In that respect, recent studies in mouse liver (Norstedt and Palmiter, 1984) have demonstrated a similar role for GH in the control of the levels of hepatic proteins. In the mouse, the liver protein MUP (major urinary protein) shows a sex-dependance. The levels of this protein are 10 times higher in the male than the female (Hastie et al, 1979). However, continuous infusion of either human or bovine GH resulted in a decrease of MUP levels toward female values. Therefore this mechanism whereby the differing secretory patterns of GH in the two sexes controlled the different levels of various hepatic proteins in male and female may be a common feature in several mammalian species.

Finally, as I am essentially concerned with the properties of the enzyme 5α-reductase, it is interesting to note that hormonal manipulation of the male rat has differing effects on the 5α-reductase of the liver and on the enzyme as it exists in androgen-dependent tissues. Thus while castration led to an increase in the levels of hepatic 5α-reductase in the male, it caused a decrease in the 5α-reductase activity of various androgen-dependent tissues. Thus it was found that 14 days following castration the specific activity of the epididymal enzyme had decreased by 83% (De Larminat et al, 1978). Similarly the specific activity of rat prostatic 5α-reductase was reduced by 40% 12 days after castration (Lee et al, 1974). The mechanisms by which androgen-deprivation leads to these opposite effects on the levels of 5α-reductase enzyme in different tissues remain unknown.
1.3. Relationship between the enzymes of steroid hormone and fatty acid metabolism

The function of 5α-reductase in the male embryo is to ensure the proper development of certain structures of the external genitalia and in this respect the conversion of testosterone to 5α-DHT can be viewed as an amplification of the androgenic signal. (see Section 1.2.4.). However, in the adult the role of 5α-reductase is not so clear-cut. For example, some classical androgen-dependant tissues - such as skeletal muscle - have little 5α-reductase activity (Krieg and Voigt, 1977). Similarly, and more relevant to this thesis, liver 5α-reduction of testosterone is a major pathway of androgen catabolism (see Section 1.1.1. Fig. 3). However, in cases of 5α-reductase deficiency, steroid clearance rates are not affected (Peterson et al, 1977), with steroids being metabolised via the 5β isomers resulting in higher 5β/5α reduced steroid ratios in the urine of affected individuals.

It would appear therefore that in the adult tissues of the male there is a certain redundancy of function of 5α-reductase. For this reason it was suggested that 5α-reductase may have a function unconnected with its role in steroid metabolism (Hodgins, 1983). It should be noted that precedents do exist for this "dual functionality" of steroid-metabolising enzymes. For example the 3α-hydroxysteroid dehydrogenase of rat liver cytosol has recently been purified to homogeneity /...
and has been shown to function as a quinone and aromatic ketone reductase (Penning et al, 1984). In the light of these considerations, it has recently been demonstrated that crotonyl CoA is a competitive inhibitor of the microsomal 5α-reductase of human prostate (Hodgins and Gaggini, 1982). The structure of crotonyl CoA and its chemical similarity to testosterone is depicted in Fig. 8

**Fig. 8** Structure of crotonyl CoA: comparison to A and B rings of Δ⁴-3 keto steroid

Crotonyl CoA is a model substrate for the enzyme enoyl CoA reductase which catalyses the final step in the microsomal elongation of fatty acids (Nugteren, 1965). This enzyme, like 5α-reductase, catalyses the NADPH-dependant reduction of a double bond α,β to a carbonyl group. The observation that crotonyl CoA competitively inhibited the 5α-reduction of testosterone by prostate microsomes led to the suggestion that the same enzyme activity catalysed the NADPH-dependant reduction of both these compounds. The affinity of 5α-reductase for crotonyl CoA (KI = 125 umol/l) is x 10⁻⁴-fold less than that for testosterone (Km =10nmol/l). I undertook some experiments in which the effects of crotonyl CoA on the 5α-reduction of testosterone by female rat liver microsomes was examined and the results obtained compared with the situation in human prostate.
1.4. Aims and scope of the present investigation

Up to date, steroid 5α-reductase has remained poorly characterised. This, in part, has been due to the loss of enzyme activity seen on removal of the enzyme from its natural environment in the membrane. In this present study, I have undertaken experiments aimed at the molecular characterisation of this enzyme activity as it exists in female rat liver microsomes. These have included a kinetic study of the membrane-bound enzyme and the subsequent solubilisation of activity from the microsomes. The solubilised enzyme activity has been further characterised by the technique of high-performance gel filtration.

Because of the tendency of the solubilised enzyme to lose activity during further manipulations I have used an alternative approach in attempting to elucidate some of the properties of steroid 5α-reductase. Thus the results of experiments are reported in which 5α-reductase was specifically labelled with a radioactive ligand. The behaviour of this radio-labelled conjugate on high-performance gel filtration is compared with that of the solubilised enzyme activity.

Finally the results of some experiments aimed at investigating the relationship between steroid 5α-reductase and crotonyl CoA reductase activities are reported.
2. MATERIALS AND METHODS

2.1 Materials

Animals:-- Mature female Wistar rats (200-250g) were supplied by the Animal House of the Departments of Biochemistry/Physiology of Glasgow University.

Radioactive Steroids:-- [1,2,6,7-\textsuperscript{3}H] testosterone (Specific Activity 94 Ci/mmol) and 5\alpha-dihydro [4-\textsuperscript{14}C] testosterone (Specific Activity - 59.2 mCi/mmol) were obtained from Amersham International. [1,2-\textsuperscript{3}H] 21-diazo-4-methyl-4-aza-5\alpha-pregnane-3, 20-dione ([\textsuperscript{3}H]-Diazo-MAPD Specific Activity 18.2 Ci/mmol) was a gift from Dr. Anne Cheung of Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, U.S.A.

Radio-inert steroids:-- testosterone, 5\alpha-dihydrotestosterone and 11-deoxycorticosterone were obtained from Steraloids Ltd. 4-Androstendione was from Organon Laboratories. Unlabelled Diazo-MAPD and 4-methyl-4-aza-3-oxo-5\alpha-pregnane-20(s)-carboxylic acid sodiumsalt (4-MAPC) were both gifts of Merck, Sharp and Dohme.

Determination of radio-activity -\textsuperscript{3}H and \textsuperscript{14}C were evaluated simultaneously by an external standard channels ratio method on a Packard Tricarb 300 Liquid Scintillation Counter. Efficiency of counting was 35-40% for \textsuperscript{3}H and 75% for \textsuperscript{14}C. Counting of samples containing tritium alone was done on a single label programme.
The efficiency of counting was 40%.

The scintillant used for counting portions of t.l.c. plates containing radio active steroid contained 5% (w/v) PPO in a solution of toluene containing 10% (v/v) methanol. Otherwise "Ecoscint" was used. This was supplied by National Diagnostics.

SDS-PAGE - Gels were cast between glass plates (20 x 17.5cm). These were supplied locally. Gels were run in a Gel Electrophoresis Apparatus (GE-2/4LS) supplied by Pharmacia. The electrophoresis power supply (EPS 500/400) was also from Pharmacia. The gel drier was from Bio-Rad.

For fluorography, "Amplify" was obtained from Amersham International. XAR-5 x-ray film was obtained from Kodak. Developer (LX-24) and fixer (Fx-40) were also from Kodak.

A solution containing Triton X-100: 1,7 diamino-heptane : H₂O (1:1:10 by vol.) was used to elute protein from the gel prior to determination of radio-activity.

Reagents:-
(a) Detergents:- n-octyl glucoside and (3-[(3-cholamido propyl)-dimethylammonio] 1-propane-sulphonate (Chaps) were from Boehringer-Mannheim, Gmb.H; Lubrol WX and sodium cholate were from Sigma Chemical Co. and sodium deoxy-cholate was supplied by British Drug House Ltd. (BDH)
(b) Other reagents:-- molecular weight markers for SDS-PAGE and gel filtration were from Sigma Chemical Co.; n-valeric, iso-valeric, butyric and crotonic acids, crotonyl Co-enzyme A, Coomassie Brilliant Blue R and bovine serum albumin (crystallised and lyophilised -- essentially globulin free) were also supplied by Sigma. NADPH and NADP+, urea and reagents for SDS-PAGE were from BDH. All other reagents including organic solvents were of analytical grade.

t.l.c. plates:-- were Polygram Sil G. uv254 (M-N).

The following apparatus was used during the course of this work. The suppliers name is in brackets.

125-watt high pressure mercury vapour lamp (Hanovia)
FPLC (Pharmacia)
HPLC (LKB instruments Ltd.)
Pye series 104 gas chromatograph (Varian)
60/80 Carbopack C/0.3% Carbowax 20M/0.1% H₃PO₄ column (Superlco Inc.)
MSE Hi-spin centrifuge (Fisons)
MSE Prepsin 50 centrifuge (Fisons)
18-55 ultracentrifuge (Beckman)
2.2 Methods

2.2.1. Preparation of rat liver microsomes

Mature female Wistar rats (weight 200 - 250g) were starved overnight to deplete livers of glycogen. The following morning the animals were killed by concussion and the following procedures were carried out at 4°C.

Livers were dissected out into homogenisation buffer (10mmol/l Tris. Cl (pH7.0), 0.25mol/l sucrose, 1mmol/l EDTA and 1mmol/l DTT). Connective tissue was removed and the pooled livers of 10-15 rats were washed in homogenisation buffer. A 33% homogenate (1g liver/2ml homogenisation buffer) was made using an MSE homogeniser (maximum setting; 4x30s. periods). The homogenate was centrifuged for 10 min. at 2000g to remove nuclei and cell debris and the supernatant from this spin was re-centrifuged at 10,000g for 10 min. to remove the mitochondrial and lysosomal fractions. The post-mitochondrial supernatant was re-centrifuged at 105,000g for 1h. The microsomal pellet obtained was washed twice with homogenisation buffer and resuspended to a final protein concentration of 10mg/ml in homogenisation buffer. The microsomes were divided into 2ml aliquots and stored at \(-120^\circ\text{C}\) in liquid \(\text{N}_2\).
2.2.2 Assay of hepatic microsomal 5α-reductase activity

5α-reductase activity was assayed according to the method described by Leshin et al (1978). The assay solution contained (final concentration) 0.1 mol/l Tris citrate (pH 7.0 (see p.74), 0.25 mmol/l NADPH, 0.38 umol/l (4.5 nCi) 5α-dihydro [4-14C] testosterone, liver microsomes (0.1 μg protein) and 1 μmol/l testosterone (197.5 pmol unlabelled testosterone and 2.5 pmol [1,2,6,7-3H] testosterone) in a total volume of 200 μl. The reaction was initiated by the addition of microsomes (diluted in 0.1 mol/l Tris citrate (pH 7.0) and incubated at 37°C for 10 min. The incubation was terminated by the addition of 500 μl of chloroform : methanol (2:1 v/v) solution. 10 μg each of testosterone, 5α-dihydrotestosterone, 11-deoxycorticosterone and 4-androstenedione were added in 20μl of ethanol and the mixture was vortexed. The aqueous and organic phases were allowed to separate and the aqueous phase was removed. The organic phase containing extracted steroid was removed and dried under vacuum. The residue was taken up in 20 μl of chloroform : methanol (2:1 v/v) and applied to a t.l.c. plate.

The chromatograph was developed with chloroform : acetone (185:15 v/v). When the t.l.c. plate was viewed under UV illumination (254nm) areas of fluorescence corresponding to testosterone (Rf 0.30), 11-deoxycorticosterone (Rf 0.38) and 4-androstenedione (Rf 0.51) were observed. 5α-dihydrotestosterone does not absorb UV light strongly at 254nm but has an almost identical Rf value to 11-deoxycorticosterone in this solvent system. This was therefore used as a marker for 5α-dihydrotestosterone and this area of the plate was cut out and added to 10 ml of scintillation fluid for determination of 3H and 14C.
Losses of $5\alpha$-dihydrotestosterone during the assay procedure were monitored by measuring the recovery of $5\alpha$-dihydro $[4^{-14}C]$ testosterone and the percentage conversion of testosterone to $5\alpha$-dihydrotestosterone was adjusted accordingly. The identity of $5\alpha$-dihydro $[1,2,6,7-^3H]$ testosterone product was confirmed by re-crystallisation to constant specific activity with authentic $5\alpha$-dihydrotestosterone. Under these assay conditions, $[1,2,6,7-^3H]$ testosterone and $5\alpha$-dihydro-$[1,2,6,7-^3H]$ testosterone accounted for 98% of the total tritium recoverable from the t.l.c. plate. Control incubations which contained no microsomes were included. The assay conditions described above are henceforth referred to as "standard assay conditions".

For assay of solubilised $5\alpha$-reductase activity the amount of protein in the assay was 1-2ug and incubation was for 0-30 min. For fractions from high performance gel filtration, the assay conditions are specified in the relevant figure legends.
2.2.3. Solubilisation of hepatic microsomal 5α-reductase.

When the ability of various detergents to solubilise 5α-reductase was examined, solubilisation was performed in 1 ml of buffer (pH 7.0) which contained (final concentration) - 10 mmol/1 Tris, 20% (v/v) glycerol, 0.1 mol/1 sodium citrate, 0.1 mol/1 KCl, 1mmol/1 EDTA, 1 mmol/1 dithiothreitol(DTT) and 100 umol/1 NADPH. (Solubilisation buffer A). The microsomal protein concentration was 6.5 - 7 mg/ml. Detergents were dissolved in solubilisation buffer A and added to varying detergent:membrane protein ratios (0.1 - 2.5).

After stirring for 1h. at 4 °C, the contents of the solubilisation mixture were centrifuged for 1h. at 105,000g. The supernatant which contained soluble 5α-reductase activity was removed and the pellet was resuspended in 1ml of solubilisation buffer A containing the appropriate concentration of detergent. Both supernatant and resuspended pellet were assayed for 5α-reductase activity and protein content. The % 5α-reductase activity in each fraction is expressed relative to the enzyme activity present prior to centrifugation and is calculated as :

\[
\text{PROTEIN IN SUPN/PLLT X SPECIFIC ACTIVITY} \times \frac{100}{\text{PROTEIN IN SOLUBILISATION MIXTURE X SPECIFIC ACTIVITY}}
\]

The ability of chaotropic agents to solubilise 5α-reductase was examined by an identical methodology with the exception that the buffer used contained (final concentrations) - 10 mmol/1 Tris, 20% glycerol, 1 mmol/1 EDTA, 1 mmol/1 DTT and 100 umol/1 NADPH. (Solubilisation buffer B). Chaotropic agents were added to the final indicated concentrations and the pH of the solution was adjusted to pH 7.0. The protein concentration was 10 mg/ml.
2.2.4 Photo-affinity labelling of hepatic microsomal 5α-reductase

Photo-affinity labelling of 5α-reductase was performed according to the method of Liang et al (1985b). All procedures were carried out at 4°C.

The reaction solution contained 40mmol/l potassium phosphate (pH 7.0), 50 umol/l NADPH, 15 nmol/l [3H] Diazo-MAPD and 2mg microsomal protein in a total volume of 4ml. The contents, in a sealed, Pyrex glass test-tube were placed 5cm from a U.V. light source (125-watt high pressure mercury vapour lamp; Hanovia). The U.V. light source was cooled by means of a water jacket. The solution was irradiated for 1h. with shaking every 10min. Following irradiation, unlabelled Diazo-MAPD was added to a final concentration of 1.5 umol/l and NADP+ was added to a final concentration of 5 mmol/l. The mixture was incubated for a further 15min. to allow for displacement of non-covalently bound [3H] Diazo-MAPD.

2mg of microsomal protein were added as carrier and the microsomes were isolated by centrifugation for 1h. at 105000g. The microsomal pellet was resuspended and washed in 4ml. of a buffer containing 40mmol/l potassium phosphate (pH 7.0), 20 umol/l unlabelled Diazo-MAPD and 1mmol/l NADP+. Following re-isolation the microsomal pellet was suspended in 1ml. of Solubilisation buffer A containing 0.87% (w/v) n-octyl glucoside (final detergent:protein ratio = 1.25). The mixture was stirred gently for 1h. and following centrifugation for 1h. at 105000g, the supernatant was carefully removed and the pellet was resuspended in 1ml. of Solubilisation buffer A containing 0.87% (w/v) n-octyl-glucoside. Aliquots of the /...
supernatant and pellet were removed for determination of protein and radio-activity.

Control incubations which contained excess unlabelled Diazo-MAPD were included for evaluation of non-specific binding of $[^3H]$ Diazo-MAPD. In another control, NADPH was omitted from the incubation mixture and in some cases irradiation was not performed.

2.2.5 High-performance gel filtration of solubilised 5α-reductase activity and solubilised, photo-affinity labelled 5α-reductase.

For analysis of enzyme activity, 200-400μl of solubilised extract (prepared as described in 2.2.3) were filtered through a 0.22 μm filter and applied to the column. 1-2mg. of protein were loaded. Columns were pre-equilibrated and eluted with the appropriate solubilisation buffer containing detergents and chaotropic agents at the concentrations indicated in the relevant figure legends. Gel filtration was performed at ambient temperature and was completed within 90min. On collection, fractions were transferred to the cold room, and were assayed within 1h. for protein content and 5α-reductase activity.

When the behaviour of photo-affinity labelled 5α-reductase was examined by high performance gel filtration, 100μl of solubilised, labelled extracts (prepared as described in 2.2.4) were filtered through a 0.22μm filter and/...
applied to the column. 100-370 ug of protein (up to
40 nCi of tritium) were loaded. Columns were pre-
equilibrated and eluted with the appropriate solubil-
isation buffer containing detergents at concentrations
indicated in the relevant figure legends. Gel filtration
fractions were assayed for protein content and 220 or
500 ul aliquots were removed for determination of radio-
activity.

Calibration was done with the following molecular
weight standards - apoferritin (433k), alcohol dehydr-
ogenase 150k), BSA (65k), carbonic anhydrase (29k) and
cytochrome C (12·3k). Void volume (Vo) was determined
with Blue Dextran 2000. Molecular weight markers were
dissolved and eluted in the appropriate solubilisation
buffers.

2.2.6.1 Sodium dodecyl sulphate polyacrylamide gel
electrophoresis (SDS-PAGE) of photo-affinity labelled
microsomal fractions.

This was performed according to the method of Laemmli
(1970). The composition of the stacking and running
gels is indicated in Tables I and II.
TABLE I

Stacking gel composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Acrylamide stock solution</td>
<td>3.00</td>
</tr>
<tr>
<td>** Stacking gel buffer</td>
<td>5.00</td>
</tr>
<tr>
<td>Distilled H\textsubscript{2}O</td>
<td>12.00</td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulphate</td>
<td>0.164</td>
</tr>
<tr>
<td>† TEMED</td>
<td>0.020</td>
</tr>
</tbody>
</table>

TABLE II

Running gel composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock solution</td>
<td>13.40</td>
</tr>
<tr>
<td>§ Running gel buffer</td>
<td>12.40</td>
</tr>
<tr>
<td>Distilled H\textsubscript{2}O</td>
<td>24.10</td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulphate</td>
<td>0.164</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.025</td>
</tr>
</tbody>
</table>

* The acrylamide stock solution contained 29.2 g acrylamide and 0.8 g, N, N\textsuperscript{1}—Methylene bis acrylamide in 100 ml of distilled H\textsubscript{2}O.

