ANDROGEN METABOLISM IN THE HUMAN PROSTATE GLAND

by

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SUMMARY

Androgen Metabolism in the Human Prostate Gland

1. Testosterone 5α-reductase, the enzyme responsible for the production of dihydrotestosterone, was partially characterised in human benign hyperplastic prostatic tissue removed at surgery. Biochemical experiments were carried out with fresh tissue or with tissue frozen in liquid nitrogen and stored for a limited period at -70°C.

2. Reductase activity was located in microsomal and nuclear fractions isolated from human hyperplastic prostatic tissue. In both these fractions, and in prostatic homogenates, activity increased linearly with time for 40min under the experimental conditions used. From then on the enzyme lost its activity rapidly and became inactive within 80min. This inactivation could not be explained by lack of cofactor or substrate nor by denaturation by some proteolytic enzymes. In homogenates, and in nuclear and microsomal fractions, NADH was unable to replace NADPH as cofactor.

3. The 5α-reductase activity in nuclear and microsomal fractions was sensitive to thiol group blocking agents. The most effective was p-chloromercuribenzoate which caused total inhibition at a concentration of 5×10^{-5}M. Iodoacetamide, iodoacetic acid, and N-ethylmaleimide also inhibited but higher

concentrations (10^{-2}M) were needed. Reductase activity inhibited by p-chloromercuribenzoate could be reactivated by the addition of dithiothreitol.

4. In both nuclear and microsomal fractions divalent cations inhibited testosterone reduction at low concentrations (5×10^{-5}M) in the following order of effectiveness:-

$$Hg^{2+} = Cu^{2+} > Zn^{2+} = Cd^{2+} \gg Mn^{2+}.$$

Stimulation of reduction by low concentrations of Zn^{2+}, observed by other researchers, could not be confirmed in either human hyperplastic or rat prostatic nuclear fractions. Inhibition of 5 α-reductase activity by the addition of zinc to both nuclear and microsomal fractions could be reversed by dithiothreitol, EDTA and o-phenanthroline.

5. Lineweaver-Burk analysis of the kinetics of zinc inhibition indicated that in both nuclear and microsomal fractions, zinc inhibited in a competitive manner with respect to cofactor but in a non-competitive manner with respect to substrate.

6. EDTA caused slight stimulation, o-phenanthroline slight inhibition and citrate had no effect on 5 α-reductase activity of both nuclear and microsomal fractions.

7. A high zinc content was detected in the hyperplastic prostate and wide variations in concentration occurred within any one gland. Much lower levels were detected in well differentiated adenocarcinomatous samples and wide variations in

concentration were again detected within any one gland.

Zinc in the human hyperplastic prostate was found to be concentrated in the extracellular, nuclear and cytosol fractions.

8. Zinc added in increasing concentrations to homogenate incubations was found to inhibit 5α-reductase activity. The extent of inhibition was similar to that observed when zinc was added to incubations of nuclear and microsomal fractions.

9. The results of a comparison of the zinc content and 5 α-reductase activity in human hyperplastic prostatic tissue homogenates indicated that a significant inverse relationship existed between zinc and 5α-reductase activity. In one prostatic sample which was infiltrated with an anaplastic malignancy no such relationship was observed.

10. Addition of reagents (EDTA, o-phenanthroline and dithiothreitol) known to reverse zinc inhibition of 5α-reductase in nuclear and microsomal incubations did not stimulate reduction in prostatic homogenates to a marked extent.

11. Prostatic cytoplasm, which was shown to contain a high proportion of the intracellular zinc, did not inhibit 5 α-reduction when it was added to microsomal incubations. Heated cytosol fraction, however, was capable of inhibiting 5 α-reductase activity. The inhibitory factor present in heated cytosol could be removed by passage through a cation exchange column, a process known to remove zinc.

12. An hypothesis is presented which involves zinc in a possible control of the accumulation of prostatic secretion by blocking the conversion of testosterone to dihydrotestosterone. Evidence for and against this hypothesis is discussed.

ABBREVIATIONS

Abbreviations used are those recommended by the Editorial Board of the Biochemical Journal (1975) with the addition of the following:-

A.E.S.:	Automatic External Standard
BOMT:	6 α-Bromo-17β -hydroxy-17α -methyl-4-oxa-5α -androstane-3-one
E.C. No.:	Enzyme Commission Number
PTFE:	Poly-tetrafluorethylene
T.K.M. Solution:	0.05M Tris-HCl (pH 7.0 at 20°C), 0.025M KCl and 5×10^{-3}M $MgCl_2$
5 α-Reductase:	NADPH-Dependent Δ^4-3-Ketosteroid-5 α-Oxidoreductase

STEROID NOMENCLATURE

Steroid nomenclature is according to the IUPAC-IUB (1967) "Revised Tentative Rules for Steroid Nomenclature" as reproduced in Steroids (1969) 13, 227-310.

Trivial	IUPAC-IUB
3α-Androstanediacetate	5α-Androstane-3α,17β-diacetate
3α-Androstanediol	5α-Androstane-3α,17β-diol
3β-Androstanediol	5α-Androstane-3β,17β-diol
Androstanedione	5α-Androstane-3,17-dione
Androstenedione	4-Androstene-3,17-dione
Androsterone	3α-Hydroxy-5α-androstan-17-one
Cortexolone	17,21-Dihydroxy-4-pregnene-3,20-dione
Corticosterone	11β,21-Dihydroxy-4-pregnene-3,20-dione
Cortisol	11β,17,21-Trihydroxy-4-pregnene-3,20-dione
Cortisone	17,21-Dihydroxy-4-pregnene-3,11,20-trione
Dehydroepiandrosterone (DHA)	3β-Hydroxy-5-androsten-17-one
Deoxycorticosterone	21-Hydroxy-4-pregnene-3,20-dione
Dihydrotestosterone	17β-Hydroxy-5α-androstan-3-one
Epitestosterone	17α-Hydroxy-4-androsten-3-one
Progesterone	4-Pregnene-3,20-dione
17α-Hydroxyprogesterone	17α-Hydroxy-4-pregnene-3,20-dione
Testosterone	17β-Hydroxy-4-androsten-3-one

ENZYME COMMISSION NUMBERS

See Enzyme Nomenclature (1965), Elsevier Publishing Co., Amsterdam

	E.C. No.
Fructose-1,6-Diphosphate Aldolase	4.1.2.13.
Glucose-6-Phosphate Dehydrogenase	1.1.1.49.
Glutathione Reductase	1.6.4.2.
3α-Hydroxysteroid Dehydrogenase	1.1.1.50.
Lipoamide Dehydrogenase	1.6.4.3.
Thioredoxin Reductase	1.6.4.-.

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A. INTRODUCTION

1. The Prostate as an Accessory Reproductive Gland

The prostate is one of the male accessory reproductive glands. These organs function as exocrine glands, manufacturing, storing and excreting the materials which at ejaculation form seminal plasma, the suspension medium for spermatozoa.

The accessory reproductive glands have many characteristics in common; each possesses a secretory epithelium with an underlying layer of connective tissue and smooth muscle fibres. There are marked dissimilarities, however, in gross structure and in the character of the epithelium, and the chemical nature of the excretions, both between different glands within a species, and within the same gland between different species. In man, seminal plasma is made up of the excretory fluids produced in the epididymis, vas deferens, ampullae, seminal vesicles, prostate, bulbourethral (Cowper's) and urethral (Littre's) glands. In the dog and cat a relatively large prostate is present but seminal vesicles are absent. The bull, on the other hand, has a small prostate but very large seminal vesicles (Mann, 1964).

The accessory glands are target tissues for androgens. This was first shown by the classical studies of John Hunter (1792), who laid the foundation for an understanding of the relation between the presence of the testes and the functional state of the accessory reproductive glands:- "the prostate, Cowper's gland and the glands along the urethra ..... are in the perfect male large and pulpy,

secreting a considerable quantity of slimy mucus which is salt to the taste ——— while in the castrated animal these are small, flabby, tough and ligamentous and have little secretion." Although this implied a messenger travelling from the testes to the accessory reproductive glands it took a further hundred years for the concept of a hormone to evolve. It was in 1902 that Bayliss and Starling defined a hormone as a chemical substance which is produced in one part of the body, enters the circulation, and is carried to distant organs and tissues to modify their structure. It is now known that testosterone, which is produced in the testes, is the male sex hormone. This hormone acts on the seminiferous tubules and the accessory reproductive glands at the same time, thus allowing the simultaneous production of seminal plasma and sperm.

A major difference between the prostate and other accessory reproductive glands is its susceptibility to hyperplasia and neoplasia in ageing men. The need to understand the biochemical nature of these diseases and the hope for medical relief of their symptoms has created much interest in and financial support for prostatic research. Much research to date, however, has focused on experiments with animal tissue, which constitutes an unsatisfactory model for the human gland. This study examines some aspects of androgen metabolism and its control in the human prostate gland.

2. The Human Prostate : Hyperplasia and Neoplasia

In the normal adult man the prostate gland surrounds the

urethra immediately beneath the neck of the bladder, it is about the size of a chestnut and weighs approximately 20g. It has a tubulo-alveolar epithelium which is connected to multiple ducts used for the discharge of prostatic secretion into the urethra. This discharge is under the control of the sympathetic nervous system which, upon stimulation, causes contraction of smooth muscle fibres embedded in connective tissue surrounding the epithelium (Price, 1963).

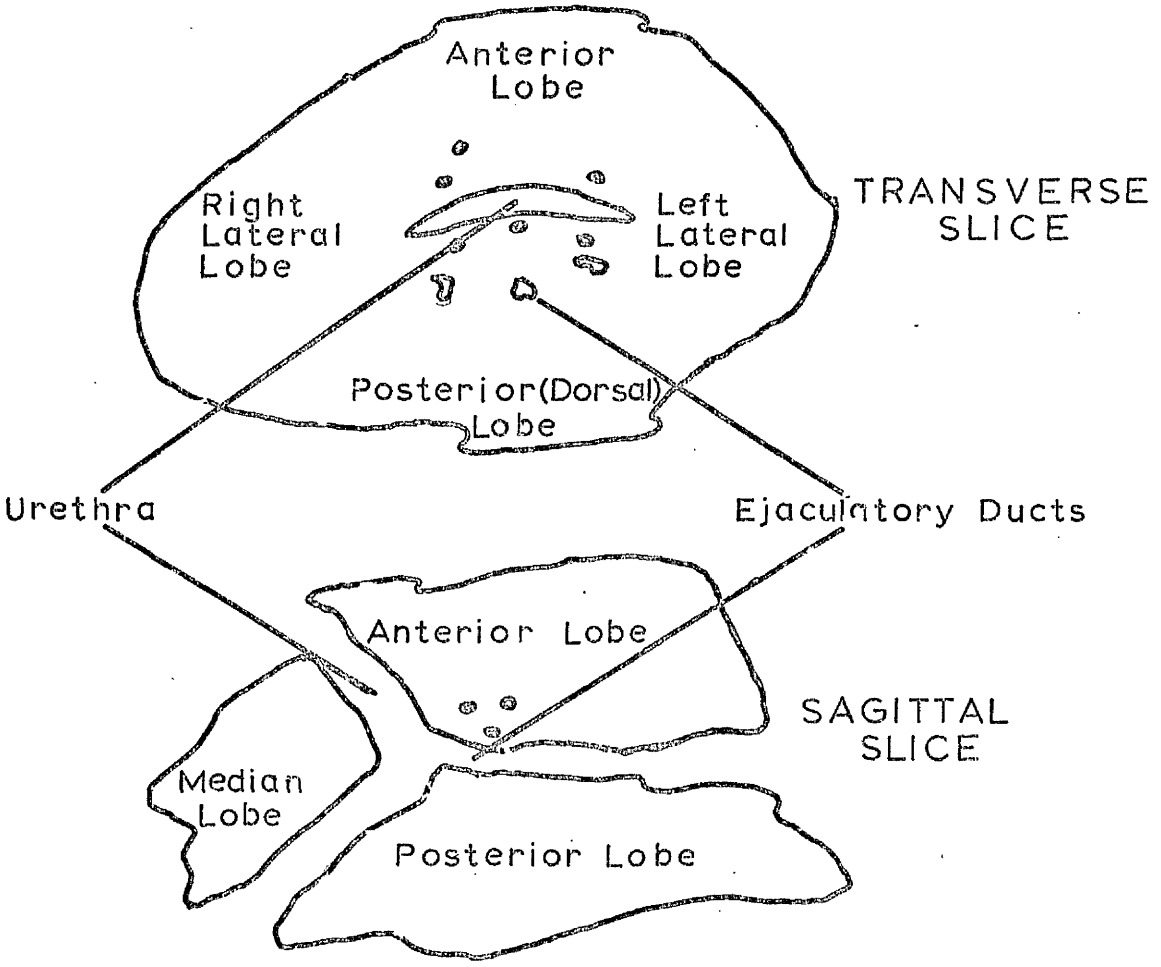
Anatomically the prostate can be divided into five lobes; posterior, middle, anterior and two lateral lobes (Figure 1). These divisions are related to the structures which pass through the gland. The posterior lobe lies behind the urethra and the ejaculatory ducts; the middle lobe lies between the urethra and ejaculatory ducts; the anterior lobe is in front of the urethra; and the lateral lobes, which in many glands are by far the largest, lie on either side of the urethra (Franks, 1954). These divisions have no functional or histological basis.