** The stacking gel buffer was 0.5 mol/l Tris Cl (pH 6.8) containing 0.4\% (w/v) SDS.

† TEMED was N, N, N\textsuperscript{1}, N\textsuperscript{1}—tetramethylene diamine

§ The running gel buffer was 1.5 mol/l Tris Cl (pH 8.8) containing 0.4\% (w/v) SDS.

The stacking gel was therefore 4\% acrylamide and the running gel 10\% acrylamide. /...
Prior to application of samples to the gel wells they were incubated with an equal volume of sample buffer at 100°C for 5 min. The composition of sample buffer is shown below in Table III

**TABLE III**

<table>
<thead>
<tr>
<th>Composition of sample buffer</th>
<th>Vol. (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mol/l Tris. Cl(pH6.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>4.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.8</td>
</tr>
<tr>
<td>10%(w/v) SDS</td>
<td>1.6</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>0.4</td>
</tr>
<tr>
<td>0.05%(w/v) bromophenol blue</td>
<td>0.2</td>
</tr>
</tbody>
</table>

For stacking, electrophoresis was performed for 1h at 15 mA and separation of proteins in the running gel was achieved by electrophoresis for 4 - 5h at 20 mA. The gel running buffer contained 0.192 mol/l glycine, 0.1%(w/v) SDS in 0.025 mol/l Tris. Cl(pH8.3). 4 litres of running buffer were made up fresh prior to each electrophoresis experiment.

Protein separated by electrophoresis was visualised by fixing and staining the gel in a solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and 0.2% (w/v) Coomassie Blue for 1h. Gels were destained overnight in 5% (v/v) methanol, 7.5% (v/v) acetic acid and dried down under /...
vacuum onto chromatography paper.

2.2.6.2. Fluorography

For fluorography, the relevant portions of the gel were processed as follows. Following electrophoresis gels were fixed overnight in a solution of 50% (v/v) methanol, 10% (v/v) acetic acid. Gels were then placed in a solution of "Amplify" and shaken for 20 - 30 min at room temperature. After drying the gel was exposed to x-ray film at -70°C for up to 2 weeks. Film was immersed in developer for 10 min transferred to an acetic acid stop-bath for 30 s and then placed in fixative for 10 min. The developed films were then left to dry prior to inspection.

2.2.6.3. Counting of Gel Slices

Relevant tracks of the gel were cut into 2 mm slices with an apparatus consisting of fixed, mounted razor blades. Gel slices were added to scintillation vials containing 1 ml of the gel swelling solution - 1,7 diamino-heptane: Triton X-100 : distilled H₂O (1:1:10, by vol). The following morning 5 ml "Ecoscint" was added and the vials were taken for determination of radio-activity.
2.2.7. Assay of microsomal crotonyl CoA reductase activity

(a) g.l.c. Assay of crotonyl CoA reductase - this was performed according to the method of Nagi et al (1983). The reaction mixture contained (final concentration) - 0.1 mol/l Tris. Cl (pH7.4), 1 mmol/l NADPH and 150 umol/l crotonyl CoA in a total volume of 1 ml. The reaction was initiated by the addition of microsomal protein (0.02-1 mg) in 0.1 mol/l Tris. Cl (pH7.4). The mixture was incubated at 37°C for up to 15 min. and the assay was terminated by addition of 0.5 ml 15% (w/v) KOH in methanol and following the addition of 1 ug n-pentanoic acid as internal standard, the assay contents were saponified at 65°C for 45 min. The pH of the reaction solution was adjusted to pH1 by the addition of 0.5 ml 4 mol/l HCl and the aqueous phase was extracted with 2 x 3 ml aliquots of diethyl ether. The volume of the ether extract was reduced by evaporation under a gentle stream of nitrogen gas to approximately 25 ul and 1 ul of this was injected into the gas chromatograph using a Hamilton syringe.

Nitrogen was used as carrier gas at a flow rate of 45 ml/min and the column was operated isothermally at 120°C. Fatty acids were identified by comparing their retention times with those of authentic standards. For quantitation a suitable internal standard (n-pentanoic acid) was included. The amounts of butyric and crotonic acid present was obtained by measuring the area under each peak. This was then expressed as a ratio relative to the area under the peak of the internal standard. Reference to a calibration curve/...
of amounts of butyric/crotonic acids at differing peak area ratios allowed for quantitation of product and substrate. Peak areas were measured by the triangulation method (peak height x width at half peak height).

(b) **Spectrophotometric assay of crotonyl CoA reductase activity** - this was performed by a modification of the method described by Podack and Seubert (1972). The reaction mixture contained 0.2 mol/l potassium phosphate (pH 7.0), 0.2 mol/l NADPH, 100-600 umol/l crotonyl CoA, 2 umol/l rotenone, 5% (w/v) di-methyl sulfoxide (DMSO) and 70 - 600 ug microsomal protein added in 0.2 mol/l potassium phosphate buffer. The final volume of the reaction mixture was 1ml. The contents were incubated at 30°C and the rate of NADPH oxidation was monitored by following the decrease in absorbance at 340 nm in a Pye Unicam SP6 - 550 UV/VIS recording spectrophotometer. The reaction was initiated by the addition of microsomal protein. Owing to the ability of microsomes to oxidise NADPH in the absence of crotonyl CoA, all assays were carried out against controls which contained all the components of the assay mixture except crotonyl CoA.

2.2.8. **Protein assays**

Protein was assayed according to the method described by Bradford (1976) with the modifications reported by Spector (1976). Bovine serum albumin was used as standard. When detergent-containing solutions were assayed for protein content samples were read against blanks containing the appropriate concentration of detergent. The protein /...
concentration of fractions from gel filtration experiments using Lubrol WX was measured by integrating the area under the OD280 trace for each fraction.

2.2.9. Kinetics.

Km (app) testosterone and Km (app) NADPH of microsomal 5α-reductase were derived using the direct linear plot method described by Eisenthal and Cornish-Bowden (1974). Data obtained on the rate of microsomal 5α-reductase activity at varying concentrations of testosterone and two different concentrations of NADPH were analysed by the Lineweaver-Burk (1934) double reciprocal plot.

2.2.10. Buffers.

Tris.Citrate buffer was routinely used for assay of 5α-reductase activity. This buffer consisted of a solution containing 0.1 mol/l Tris base and 0.1 mol/l sodium citrate. the buffer was adjusted to the desired pH by the addition of HCl.

2.2.11. Authentication of 5α-DHT as the product of 5α-reductase activity.

The identity of 5α-dihydro[1,2,6,7-3H]testosterone produced by the enzymic reduction of [1,2,6,7-3H]testosterone was confirmed by re-crystallisation to constant specific activity. [3H] 5α-DHT was eluted from the relevant portion of the t.l.c. plate into 5 ml of methanol. Aliquots /...
were removed and the radioactivity determined. 50 mg of unlabelled authentic 5α-DHT was added and the counts were expressed as d.p.m./mg 5α-DHT (specific activity).

The methanol extract was dried down and dissolved in 500 ul of acetone and refluxed gently. 1ml hexane was added and the mixture was boiled (removing acetone) until crystals appeared. The tube was left in the deep freeze for 2h. The supernatant was removed (S1). The crystals were redissolved in 500 ul of acetone and the procedure repeated. The second supernatant was removed (S2). The recrystallisation was repeated using chloroform:hexane (1:2) and the supernatant (S3) was removed. S1, S2, S3 and the remaining crystals were dried down under vacuum. Exact quantities of crystals from each of the four samples were weighed out, dissolved in 10ml scintillation fluid and the radioactivity determined. The results are expressed as specific activity (d.p.m./ug crystals). A constant specific activity throughout repeated recrystallisation indicated the authenticity of 5α-dihydro[1,2,6,7-3H]testosterone produced by the enzymic reaction.
3. EXPERIMENTAL

3.1. Properties of Hepatic Microsomal 5α-reductase

3.1.1. Identification of metabolites produced on incubation of rat liver microsomes with testosterone.

The pattern of metabolites produced when testosterone was incubated with rat liver microsomes depended on the amount of microsomal protein present in the assay mixture. This is illustrated by the data in Table IV.

Table IV Testosterone metabolism in relation to the amount of microsomal protein present in the assay

<table>
<thead>
<tr>
<th>Protein/assay(ug)</th>
<th>13.0</th>
<th>1.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Ratio-activity recovered as:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>(8.1;4.0)</td>
<td>(80.5;78.9)</td>
</tr>
<tr>
<td>5α-dihydrotestosterone</td>
<td>(29.9;14.0)</td>
<td>(18.2;19.1)</td>
</tr>
<tr>
<td>5α-androstanediols</td>
<td>(45.8;76.0)</td>
<td>(0.5;0.5)</td>
</tr>
<tr>
<td>*Polar metabolites</td>
<td>(11.2;3.3)</td>
<td>(0.5;0.5)</td>
</tr>
</tbody>
</table>

Standard assay conditions (see Methods; section 2.2.2.) with assays containing either 13ug or 1.3ug of microsomal protein. Each result represents a single experiment.

At the higher protein concentration, there was extensive further metabolism of 5α-DHT to androstanediol and polar metabolites (*polar metabolites are defined as those recovered between the origin and androstanediol following development of the t.l.c. plate; these are probably hydroxylated steroids /...
(Hay and Hodgins, 1973) although no further experiments were undertaken to elucidate their structure. To avoid complications in the assay procedure therefore, assays of microsomal 5α-reductase activity were carried out with 1 μg of protein. When solubilised 5α-reductase activity was assayed, 2μg of protein were present in the assay mixture. This was done to ensure measurable conversions of testosterone to 5α-DHT. In these instances testosterone and 5α-DHT still accounted for > 98% of the recovered radio-activity. Some of the fractions from the gel filtration of solubilised microsomes converted substantial amounts of testosterone and 5α-DHT to polar metabolites (see Section 3.3.4).

The identity of [3H]-5α-DHT produced by the enzymic reaction was confirmed by re-crystallisation to constant specific activity with authentic radio-inert 5α-DHT according to the procedure described in Methods section 2.2.11 – see Table V.

Table V  

<table>
<thead>
<tr>
<th>Authentication of [3H] 5α-DHT produced on enzymic 5α-reduction of [3H] testosterone by rat liver microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Activity (d.p.m./mg 5α-DHT)</td>
</tr>
<tr>
<td>Original solution</td>
</tr>
<tr>
<td>Supernatant 1</td>
</tr>
<tr>
<td>Supernatant 2</td>
</tr>
<tr>
<td>Supernatant 3</td>
</tr>
<tr>
<td>Final crystals</td>
</tr>
</tbody>
</table>

5α-DHT (50mg) was dissolved in the methanol eluate of t.l.c. plate containing putative [3H] 5α-DHT. The purity of [3H] /...
5α-DHT was checked by monitoring specific activity through three re-crystallisations.

3.1.2. Linearity of product formation with time

The results of a time course on hepatic microsomal testosterone 5α-reductase are illustrated in Fig. 9. When testosterone and NADPH were incubated with 1 µg of microsomal protein under standard assay conditions, the rate of testosterone 5α-reduction remained linear up to approximately 15 min. By this time 25% of the substrate had been converted into 5α-DHT.
Fig. 9  Time course of hepatic microsomal 5α-reductase activity; Standard assay conditions (see Methods Section 2.2.2.) with incubations terminated at the indicated times. For each time point duplicate incubations were performed and the results are expressed as the mean ± range.
3.1.3. Linearity of product formation with enzyme concentration.

Fig. 10 indicates the results of an experiment demonstrating that the rate of 5α-reduced product formation was linear up to a protein concentration of lug/assay.
Fig. 10 Conversion of T to 5α-DHT as a function of enzyme concentration. Microsomal protein was added to the final indicated concentrations and reaction mixtures were incubated for 5 min. Otherwise standard assay conditions (see Methods Section 2.2.2.). For each protein concentration duplicate incubations were performed and results are expressed as the mean ± range.
3.1.4 pH optimum of hepatic 5α-reductase.

5α-reductase activity was assayed at pHs varying from pH4 – pH9 in 0.1 mol/l Tris-citrate buffer. As Fig.11 demonstrates the microsomal enzyme displayed a broad pH optimum around 7-8.

Pre-incubation of the enzyme for 20min. in 0.1 mol/l Tris-citrate (pH4 – 9) containing 0.5 mol/l NADPH, followed by assay at pH7, resulted in a pH profile similar to the original one (see dashed line; Fig.11). This indicated that the observed pH profile of 5α-reductase was due to an instability of the enzyme at extremes of pH rather than a dependancy of the reaction mechanism on pH.
Fig. 11 Determination of the pH optimum of hepatic microsomal 5α-reductase. Enzyme activity was measured in Tris. citrate buffer at the indicated pH. Standard assay conditions (see Methods Section 2.2.2) for each pH duplicate incubations were performed and the mean values are plotted. In each case individual values for enzyme activity differed by less than 10%.
3.1.5 Estimation of the apparent Km testosterone

The apparent Km of hepatic microsomal 5α-reductase for testosterone was determined under standard assay conditions with the concentration of testosterone varied from 25 - 400 nmol/l and the concentration of NADPH fixed at 0.5 mmol/l. The data obtained were analysed by the direct linear plot method described by Eisenthal and Cornish-Bowden (1974). See Fig. 12. This method gave a median value for the Km app. (testosterone) of 370nmol/l (range 110-395nmol/l). The median value obtained for V max was 1.74nmol min⁻¹mg⁻¹ (range 0.76 - 1.86nmol min⁻¹mg⁻¹).
Fig. 12 The rate of microsomal 5α-reduction as a function of testosterone concentration. The concentration of testosterone was varied from 0 - 0.4 umol/l. The concentration of NADPH was constant at 0.5 mmol/l. Otherwise standard assay conditions (see Methods section 2.2.2). For each testosterone concentration duplicate incubations were performed and results are expressed as means. Ranges differed by <5% for each testosterone concentration. Data were analysed by the direct linear plot method.
3.1.6. Estimation of apparent Km NADPH

The apparent Km of microsomal 5α-reductase for NADPH was also determined under standard assay conditions, with the concentration of NADPH varied from 0.25 - 5umol/l and the concentration of testosterone fixed at 1umol/l. Data were again analysed by the method of Eisenthal and Cornish-Bowden (1974). A median Km app. (NADPH) of 1.5umol/l (range 0.55-2.30umol/l) was found - see Fig. 13. In this instance a median V max value of 3.37nmol min⁻¹mg⁻¹ (range 1.50 - 4.37nmol min⁻¹mg⁻¹) was found.
Fig. 13 The rate of microsomal testosterone 5α-reduction as a function of NADPH concentration. The concentration of NADPH was varied from 0-5 umol/1. The concentration of testosterone was constant at 1 umol/1. Otherwise standard assay conditions (see Methods section 2.2.2). For each NADPH concentration duplicate incubations were performed and results are expressed as means. Ranges differed by <5% for each NADPH concentration. Data were analysed by the direct linear plot method.
3.1.7. The rate of microsomal 5α-reductase at varying concentrations of testosterone and two different concentrations of NADPH

Microsomal 5α-reductase activity was measured over a range of testosterone concentrations (0-1 umol/l) at two concentrations of NADPH - 2.5 umol/l (Km) and 2.5 mmol/l (saturating). The data obtained were analysed by the Lineweaver-Burk double reciprocal plot and are shown in Fig. 14.

Inspection of Fig. 14 shows that the lines obtained intersect close to the 1/v axis. This data rules out the Hünig - Pong mechanism and suggests that this bi-substrate reaction proceeds via a sequential mechanism.
Fig. 14  Rate of microsomal 5α-reductase activity at varying concentrations of testosterone and two different concentrations of NADPH.

Testosterone concentration varied from 0-1 umol/l and NADPH concentration was fixed at either 2.5 umol/l or 2.5 mmol/l. Otherwise standard assay conditions. For each concentration of testosterone and NADPH duplicate incubations were performed and the results are expressed as the mean ± range.
3.1.8. Stability of hepatic microsomal 5β-reductase activity

Microsomal 5β-reductase activity was stable when stored in liquid N₂. No activity was lost over 18 weeks - see Table VI.