The gland can be more clearly divided into an inner periurethral area and an outer or "true" prostate (Figure 1). The periurethral tissue consists of prostatic acini, mucosal glands, the prostatic utricle, and the prostatic urethra itself (Franks, 1954). In some (but not all) glands the inner and outer regions are separated by a recognisable fibrous capsule (Jacoby, 1923).

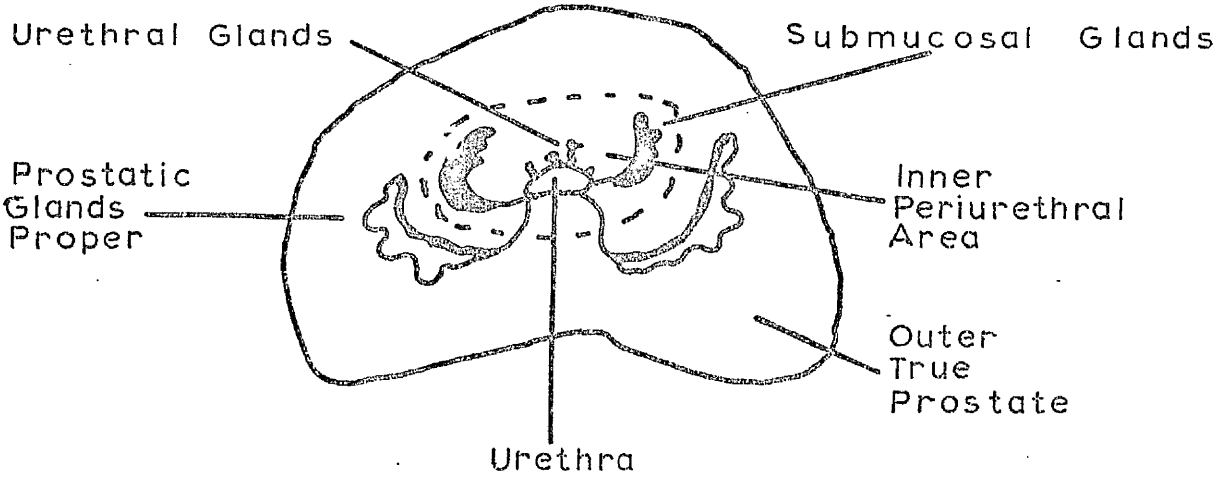
The inner and outer regions may be under different endocrine controls. The inner gland, for example, is derived from the

Figure 1. DIVISIONS OF THE HUMAN PROSTATE.

ANATOMICAL (Kerr et al, 1960)



HISTOLOGICAL (Franks, 1954)



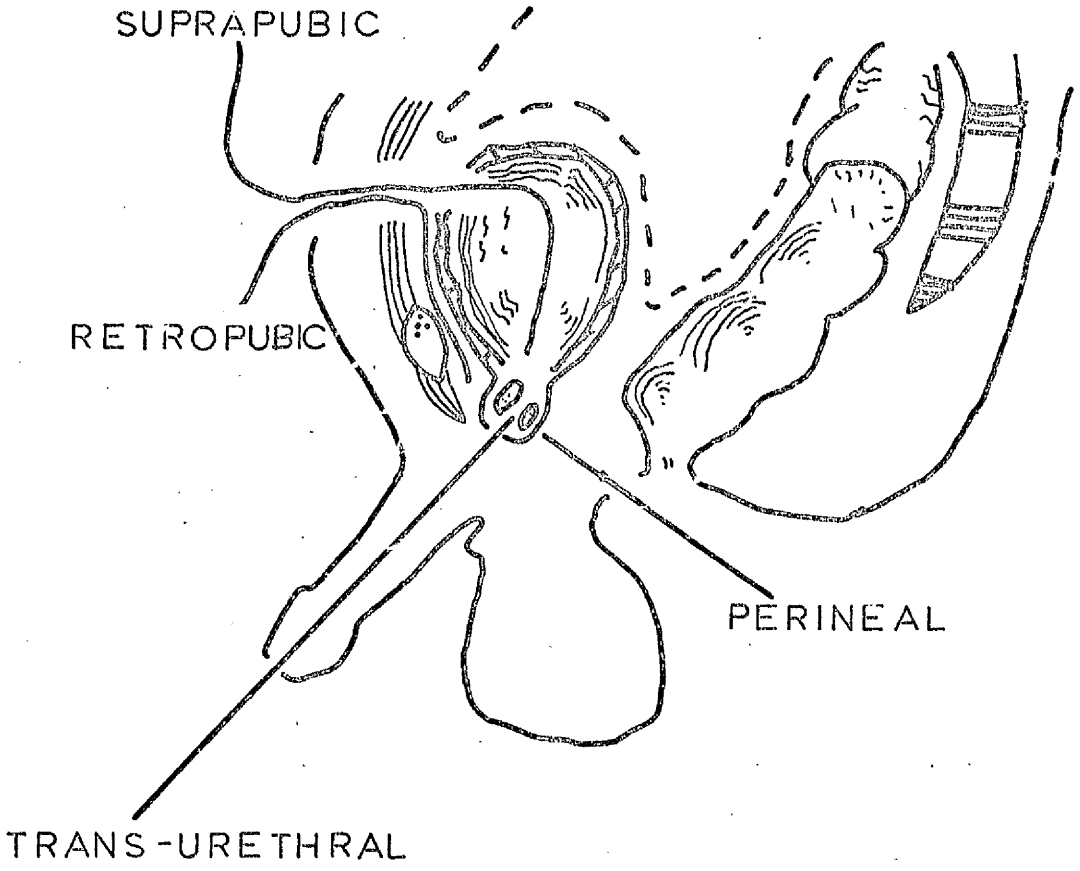
Müllerian duct system (Lowsley, 1912) and is estrogen sensitive. At birth this area of the gland is enlarged, probably due to the presence of maternal estrogens (Franks, 1954). Also, in pseudohermaphrodites the prostate is dependent on the sex of the gonad. If there are ovaries present, the prostate is represented only by the middle and lateral lobes; when both testes are present, the entire male prostate, including the posterior lobe, surrounds the urethra (Moszkowicz, 1935; Andrews, 1951).

Benign hyperplasia of the prostate affects most males over sixty years of age and causes enlargement of the inner gland. When the urethra becomes constricted surgical removal is required to relieve the kidneys and circulatory system of the damaging effects of back pressure. Surgical approaches to the prostate gland are shown in Figure 2. During progression of the disease the outer region of the gland is compressed into a hard fibrous capsule which usually remains after surgery. A diseased prostate gland may weigh up to 200g, a weight increase of 1000%. The disease is confined to a limited range of species and has been detected to date only in man, dog and lion. The histological appearance of diseased glands in these species is, however, dissimilar.

Epidemiological studies have supplied no important clues to the etiology of benign hyperplasia except that they revealed some minor differences in racial and genetic frequencies (Geller, 1974). Geller (1974) has also made a study of clinical literature on medical treatment of benign hyperplasia by a variety of steroidal

Figure 2.

SURGICAL APPROACH TO THE PROSTATE GLAND.



Adapted from Sturdy, D.E. (1974).

compounds. The only effective agents of this type appear to be the progestational anti-androgens. These, he suggests, are a very helpful prototype for the ultimate medical treatment of human benign prostatic hyperplasia.

Prostatic carcinoma is the most prevalent male cancer, and causes approximately 7% - 8% of all deaths due to tumours in men over fifty years of age in the United Kingdom (Franks, 1974). Carcinoma of the prostate is found in the androgen sensitive outer region of the gland and is similar to carcinomas arising in other glandular organs. It is usually recognised by scirrhus and adenocarcinomatous growths. The incidence of carcinoma of the prostate, unlike that of benign hyperplasia, appears to be subject to remarkable racial and geographic differences (Franks, 1974). It is interesting that a familial association between breast cancer and both prostatic and uterine malignancies has recently been demonstrated (Thiessen, 1974). Conservative treatment (hormonal or radiation) of prostatic carcinoma is the standard form of therapy. The discovery of the androgen dependent nature of the disease (Huggins and Hodges, 1941) led to the widespread clinical use of estrogens (Huggins, 1941). Administration of pharmacological amounts of estrogen lowers testicular secretion of testosterone probably through suppression of pituitary gonadotrophin release. Unpleasant side effects include breast enlargement. Although estrogen treatment causes shrinkage of the primary lesion in the prostate this is not a cure for prostatic carcinoma but only delays the fatal consequences of the disease.

This account emphasises a connection between steroid hormones and the diseases of benign hyperplasia and adenocarcinoma of the human prostate gland. Much research has therefore been based on the assumption that these diseases are caused by an upset in steroid hormone action.

3. Hormonal Metabolism and Mechanism of Action

By the early 1960s a large volume of information had accumulated on the effects, often dramatic, of steroid hormones on target tissues. These studies have been reviewed by Grant (1969) and by Dorfman and Ungar (1965). More recent research has been directed towards finding out how steroid hormones induce effects on target tissues. The most promising approach, instigated by Jensen et al. (1968) and Gorski and Morgan (1967), was to follow the progress of a steroid within the target cell. Initial experiments showed that when estradiol-17β entered its target tissue (in this case rat uterus) it bound to two distinct types of protein. Proteins to which the steroid bound with low capacity but high affinity were called specific receptors and these proteins were responsible for the transportation of bound steroid from the cytoplasm to the nucleus. Proteins, on the other hand, to which the steroid bound with high capacity but low affinity were called storage receptors (Wurtman and Jensen, 1968).