Table VI  Stability of hepatic microsomal 5β-reductase activity

<table>
<thead>
<tr>
<th>Time</th>
<th>Specific Activity (nmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.49 (±0.21)</td>
</tr>
<tr>
<td>1 day</td>
<td>4.21 (±0.18)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>3.18 (±0.29)</td>
</tr>
<tr>
<td>18 weeks</td>
<td>4.50 (±0.30)</td>
</tr>
</tbody>
</table>

Standard assay conditions. Microsomes were stored in liquid N₂ and at the indicated times thawed and assayed for 5β-reductase activity. For each time point four assays were performed and the results are expressed as the mean ± S.E.
3.1.9. Discussion

The results shown in Sections 3.1.2 and 3.1.3 indicate that the specific activity of female rat hepatic microsomal 5α-reductase is of the order of 4-5 nmol 5α-DHT formed/min/mg microsomal protein. Shown below are values found for the specific activity of female rat liver microsomal 5α-reductase by other workers:

<table>
<thead>
<tr>
<th>[Testosterone]</th>
<th>[NADPH] (nmol/min/mg)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.18 mmol/l</td>
<td>0.12 mmol/l</td>
<td>5.2</td>
</tr>
<tr>
<td>1.0 umol/l</td>
<td>50 umol/l</td>
<td>16.0</td>
</tr>
<tr>
<td>0.10 mmol/l</td>
<td>0.4 mmol/l</td>
<td>11.4</td>
</tr>
</tbody>
</table>

It should be noted that in the study reported by Liang et al (1985b), Sprague-Dawley rats were used whereas in this work Wistar rats were used for the preparation of microsomes. Similarly Liang et al (1985b) conducted their 5α-reductase assays in potassium phosphate buffer of pH 6.5. Little difference was found in this study when 5α-reductase activity was assayed in a 0.1 mol/l Tris-citrate buffer at pH 6.5 or pH 7.0 (see Fig. 11; section 3.1.4). Nevertheless, it is possible that the enzyme may exhibit a different pH profile in a buffer of different composition. Although Roy (1971) used a similar radiochromatographic assay method and assay pH (6.9) as those employed in this study, he did not specify the strain of rat which was used as the enzyme source. He also used concentrations of steroid 180-fold higher than that used in this study (0.18 mmol/l vs 1 umol/l). As this /...
far exceeds the solubility of testosterone in aqueous solutions
some doubt must exist as to the physical nature of the substrate
presented to the enzyme. Golf and Graef (1978) did use
Wistar rats for the preparation of hepatic microsomes and
an assay pH of 7. However they also employ concentrations
of steroid (0.10mmol/l) far in excess of those used in the
present study. Furthermore, Golf and Graef (1978) used a
GLC technique to detect the products of the 5α-reductase
reaction and did not specify the amount of microsomal protein
present in their assay solution. Therefore whilst Roy (1971)
reported a similar specific activity to that found in the
present study and Golf and Graef (1978) and Liang et al
(1985b) report 2-fold and 3-fold higher specific activities
respectively, these inconsistencies can be explained by
differences in assay conditions, methodologies and strain
of rat used. The value of 4-5nmol/min/mg reported for
the specific activity of female rat hepatic microsomal enzyme
should be compared with a value of 1.24nmol/min/mg for male
rat hepatic microsomal 5α-reductase (Mode and Norstedt, 1982)
and confirms previous findings (reviewed by Colby, 1980)
that 5α-reductase activity is higher in the female rat than
the male. The findings of a broad pH optimum around pH7-8
agreed with the findings of McGuire and Timklns (1960). It
stands in stark contrast, however, to the data reported by
Roy (1971) who found a pH optimum for 5α-reductase of pH 5.5.
Roy reported that this may not have reflected the true pH
optimum of 5α-reductase, but rather the result of reduced
oxidation of NADPH co-factor by microsomes at acid pH. Thus
at neutral pH the rapid oxidation of NADPH by microsomes may/.
have resulted in the supply of co-factor becoming rate limiting. Indeed Roy (1971) reported that the rate of NADPH oxidation at pH 6.9 was 2.5-fold higher than that at pH 5.6. Thus at the high levels of protein present in the assay (500μg), the differential rate of microsomal NADPH oxidation at different pH values may have resulted in the observed acidic pH profile. This observation by Roy (1971) may be potentially significant as other workers have reported an acidic pH optimum for 5α-reductase from different tissue sources. These include the microsomal enzyme from human neonatal foreskin – pH 5.6 (Voigt et al, 1970), from the human prostate – pH 5.0 (Hodgins and Gaggini, 1983; Liang et al, 1985a), the nuclear and microsomal enzymes of rat seminal vesicles – pH 5.7 – 5.9 (Suzuki and Tamoki, 1974), the testosterone 5α-reductase of human epididymis pH 5.5 (Fisher et al, 1978). Moore et al (1975) have also reported a pH optimum of 5 – 6 for fibroblasts derived from genital skin (foreskin, labia majora and scrotum). It is possible that the observed acidic pH optimum of each of these enzymes may indeed be an artefact due to decreased oxidation of NADPH at acidic pH. However, Fisher et al (1978) reported that the cortisol 5α-reductase activity of human epididymis had a more neutral pH optimum (6.5) and Moore and Wilson (1976) reported that the microsomal enzyme derived from non-genital skin fibroblasts (inguinal biopsy site) did not show a peak of activity at pH 5.5, but displayed a broad pH optimum at pH 7 – 9.

One other reason may be suggested for the acidic pH profile demonstrated for 5α-reductase in some tissues by other workers. NADP⁺ is known to inhibit 5α-reductase activity/...
Thus the increased activity of NADPH oxidising enzymes at neutral pH would cause an increase in the concentration of NADP⁺ present in the assay mixture and this in turn could lead to an inhibition of 5α-reductase activity. The acid pH optimum of microsomal skin 5α-reductase (Voigt et al, 1970) has been explained by proposing that a reaction mechanism which abstracts protons from the medium (see Fig 7; section 1.2.5) would be favoured, by the law of mass action, by an acidic pH optimum. The precipitous drop in enzymic activity seen below pH 5 may be due to the instability of NADPH at the pH. Indeed Voigt et al (1970) demonstrated that at the end of their assay only 10% of the added NADPH was present at pH 4.8.

The neutral pH optimum found for the rat hepatic enzyme in this study compared with that found by different workers in the tissues described above may reflect a difference between the liver enzyme and the enzyme present in these other tissues. Indeed the results of the pre-incubation experiment (dashed line; Fig. 11; section 3.1.4) indicate that the observed pH profile of rat hepatic microsomal 5α-reductase may be due to inactivation of the enzyme at extremes of pH rather than a dependency of the reaction mechanism on pH.

The kinetic data reported in sections 3.1.5.(see Fig.12) and 3.1.6.(see Fig. 13) indicate values for the Km (app) testosterone of 370 nmol/l and for the Km (app) NADPH of 1.5umol/l. Listed below are values obtained by other workers for the...
kinetic constants of 5α-reductase enzymes.

Table VII. Reported Kinetic constants for 5α-reductases

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Steroid</th>
<th>NADPH</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Km*)</td>
<td>(Km*)</td>
<td></td>
</tr>
<tr>
<td>Rat liver (M)</td>
<td>Androstenedione 30</td>
<td>500</td>
<td>McGuire &amp; Tomkins(1960)</td>
</tr>
<tr>
<td>&quot; &quot; (M)</td>
<td>Cortisone 140</td>
<td></td>
<td>McGuire et al(1960)</td>
</tr>
<tr>
<td>&quot; &quot; (M)</td>
<td>Cortisol 150</td>
<td></td>
<td>McGuire et al(1960)</td>
</tr>
<tr>
<td>&quot; &quot; (M)</td>
<td>Testosterone 154</td>
<td></td>
<td>Nozu &amp; Tamoaki(1974)</td>
</tr>
<tr>
<td>&quot; &quot; (M)</td>
<td>Testosterone 2</td>
<td></td>
<td>Roy (1971)</td>
</tr>
<tr>
<td>Rat prostate(W)</td>
<td>Testosterone 0.79</td>
<td>20</td>
<td>Liang et al(1985a)</td>
</tr>
<tr>
<td>&quot; &quot; (N)</td>
<td>Testosterone 0.90</td>
<td>21</td>
<td>Liang &amp; Heiss(1981)</td>
</tr>
<tr>
<td>&quot; &quot; (N)</td>
<td>Progesterone 0.35</td>
<td></td>
<td>Frederiks &amp; Wilson(1971)</td>
</tr>
<tr>
<td></td>
<td>Deoxycorticosterone 1.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat prostate(N)</td>
<td>Testosterone 1.05</td>
<td></td>
<td>Nozu &amp; Tomaoki(1974)</td>
</tr>
<tr>
<td>Human breast(M)</td>
<td>Testosterone 0.90</td>
<td></td>
<td>Hodgins &amp; Gaggini(1983)</td>
</tr>
<tr>
<td>Human prostate(M)</td>
<td>Testosterone 0.01-0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testosterone 1.10</td>
<td></td>
<td>Voigt et al(1970)</td>
</tr>
<tr>
<td>Human foreskin(M)</td>
<td>Testosterone 0.08</td>
<td>40</td>
<td>Leshin et al(1978)</td>
</tr>
<tr>
<td>Genital skin fibroblasts</td>
<td>Testosterone 0.12</td>
<td></td>
<td>Fisher et al(1978)</td>
</tr>
<tr>
<td>Human epididymis(M)</td>
<td>Testosterone 3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human prostate(W)</td>
<td>Testosterone 0.037</td>
<td>2.0</td>
<td>Houston and Habib(1984)</td>
</tr>
<tr>
<td>Rat anterior (M)</td>
<td>Progesterone 0.193</td>
<td></td>
<td>Bertics &amp; Karavolas(1984)</td>
</tr>
<tr>
<td>pituitary</td>
<td>Progesterone 0.113</td>
<td></td>
<td>Bertics &amp; Karavolas(1984)</td>
</tr>
</tbody>
</table>

** M = microsomal; N = nuclear; W = whole particulate

* Km's expressed in umol/l

As can be seen early studies on the rat liver microsomal enzyme (McGuire and Tomkins, 1960; McGuire et al, 1960; /..
Nozu and Tamaoki (1974) gave Km values for steroid far in excess of that reported in this study (approx. 100-500 fold greater). In the first two studies (McGuire and Tomkins, 1960; McGuire et al, 1960) steroid 5α-reductase activity was assayed spectrophotometrically by monitoring the decrease in absorbance at 240nm which occurs on reduction of the 4-5 double bond. The concentration of steroid substrate was varied from 10-250umol/l - a concentration range approximately 1000-fold higher than that used in the present system. Clearly this assay system was much less sensitive than that used in the present study and again the concentrations of steroid present far exceeded their aqueous solubility. Roy (1971) reported a Km(app) testosterone of 2umol/l or approximately 5-fold higher than that reported in this study. Although Roy does not specify the concentration range over which his kinetic measurements were made, his assays of rat liver microsomal 5α-reductase activity were routinely performed at 67umol/l. The maximum concentration of steroid used in this study in the determination of the Km(app) testosterone was 400nmol/l (see Fig. 12; section 3.1.5). A valid criticism of the data reported in this study is that the maximum concentration of substrate used is only just higher than the Km(app) value obtained. This means that the concentration of testosterone may not have been high enough to give an accurate value for Vmax. This would consequently lead to an underestimation of Km(app) testosterone. Therefore if Roy (1971) used higher concentrations of testosterone in his kinetic studies, his value for the Km(app) of testosterone (2umol/l) may be /..
a more accurate determination. The values for the $K_m(app)$ NADPH for rat liver microsomal $5\alpha$-reductase reported by other workers—500umol/l (McGuire and Tomkins, 1960), 200umol/l (Roy, 1971)—are much higher than those reported in the present study. Roy (1971) has suggested that this may be an over-estimate of the $K_m(app)$ NADPH value for $5\alpha$-reductase caused by the consumption of NADPH by other microsomal NADPH-oxidising enzymes (e.g., NADPH-cytochrome C reductase). Thus at the higher concentrations of microsomal protein used in these studies (200 ug/assay in the case of Roy's report) significant oxidation of NADPH may have occurred, reducing the amount of NADPH available to $5\alpha$-reductase. Indeed Roy (1971) has forwarded this as an explanation for the larger $K_m(app)$ NADPH of the rat liver enzyme than the prostate enzyme reported by him and subsequently by other workers (Frederiksen and Wilson, 1971; Liang and Heiss, 1981).

The $V_{max}$ value reported in Section 3.1.5 when the concentration of NADPH was fixed at 0.5 mmol/l and testosterone was the variable substrate (1.74 nmol/min/mg) was about two-fold lower than that reported when the concentration of testosterone was fixed at 1umol/l and NADPH was the variable substrate (3.37 nmol/min/mg). As indicated above in the former case the highest concentration of testosterone used was only just above the determined $K_m(app)$ value and this could have accounted for the discrepancies in the values obtained. Indeed the latter value for $V_{max}$ is closer to the value obtained for the specific activity of $5\alpha$-reductase of 4-6 nmol/min/mg from the time course (see Fig.9; Section 3.1.2), the reaction
velocity versus enzyme concentration plot (see Fig. 10; Section 3.1.3) and the pH optimum determination (see Fig. 11; Section 3.1.4). The value reported for the Km(app) testosterone in this present study falls in the range 0.08-3.60 umol/l reported for the Km(app) steroid for 5α-reductases from other tissues—see Table VII.

Evidence has been provided in this study of a sequential reaction mechanism for rat hepatic microsomal 5α-reductase (see Fig. 14; Section 3.1.7). This finding has not been reported previously, however Bertics et al (1984) reported that 4-aza-4-methyl-5α-pregnane-3,20-dione inhibited rat pituitary progesterone 5α-reductase competitively with respect to testosterone (Ki=7.2 nmol/l) and uncompetitively with respect to NADPH (Ki=17.9 nmol/l). Bertics et al (1984) interpreted this data as being consistent with a reaction mechanism whereby NADPH binds to 5α-reductase before steroid. In a similar vein Liang et al (1983) demonstrated that the binding of [1,2-3H] 17-N,N-diethyl carbamoyl-4-methyl-4-aza-5α-androstan-3-one to hepatic microsomal 5α-reductase required the obligatory presence of NADPH. This data suggested a binding site on the 5α-reductase for both NADPH and steroid. An alternative reaction mechanism for 5α-reductase has been suggested however. This will be discussed in the final section (see Section 4).
3.2. Solubilisation of hepatic microsomal testosterone 5α-reductase.

3.2.1. Definition of solubilised enzyme activity.
For the purposes of this section solubilised enzyme activity is defined as the activity recovered from the supernatant following centrifugation of microsomes treated with solubilisation solution at 105000g for 1h. This is one of the criteria used by Razin (1972) to define soluble enzyme activity.

3.2.2. Solubilisation of 5α-reductase by chaotropic agents.
The ability of salt solutions and urea to solubilise microsomal 5α-reductase was examined. The results are illustrated in Table VIII. As can be seen each of these treatments solubilised less than 6% of the total microsomal 5α-reductase activity. Thus 0.5mol/l KCl + 0.5 mol/l sodium citrate released only 5.6% of the total 5α-reductase activity into the supernatant. Furthermore solubilisation of microsomes with these agents left substantial amounts of 5α-reductase activity in the pellet (see Table VIII).
Treatment of microsomes with higher concentrations of urea (4mol/l and 8mol/l) caused inactivation of 5α-reductase (J.Mackie; final year Dissertation in Molecular Biology; 1985). The fact that treatment of membranes with salt and urea solutions resulted in the solubilisation of small amounts of 5α-reductase activity suggested that the enzyme protein was embedded in the hydrophobic part of the membrane. Such proteins require detergent /...
treatment for effective solubilisation. For this reason experiments were undertaken to evaluate the ability of various detergents to solubilise microsomal 5α-reductase.
Table VIII The solubilisation of microsomal 5α-reductase activity by chaotropic agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Before Centrifugation</th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M KCl</td>
<td>10 9.17 91.7 1.1 1.00 1.10 1.2</td>
<td>10.4 3.41 35.5 38.7</td>
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<tr>
<td>+ 0.1M Na₃Cit.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0.3M KCl</td>
<td>10 9.83 98.3 1.9 2.59 4.92 5.0</td>
<td>9.4 4.39 41.3 42.0</td>
<td></td>
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<tr>
<td>+ 0.3M Na₃Cit.</td>
<td></td>
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</tr>
<tr>
<td>0.5M KCl</td>
<td>10 9.60 96.0 2.2 2.44 5.37 5.6</td>
<td>9.7 3.20 31.0 32.3</td>
<td></td>
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<tr>
<td>+ 0.5M Na₃Cit.</td>
<td></td>
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<tr>
<td>2M urea</td>
<td>10 9.58 95.8 3.3 0.90 2.97 3.1</td>
<td>8.8 4.68 41.2 43.0</td>
<td></td>
</tr>
</tbody>
</table>

* S.A. = Specific activity in nmol min⁻¹ mg⁻¹.
+ T.A. = Total activity in nmol min⁻¹.
Pt. = Protein

For solubilisation protocol see Methods section 2.2.3. Chaotropic agents were present in 1ml of solubilisation buffer B. The microsomal protein concentration present during solubilisation was 10mg/ml. Enzyme assays were performed in triplicate on microsomes in solubilisation solution prior to centrifugation and on the supernatant and resuspended pellets obtained following centrifugation. Specific activities are reported as the mean values obtained. Standard errors varied by less than 5% of the mean value. The mean % total enzyme activity recovered in each pellet and supernatant is expressed relative the mean total enzyme activity present prior to centrifugation. Standard assay conditions with 2ug protein/assay.
3.2.3.1. Inhibition of microsomal 5α-reductase by various detergents.

The carry over of detergent from solubilisation experiments into the assay mixture may result in inhibition of 5α-reductase activity. (Houston et al; 1984). It is important therefore to ensure that 5α-reductase activity is not inhibited by the concentrations of detergents likely to be present during the assay. I investigated the ability of three detergents—Lubrol WX (a non-ionic polyoxyethylene alcohol ether), Chaps (a zwitterionic bile salt derivative) and n-octyl glucoside (a non-ionic glycoside) to solubilise 5α-reductase. Fig. 15 is a dose response curve of the inhibition of microsomal 5α-reductase by these three detergents. From this data it can be seen that the approximate *ID$_{50}$s for Lubrol WX, Chaps and n-octyl glucoside are 0.025% (w/v), 0.05% (w/v) and 0.27% (w/v).

Assay of solubilised protein involves a considerable dilution of the preparation (approx. 1 → 1000). At these levels the final concentration of detergent in the assay solution is of the order of 10$^{-3}$% (w/v). As can be seen from Fig. 15 at these concentrations Lubrol WX, Chaps and n-octyl glucoside have no effect on 5α-reductase activity.

* ID$_{50}$ is that concentration of detergent required to inhibit 5α-reductase activity by 50%.
Fig. 15 - Inhibition of hepatic microsomal testosterone 5α-reductase by various detergents. Standard assay conditions with incubations containing varying concentrations of n-octyl glucoside, Chaps or Lubrol WX as indicated. Results are expressed as % of control enzyme activity (no detergent). For experiments with Lubrol WX and Chaps the control enzyme activity was $2.48 \pm 0.15 \text{ nmol min}^{-1} \text{ mg}^{-1} \left[ \text{mean} \pm \text{ S.E.}; n=3 \right]$. For experiments with n-octyl glucoside the control enzyme activity was $1.71 \pm 0.10 \text{ nmol min}^{-1} \text{ mg}^{-1} \left[ \text{mean} \pm \text{S.E.}; n=3 \right]$. For each detergent concentration, triplicate assays were performed and the results are expressed as the mean $\pm$ S.E.
3.2.3.2. Solubilisation of 5\(\alpha\)-reductase by detergents.

The ability of Lubrol WX, Chaps and n-octyl glucoside to solubilise microsomal 5\(\alpha\)-reductase at various detergent:protein ratios (D:P) ratios was examined. The data are presented in Table IX, summarised in Fig. 16 and discussed below.

Chaps was effective in solubilising 5\(\alpha\)-reductase only at the lowest D:P ratio examined (0·1). It should be noted that the concentration of Chaps used in this experiment (0·07\%) is below the CMC of Chaps. At this low concentration of Chaps substantial 5\(\alpha\)-reductase activity remained in the pellet. As Chaps was used at increasing D:P ratios enzyme activity was progressively decreased in the pellet. However, no augmentation of solubilised 5\(\alpha\)-reductase activity was observed.

When Lubrol WX was examined it was found that at the lowest D:P ratio examined (0·2), a "fluffy layer" was present over a more compact pellet. This was carefully removed from the pellet and clear supernatant and was found to contain 7\% of the total 5\(\alpha\)-reductase activity. This was considered to be insoluble enzyme activity and was added to the 83·5\% total enzyme activity recovered in the compact pellet to give a total of 90·5\% of enzyme remaining insoluble after treatment with Lubrol WX at a D:P ratio of 0·2. When the D:P ratio was increased to 0·4, a clear supernatant containing 26\% of the total enzyme activity was obtained. Further increases in the D:P ratio resulted in a sharp decrease in the yield of 5\(\alpha\)-reductase activity recoverable in the supernatant.
n-octyl glucoside prove to be the most effective detergent of the three examined in solubilising enzyme activity. At an optimum D:P ratio of 1.25, 30.7% of the total enzyme activity was solubilised. At this D:P ratio little enzyme activity remained in the pellet.