These findings stimulated much interest and a general picture has now emerged for the mechanism of action of all steroid hormones. (See Gorski et al., 1968; Smellie, 1971; Raspé, 1971; Jensen and De Sombre, 1972; O'Malley and Mearns, 1974;

Liao, 1974; and King and Mainwaring, 1974 for reviews.) After a steroid hormone enters the cell of a target tissue, a large proportion of this hormone becomes bound to storage receptors. A smaller proportion becomes bound to specific receptors, which upon centrifugation sediment at about 8S. During binding of steroid to specific receptors the receptor protein undergoes a conformational change to a form that sediments at 4S - 5S, permitting or causing its translocation to the nucleus. The overall process has been shown to be energy dependent. Once in the nucleus, an extremely low concentration of hormone, receptor, or hormone-receptor complex initiates the passage of a large amount of information stored within the double-helical structure of DNA back to the cytoplasm. This amplified message leaves the nucleus as messenger RNA and is translated into proteins by the ribosomes. These steroid induced proteins mediate many of the cellular processes in steroid-sensitive tissues. This picture is, however, an oversimplification. The synthesis of tyrosine aminotransferase, for example, may be controlled by corticosteroids acting at a translational level (Tomkins, 1969). There are, in addition, no well substantiated reports that high affinity receptors exist in the cytoplasm of skeletal muscle, an androgen responsive tissue (King and Mainwaring, 1974).

Estradiol-17β, which is not extensively metabolised within target cells, has been widely used in studying the mechanism of steroid hormone action. The mechanism of action of androgens

is less readily understood, however, since these hormones may be extensively metabolised within target cells. *In vitro* incubations of radioactive testosterone with slices, minces, or homogenates of human hyperplastic prostatic tissue have produced a multiplicity of products. Chamberlain *et al.* (1969), for example, identified ten metabolites during incubations of human prostatic homogenates with 80nM testosterone-4-^{14}C. These may have been produced by at least five steroid transforming enzymes: 3α -, 3β -, and 17β -hydroxysteroid dehydrogenases and 5α - and 5β -reductases. The design of this experiment, in which a high substrate concentration was incubated for a long time (2h), allowed measurement of the total catabolic potential of the prostatic homogenate.

The 3α - and 3β -hydroxysteroid dehydrogenases were found predominantly in the cytosol fraction, whereas the 17β -hydroxysteroid dehydrogenase was located in the mitochondrial fraction. The 5α -reductase was located in the microsomal and cytosol fractions, and the 5β -reductase was found only in the cytosol fraction. Further experiments failed to confirm the existence of a cytoplasmic 5α -reductase (Ofner *et al.*, 1970). Some of these enzymes had been detected by earlier incubation studies of testosterone with human hyperplastic prostatic tissue. Wotiz and Lemon (1954) and Kinson (1962) showed 17β -hydroxysteroid activity; Farnsworth and Brown (1963) and Acevedo and Goldzieher (1965) showed 3α -hydroxysteroid activity. Farnsworth and Brown (1963) also made the original discovery that a 5 α-reductase was present in this tissue.

In vivo perfusion studies in a human subject with prostatic hyperplasia have confirmed the existence of all these enzymes except for the 5β-reductase (Morfin *et al.*, 1970). In a further study, Ofner *et al.* (1970) were also unable to confirm their earlier reports of a soluble 5 α-reductase. Many more recent incubation studies (Shimazaki *et al.*, 1965; Gloyna and Wilson, 1969; Siiteri and Wilson, 1970; Becker *et al.*, 1972; Harper *et al.*, 1974 and Jenkins and McCaffery, 1974) have confirmed the existence of an extremely active 5 α-reductase with an obligatory requirement for NADPH as cofactor in human prostatic tissue. Siiteri and Wilson (1970) demonstrated that, although the concentration of testosterone and androstenedione do not differ between normal and hyperplastic human prostate glands, there is a five-fold increase in the concentration of dihydrotestosterone in the hyperplastic as compared with the normal gland. Furthermore, the concentration of dihydrotestosterone in the peripheral areas of both normal and early hyperplastic glands were two and three times greater than the levels found in the outer regions of these glands. A comparison of the ability of prostatic slices to convert testosterone to dihydrotestosterone showed that normal and hyperplastic tissue behaved similarly. Identical results were obtained from dog prostates (Gloyna *et al.*, 1970). These findings suggest a relationship between dihydrotestosterone and benign hyperplasia, but it has not yet been established whether dihydrotestosterone accumulation is a cause or an effect of the disease.

Jenkins and McCaffery (1974) found that although no definite relationship exists between 5α -reductase activity and the histology of prostatic samples, the ability of adenocarcinomatous tissue to reduce testosterone decreased as differentiation decreased. In contrast, Becker et al. (1972) reported that in hyperplastic tissue the amount of 5α -reductase was dependent upon the proportion of epithelial elements in the tissue. Dihydrotestosterone, while of major importance, is not the only testosterone metabolite present in human hyperplastic tissue. It has been demonstrated that testosterone and androstenedione are interconvertible (Wotiz and Lemon, 1954; Farnsworth et al., 1962; Acevedo and Goldzieher, 1964 and 1965; Ofner et al., 1965). Human prostatic tissue can also demethylate testosterone to compounds of the 19-nor series and form 2-methoxyestrone (Farnsworth, 1965 and 1966). Further reduction of dihydrotestosterone has been studied by Jenkins and McCaffery (1974). They observed that the ratio of the isomers of androstanediol are abnormal in prostatic disease. The 3α /3β ratio, which in a normal gland is 2.8/1, becomes 4.8/1 in hyperplastic tissue. The 3α - and 3β - androstanediols can be further hydroxylated at the 6 and 7 positions (Ofner, 1974). Other hydroxylations occurring in C-19 steroids are 2β -hydroxylation which was detected in minced normal human prostatic tissue (Acevedo and Goldzieher, 1964) and 6β - and 2β - hydroxylations of androstenedione which were detected in minced preparations of hyperplastic and adenocarcinomatous human prostatic tissue (Acevedo and Goldzieher, 1965). The relationship of

minor steroid metabolites to prostatic disease, however, remains uncertain.