One other detergent, sodium deoxycholate proved to be ineffective at solubilising 5α-reductase activity. Indeed at low concentrations (0.14% w/v) this bile salt directly inhibited 5α-reductase activity. [The structures of the detergents used in these solubilisation studies are shown in Table X].
For solubilisation protocol see Methods Section 2.2.3. Detergents were present at the indicated concentration in 1 ml of solubilisation buffer A. The microsomal protein concentration during solubilisation was 7 mg/ml (10 mg/ml for Chaps). Triplicate enzyme assays were performed on each microsome-solubilising solution suspension prior to centrifugation and on each supernatant and pellet obtained. Specific activities are reported as the means. Individual values obtained for each experiment differed by less than 10%. The mean % total enzyme activity recovered in each supernatant and pellet is expressed relative to the mean total enzyme activity present prior to centrifugation. Standard assay conditions with 2 ug protein/assay.
<table>
<thead>
<tr>
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</tr>
</tbody>
</table>

**TABLE IX**

The solubilization of hepatic microsomal 5'-reductase activity by detergents.
Fig. 16

Optimum detergent : protein ratios for solubilisation of hepatic microsomal 5\% - reductase by Chaps, Lubrol WX and n-octyl glucoside. This figure summarises some of the data presented in Table XI. The mean % total enzyme activity recovered in the supernatant is plotted in relation to the D/P ratio for each detergent.
Table X  Structural Formulae of Detergents used to
Solubilise microsomal $5\alpha$-reductase

Chaps

n-Octyl glucoside

Lubrol WX

Na deoxycholate
3.2.4. Stability of solubilised 5α-reductase

5α-reductase activity solubilised by chaotropic agents (Table VI) appeared to be stable to freezing and thawing with little loss of enzyme activity on storage at -20°C for 1 week. Similarly Chaps - and Lubrol WX - solubilised enzyme activity was stable at -20°C. However this was not the case for the n-octyl glucoside solubilised enzyme. Almost all n-octyl glucoside solubilised enzyme activity was lost after 5 days whether the enzyme was stored at 4°C, -20°C or at -120°C in liquid N₂. - see Table XI.

Table XI Instability of n-octyl glucoside - solubilised 5α-reductase activity.

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Sp.Act (n mol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.45(±0.04)</td>
</tr>
<tr>
<td>1</td>
<td>0.93(±0.03) 1.36(±0.02) 1.59(±0.05)</td>
</tr>
<tr>
<td>4</td>
<td>0.04(±0.007) 0.102(±0.010) 0.092(±0.028)</td>
</tr>
</tbody>
</table>

Microsomes were solubilised with n-octyl glucoside at a D/P ratio of 1.25 as described in Methods section 2.2.3. 5α-reductase activity was assayed and the solubilised microsomes were then divided into three aliquots and stored at either 4°C, -20°C or -120°C. After 1 and 4 days the solubilised microsomes were thawed and assayed for 5α-reductase activity. Enzyme assays were /...
performed in triplicate and the data are presented as the mean ± S.E. Standard assay conditions with 2ug protein/assay.
3.2.5. Discussion

Treatment of hepatic microsomal membranes with chaotropic agents solubilised small amounts of 5α-reductase activity. (see Table VIII; section 3.2.2.) There are no other reports in the literature concerning the solubilisation of hepatic microsomal 5α-reductase by treatment of membranes with salt or urea. Recently however, Houston and Habib (1984) have demonstrated that homogenisation of human prostatic tissue in a medium which contained 0.5mol/l KCl and 0.5mol/l sodium citrate solubilised 50% of the total 5α-reductase activity. Earlier Scheer and Robaire (1983) had demonstrated that 50% of the total rat epididymal 5α-reductase activity could be solubilised by treatment with the same concentrations of KCl and sodium citrate. In this case the solubilisation buffer contained 20% (v/v) glycerol and 0.1mmol/l NADPH. It may be that the comparatively small yield of hepatic 5α-reductase solubilised by salt or urea treatment reflects a difference in the location of 5α-reductase in the membrane of hepatic microsomes with the enzyme being deeply embedded in the hydrophobic part of the membrane. In the human prostate and rat epididymis the enzyme may have a more peripheral location in the membrane and thus treatment with salt solutions solubilised significant amounts of activity.

An interesting finding was that solubilisation of microsomes with salts or urea was always associated with a loss of enzyme activity - that is % enzyme activity recovered in pellet + supernatant < 100 (see Table VIII Section 3.2.2.). Values /...
for recoveries of enzyme activity following centrifugation
varied from 28.1% (0.5mol/l KCl) to 47% (0.3mol/l KCl +
0.3mol/l sodium citrate). It may be that centrifugation
separated into the supernatant and pellet components
whose interaction were necessary for maximum 5α-reductase
activity. This could be tested by attempting to restore
enzyme activity by recombination of supernatant and pellet.

As a preliminary to the study of the solubilisation of
hepatic microsomal 5α-reductase by detergents we investigated
the inhibition of enzyme activity by the detergents used.
If present at a sufficiently high concentration (5mg/ml -
see Fig. 15; section 3.2.3.1), Lubrol WX, Chaps and n-octyl
glucoside all significantly inhibited 5α-reductase activity.
Houston et al (1985) using Lubrol PX, reported that the
presence of this detergent in the assay inhibited human
prostatic 5α-reductase. Inhibition is evident at a deter­
genent concentration of 20ug/ml and is essentially complete
at concentrations of 100ug/ml. In this study an ID50 for
Lubrol WX inhibition of rat hepatic microsomal 5α-reductase
of 250ug/ml is reported. The apparent discrepancies
between these figures may be the result of decreased sensiti­
vity of the rat hepatic enzyme to inhibition or it may
simply be that Lubrol PX is a more effective inhibitory
agent than the related detergent Lubrol WX.

The successful solubilisation of hepatic microsomal 5α-
reductase by Lubrol WX confirmed the previous findings of
Golf and Graef (1978). These authors used Lubrol WX at
higher D:P ratios (0.67 – 1.00) than those found to be /...
successful in the present study. They used a solubilisation buffer which contained 0.1mol/l KCl, 0.1mol/l sodium citrate and 40% (v/v) glycerol. The authors did not report a yield of solubilised enzyme. It may be that the higher concentrations of glycerol used in Golf and Graef's study allow for greater stability of hepatic microsomal 5α-reductase at higher D:P ratios.

Lubrol WX has also proved successful in solubilisation of the rat epididymal microsomal and nuclear enzymes (Scheer and Robaire, 1983). Thus at a D:P ratio of 0.5 - 0.7 and in a buffer containing 0.1mol/l KCl, 0.1mol/l sodium citrate and 20% (v/v) glycerol, they reported 25% of the total 5α-reductase activity solubilised.

This work provides the first report of the successful solubilisation of rat hepatic microsomal 5α-reductase by treatment with the non-ionic detergent n-octyl glucoside. Previously, however Bertics and Karavolas (1985) had demonstrated that the pituitary microsomal progesterone 5α-reductase could be solubilised by a combination of n-octyl glucoside (final D:P ratio 2.5) and 1mol/l KCl. The composition of the solubilisation buffer used by Bertics and Karavolas (1985) was similar to that used in the present study except that it did not contain sodium citrate and the final concentration of NADPH was 5mmol/l. Nevertheless, they report a similar value (38%) for the total 5α-reductase activity solubilised compared to the...
value obtained for the solubilisation of hepatic microsomal 5\textsuperscript{-reductase activity at optimum D:P ratios of n-octyl glucoside (30.7% - see Table IX).

Chaps proved to be disappointing in respect of 5\textsuperscript{-reductase solubilisation. Indeed this detergent appeared to directly inhibit microsomal 5\textsuperscript{-reductase activity as it was added to microsomal suspensions in increasing concentrations. There are no reports on the solubilisation of 5\textsuperscript{-reductases from other tissues using Chaps.

The inhibition of microsomal 5\textsuperscript{-reductase by deoxycholate confirms the previous findings of Roy (1971).

As was the case when the solubilisation of microsomal 5\textsuperscript{-reductase with chaotropic agents was studied, solubilisation of hepatic microsomal 5\textsuperscript{-reductase with detergents also caused a loss of 5\textsuperscript{-reductase activity. Again, this may be explained as the separation into the supernatant and pellet of components whose interaction are necessary for maximum 5\textsuperscript{-reductase activity. A more likely explanation however, may be the inactivation known to occur to membrane-bound enzymes at increasing D:P ratios. (Hjelmeland and Chrambach, 1984). Indeed in the case of 5\textsuperscript{-reductases, the yield of solubilised enzyme decreased as the n-octyl glucoside: microsomal protein ratio was increased (Bertics and Karavolas, 1985). Similarly (Scheer and Robaire, 1983) demonstrated that the yield of solubilised rat epididymal microsomal 5\textsuperscript{-reductase decreased as the ratio of Tween 80 or Lubrol WX: microsomal protein was increased.
In this study it is reported that 21-63% of n-octyl glucoside solubilised microsomal 5α-reductase activity was lost over one day (see Table XI; section 3.2.4). The amount of enzyme activity remaining depended on the temperature at which the solubilised microsomes were stored, with enzyme stability being favoured by lower temperatures. Bertics and Karavolas (1985) reported that the n-octyl glucoside solubilised rat pituitary progesterone 5α-reductase was stable for 1 day at 4°C. This preparation however had previously been extensively dialysed against non-detergent containing buffer and it would seem reasonable to postulate that the stability of the enzyme may be inversely related to the concentration of n-octyl glucoside present in the solubilised preparation.

For the solubilisation studies reported in Table VIII (section 3.2.2) and Table IX (section 3.2.3.2) a new batch of rat liver microsomes was prepared. The specific activity of this preparation (8-9nmol/min/mg) was approximately two-fold higher than that described in Section 3.1. It appeared therefore that there was some inter-batch variation in microsomal 5α-reductase activity.
3.3. Gel filtration of solubilised 5α-reductase activity

Microsomal 5α-reductase activity which had been solubilised either by treatment with chaotropic agents (Table VIII; section 3.2.2) or by use of detergents (Table IX; section 3.2.3.2) was subjected to high-performance gel filtration on a Superose 6 gel filtration column – see Methods 2.2.5.

3.3.1. Gel filtration of Salt-, urea- and CHAPS-solubilised 5α-reductase activity

On gel filtration of these solubilised supernatants no enzyme activity was recovered in any of the column fractions. Activity could not be restored by assaying for enzyme activity in the presence of 1mg/ml sonicated phosphatidylcholine liposomes. In a further attempt to restore enzyme activity various column fractions were combined and assayed for 5α-reductase activity. Details of the recombined column fractions tested for 5α-reductase activity are shown in Table XII.

Table XII Recombinations of column fractions from gel filtration of salt-, urea- and CHAPS-solubilised microsomes

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>200ul each of fractions 9-15</td>
</tr>
<tr>
<td>B</td>
<td>200ul each of fractions 16-20</td>
</tr>
<tr>
<td>C</td>
<td>200ul each of fractions 21-28</td>
</tr>
<tr>
<td>D</td>
<td>200ul A + 200ul B</td>
</tr>
<tr>
<td>E</td>
<td>200ul A + 200ul C</td>
</tr>
</tbody>
</table>
Table XII contd./..

F  200u1 B + 200u1 C
G  200u1 A + 200u1 B + 200u1 C

100u1 each of the recombination (A-G) was assayed in triplicate for 5α-reductase activity in the presence and absence of 1mg/ml sonicated phosphatidyl choline liposomes. Standard assay conditions.

Attempts to restore 5α-reductase activity by this method were uniformly unsuccessful. The OD280 profiles obtained on gel filtration of these three solubilised extracts are shown in Fig. 17 (a) - (c).
Fig. 17(a) OD280 profile obtained on gel filtration of Chaps - solubilised microsomes.

10mg of microsomal protein were solubilised in 1ml of solubilisation buffer A containing 0.1% (w/v) Chaps (final D:P ratio = 0.1) according to the procedure described in Methods section 2.2.3. 250ul of the resulting supernatant (approx. 1.00mg protein) was applied to a Superose 6 gel filtration column. The column was equilibrated and eluted with solubilisation buffer A containing 1.0% (w/v) Chaps. Eluent flow rate was 0.4 ml/min and fraction sizes were 1ml. No 5\(\alpha\)-reductase activity was recovered in any of the column fractions. The OD280 profile obtained is shown.

Fig. 17(b) OD280 profile obtained on gel filtration of salt-solubilised microsomes.

10mg of microsomal protein were solubilised in 1ml of solubilisation buffer B containing 0.1 mol/1 KCl and 0.1 mol/1 sodium citrate according to the procedure described in Methods section 2.2.3. 250ul of the resulting supernatant (approx. 0.3mg protein) was applied to a Superose 6 gel filtration column. The column was equilibrated and eluted with solubilisation buffer B containing 0.1 mol/1 KCl and 0.1 mol/1 sodium citrate. Eluent flow rate was 0.4 ml/min and fraction sizes were 1ml. No 5\(\alpha\)-reductase activity was recovered in any of the column fractions. The OD280 profile obtained is shown.
Fig. 17(c) OD280 profile obtained on gel filtration of urea-solubilised microsomes.

10mg of microsomal protein were solubilised in 1ml of solubilisation buffer B containing 2 mol/l urea according to the procedure described in Methods section 2.2.3. 250μl of the resulting supernatant (approx. 0.9mg protein) was applied to a Superose gel filtration column. The column was equilibrated and eluted with solubilisation buffer B containing 2 mol/l urea. Eluent flow rate was 0.4ml/min and fraction sizes were 1ml. No 5α-reductase activity was recovered in any of the column fractions. The OD280 profile obtained is shown.
Fig. 17

(a) Chaps
(b) Salt
(c) Urea

Vc (ml) 22 26
H8
3.3.2. Gel filtration of Lubrol WX - solubilised 5α-reductase activity

The enzyme profile obtained on gel filtration of the Lubrol WX - solubilised enzyme activity is shown in Fig. 18. As can be seen all the 5α-reductase activity eluted at or near the void volume. Table XIII lists the specific activity found for column fractions containing active enzyme.

Table XIII  Specific activity of 5α-reductase in column fractions from gel filtration of Lubrol - solubilised 5α-reductase

<table>
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<tr>
<th>Fraction</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
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<tbody>
<tr>
<td>Original solubilised enzyme</td>
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<td>1</td>
</tr>
<tr>
<td>Fraction No. 11</td>
<td>1.64</td>
<td>x 3.3</td>
</tr>
<tr>
<td>Fraction No. 12</td>
<td>0.29</td>
<td>x 0.6</td>
</tr>
<tr>
<td>Fraction No. 13</td>
<td>0.12</td>
<td>x 0.2</td>
</tr>
</tbody>
</table>

* in nmol/min/mg

The original solubilised enzyme activity which was applied to the gel filtration column was assayed in triplicate and column fractions were assayed in duplicate according to the procedure described in the legend to Fig. 18. The mean specific activities found are reported.

In the first fraction obtained from the gel filtration column (No. 11) there is indeed some purification of 5α-reductase activity (∼3-fold). The recovery of enzyme/...
activity from the gel filtration column was approx. 15% of the total activity applied to the column in the soluble supernatant.
Fig. 18 - High performance gel filtration of Lubrol WX solubilised hepatic microsomal 5α-reductase

7mg of microsomal protein were solubilised in 1 ml of solubilisation buffer A containing 0.28% (w/v) Lubrol WX (final D:P ratio 0.4) according to the procedure described in Methods section 2.2.3. 200ul of the resulting supernatant (approx. 1.25mg protein) was applied to a Superose 6 FPLC gel filtration column. The column was equilibrated and eluted with solubilisation buffer A containing 0.3% (w/v) Lubrol WX. Eluent flow rate was 0.4ml/min and fraction sizes were 1ml. 50ul aliquots of column fractions were assayed in duplicate for 5α-reductase activity. The assay mixture contained (in a total volume of 500ul) 0.1 mol/l Tris Cl (pH 7.0), 1 umol/l testosterone, 10^4 d.p.m.(0.38 umol/l) 5α-DHT and 0.5 mmol/l NADPH. Incubations were at 37°C for 20 min.

The mean 5α-reductase activity for each fraction is expressed as nmol min⁻¹ ml⁻¹. The Lubrol WX - solubilised microsomal extract was incubated at ambient temperature for the duration of the gel filtration run (~ 1½hr) and was assayed along with and under identical conditions to those pertaining for the column fractions. The activity of this unfractionated enzyme represented the control enzyme activity of the applied extract and was 0.49± 0.04 nmol min⁻¹ mg⁻¹ [mean ± S.E.; n=3]. It did not differ significantly from the activity of freshly solubilised microsomal 5α-reductase activity. Two experiments were performed with Lubrol WX - solubilised microsomes and similar results were obtained in both instances.
3.3.3. Gel filtration of n-octyl glucoside solubilised 5α-reductase activity

As was the case with the Chaps-, salt- and urea- solubilised 5α-reductase (see section 3.3.1) on gel filtration of the n-octyl glucoside solubilised enzyme no activity was recovered in any of the column fractions when the eluent buffer contained 0.87% (w/v) n-octyl glucoside. Activity was not restored by assaying the column fractions in the presence of 1mg/ml sonicated phosphatidyl choline solution added to the enzyme assay. As with the Chaps-, salt- and urea- solubilised enzymes (see section 3.3.1), column fractions were recombined in an attempt to restore 5α-reductase activity. Details of the recombined column fractions tested for 5α-reductase activity are given below in Table XIV.

Table XIV Recombinations of column fractions from gel filtration of n-octyl glucoside solubilised microsomes

<table>
<thead>
<tr>
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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>200ul each of fractions 13-17</td>
<td>200ul each of fractions 18-23</td>
<td>200ul each of fractions 24-29</td>
<td>200ul A + 200ul B</td>
<td>200ul B + 200ul C</td>
<td>200ul A + 200ul C</td>
<td>200ul A + 200ul B + 200ul C</td>
</tr>
</tbody>
</table>

100ul each of the recombinations (A-G) was assayed in triplicate for 5α-reductase activity in the presence and/...
absence of 1mg/ml phosphatidyl choline. Standard assay conditions.

As with the Chaps-, salt- and urea solubilised enzymes, attempts to restore 5α-reductase activity by recombination of fractions were unsuccessful. It was observed however that the OD280 profile obtained on gel filtration of the n-octyl glucoside solubilised enzyme was different from that of the Lubrol WX solubilised enzyme. The OD280 profile of the n-octyl glucoside solubilised microsomes is shown in Fig. 19. The OD280 profile from the Lubrol WX gel filtration in Fig. 18 is super-imposed for comparison.

Notice in the case of the n-octyl glucoside solubilisation and gel filtration the absence of the early-eluting material containing enzyme activity which was seen for the Lubrol WX solubilisation and gel filtration. The observations suggested that any 5α-reductase activity recovered on gel filtration was present in a large aggregated form and conditions which caused dissaggregation of this complex led to a loss of enzyme activity. It has been hypothesised that non-ionic detergents such as Triton and Lubrol do not disaggregate proteins as effectively as other detergents (Hjelmeland and Chrambach, 1984) and this may be why the only time enzyme activity is recovered is as large aggregates on Lubrol WX solubilisation and gel filtration. Further support for this theory was obtained from a series of experiments which examined the enzyme profile obtained when the n-octyl glucoside solubilised enzyme was again subjected to gel filtration with the modification that the elution buffer contained 0.05% w/v n-octyl glucoside. This is /...
Fig. 19 - Comparison of the OD280 profiles obtained on gel filtration of Lubrol WX and n-octyl glucoside solubilised microsomal extracts.

7mg of microsomal protein were solubilised in 1 ml of solubilisation buffer A containing 0.87% (w/v) n-octyl glucoside (final detergent: protein ratio = 1.25) according to the procedures described in Methods section 2.2.3. 200μl of the resulting supernatant (approximately 1mg protein) was applied to the Superose 6 FPLC column. The column was equilibrated and eluted with solubilisation buffer A containing 0.87% (w/v) n-octyl glucoside. Eluant flow rate was 0.4 ml min⁻¹ and fraction sizes were 1ml. The OD280 profile obtained is compared with that seen in Fig. 18 for the Lubrol WX-solubilised microsomal extract.
Fig. 20 High-performance gel filtration of n-octyl glucoside solubilised hepatic microsomal 5α-reductase.

7mg of microsomal protein were solubilised in 1ml of solubilisation buffer A containing 0.87% (w/v) n-octyl glucoside (final detergent:protein ratio = 1.25) according to the procedures described in Methods section 2.2.3. 400μl of supernatant (approx. 2mg protein) was applied to the Superose 6 gel filtration column. Elution conditions were as described in Fig.19 except that the elution buffer contained 0.05% (w/v) n-octyl glucoside. 25μl aliquots of column fractions were assayed in duplicate for 5α-reductase activity under standard assay conditions. The mean 5α-reductase activity found for each fraction is expressed as nmol min⁻¹ ml⁻¹. The column fractions were assayed for protein content and this is also plotted. The n-octyl glucoside-solubilised microsomal extract was incubated at ambient temperature for the duration of the gel filtration run (∼1½hr) and was assayed along with and under identical conditions to those pertaining for the column fractions. The activity of this unfractionated enzyme represented the control enzyme activity of the applied extract and was 1.25± 0.30 nmol min⁻¹ mg⁻¹ [mean± S.E. ; n=3]. It did not differ significantly from the activity of freshly solubilised microsomes. Three experiments were performed with n-octyl glucoside solubilised microsomes and similar results were obtained in each instance.
well below the published CMC value for n-octyl glucoside (0.73% w/v) (Hjelmeland and Chrambach, 1984). Three experiments were done in total and similar results were obtained in each instance. The results of a representative experiment are shown in Fig. 20. Notice the re-appearance of early-eluting material containing approx. 70% of the applied enzyme activity. Notice also the presence of enzyme activity well included on the gel filtration column. This amounts to some 10% of the total applied enzyme activity and was observed in each of these separate experiments. Table XV lists the specific activity found for some of the column fractions containing active enzyme.

Table XV Specific activity of 5α-reductase in column fractions from n-octyl-glucoside-solubilised microsomes

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original solubilised enzyme</td>
<td>1.25</td>
<td>1</td>
</tr>
<tr>
<td>Fraction No. 8</td>
<td>4.60</td>
<td>x 3.7</td>
</tr>
<tr>
<td>Fraction No. 9</td>
<td>3.20</td>
<td>x 2.6</td>
</tr>
<tr>
<td>Fraction No. 10</td>
<td>3.40</td>
<td>x 2.7</td>
</tr>
<tr>
<td>Fraction No. 23</td>
<td>2.85</td>
<td>x 2.3</td>
</tr>
<tr>
<td>Fraction No. 24</td>
<td>2.05</td>
<td>x 1.6</td>
</tr>
<tr>
<td>Fraction No. 25</td>
<td>4.80</td>
<td>x 3.8</td>
</tr>
<tr>
<td>Fraction No. 26</td>
<td>1.45</td>
<td>x 1.2</td>
</tr>
</tbody>
</table>

* in nmol/min/mg

The original solubilised enzyme activity which was applied to the gel filtration column was assayed in triplicate and column fractions were assayed in duplicate according to the procedure described in the legend to Fig. 20. The mean specific activities found are reported. Individual values varied by < 10%. In the column fractions listed in...
Table XV purification factors for 5α-reductase activity ranging from 1.2 - 3.8 were found.

That the concentration of n-octyl glucoside in the FPLC elution buffer was a critical factor in the recovery of 5α-reductase activity was illustrated by a series of experiments which are described in Fig. 21. N-octyl glucoside solubilised extracts were incubated with solubilisation buffer A containing different concentrations of n-octyl glucoside. Conditions were chosen so as to represent those pertaining on gel filtration of n-octyl glucoside solubilised microsomes using elution buffers containing n-octyl glucoside at the concentrations indicated in Fig. 21. As can be seen 5α-reductase activity was stable on pre-incubation in buffer containing 0.45% (w/v) n-octyl glucoside, there being little loss of enzyme activity over a two-hour period. However, if the concentration of n-octyl glucoside was increased by only 0.05% (w/v) there was a rapid loss of enzyme activity with only 30% of the control activity remaining after 2 hr. If the concentration of n-octyl glucoside was raised by a further 0.05% (w/v) to 0.55% (w/v) there was an instantaneous loss of enzyme activity and no activity remained after 2 hr. When n-octyl glucoside solubilised microsomes were subjected to gel filtration and elution with a buffer containing 0.45% (w/v) n-octyl glucoside, then an enzyme and protein profile similar to that obtained on elution with a buffer containing 0.05% (w/v) n-octyl glucoside was seen. This is shown in Fig. 22. Again the bulk of the enzyme activity (83% of the total applied) eluted early at or near the void volume. Note however that in this experiment the included enzyme /...
7mg of microsomal protein were solubilised with n-octyl glucoside at a D/P ratio of 1.25 according to the procedures described in Figs. 19 and 20 and in Methods section 2.2.3. 25ug of the solubilised protein was added to 975ul of solubilisation buffer A containing 0.45%, 0.50% or 0.55% (w/v) n-octyl glucoside and incubated at room temperature. This gives D/P ratios in the pre-incubation solutions of 18, 20 and 22 respectively. At the indicated time intervals, aliquots (80ul; 2ug protein) were removed and assayed for residual 5α-reductase activity. (Standard assay conditions). Assays were performed in duplicate and the results are expressed as the mean± range. For solubilised microsomes incubated at 0.45% (w/v) n-octyl glucoside, the specific activity of 5α-reductase at t=0min was 2.81± 0.20 nmol min⁻¹ mg⁻¹ [mean± range ; n = 2]. This is normalised to 100% enzyme activity and all other values are expressed relative to this for comparison. This activity was not significantly different from that of freshly solubilised microsomal 5α-reductase assayed under standard assay conditions 2.30± 0.07 nmol min⁻¹ mg⁻¹ [mean±S.E. n = 4].
Fig. 22 High performance gel filtration of n-octyl glucoside solubilised hepatic microsomal 5α-reductase.

7mg of microsomal protein were solubilised with n-octyl glucoside at a D/P ratio of 1.25 according to the procedures described in Figs. 19 and 20 and Methods section 2.2.3. FPLC elution conditions were as described for Fig. 20, except that the elution buffer contained 0.45% (w/v) n-octyl glucoside. 400μl of supernatant (approx. 2mg protein) was applied to the Superose 6 FPLC column. 25μl aliquots of column fractions were assayed in duplicate for 5α-reductase activity under standard assay conditions. The mean enzyme activity found for each fraction is expressed as nmol min⁻¹ ml⁻¹. The column fractions were assayed for protein content and this is also plotted. The n-octyl glucoside-solubilised microsomal extract was incubated at ambient temperature for the duration of the gel filtration run (≈1 h) and was assayed along with and under identical conditions to those pertaining for the column fractions. The activity of this unfraccionated enzyme represented the control enzyme activity of the applied extract and was 3.27 ± 0.04 nmol min⁻¹ mg⁻¹ [mean ± S.E.; n = 3]. It did not differ significantly from the 5α-reductase activity of freshly solubilised microsomes.
activity which was seen on elution with a buffer containing 0.05% (w/v) n-octyl glucoside is not seen when this higher concentration of n-octyl glucoside is used although there was a peak evident at Ve = 18ml.

When n-octyl glucoside - solubilised enzyme was eluted from the gel filtration column with a buffer containing 0.5 (w/v) n-octyl glucoside (a concentration of n-octyl glucoside which caused a rapid decrease in 5α-reductase activity when pre-incubated with n-octyl glucoside solubilised microsomes - see Fig. 21) then an enzyme and protein profile similar to that seen on elution with 0.87% (w/v) n-octyl glucoside was obtained - see Fig. 19. Thus there was no enzyme activity recovered in any fractions with an absence of early-eluting material and the associated peak of enzyme activity. It seemed, from this series of experiments, that further characterisation of enzyme activity would be hampered by the fact that conditions which resulted in the true solubilisation of the enzyme protein would result in the loss of the bulk of the enzyme activity. We therefore decided to adopt an alternative approach in an attempt to further characterise 5α-reductase. (see Results Section 4).
3.3.4. Transformation of testosterone and 5α-dihydrotestosterone by other microsomal steroid metabolising enzymes.

Before leaving this section it must be stated that some of the column fractions from the gel filtration experiments described in this section were able to metabolise $^3$H-testosterone to products other than $^3$H 5α-DHT and were also capable of metabolising the $^{14}$C 5α-DHT present in the assay mixture. In these instances, $^3$H and $^{14}$C were recovered from an area in the t.l.c. plate just behind the position of testosterone and also from an area close to the origin. In the salt-, Chaps-, Lubrol WX- and n-octyl glucoside solubilised enzymes the peaks of activity were always present in Fraction Nos. 20 and 21. In the urea-solubilised microsomes this enzyme activity was not detected. Although no further attempts were made to characterise these enzyme activities they probably represent the microsomal cytochrome P450 steroid hydroxylases and the 3-hydroxy steroid dehydrogenases which convert testosterone and 5α-DHT to androstanediols and polar hydroxylated steroid metabolites. A typical enzyme profile is shown in Fig. 23. The results in this figure indicate the radioactivity ($^3$H and $^{14}$C) recovered from the area of the t.l.c. plate close to the origin ($R_f < 0.2$) and thus indicated those column fractions which contained the steroid hydroxylating activity.
Fig. 23 Metabolism of $[^3\text{H}]$-testosterone and $5\alpha$-dihydro-$[^{14}\text{C}]$-testosterone to polar compounds.

Aliquots of column fractions from gel filtration were incubated in duplicate with $[^3\text{H}]$-testosterone and $5\alpha$-dihydro-$[^{14}\text{C}]$-testosterone under identical conditions to those described in the legend to Fig. 20. Following t.l.c. relevant portions of the t.l.c. plate close to the origin ($R_f<0.2$) were removed and the radioactivity determined. The incubation mixtures contained $3.97 \times 10^5$ d.p.m. $[^3\text{H}]$-testosterone and $7.16 \times 10^3$ d.p.m. $5\alpha$-dihydro-$[^{14}\text{C}]$-testosterone.
3.3.5. Discussion

There are only two reports in the literature describing the chromatographic behaviour of 5α-reductase.

In 1974, Moore and Wilson described the behaviour of solubilised nuclear and microsomal rat prostatic 5α-reductase on gel filtration. The enzyme activity was solubilised by a combination of digitonin (D/P ratio = 2) and 3M KCl. When the solubilised microsomal 5α-reductase enzyme activity was applied to a Bio-Gel A-1.5m gel filtration column 35% of recovered enzyme activity was excluded from the column. The remaining enzyme activity eluted in a peak corresponding to a molecular weight of 250-350k. If KCl was omitted from the solubilisation and column elution buffers then the amount of enzyme activity excluded from the column increased to 75%. Similar results were obtained with the solubilised nuclear enzyme, although in the absence of KCl no enzyme activity was recovered from the gel filtration column. The authors suggested that this was due to the precipitation of the enzyme on the column.

In 1978, Golf and Graef described the results of some experiments which examined the behaviour of solubilised rat hepatic microsomal 5α-reductase on affinity chromatography. In these studies the authors employed a testosterone-linked Sepharose 4B column and applied Lubrol WX - solubilised 5α-reductase activity (D/P ratio 0.6-1.0) to this column. /.
They obtained four fractions which they identify as I - "membraneous fragments"; II - NADPH; cytochrome oxidoreductase; III - testosterone 5α-reductase; the authors also indicated that sonicated phosphatidyl choline could replace fraction IV. A re-combination of all four fractions was necessary for maximal 5α-reductase activity. The authors indicated that the testosterone concentration bound to Sepharose was very important for the reproducibility of these results.

As well as the report by Moore and Wilson (1974) of the presence of species of 5α-reductase in different states of aggregation, Larner and Wiebe (1983) described similar properties in a different microsomal steroid metabolising enzyme, the 5-ane-3β-hydroxy steroid oxidoreductase from rat Leydig cells. They reported that the partially purified enzyme can exist as a molecule with an Mr of 35000 or as aggregates of Mr 150000, a finding not dissimilar to that described above for microsomal 5α-reductase. Note that the well-included enzyme activity apparent on elution with 0.05% n-octyl glucoside is outwith the resolving power of the gel filtration column and it is highly likely that there is some form of interaction between this species and the column material. Indeed Clezardin et al (1986) found that monoclonal IgM (Mr 900000) can either elute at the void volume (Ve = 10ml.) or at Ve = 21ml. depending on the composition of the gel filtration elution buffer. These workers also used a Superose 6 column.

Two other anomalies must be raised in relation to the n-octyl glucoside - solubilisation/gel filtration experiments.
Firstly, when n-octyl glucoside-solubilised enzyme was eluted from the gel filtration column with a buffer containing 0.45% (w/v) n-octyl glucoside (which is a sub-CMC concentration of n-octyl glucoside) then again the bulk of the enzyme activity applied eluted at the void volume. However, in this instance, the well-included enzyme activity seen on elution with 0.05% (w/v) n-octyl glucoside is not evident. In order to explain these findings it can be postulated that the well included form of the enzyme is directly inhibited by 0.45% (w/v) n-octyl glucoside but not by 0.05% (w/v) n-octyl glucoside. (This in contrast to the inhibition of enzyme activity by disaggregation when the concentration of n-octyl glucoside is raised above the CMC). Indeed there were some results described in this thesis which lent tentative support to this conclusion. Careful inspection of Fig. 21 (Section 3.3.3.) indicates that pre-incubation of n-octyl glucoside solubilised microsomes in 0.45% (w/v) n-octyl glucoside did lead to a slight, but perceptible loss of enzyme activity (10-20%). This loss of enzyme activity is not seen when solubilised enzyme is pre-incubated in 0.05% (w/v) n-octyl glucoside. About 10% of the total enzyme activity was found to be well-included (see Fig. 20 Section 3.3.3).

The second phenomenon which requires some explanation is the complete loss of enzyme activity on elution with 0.87% (w/v) n-octyl glucoside despite the fact that successful solubilisation of enzyme activity occurs at 0.87% (w/v). One explanation for this may be that it is D:P ratios which are important when considering inactivation of enzyme (see this Section and Fig. 16; Section 3.2.3.2). Gel filtration involves dilution of protein sample which in turn /...
leads to an increase in the D:P ratio in each fraction from the gel filtration column. It is possible that the rapid loss of enzyme activity may be associated with this phenomenon.

The observation of steroid hydroxylase and hydroxysteroid dehydrogenase activity in some column fractions will be discussed in detail in the final section of this thesis — "4. General Discussion and Conclusions."

As mentioned at the end of Section 3.3.3, the lability of 5β-reductase enzyme activity during further manipulations led us to attempts to characterise the enzyme by alternative techniques. The results of these experiments are reported in the following section (3.4).
3.4. Photo-affinity labelling of hepatic microsomal $5\alpha$-reductase with Diazo-MAPD

3.4.1. The specificity of binding of diazo-MAPD to microsomal $5\alpha$-reductase

Diazo-MAPD has been reported to bind specifically to rat liver and prostate microsomal $5\alpha$-reductase (Liang et al., 1985b). If this is the case then radioactively labelled Diazo-MAPD could be linked to $5\alpha$-reductase and used as a marker for the enzyme under conditions where $5\alpha$-reductase normally loses its activity. A series of experiments were carried out to investigate the specificity of binding of [1,2-$^3$H] - Diazo-MAPD to rat liver microsomes. The results of these experiments are presented in Fig. 24 and discussed below.

In the control incubation (a) in Fig. 24 the binding of [${}^3$H] - Diazo-MAPD was $600\pm 95$ dpm/ug solubilised protein. When UV irradiation was performed in the presence of a 1000-fold excess of unlabelled diazo-MAPD binding was reduced by 87% to $80\pm 10$ dpm/ug solubilised protein. This provides an indication as to the extent of non-specific binding of [${}^3$H] - Diazo-MAPD. When NADPH was omitted from the incubation mixture or when irradiation was not performed the binding of [${}^3$H] - Diazo-MAPD to rat liver microsomes was 29% and 21% of that in the control incubations. The results described in Fig. 24 indicated that [${}^3$H] - Diazo-MAPD was binding to hepatic microsomes in a specific, NADPH-dependant manner and that this binding required UV irradiation.
Fig. 24 Photo-affinity labelling of hepatic microsomal \( \alpha \)-reductase.

For conditions during photo-labelling see Methods – section 2.2.4. For the test incubation and three controls, results are expressed as \(^3\)H d.p.m.-bound/\( \mu \)g solubilised protein and are the means \( \pm \) S.E. of three separate experiments.
3.4.2. Gel filtration of solubilised, photo-affinity labelled microsomal 5α-reductase

As with n-octyl glucoside solubilised enzyme activity, the n-octyl glucoside solubilised, photo-affinity labelled 5α-reductase was analysed by high performance gel filtration. Microsomes were labelled with $[^3H]$-Diazo-MAPD in the presence or absence of a 1000-fold excess of unlabelled Diazo-MAPD and in the absence of NADPH [Fig. 24 (a), (b) and (c)]. The three preparations were solubilised and applied to the Superose 6 gel filtration column. The radio-active profiles obtained for each of the three preparations is shown in Fig. 25 (a) - (c). For each of the three preparations 2 peaks of radio-activity were obtained eluting at Ve 18ml and Ve 23ml. This former peak corresponds to a Mr of 42000. The latter peak has a larger elution volume than cytochrome C [Ve 21ml; Mr = 12300].