Steroids of adrenal origin may also be metabolised by hyperplastic prostatic tissue. For example, dehydroepiandrosterone (DHA) and DHA sulphate can be converted to dihydrotestosterone and androstanediols *in vitro*. Radioactive DHA sulphate infused into patients undergoing prostatectomy has been shown to be taken up by the hyperplastic tissue (Harper *et al.*, 1974). Collins *et al.* (1970) detected C-19 steroid sulphatase activity in human prostatic tissue by isolating DHA from incubations with DHA sulphate, and by isolating testosterone from incubations with testosterone sulphate. They also found that androstenedione was metabolised to androstanedione and androsterone.

A number of studies (Shimazaki *et al.*, 1965; Farnsworth, 1970; and Jenkins and McCaffery, 1974) have shown that estrogens at very high concentrations can inhibit human prostatic 5α-reductase activity *in vitro*. Giorgi *et al.* (1972a) have, however, shown that at much lower concentrations estradiol-17β may affect the uptake of androgens by human prostatic tissue. Of more interest is the demonstration by Jenkins and McCaffery (1974) that progesterone is a strong inhibitor of 5 α-reductase activity *in vitro*. They suggest that this steroid could have a marked effect on the metabolism of testosterone if it were administered *in vivo*.

Giorgi *et al.* (1971, 1972a and b, 1973 and 1974) have used a superfusion technique to study androgen metabolism in both human and dog prostatic tissue. This type of experimental procedure

creates an artificial environment nearer to the *in vivo* situation than does the standard *in vitro* incubation method. Results from these superfusion studies confirmed that dihydrotestosterone is the major metabolite of testosterone, and indicated that androgen uptake is higher in hyperplastic than in normal prostatic tissue. She suggests that this difference may be explained by an excessive number of non-specific binding sites in hyperplastic tissue which might reduce the steroid available for metabolism and release. At the same time the amount of androgen reaching the specific receptors might be affected. Although the former conclusion seems logical, it is probably unlikely that binding to non-specific receptors would have any effect on binding to specific receptors, since these proteins have an affinity for steroids of the order of 10^4 times greater than storage receptors. In adenocarcinomatous prostatic tissue decreased levels of non-specific binding sites and 5 α-reductase activity were detected during superfusion.

Specific receptors have been known for some time to be of great importance in the mechanism of action of steroid hormones. Now that methods to study specific steroid binding have been established, many researchers have located specific receptors in a whole range of target tissues of many species. Using Sephadex-gel chromatography, Hansson and Tveter (1971) have shown that components in human hyperplastic prostatic cytosol fractions are able to bind dihydrotestosterone and this binding can be reduced by the anti-androgens cyproterone and SK

and F 7690. Androphilic macromolecules were also detected in nuclear 1M NaCl extracts. Mainwaring and Milroy (1973) used a more sensitive technique for the detection of specific receptors in human hyperplastic prostatic tissue. The cytosol fraction was pre-incubated with labelled steroid at $0^{o}C$ and then subjected to isopycnic centrifugation in a 5% - 20% sucrose gradient. This method allows the separation of binding components with different sedimentation coefficients. Specific binding of ^{3}H-dihydrotestosterone to a protein of sedimentation coefficient 8S was detected. Other ^{3}H-steroids including estradiol-17β , cortisol, androstenedione, and testosterone were not bound to the 8S protein to any significant extent, but non-specific low affinity binding was observed. At high concentrations, cyproterone acetate suppressed the specific binding of ^{3}H-dihydrotestosterone.

Although the specific androgen receptor in the rat prostate has been extensively studied (Liao, 1974) the same is not true for the human gland. This bias may be due both to the difficulty in obtaining normal human prostatic tissue and the experimental difficulties involved in working with human hyperplastic prostatic tissue. Mainwaring and Milroy (1973) were unable to detect receptors in all the samples of human hyperplastic prostate glands they studied. They offer two explanations for this variation. Human hyperplastic prostatic tissue is very difficult to homogenise, and the receptors may have been destroyed during homogenisation. Secondly dilution of the trace amounts of ^{3}H-dihydrotestosterone may be caused by the high level of dihydrotestosterone in the tissue.

The possible presence of contamination from plasma sex steroid globulin, which has characteristics almost identical with the specific prostatic androgen receptor (Vermeulen and Verdonck, 1968) further complicates the study of androgen receptors in the human prostate gland. Steins et al. (1974) and Cowan et al. (1975) have been unable to determine whether specific dihydrotestosterone binding in cytosols isolated from hyperplastic prostatic tissue was due to sex steroid binding globulin or to a specific receptor. Recently a specific receptor for 5 α-androstane-3α ,17α -diol has been identified in both normal and hyperplastic dog prostatic tissue (Evans and Pierrepoint, 1975). In the dog prostate 5α -androstane-3 α, 17α -diol may therefore be the active androgen.