Although present in all the three experiments, the peaks eluting at Ve 18ml and Ve 23ml, contain much less radio-activity in the experiments in which microsomes were photo-affinity labelled in the presence of excess unlabelled Diazo-MAPD and when microsomes were labelled in the absence of NADPH. [See Fig. 25]. In addition, in these two experiments the presence of additional peaks of radioactivity was noted. Thus in the case of microsomes labelled in the presence of excess radio-inert Diazo-MAPD peaks eluting at Ve 28ml and Ve 31-32ml were observed. This latter peak was also observed when microsomes /...
labelled in the absence of NADPH were solubilised and gel filtered and it corresponded to the elution position of free $[^{3}\text{H}]$ Diazo-MAPD. [Fig. 25]

In an additional experiment, n-octyl glucoside-solubilised, photo-affinity labelled microsomes were applied to a TSK G3000 SW high-performance gel filtration column. This column provides better resolution of proteins over the molecular weight range $5-300 \times 10^3$. The results of this experiment are shown in Fig. 26 and described below. Notice, as was the case with gel filtration on the Superose 6 column, two peaks of radio-activity were obtained eluting at $Ve = 18\text{ml}$ and $Ve = 27-28\text{ml}$. This former peak corresponded to a Mr of 52000 whereas the latter peak again shows a larger elution volume than the cytochrome C Mr. marker [$Ve = 23\text{ml}$]. When the TSK G3000 SW column was used, free steroid eluted at $Ve = 32\text{ml}$.

In three separate experiments which resembled those described in results Section 3.3.3, the radioactive profile obtained when solubilised, photo-affinity labelled microsomes were applied to the Superose 6 column and eluted with buffer containing sub-CMC concentrations of n-octyl glucoside was examined. The results obtained in one of these experiments are shown in Fig. 27 and should be compared with the radioactive profile seen in Fig. 25. In both instances a peak of radioactivity eluted at $Ve = 23-24\text{ml}$. However, when the concentration of n-octyl glucoside in the FPLC elution buffer was reduced below the CMC to 0.05% (w/v) the peak evident at $Ve = 18\text{ml}$ in Fig. 25 was not seen. Instead a broad peak of/...
Fig. 26 - High performance gel filtration of n-octyl glucoside solubilised, photo-affinity labelled microsomes.

Photo-affinity labelling and HPLC conditions were as described in the legend to Fig. 28(a). 200μl of supernatant (containing 370μg protein; 88000 d.p.m.) was applied to a TSK G3000 SW high performance gel filtration column. Elution conditions are identical to those described in the legend to Fig. 25.
Fig. 27 - High performance gel filtration of n-octyl glucoside solubilised, photo-affinity labelled microsomes.

All conditions are identical to those described in Fig. 25, with the exception that the FPLC elution buffer contained 0.05% (w/v) n/octyl glucoside. 300ug protein (55800d.p.m.) was applied to the Superose 6 column.
early-eluting radioactivity was evident along with early eluting protein. This early-eluting material was not seen in Fig. 25 but was evident when solubilised 5β-reductase enzyme was eluted with buffer containing 0.05% (w/v) n-octyl glucoside. (see Fig. 20 : results section 3.3.3). Also notice that the position of elution of the major radioactive peaks in Fig. 27 co-incides with the elution position of solubilised enzyme activity in Fig. 20.

The enzyme profile from Fig. 20 is super-imposed on the radioactive profile of Fig. 27 for comparison. (see Fig. 28).
Fig. 28 A comparison of the gel filtration profiles of n-octyl glucoside solubilised 5α-reductase activity and n-octyl glucoside solubilised, photo-affinity labelled microsomes.

The enzyme profile is that shown in Fig. 20 and the radioactivity profile is that depicted in Fig. 27. Experimental conditions are described in the relevant figure legends.
3.4.3. SDS-PAGE of photo-affinity labelled microsomal 5\(\alpha\)-reductase

This technique was used in an attempt to detect the radio-labelled protein produced by photo-affinity labelling of microsomes with \(^3\text{H}\) Diazo-MAPD. Two approaches were used: Following electrophoresis, relevant gel tracks were cut into slices, the gel pieces dissolved and taken for determination of radioactivity (see Methods section 2.2.6.3). Some of the gel was also processed for fluorography (see Methods section 2.2.6.2) and the developed fluorograph was examined for the presence of radio-labelled protein. The results of a gel slicing experiment are shown in Fig. 29 and are described below.

Following electrophoresis of solubilised, photo-affinity labelled microsomes all the radioactivity is recovered at or near the dye front. The results of a fluorograph are shown in Fig. 30 and confirm the findings described in Fig. 29 with bands representing tritium present near or close to the dye front. This may represent the labelling of a small Mr (10-15k) protein although two other explanations could be forwarded for the results obtained in the gel slicing and fluorography experiments.

(a) During sample preparation for electrophoresis, tritium exchange with sample buffer was occurring and (b) \(^3\text{H}\) Diazo-MAPD is not covalently bound to 5\(\alpha\)-reductase protein and thus free \(^3\text{H}\) Diazo-MAPD, having a low Mr (380) runs at the dye front on electrophoresis. Experiments were undertaken to examine these possibilities.
Fig. 29 SDS – PAGE of n-octyl glucoside solubilised, photo-affinity labelled microsomes.

150ug of solubilised protein was loaded to each of two gel wells. Gels were run, the relevant portions sliced and the $^3$H d.p.m. determined according to the procedure described in Methods section 2.2.6.3.
Fig. 30 Fluorograph of photo-affinity labelled rat liver microsomes

Microsomes were photo-affinity labelled with \(^{\text{3}}\)H D iazo-MAPD according to the procedure described in Methods, section 2.2.4. 10, 25, 50 and 100ug of microsomal protein were loaded into the gel wells. Gels were processed for fluorography as described in Methods, section 2.6.6.2. The position of molecular weight markers is indicated. Lane 1 - 100ug microsomal protein; 2 - 50ug; 3 - 25ug; 4 - 10ug.
3.4.4. Extraction of $[^3\text{H}]$Diazo-MAPD from photo-affinity labelled microsomes

The ability of organic solvents to extract $[^3\text{H}]$Diazo-MAPD from labelled microsomal preparations was examined. The results of a representative experiment are shown in Table XVI and described below.

Table XVI The extraction of $[^3\text{H}]$Diazo-MAPD from photo-affinity labelled microsomes

195µg (28740 dpm) of labelled, n-octyl glucoside solubilised microsomes were precipitated with an equal volume of 10% (w/v) TCA. An aliquot of the supernatant was removed for the determination of $[^3\text{H}]$ d.p.m. The pellet was washed with ethanol and then with ethyl acetate. Aliquots of these organic extracts were removed for determination of $[^3\text{H}]$ d.p.m.

<table>
<thead>
<tr>
<th></th>
<th>$[^3\text{H}]$ d.p.m</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labelled Microsomes</td>
<td>28470</td>
<td>100</td>
</tr>
<tr>
<td>TCA supernatant</td>
<td>8025</td>
<td>28.1</td>
</tr>
<tr>
<td>Ethanol wash</td>
<td>19475</td>
<td>68.4</td>
</tr>
<tr>
<td>Ethyl acetate wash</td>
<td>520</td>
<td>1.8</td>
</tr>
</tbody>
</table>

The results illustrated above in Table XVI suggest that almost all the $[^3\text{H}]$Diazo-MAPD can be extracted from photo-affinity labelled microsomes. Following precipitation of microsomal protein with TCA almost one-third of the $[^3\text{H}]$ d.p.m. remain in the supernatant. When the TCA precipitate is washed with /..
ethanol, the remainder of the radio-active ligand can be removed from the microsomes.
3.4.5. Discussion

In 1985, Liang et al described a diazo steroid which specifically labelled hepatic microsomal 5\(\alpha\)-reductase. The structure of this compound - 21-Diazo-4-methyl-4-aza-5\(\alpha\)-pregnane-3, 20-dione (Diazo-MAPD) is shown below.

Fig. 31 The structure of Diazo-MAPD
The chemical basis of binding of Diazo-MAPD to microsomal 5α-reductase.

Resonance structures can be drawn for the A ring of Diazo-MAPD as shown in Fig. 32.

Fig. 32. The resonance structures of the A ring of Diazo-MAPD

The mechanism of testosterone 5α-reduction is believed to involve the addition of hydride ion to C5 – see section 1.2.5. and Fig. 7. The similarity of the transition state structure involved in 5α-reduction and of the A ring of Diazo-MAPD is shown below in Fig. 33.

Fig. 33. Structural similarity of the suggested enol intermediate formed during testosterone 5α-reduction and the A-ring of Diazo-MAPD
Thus it has been postulated that Diazo-MAPD is in fact a transition state analogue and this could explain the specificity of Diazo-MAPD binding to 5α-reductase. Indeed Diazo-MAPD has been shown to inhibit hepatic microsomal 5α-reductase, $K_i = 8.7$nmol/l (Liang et al, 1985b). This inhibition has been shown to be competitive with respect to testosterone. On UV irradiation of microsomal suspensions in the presence of Diazo-MAPD this inhibition was irreversible. It has been suggested that on UV irradiation the diazo group on the 17β side chain decomposed to form reactive carbene and ketene intermediates (Liang et al, 1985b) – see Fig. 34.

![Fig. 34 The decomposition of the diazo functional group of diazo-MAPD to form carbene and ketene intermediates.](image)

These reactive intermediates presumably form covalent linkages with adjacent amino acid side chain groups on the enzyme protein.
Liang et al (1985(b)) provided further evidence that
Diazo-MAPD bound specifically to microsomal 5α-reductase.
They demonstrated that the level of binding in the microsomes
of different rat tissues correlated with the known levels
of 5α-reductase in that tissue. Therefore compared to
female rat liver microsomes, binding to male rat liver
microsomes, to prostate microsomes and to skeletal muscle
microsomes was 43%, 1% and 0.2% respectively.

Also, substrates of 5α-reductase (testosterone and progesterone)
inhibited the binding of [3H]-Diazo-MAPD to microsomes,
whereas 5α-reduced steroid - 5α-dihydrotestosterone and
5α-androstandiol (which are substrates for cyt.P450 hydroxylase
and 3-hydroxysteroid dehydrogenase) had no effect on binding.

The finding that the binding of [3H] Diazo-MAPD to microsomes
was stimulated by NADPH and was UV-irradiation-dependant
(see Fig. 24; section 3.4.1) agreed with the results reported

The results of the photo-affinity labelling and gel filtration
experiments reported in Section 3.4.2 lend support to the
hypothesis that there exists an aggregatory and non-aggregatory
form (perhaps a proteolytic fragment) of 5α-reductase. The
similarity of enzymic and radio-active profiles obtained on
gel filtration (see Fig. 28; section 3.4.2) is particularly
convincing in this respect. When a CMC (0.87% (w/v))
solution of n-octyl glucoside was used for column elution,
the broad early-eluting radioactive material is replaced /...
by a major peak eluting with an estimated Mr of 42000 (Superose 6 - see Fig. 25; section 3.4.2) or 52000 (TSK G3000 SW - see Fig. 26; section 3.4.2). This would suggest that it is this species of the enzyme which aggregates on elution with a sub-CMC (0.05% (w/v)) concentration of n-octyl glucoside.

Liang et al (1985b) reported that on high performance gel filtration of photo-affinity labelled microsomes, radioactivity eluted in one major peak with Mr = 50000. It should be noted however that these workers used Lubrol PX for microsome solubilisation and applied whole extracts (i.e. uncentrifuged preparations) to their gel filtration column. It may be that these differences in methodology account for the difference in radio-active profiles obtained on gel filtration.

I reported that [³H]-Diazo-MAPD could be extracted from labelled microsomes by acid precipitation of microsomal protein and washing of the resulting pellet with organic solvent (see Table XVI; section 3.4.4). This observation must be reconciled with the results reported in this thesis (see Fig. 24; section 3.4.1) and also demonstrated by Liang et al (1985b) that binding of [³H]-Diazo-MAPD to microsomes is dependant on UV irradiation.

Inspection of the data presented in Table XVI; section 3.4.4 indicates that following TCA precipitation of photo-affinity labelled microsomes 28% of the ligand is recovered in the supernatant. Inspection of Fig. 24; section 3.4.1 indicates that this level of radio-activity can be accounted for by/...
non-specific binding of ligand. The extraction of the remainder of the label by washing with organic solvent may be explained if $[^3\text{H}]-\text{Diazo-MAPD}$ does not bind to the $5\alpha$-reductase enzyme protein per se but rather to lipid tightly associated with and closely related to the enzyme active site. This lipid-linked radio-active ligand would be extractable by organic solvent treatment. Indeed Levitski (1986) has described the covalent linkage of a ligand specific for the $\beta$-adrenergic receptor to phospholipid tightly associated with the receptor rather than to the receptor itself. This would be compatible with the results of the gel-slicing and fluorography experiments described in section 3.4.3. which demonstrated the presence of tritium close to the solvent front, with the $[^3\text{H}]-\text{Diazo-MAPD}$ linked to a low molecular weight microsomal lipid.

The final section of the experimental work undertaken in this thesis describes some experiments which investigated a possible relationship between testosterone $5\alpha$-reductase and microsomal enoyl CoA reductase, an enzyme involved in the malonyl CoA-dependant elongation of fatty acids. (Nugteren, 1965) - see Section 3.5.
3.5. Effect of crotonyl CoA on hepatic microsomal 5α-reductase activity.

3.5.1. Inhibition of rat hepatic microsomal 5α-reductase by crotonyl CoA.

The ability of crotonyl CoA to inhibit the rat hepatic conversion of testosterone to 5α-DHT was tested over the concentration range 0-10 mmol/l. Two separate experiments were done and similar results were obtained in both instances. The results of one experiment are illustrated in Fig. 35. The experimental conditions are described in the accompanying figure legend. Fig. 35 shows that crotonyl CoA only inhibited hepatic microsomal 5α-reductase when present at high concentrations. Thus when [crotonyl CoA] = 10 mmol/l (representing an inhibitor : substrate ratio of $4 \times 10^6$), there was about a 20% inhibition of 5α-reductase activity. Under identical assay conditions and at a final [crotonyl CoA] of 1 mmol/l, no inhibition of 5α-reductase was apparent. (see Fig. 35).

The ability of crotonyl CoA to inhibit the conversion of testosterone to 5α-DHT by human prostate microsomes has also been examined (Hodgins, 1983). The results of a dose-response curve for the prostatic microsomal enzyme are shown in Fig. 36. A comparison of Fig. 35 and 36 indicated that the human prostatic enzyme was far more sensitive to crotonyl CoA inhibition with the ID$_{50}$ (half-maximal inhibition) at around 300 umol/l. The inhibitor:substrate ratio at this point was $1.25 \times 10^4$. 
Fig. 35 Inhibition of hepatic-microsomal 5α-reductase activity by crotonyl CoA

For inhibition of rat hepatic microsomal 5α-reductase, the [Testosterone] = 2.45 nmol/l and [NADPH] = 1 mmol/l. Otherwise standard assay conditions. Crotonyl CoA was added to the final indicated concentration. In the control incubation, the % conversion of T to 5α-DHT was 16.35 (± 2.05%) (n = 2; mean ± range). For each concentration of crotonyl CoA, duplicate incubations were performed and the results are expressed as the mean ± range. ▲ = 1 mmol/l α-methyl crotonyl CoA; ■ = 1 mmol/l β-methyl crotonyl CoA.
Fig. 36  Inhibition of prostatic microsomal 5α-reductase activity by crotonyl CoA.

For inhibition of prostatic microsomal 5α-reductase activity, the [Testosterone] = 25 nmol/l, the [NADPH] = 2 mmol/l and 11 ug of microsomal protein were present in a total volume of 200 ul 0.1 M Tris/Citrate buffer (pH 7.5). Incubation was at 37°C for 15 min. Crotonyl CoA was added to the final indicated concentration. In the control incubation [CrCoA = 0], the % conversion of T to 5-DHT was 13.9 ± 1.2%; (n=2: mean ± range). For each concentration of crotonyl CoA, duplicate incubations were performed and the results are expressed as the mean ± range. (After P. Gaggini personal comm.).
3.5.2. Metabolism of crotonyl CoA by rat hepatic microsomes.

One reason for the differential sensitivity of the 5α-reductase from these two tissues to crotonyl CoA inhibition may have been that crotonyl CoA was being metabolised by rat liver microsomes. Experiments were therefore undertaken to establish an assay for microsomal crotonyl CoA reductase activity. The results of these experiments are reported below.

Rat hepatic microsomal crotonyl CoA reductase activity was assayed spectrophotometrically as described in Methods section 2.2.7.(b) In all, three microsomal preparations were assayed for endogenous and crotonyl CoA-induced NADPH oxidation. Seven observations of endogenous and crotonyl CoA-induced NADPH oxidation were made in total and the results are expressed as the mean ± S.E.

<table>
<thead>
<tr>
<th>ENDOGENOUS RATE</th>
<th>CROTONYL CoA-INDUCED RATE</th>
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<tr>
<td>11.2(±2.1) nmol/min/mg</td>
<td>22.5(±1.8) nmol/min/mg</td>
</tr>
</tbody>
</table>

[n=7 ± S.E.] [n=7 ± S.E.]

Subtracting the mean endogenous rate of NADPH oxidation from the mean crotonyl CoA-induced rate gave an average net crotonyl CoA reductase activity of 11.3 nmol/min/mg. The presence of testosterone in the assay solution (final concentration = 1 μmol/1) did not affect the rate of crotonyl CoA-induced NADPH oxidation.

Microsomes are able to convert unsaturated acyl CoA substrates into products other than the corresponding saturated acid. Nugteren (1965) demonstrated the conversion of trans-2-hexadecenoyl CoA into both palmitic and β-hydroxy palmitic acid. This latter product is formed by the action of a /...
microsomal enoyl hydratase activity (E.C. 4.2.1.7.) (Seubert et al., 1968). Indeed it was apparent that microsomes metabolised crotonyl CoA in the absence of NADPH (as judged by the decrease in absorbance at 280nm – Dodds et al., 1981). See Fig. 37

**Fig. 37 Metabolism of crotonyl CoA by rat liver microsomes in the absence of NADPH.**

Assay conditions as for those described for the spectrophotometric determination of crotonyl CoA reductase activity (see Methods section 2.2.7.(b) except that NADPH was omitted from the incubation mixture. The crotonyl CoA concentration /...
was 100 μmol/l and the reaction was initiated by the addition of 70μg microsomal protein.