4. Testosterone 5 α-Reductase and its Significance in Androgen Action

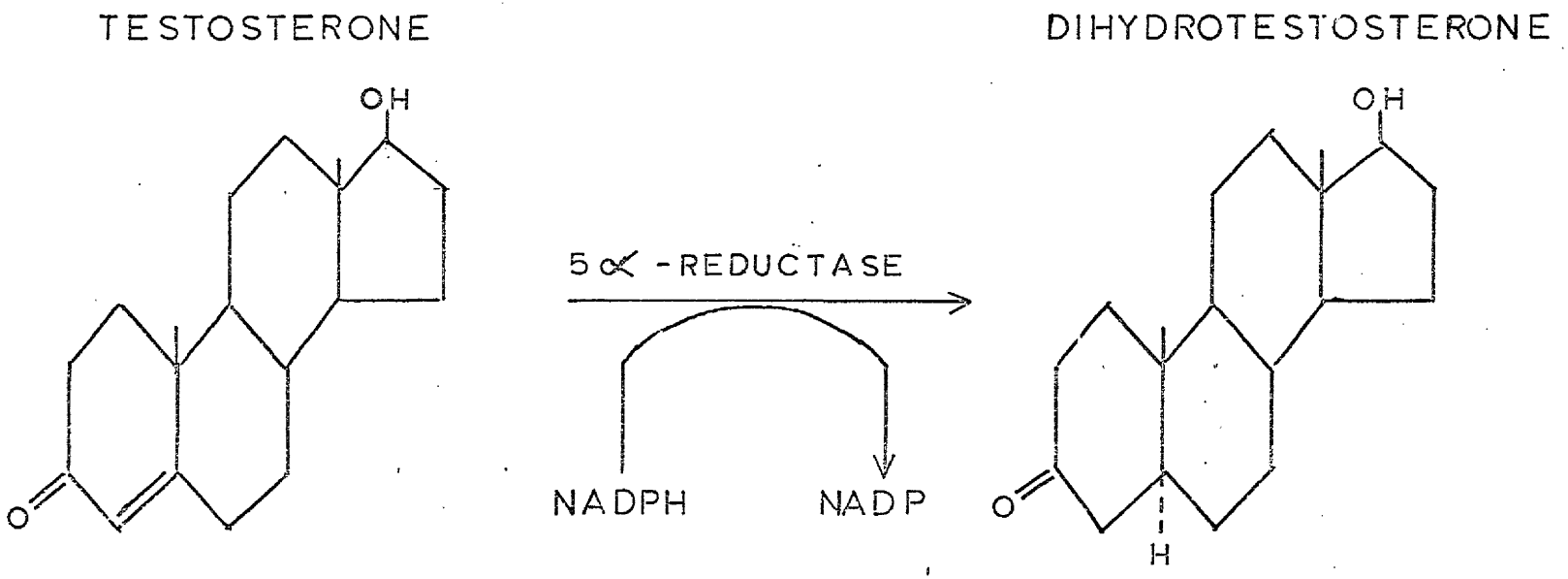
The first evidence which indicated the importance of dihydrotestosterone in androgen action came as a result of studies on the intracellular localisation of ^{3}H-testosterone after its *in vivo* perfusion in male ducks. Wilson and Loeb (1965) found that radioactivity originating from ^{3}H-testosterone was bound to a protein component of nuclei isolated from the duck preen gland. Extending this study to the rat prostate gland, Bruchovsky and Wilson (1968) showed that prior to entering the nucleus ^{3}H-testosterone had been converted to ^{3}H-dihydrotestosterone. This finding was confirmed independently by Anderson and Liao (1968). The conversion of testosterone to dihydrotestosterone is outlined

in Figure 3.

The 5α-reductase enzyme responsible for the conversion of testosterone to dihydrotestosterone has been extensively studied in many androgen dependent and androgen responsive tissues. In androgen dependent tissues androgens initiate both growth and DNA synthesis, while in androgen responsive tissues no androgen stimulated DNA synthesis occurs (Mingeull and Sierralta, 1975). Collectively these tissues are referred to as androgen sensitive tissues and include the liver, testes, adrenals, skin, epididymis, seminal vesicles, brain, kidney and prostate.

Frederiksen and Wilson (1971) partially characterised the 5α-reductase from the rat prostate gland. They reported that the 5α-reductase was distributed equally between the nuclear and microsomal fractions, it had an absolute requirement for NADPH as source of reducing hydrogen, a pH optimum of 6.6, remained stable to freezing but became unstable upon heating at 37°C for long periods. The enzyme was also found to be potentially inhibited by divalent cations. The substrate specificity for a variety of Δ^4-3-ketosteroids was investigated. Epitestosterone, 20α-hydroxy-4-pregnen-3-one, 17α-hydroxyprogesterone and progesterone were more effective substrates than testosterone. Deoxycorticosterone, cortexolone and androstenedione were of the same order of activity as testosterone. Androstenediol, although a substrate, was approximately half as effective as testosterone. Corticosterone, cortisol and cortisone were totally inactive as

Figure 3. CONVERSION OF TESTOSTERONE TO DIHYDROTESTOSTERONE



substrates for reduction over the concentration range studied.

On the bases of pH optima and K_m it was concluded that the microsomal and nuclear 5α-reductase activities belonged to a single enzymic entity. This may be contrasted with the situation in the liver where at least five 5α-reductase enzymes have been detected, each having a limited substrate specificity (McGuire and Tomkins, 1960; McGuire et al., 1960).

The rat prostatic nuclear 5α-reductase has been located on the nuclear membrane (Moore and Wilson, 1972). As the result of chemical analysis, light microscopy, and examination of the subcellular distribution of several marker enzymes, it was concluded that the nuclear fraction used in this study was free from major contamination from other cytoplasmic constituents. The pure nuclear fraction was subjected to sonication followed by density gradient centrifugation. On the bases of buoyant density, the chemical composition of the fraction and detection of a flotation pattern similar to that of NADH-cytochrome c reductase, a known enzyme of the nuclear membrane, it was concluded that the 5α-reductase in prostatic nuclei is located on the nuclear membrane. The enzyme is partially inhibited by Triton X-100 (Liao, 1974), a detergent known to remove the outer nuclear membrane (Blobel and Potter, 1966; Sadowski and Steiner, 1968). It is not yet known whether the location of the 5α-reductase on the outer nuclear membrane serves any physiological function. The 5α-reductase from rat prostate has been further purified after digitonin and potassium chloride

extraction (Moore and Wilson, 1974). In this study, the 5α-reductase in both the nuclear and microsomal extracts had an apparent molecular weight in the order of 250,000 – 350,000 as estimated by gel filtration and a sedimentation coefficient of 13.5S - 15S. The localisation of rat prostatic 5α-reductase in nuclear and microsomal fractions has been confirmed by Nozu and Tamaoki (1973). They also observed that cytosol caused inhibition of ^{3}H-testosterone reduction in microsomal incubations but not in nuclear incubations. This appears to be the only detectable difference between microsomal and nuclear rat prostatic 5α-reductase. In both these fractions the 5 α-reductase has been shown to carry out the stereospecific transfer of the 4-pro-S-proton of NADPH to the double bond at C4 - C5 of testosterone (Nozu and Tamaoki, 1974).