The rate of NADPH-independant, microsomal crotonyl CoA metabolism was measured in duplicate in two separate microsomal preparations and a value of 17.1±2.8 nmol/min/mg (mean± S.E.) was obtained ( E²₈₀ crotonyl CoA = 5 X 10⁻³ ). It was therefore decided to try and detect the products of hepatic microsomal crotonyl CoA metabolism. This was done by the g.l.c. analysis described in Methods section 2.2.7.(a).

3.5.3. Products of rat hepatic microsomal crotonyl CoA metabolism.

The separation of butyric(4;0), crotonic(4:1) and pentanoic(5:0) acids by g.l.c. is shown below in Fig38.

Fig.38 The separation of 4:0, 4:1 and 5:0 by gas-liquid chromatography.
Under the experimental conditions the retention times for these compounds were 2.3, 4.7 and 7.3 min. respectively and were reproducible from experiment to experiment.

In Table XVII below, the results of eight separate experiments examining the amounts of butyric and crotonic acids formed in the presence and absence of microsomes are shown. The assay conditions are described in Methods section 2.2.7(a).

Table XVII  Rat hepatic microsomal metabolism of crotonyl CoA

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<tr>
<td></td>
<td>%4:0</td>
<td>%4:1</td>
</tr>
<tr>
<td>14.0</td>
<td>14.0</td>
<td>11.6</td>
</tr>
<tr>
<td>14.0</td>
<td>50.2</td>
<td>21.7</td>
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<tr>
<td>11.6</td>
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<tr>
<td>7.4</td>
<td>52.5</td>
<td>23.8</td>
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<tr>
<td>8.1</td>
<td>63.2</td>
<td>15.4</td>
</tr>
<tr>
<td>12.5</td>
<td>57.8</td>
<td>(25.5±0.8)</td>
</tr>
<tr>
<td>(11.7±0.9)</td>
<td>(54.8±1.9)</td>
<td>(Mean±S.E.)</td>
</tr>
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For each experiment the amount (ug) of butyric and crotonic acids present was obtained from the ratio of the peak area for 4:0 or 4:1 with that of the 5:0 internal standard. The results are expressed as a % of the crotonyl CoA added to the assay mixture. The following points should be noted:

(i) even in the absence of microsomes a considerable amount of 4:0 is present (mean value 11.7%).
(ii) in the blank, the total amount of 4:0 and 4:1 recovered amounts to only 66.5% (11.7 + 54.8) of the added crotonyl CoA.
(iii) in the presence of microsomes the amount of 4:0 recovered from the assay mixture is increased to a mean value of 25.5% whereas very little crotonic acid is present (mean 5.8%). No extra peaks were observed when the products of /...
crotonyl CoA metabolism by microsomes were analysed by this method.

3.5.4. Discussion

Microsomal enoyl CoA reductase catalyses the final step in the elongation of fatty acids (Nugteren, 1965) namely the saturation of trans-2-enoyl CoA derivatives. Early studies (Podack and Seubert, 1972) described some of the properties of a microsomal enzyme which was able to catalyse the reduction of trans-2, 3-hexenoyl CoA and trans-2, 3-decenoyl CoA. This enzyme could use either NADH or NADPH as co-factor and exhibited a pH optimum at pH 7.9. More recent studies however have indicated the presence, in microsomes, of two enoyl CoA reductases (Cinti et al, 1982; Prasad et al, 1983; Nagi et al, 1983; Prasad et al, 1985). These workers have demonstrated the presence of an NADPH-dependant enoyl CoA reductase and another enoyl CoA reductase which can utilise either NADPH or NADH. Crotonyl CoA is metabolised by the NADPH-dependant enzyme. This enzyme has been solubilised from microsomes by treatment with sodium cholate and has been purified to near homogeneity by DEAE-52,2'5'-ADP-sepharose and NADP-agarose chromatography. The Mr on SDS-PAGE is 51000.

It is reported in this present study (see Section 3.5.1 and Figs. 35 and 36) that crotonyl CoA is not as effective an inhibitor of the rat liver microsomal 5α-reductase as it is of the human prostatic microsomal 5α-reductase. Crotonyl CoA was reported to be a competitive inhibitor of prostatic microsomal 5α-reductase (with respect to testosterone), $K_i = \ldots$
Ki = 125µmol/l. Interestingly, Nagi et al (1983) reported a Km value of 125µmol/l for crotonyl CoA for microsomal enoyl CoA reductase. The Km (testosterone) value for the prostatic microsomal enzyme was reported at 10 nmol/l (Hodgin and Gaggini, 1983). This gives a $K_i$/(crotonyl CoA)/Km (testosterone) ratio of $1.25 \times 10^4$. If one assumed that the rat liver enzyme displayed the same relative affinities for testosterone and crotonyl CoA as the human prostatic enzyme, then assuming a Km(testosterone) for the rat liver enzyme of 370 nmol/l (see Section 3.1.5), a $K_i$ (crotonyl) CoA of approx. 4.6 mmol/l would be expected. Thus under experimental conditions where the [testosterone] < Km (as pertains for the dose response curve described in Fig. 35), this concentration of crotonyl CoA would be expected to give 50% inhibition of testosterone 5α-reductase. As indicated in section 3.5.1, crotonyl CoA, at a final concentration of 10 mmol/l inhibited rat hepatic testosterone 5α-reductase by only 20%.

We reasoned that β-methyl crotonyl CoA would be a more effective inhibitor of 5α-reductase due to it's greater structural analogy to the A and B rings of testosterone — see Fig. 39.
However at a final concentration of 1 mmol/l, β-methyl crotonyl CoA did not inhibit testosterone 5α-reductase activity. α-methyl crotonyl CoA also failed to inhibit 5α-reductase activity (see Fig 35; Section 3.5.1)

It was postulated that the reason crotonyl CoA failed to inhibit testosterone 5α-reductase was due to its rapid metabolism by microsomes. The microsomal metabolism of crotonyl CoA was therefore examined. When crotonyl CoA reductase activity was measured by spectrophotometrically monitoring the rate of crotonyl CoA induced NADPH oxidation an average crotonyl CoA reductase activity of 11.3 nmol/min/mg was obtained. At a crotonyl CoA concentration of 200 umol/l, Nagi et al (1983) reported a V max value of 10 nmol/min/mg. As they also reported a Km value for crotonyl CoA of 125 umol/l it /..
is likely that the V max value is an underestimate. In this present study the observed rate of 11.3 nmol/min/mg was obtained at a crotonyl CoA concentration of 500 umol/l.

The metabolism of crotonyl CoA by microsomes in the absence of NADPH was also observed. At a concentration of 100 umol/l crotonyl CoA the rate of this NADPH-independent crotonyl CoA metabolism was measured at 17.1 nmol/min/mg. Bernert and Sprecher (1977) have demonstrated the conversion of trans-2,3-octadec-enoic acid to β-hydroxyoctadecenoic acid by a reverse dehydrase present in rat liver microsomes. At a substrate concentration of 200 umol/l (which according to their data is not saturating) they report this activity at 30 nmol/min/mg. Furthermore in the presence of NADPH they demonstrate the simultaneous conversion of trans-α,β-unsaturated fatty acyl CoAs to the β-hydroxy and saturated derivatives by microsomes. These authors found that the relative activities of the reverse dehydrase and enoyl CoA reductase enzymes depended on whether albumin was present in the assay solution. In the presence of albumin, the enoyl CoA reductase activity was over three times greater than the reverse dehydrase activity. However when albumin was omitted from the assay medium then reverse dehydrase activity was 2-3 times greater than enoyl CoA reductase activity. In this present study, crotonyl CoA reductase activity was assayed in the absence of albumin. Enoyl CoA reductase activity is reported as 11.3 nmol/min/mg, whereas NADPH-independent crotonyl CoA metabolism (presumably reverse dehydrase activity) is reported at 17.1 nmol/min/mg (1.5-fold higher). Note /.
however that in this present study crotonyl CoA reductase activity was measured at a substrate concentration five times higher than the NADPH-independent microsomal crotonyl CoA metabolism, (500 umol/l versus 100 umol/l). As the apparent Km (crotonyl CoA) for the microsomal reverse dehydrase reaction is unknown, it may be that if enoyl CoA reductase and reverse dehydrase reactions were measured at the same concentration of crotonyl CoA an ever greater difference in the enzyme activities would be found. Although these findings of NADPH-independent crotonyl CoA metabolism by microsomes reported in this study and the description of a reverse dehydrase activity in rat liver microsomes reported by other workers could explain the inability of crotonyl CoA to inhibit microsomal testosterone 5α-reductase this is unlikely if the data presented in this thesis are examined carefully. Thus it was found that at 1mmol/l crotonyl CoA did not inhibit 5α-reductase activity under standard assay conditions (200 ul assay volume; 1 ug microsomal protein). Under these conditions, there are 200 nmol of crotonyl CoA present. Assuming under these conditions that the rates of crotonyl CoA reductase and crotonyl CoA reverse dehydrase proceed at the values reported in this study (11.3 and 17.1 nmol/min/mg respectively; total = 28.4 nmol/min/mg), then after a 10 min incubation period a total of $\frac{1}{1000} \times 28.4 \times 10 = 284$ pmol of crotonyl CoA would be metabolised. The incubation solution contained 200 nmol of crotonyl CoA (1mmol/l final concentration in a total volume of 200 ul). Therefore from the above calculations it would appear that less than 0.3% of the crotonyl CoA would be metabolised by microsomes. It would appear therefore that the suggestion that crotonyl CoA is unable to inhibit microsomal 5α-reductase because of its /...
rapid and extensive metabolism by microsomes is untenable.

Related to the discussion of the relationship between hepatic microsomal 5α-reductase and enoyl CoA reductase is the report by Nagi et al (1983) and Prasad et al (1985) of the successful solubilisation of enoyl CoA reductase by treatment with a solution containing 20% (v/v) glycerol, 0.1 mol/l Tris Cl (pH 7.7), 1 mmol/l DTT, 0.1 mmol/l PMSF and sodium cholate (D/P 0.5 – 1.5). Following treatment of microsomes with this solution (stirred for 1h. at 4°C) and centrifugation at 120000g for 45 min., 70% of the total enzyme activity was recoverable in the supernatant. In the course of the present study it was found that treatment of microsomes with exactly the same solubilisation cocktail did not solubilise any testosterone 5α-reductase activity from the microsomes. Although this finding would appear to provide indirect evidence for the hypothesis that testosterone 5α-reductase and enoyl CoA reductase are in fact separate enzymes, recent findings (Prasad et al, 1985) provides evidence which may explain the inability of crotonyl CoA to influence testosterone 5α-reductase. Thus in the course of the purification of enoyl CoA reductase they noted that the enzyme would catalyse the NADPH-dependant reduction of α,β-unsaturated enoyl CoAs of varying chain lengths (crotonyl CoA to hexadecenoyl CoA). However it was noted that hexenoyl CoA did not inhibit the reduction of hexadecenoyl CoA or decenoyl CoA, but did inhibit the reduction of crotonyl CoA. The authors suggested that these findings were compatible with several possibilities -
(i) their final enzyme preparation could in fact contain two separate enzymes with a similar molecular weight — one which used short chain substrates and one which used long chain substrates.

(ii) could be a single enzyme with an active site for short chain substrates and a site for long chain substrates.

(iii) could be an enzyme with one active site but when long chain substrates are bound, a confirmational change occurs such that short chain substrates no longer bind.

Which ever of these alternatives proved to be the case they could provide a rational explanation for the inability of crotonyl CoA to inhibit testosterone 5α-reductase. In this model testosterone would be "seen" by the enzyme(s) as a long chain fatty acyl CoA substrate and thus according to the data of Prasad et al (1985) its reduction would not be inhibited by crotonyl CoA. The inhibition of prostatic 5α-reductase by crotonyl CoA could be explained by the fact that the prostate contains a single enoyl CoA reductase with a broad chain length specificity for short- and long- chain enoyl CoAs. One way this theory could be tested would be to determine whether hexadecenoyl CoA could inhibit rat liver testosterone 5α-reductase and whether it could also inhibit prostatic 5α-reductase.

Results of the GLC analysis of the microsomal metabolism of crotonyl CoA showed some rather unusual features. Firstly, even in the absence of microsomes, substantial amounts of butyrate were formed — see Table XVII; section 3.5.3. Therefore it would appear that there was substantial non-enzymic reduction of the double bond of crotonyl CoA during the assay/...
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work-up procedure. This phenomenon was not reported by other workers using exactly the same assay procedure (see for example Nagi et al, 1983). It was not due to the contamination of either crotonyl CoA or the pentanoic acid by butyric acid. Also in the control (no microsomes) incubation only 66.5% of the added substrate is recovered (11.7% 4:0 + 54.8% 4:1). This leaves 33.5% of the added substrate unaccounted for. It is known that under aqueous acid conditions, double bonds undergo hydration (Conrow and McDonald, 1971). As the assay procedure involves adjusting the pH of the incubation solution to pHl prior to organic extraction of reactants and products it is possible that this may cause any unreacted crotonic acid to be converted to $\beta$-hydroxy butyric acid. This compound is not detected by the GLC column used in the present study (Cinti et al, 1982). Thus the apparent loss of crotonyl CoA may be accounted for by its non-enzymic conversion to $\beta$-hydroxybutyrate. In the presence of microsomes the amount of butyric acid recovered is more than doubled (25.5% compared to 11.7%). However, the corresponding decrease in crotonic acid recovered is much greater (5.8% compared to 54.8%). Thus it would appear that crotonyl CoA is also being metabolised by the microsomal reverse dehydrase enzyme to $\beta$-hydroxy butyrate which as mentioned above is undetectable in this assay system. The residual crotonic acid recovered may reflect simply the amount of crotonyl CoA which is not metabolised by the microsomes on the other hand it may reflect the activity of another microsomal enzyme - thioesterase which acts to convert /...
crotonyl CoA to crotonic acid. Crotonic acid, stripped of its CoA moiety would cease to be a substrate for the other microsomal enzymes.
4. GENERAL DISCUSSION AND CONCLUSIONS

It is interesting to compare the properties of hepatic microsomal 5\(\beta\)-reductase (outlined in section 3.1) described in this present study with related steroid metabolising enzymes.

The soluble 5\(\beta\)-reductase of rat liver has been purified to homogeneity (Mode and Rafter, 1985). The Mr of the enzyme on gel filtration was 42000 and the specific activity reported for the enzyme from female rat liver was 0.1 nmol/min/mg protein, about 50-fold lower than that reported for the microsomal 5\(\alpha\)-reductase in this present study. The 5\(\beta\)-reductase had a pH optimum at pH 7-8, similar to that found for the 5\(\alpha\)-reductase and the purified enzyme had a Km(app) for steroid substrate of 10.2 umol/l - an order of magnitude higher than that reported for 5\(\alpha\)-reductase.

Although this present study has described the properties of an NADPH-dependant 5\(\alpha\)-reductase the presence of an NADH -dependant microsomal 5\(\alpha\)-reductase in rat liver microsomes has also been described (Graef et al, 1978; Golf and Graef, 1982; Graef and Golf, 1984; Golf et al, 1985). This NADH -dependant enzyme activity was differentiated from the NADPH-dependant activity on the basis of differential inhibition by nucleotide analogues (Golf and Graef, 1982), by differing conditions for solubilisa-...
solubilisation (Graef et al., 1978), by a circadian rhythm demonstrated by the NADH-dependant activity (Graef and Golf, 1984) and by the dependance of this enzyme on the presence of phosphate in the assay solution (Leybold and Staudinger, 1962). The pH optimum reported for the enzyme was 6.5 and the specific activity at 38.4 nmol/min/mg was 10-fold greater than that reported for NADPH 5α-reductase in this study. Interestingly these workers reported incorporation of tritium into steroid from NADPH labelled in the 4β-position with tritium. This is the same stereoselectivity for hydrogen transfer as that previously demonstrated for the NADPH-dependant 5α-reductase activity (McGuire and Tomkins, 1960b; Bjorkhem, 1969b; Abul-Hajj, 1972).

A neutral pH optimum was reported for hepatic microsomal 5α-reductase. This was contrasted with the acidic pH optimum reported for 5α-reductases from different tissues and also with the one report describing an acidic pH optimum for the rat liver enzyme. (see Section 3.1.9) It was suggested that an acidic pH optimum may reflect the reaction mechanism being favoured by the mass action law by a high concentration of protons. It is interesting in this light to note the pH profile exhibited by the microsomal 5-ane-3β-hydroxysteroid oxidoreductase (Larner and Wiebe, 1983). This enzyme catalyses the reversible reduction of 5α-dihydrotestosterone to 5α-androstan-3β, /..
17β-diol. The reduction reaction showed a pH optimum of 6.6, whereas the oxidation reaction had an alkaline pH optimum of 8.0-8.5. These findings are compatible with the hypothesis that the observed pH optima were related to the reaction mechanism.

Although it has been strongly suggested by data presented in this present study and by the work of other laboratories (see Section 3.1.9) that the 5α-reductase enzyme contained a binding site for both NADPH and steroid and that the reaction proceeded by the direct transfer of hydride from co-factor to steroid, an alternative reaction mechanism was suggested by Golf and Graef (1978). They proposed that NADPH transferred reducing equivalents to co-enzyme Q₁₀ via NADPH:cytochrome oxidoreductase and then to steroid via 5α-reductase. In support of this they cited data showing the inhibition of testosterone 5α-reductase activity by antisera raised to NADPH cytochrome oxidoreductase and also demonstrated that QH₂ can replace NADPH as the electron donor for testosterone 5α-reduction. It is however difficult to reconcile this conclusion with the observation by other workers of direct incorporation of tritium into steroid from NADP³⁻H. If the mechanism described by Golf and Graef were to pertain then it would be expected that equilibration of label (from NADP³⁻H) with protons present in the assay solution would occur and little label would be expected to appear in the steroid. However, it may be rationalised that ³⁻H transfer from NADP³⁻H to the FAD moiety of NADPH cytochrome oxidoreductase then to Q₁₀ and finally to steroid may occur. Evidence presented in this thesis (see Fig 23; section 3.3.4) /..
indicated that testosterone 5α-reductase activity can be separated from steroid hydroxylase activity. Fractions capable of hydroxylating steroids must contain NADPH cytochrome oxidoreductase and therefore the dependence of 5α-reductase on the transfer of reducing equivalents from NADPH:cytochrome oxidoreductase would appear to be doubtful. It would appear therefore that the balance of evidence suggested that in rat liver - as in rat prostate, seminal vesicles and epididymis (Cooke and Robaire, 1984) - NADPH and steroid bound to the same enzyme protein and there was direct transfer of hydride from co-factor to substrate. This type of mechanism was demonstrated for other steroid metabolising enzymes including the 5β-reductase of rat liver cytosol (Abul-Hajj, 1972), the hepatic microsomal 3β-hydroxysteroid dehydrogenase (Bjorkhem and Daniellson, 1970) and the soluble 3α-hydroxy steroid dehydrogenase (Bjorkhem and Daniellson, 1970).

In detailing the importance of detergent:membrane protein ratios and choice of detergent for the solubilisation of membrane proteins with retention of function, Hjelmeland and Chrambach (1984) described four outcomes which may arise -

(i) Treatment of membranes with detergent led to the progressive solubilisation of functional protein as the D:P ratio increased. Biological activity reached a plateau and remained stable at high detergent concentrations.
(ii) biological activity rose and then fell as detergent concentration was increased leading to an optimum D/P ratio.

(iii) all biological activity remained in the pellet even at high D/P ratios.

(iv) all activity was lost upon treatment of membranes with detergent.

It was clear on inspection of Fig. 16 (section 3.2.3.2) that for n-octyl glucoside, Lubrol WX and Chaps solubilisation of hepatic microsomal 5α-reductase category (ii) described by Hjelmeland and Chrambach applied. Sodium deoxycholate fell into category (iv).

It was interesting to note that although sodium deoxycholate was ineffective in solubilisation of 5α-reductase the closely related zwitterionic bile acid Chaps did show limited success.

The importance of a correct n-octyl glucoside: microsomal protein ratio for effective solubilisation of 5α-reductase was indirectly corroborated by other workers. Bertics and Karavolas (1984) reported that when n-octyl glucoside was used at a D:P ratio of 3.5, less than 5% of hypothalamic progesterone 5α-reductase was solubilised. In a subsequent publication however, Bertics and Karavolas (1985) demonstrated that under otherwise identical conditions n-octyl glucoside at a D:P ratio of 2.5 was able to solubilise up to 45% of rat pituitary progesterone /...
Hjelmeland and Chrambach (1984) have also described the physical characteristics of detergent interaction with membranes as the D:P ratio is progressively increased. Thus at low detergent:protein ratios (< 0.1) detergent bound to membranes and as the D:P ratio was increased to 1-2, membrane lysis occurred and lipid-protein detergent and lipid-detergent complexes were produced. It was only when the D:P ratio was increased to 10-20 that effective membrane solubilisation occurred with the production of detergent-protein and detergent-lipid micelles. It was immediately obvious that at the D:P ratios which proved effective for the solubilisation of hepatic microsomal 5\textsuperscript{7}-reductase (see Table IX; section 3.2.3.2) - by the criterion of non-sedimentation of enzyme activity following high-speed centrifugation - that judged by the model of Hjelmeland and Chrambach complete membrane solubilisation did not occur. Indeed in solubilisation experiments of hepatic microsomal 5\textsuperscript{7}-reductase with Lubrol WX at the lowest D:P ratio of 0.2 the supernatant was not clear, but contained opaque material which slowly sedimented to form a "fluffy layer" over a compact pellet (see section 3.2.3.2). Bertics and Karavolas (1984) observed the same type of phenomenon on solubilisation of pituitary progesterone 5\textsuperscript{7}-reductase with n-octyl glucoside at a final concentration of 15 mmol/1. As this is below the published CMC of n-octyl glucoside /.
(25mmol/l: Hjelmeland and Chrambach; 1984) this finding may not be altogether surprising. However, when Chaps was used at a D:P ratio of 0.1, its final concentration was below its CMC value and the supernatant obtained following centrifugation appeared transparent.

An interesting finding was the differential stability of the Lubrol WX-, Chaps- and n-octyl glucoside - solubilised enzyme (see Table XI; section 3.2.4). This suggested that the nature of the detergent shell around the solubilised enzyme was important in maintaining the activity of solubilised enzyme.

Although the success or failure of various combinations of detergents in solubilising hepatic microsomal 5α-reductase was not investigated in the course of this present study, testosterone 16α-hydroxylase was solubilised from mouse liver microsomes by treatment with a combination of 0.5% (w/v) sodium cholate and 0.05% Chaps (Devore et al, 1985) and this is an area which deserves further investigation.

The 5α-reductase of Mycobacterium smegmatis can be released from the subcellular membranes of the bacterium by sonication (Lestrovaja et al, 1977), with about 50% of the enzyme activity being recovered in the supernatant following high-speed centrifugation of sonicated cell suspensions. The porcine testicular microsomal 17β-hydroxysteroid dehydrogenase was also solubilised by sonication and in this form the enzyme is well included /..
(eluting at 1.80 void volumes) on gel filtration (Inano and Tamaoki, 1973). Although the solubilisation of hepatic microsomal 5\(\alpha\)-reductase by sonication was not investigated, the results presented in this present study (see Section 3.2) indicating that 5\(\alpha\)-reductase was deeply embedded in the microsomal membrane suggested that sonication would lead to the production of an enzyme species still bound to membranous fragments.

As well as examining the gel filtration properties of 5\(\alpha\)-reductase, it was also noted that certain of the fractions obtained on gel filtration of solubilised microsomal extracts metabolised testosterone and 5\(\alpha\)-dihydrotestosterone to more polar (probably hydroxylated) steroids. Therefore these fractions must contain both NADPH:cytochrome c (cytochrome P450) oxidoreductase (E.C.1.6.2.4) (Yasukochi and Masters, 1976) and cytochrome P450. These two enzymes form the microsomal "mixed function oxidase" system which is responsible for the stereo specific hydroxylation of the steroid nucleus. Testosterone was shown to be converted to 2\(\beta\), 6\(\alpha\), 6\(\beta\), 7\(\alpha\), 15\(\alpha\), 16\(\alpha\) and 16\(\beta\)-hydroxytestosterone (Ford et al, 1975) on incubation with mouse liver microsomes. The purification of several forms of cytochrome P450 each displaying a specific stereo selectivity for steroid hydroxylations was reported (Cheng and Schenkman, 1982; Johnson, 1982; Agosin et al, 1979; Gungerich et al, 1982; Harada and Negishi, 1984). For example a P450 15\(\alpha\)(a testosterone 15\(\alpha\)-hydroxylase was solubilised from mouse liver microsomes and purified to homogeneity (Harada & Negishi, 1984). The Mr of P450 15\(\alpha\) on SDS-PAGE was 48000 and when /.
reconstituted with NADPH:cytochrome P450 oxidoreductase was able, in the presence of NADPH, to hydroxylate testosterone at a rate of 1349 nmol/min/mg microsomes. The authors report that this was a rate 1000-fold higher than that in the original solubilised preparation. The purified NADPH: cytochrome P450 oxidoreductase of rat liver microsomes has a Mr of 78000 (Yasukochi and Masters, 1976). Despite the differences in Mr of the NADPH:cytochrome P450 oxidoreductases and the cytochrome P450s (Mr 50000) these two enzyme activities must be present together in those fractions from the gel filtration column which show hydroxylating activity.

For demonstration of maximal hydroxylation activity the two enzyme activities were re-constituted in the presence of dilauroyl phosphatidyl choline (Harada and Negishi, 1984; Devore et al, 1985). It was not reported that the presence of phospholipid was required to demonstrate hydroxylation and indeed in this present study hydroxylation of testosterone in the absence of phospholipid in the assay was demonstrated.

Liang et al (1985b) undertook a Scatchard analysis of the binding of \[^3H\] diazo-MAPD to female rat liver microsomes and reported saturation of binding at approx. 400 nmol/1 \[^3H\] diazo-MAPD and that there were 125 pMol binding sites/mg protein. In the same publication, they reported Vmax value of 16 nmol/min/mg for female rat liver microsomal 5α-reductase. These data allow one to calculate a turnover number for the enzyme \( K = \frac{V_{\text{max}}}{E_t} \), where \( E_t \) = the concentration of enzyme active sites (Barnum, 1969). Thus in the case of 5α-reductase \( K = \frac{16\text{nmol/min/mg}}{125\text{pMol/mg}} = 128/\text{min} \). The binding data provided by Liang et al (1985b) also allow for calculation of the /..
relative abundance of 5α-reductase enzyme protein in the microsomes. Assuming a Mr for microsomal 5α-reductase of 50000 (the Mr of the radio-labelled conjugate reported by Liang et al, (1985b)) and assuming one steroid binding site per enzyme molecule, then 125 pmol of enzyme/mg microsomal protein represents (125x50000) pg or 6.25 ug enzyme protein/mg microsomal protein i.e. 5α-reductase represents approx. 0.6% of the total microsomal protein. Mode and Rafter (1985) raised anti-serum to purified rat liver cytosolic 5β-reductase and quantitated the amount of enzyme present in rat liver cytosol by Western blotting. They reported that cytosolic 5β-reductase constituted 0.3% of the total cytosolic proteins of rat liver.

As the photo-affinity binding experiments reported in this present study were performed at a [dMAPD] = 15 nmol/l (see Section 3.4) I was unable to corroborate the value obtained by Liang et al (1985b) for the relative abundance of microsomal 5α-reductase.

Experiments were undertaken to investigate the hypothesis that hepatic microsomal 5α-reductase and enoyl CoA reductase were in fact the same enzyme protein. Results of competition experiments suggested that this was not the case although it was pointed out that in the light of recent data of Cinti and co-workers,(Cinti et al, 1982; Prasad et al, 1983; Nagi et al, 1983; Prasad et al, 1985) further experiments should be undertaken in order to confirm this finding.

The ability of steroid-metabolising enzymes to act on /...
substrates other than steroids is well documented. For example, mouse liver testosterone 15α-hydroxylase can also catalyse benzphetamine N-demethylation, 7-ethoxy-coumarin 0-de-ethylation, aniline 4-hydroxylation, benzo(α) pyrene 3-hydroxylation, acetanilide 4-hydroxylation and, interestingly in the light of the present investigation, lauric acid hydroxylation at positions 11 and 12 (Harada and Negishi, 1984). The cytosolic 3α-hydroxysteroid dehydrogenase of rat liver has been purified to homogeneity (Penning et al, 1984). As well as being responsible for the oxidation of androsterone this enzyme will also catalyse the oxidation of 1-acenaphthenol and benzene (1,2) di-hydro diol. Further, in the presence of either NADH or NADPH it will promote the reduction of certain quinones, aromatic aldehydes and ketones. Another intriguing finding from the point of view of this study is the observation that arachidonic acid inhibits this enzyme competitively with respect to androsterone (1C50 = 15 umol/l). In each of these two examples therefore the steroid-metabolising enzymes were able to act on fatty acid substrates and it may be the case that other steroid metabolising enzymes are able to metabolise fatty acids.

In the light of the findings reported in this study and of recent reports from other laboratories, several avenues of study remain open for investigations into the enzymological properties and physiological significance of testosterone 5α-reductase.
Purification of active 5\(\alpha\)-reductase necessitates the true solubilisation of the enzyme under conditions in which it maintains its activity during further manipulations. One suggestion which was made (see Section 3.2.5) was that combinations of detergents could be tested in an effort to achieve this aim.

It was found that when 5\(\alpha\)-reductase activity was lost during gel filtration, (see Section 3.3) enzyme activity could not be restored by the addition of phosphatidyl choline to the assay solution. In a study of the epididymal microsomal 5\(\alpha\)-reductase, Cooke and Robaire, (1985) demonstrated that the activity of the solubilised enzyme was stimulated only by the addition of phospholipids of defined chemical structure (the most effective was dilauroyl-phosphatidyl choline).

It may be that the activity of rat liver 5\(\alpha\)-reductase could be restored by recombination with phospho-lipid micelles of defined chemical structure, whereas simply assaying for enzyme activity in the presence of sonicated suspensions of crude phosphatidyl choline (as was done in this present study) is unsuccessful.

The use of \([^{3}\text{H}]\text{-Diazo-MAPD}\) as an affinity label for 5\(\alpha\)-reductase also opens up other potential fields of study. The enzyme specie(s) could be purified using this technique. Although under these conditions the enzyme itself has lost all activity the protein itself could be used to raise an anti-serum. Such an anti-serum could prove a useful tool/...
in investigations of both the normal and of mutant $5\alpha$-reductase (eg. those of FIMPII patients – see Section 1.2.4).

Further characterisation of $5\alpha$-reductase is by no means a purely academic exercise as further physiological questions regarding the $5\alpha$-reduction of testosterone remain to be answered. Recently, Andriole et al (1987) demonstrated that administration of $17\beta$-N, N-diethylcarbamoyl-4-methyl-4-aza-$5\alpha$-androstan-3-one (4-MA) to mice bearing the human androgen-responsive genito-urinary tumour (PC-82) significantly inhibited tumour growth. In a completely different vein, Rittmaster et al, (1987) observed that the same compound (4-MA) topically applied daily over a period of 27 months, prevented the development of baldness in the stump-tailed macaque. Although 4-MA is a powerful inhibitor of $5\alpha$-reductase ($K_i = 5.3\text{ nmol/l}$) Liang et al (1984), it also has a weak affinity for the androgen receptor. It is impossible therefore, to state unequivocally by which mechanism 4-MA operates.

In relation to the involvement of $5\alpha$-reductase in the development of other skin disorders, Dijkstra et al (1987) recently reported that the levels of $5\alpha$-reductase activity amongst various human skin substructures was highest in the sebaceous glands. They suggested, therefore, that the higher levels of $5\alpha$-reductase found in acne-bearing skin (see Section 1.2.4) were simply a reflection of the hypertrophy of sebaceous glands known to occur in this condition.
In a recent detailed study of the effects of phospholipases on epididymal 5α-reductase activity Cooke and Robaire (1987) have shown that treatment of nuclear and microsomal membranes with 5α-reductase led to a decrease in the 5α-reductase activity of both intra-cellular compartments. The possibility exists therefore for the control of 5α-reductase in vivo by the activation or inhibition of cellular phospholipases and subsequent modulation of 5α-reductase activity. This may indeed be one mechanism whereby altered levels of intra-cellular 5α-DHT are generated in some pathological situations.
IN SUMMARY

(1) Hepatic microsomal 5\(\alpha\)-reductase of female rat liver microsomes has been characterised with respect to pH optimum and the enzyme has been shown to display normal Michaelis-Menten kinetics. Values for the \(K_m\) (app) testosterone and \(K_m\) (app) NADPH were reported at 370 nmol/l and 1.5 umol/l respectively. Further kinetic evidence suggestive of a sequential reaction mechanism was reported.

(2) Three detergents - Lubrol WX, Chaps and n-octyl glucoside - were shown to be successful in solubilising 5\(\alpha\)-reductase activity. Of these n-octyl glucoside proved to be the most effective, solubilising over 30.5% of the total enzyme activity at a D:P ratio of 1.25.

(3) Solubilised enzyme has been further characterised by gel filtration. On gel filtration of the Chaps-solubilised enzyme all activity was lost. When the Lubrol-WX solubilised enzyme was subject to gel filtration enzyme activity was recovered at the void volume. When the n-octyl glucoside enzyme was gel filtered and eluted with a buffer containing 0.87% (w/v) n-octyl glucoside again all enzyme activity was lost. When the enzyme was eluted with a buffer containing 0.05% (w/v) n-octyl glucoside 70% of the applied activity eluted early at or near the void volume. 10% of the total enzyme activity was well included.
Microsomal 5\(\alpha\)-reductase has been photo-affinity labelled with the specific ligand \(^3\text{H}\) Diazo-MAPD. The binding has been shown to be NADPH- and UV irradiation-dependant. Photo-affinity labelled n-octyl glucoside solubilised microsomes have also been characterised by high-performance gel filtration. On elution with 0.05\% (w/v) n-octyl glucoside, early-and late-eluting peaks of radioactivity were observed. The radio-active profile obtained is similar to the corresponding enzymic profile. On elution with 0.87\% (w/v) n-octyl glucoside the early-eluting radio-activity is replaced by a single peak eluting with a molecular weight of 40-50k.

Indirect evidence is presented that binding of radio-active ligand may be to a non-protein component of the microsomes.

Crotonyl CoA inhibits hepatic microsomal 5\(\alpha\)-reductase activity only at high inhibitor/substrate ratios. However, the hypothesis that there is a relationship between the enzymes testosterone 5\(\alpha\)-reductase and enoyl-CoA reductase(s) requires further investigation.
REFERENCES

Abul-Hajj, Y.-J. (1972) Stereospecificity of hydrogen transfer from NADPH by steroid $\Delta^4-5\alpha$- and $\Delta^4-5\beta$-reductase, Steroids 20, 215-222


Bjorkhem, I. (1969b) Mechanism and stereochemistry of the enzymatic conversion of a $\Delta^4$-3-oxosteroid into a 3-oxo-5$\alpha$-steroid. Eur. J. Biochem. 8, 345-351.

Bjorkhem, I. and Daniellson, K. (1970) Stereochemistry of hydrogen transfer from pyridine nucleotides catalysed by $\Delta^4$-3-oxosteroid 5$\beta$-reductase and 3 hydroxysteroid dehydrogenase from rat liver. Eur. J. Biochem. 12, 80-84.


Bruchovsky, N. and Wilson, J.D. (1968b) The intra-nuclear binding of testosterone and 5α-androstane-17βol-3-one by rat prostate. J. Biol. Chem. 243, 5953-5960.


Dorfman, R.I. and Sharma, D.C. (1965) An outline of the biosynthesis of corticosteroids and androgens. Steroids, 6, 229-236


Doughty, C., Booth, J.E., McDonald, P.G. and Parrott, R.F. (1975) Inhibition by the anti-oestrogen MER-25, of defeminisation induced by the synthetic oestrogen RU2858. J. Endocrinol. 67, 459-460


Liang, T. and Heiss, C.E. (1981) Inhibition of 5α-reductase, receptor binding and nuclear uptake of androgens in the prostate by a 4-methyl-4-azasteroid. J. Biol. Chem. 256, 7998-8005.


Moore, R.J. and Wilson, J.D. (1972) Localisation of the reduced nicotinamide adenine dinucleotide phosphate : $\Delta^4$-3-ketosteroid 5$\alpha$-oxidoreductase in the nuclear membrane of the rat ventral prostate. J. Biol. Chem. 247, 958-967.

Moore, R.J. and Wilson, J.D. (1974) Extraction of the reduced nicotinamide adenine dinucleotide phosphate : $\Delta^4$-3-ketosteroid 5$\alpha$-oxidoreductase of rat prostate with digitonin and potassium chloride. Biochemistry 13, 450-456.


Moore, R.J., Griffin, J.E. and Wilson, J.D. (1975) Diminished 5$\alpha$-reductase activity in extracts of fibroblasts cultured from patients with familial incomplete male pseudohermaphroditism Type 2. J. Biol. Chem 250, 7168-7172.


Segal, I.H. (1975) In Enzyme Kinetics; Wiley: New York p.110


Sodersten, P. (1975) Mounting behaviour and lordosis behaviour in castrated male rats treated with testosterone propionate or oestradiol benzoate or dihydrotestosterone in combination with testosterone propionate. Horm. Behav. 6, 159-164.


Wilson, J.D. (1973) Testosterone uptake by the urogenital tract of the rabbit embryo. Endocrinology 92, 1192-1199.