Although there must be an intimate relationship between the specific androgen receptor and the 5 α-reductase these functions belong to different proteins. The specific receptor and the 5α-reductase are located in different subcellular fractions (Mainwaring, 1970) and the anti-androgens cyproterone acetate and BOMT selectively block high affinity binding to dihydrotestosterone without impairment of 5 α-reductase activity (Mainwaring, 1972).

The 5 α-reductase of the rat prostate is androgen dependent. It decreases following castration and is subsequently increased after testosterone treatment (Moore and Wilson, 1973; Gustafsson and Pousette, 1974). Different types

of regulation appear to exist in liver and kidney tissue. In the liver, 5α -reductase markedly increased after castration of males. This is not unexpected since a higher rate of 5 α-reduction occurs in liver nuclei in female rats than in males. In kidney nuclei, the enzyme was found to be almost unaffected by age, sex, castration and treatment with testosterone, but a marked change occurred after treatment with estradiol-17 β (Gustafsson and Pousette, 1974).

It is possible that androgens exert their control by different mechanisms in androgen dependent tissues, such as prostate, and in androgen responsive tissues, such as liver (Tomkins, 1957) and kidney (Verhoeven and De Moore, 1971). This may account for the different constraints imposed upon the 5α-reductase in different tissues. Furthermore, *in vitro* measurements in liver and kidney revealed high levels of 5α-reductase whereas *in vivo* determinations showed a much lower production of dihydrotestosterone. In the rat prostate, however, high activity of the 5α -reductase can be observed both *in vitro* and *in vivo* (King and Mainwaring, 1974).

Verhoeven *et al.* (1974) studied 5 α-reductase activity in fractions isolated from prostate, submaxillary gland, lung, kidney and midbrain. The total 5α -reductase activity varied as follows:-

lung>prostate>kidney>submaxillary gland>midbrain.

The particulate fractions were the major sites of 5α -reductase activity in all tissues except the submaxillary gland. The nuclear fractions contributed 19% of the total 5 α-reductase

activity in kidney and 15% in prostate. Verhoeven et al. (1974) are of the opinion that 5 α-reductase activity found in prostatic nuclear fractions by other workers may be overestimated because nuclear enzyme activity may be dependent, to some extent, upon nuclear purity.

There is considerable evidence to substantiate the hypothesis that dihydrotestosterone is the active androgen which maintains certain androgen sensitive tissues. In some bioassays this hormone has a more potent androgenic activity than testosterone (Dorfman and Shipley, 1956; Hilgar and Hummel, 1964). Moreover, dihydrotestosterone was found to be selectively retained in rat prostatic nuclei, where it stimulated specific RNA synthesis (Davies et al., 1972; Anderson et al., 1972). Dihydrotestosterone stimulated RNA synthesis has also been observed in nuclei isolated from human hyperplastic prostatic tissue (Davies and Griffiths, 1973). In many, but not all, androgen sensitive tissues, specific high affinity receptors have been detected for dihydrotestosterone, but not for testosterone (Mainwaring and Mangan, 1973).

The complex situation concerning androgen action in target organs has recently been clarified in a study by Imperato-McGinley et al. (1974), who have located a group of human male pseudohermaphrodites in a village in the Dominican Republic. These subjects are born with ambiguity of the external genitalia and have marked virilisation at puberty. Biochemical data revealed abnormally low levels of 5α-reductase activity. By studying the clinical

details linked to this deficiency, it has been possible to distinguish between organs that are sensitive to testosterone and others that are sensitive to dihydrotestosterone. The authors hypothesise that during embryogenesis and again at puberty, both testosterone and dihydrotestosterone are necessary for complete male external differentiation and development. Testosterone, secreted in utero by the testes, acts directly on the Wolfian ducts to cause differentiation to the vas deferens, epididymis and seminal vesicles, whereas testosterone acts as a pre-hormone in the urogenital sinus and urogenital tubercle, where its conversion to dihydrotestosterone results in differentiation of the external genitalia and prostate. The androgen induced changes at puberty, in particular, increase in muscle mass, the growth of phallus and scrotum and deepening of the voice appear to be mediated by testosterone and occur in the affected subjects. Development of the prostate gland, increase in facial hair, temporal recession of the hair line and acne do not occur and appear to be mediated by dihydrotestosterone. Postpubertal psychosexual orientation is male, indicating that testosterone and not dihydrotestosterone is the important messenger.

Although the conversion of testosterone to dihydrotestosterone is of ultimate importance in target tissues such as the prostate, the above study indicates that this conversion is not ubiquitous for androgen action in man, and a complex interplay exists between testosterone and dihydrotestosterone. Androgen metabolism within a target tissue may play an elaborate role in the control

of different cellular functions. For example, it has been shown that in prostatic organ culture dihydrotestosterone stimulates cell division, whereas 3 β-androstanediol stimulates cellular secretions (Baulieu et al., 1966; Lasnitzki, 1970).

Dihydrotestosterone is not the only active hormone synthesised from a pre-hormone in a target tissue. Progesterone is metabolised, by a 5 α-reductase enzyme, to 5 α-pregnane-3,20-dione in chick oviduct, and this product binds to a specific 8S receptor enabling it to stimulate the synthesis of avidin (Strott, 1974). In the kidney, 25-hydroxycholecalciferol is activated by a 25(OH)D_3-1-hydroxylase enzyme converting it to 1,25-dihydroxy-cholecalciferol (Fraser and Kodicek, 1970).

5. Zinc and the Prostate

Zinc, the twenty fifth most abundant element, was first shown to have biological significance in 1869 when it was found to be required for the growth of the mould, Aspergillus niger (Raulin, 1869). It is known to be an integral part of a number of enzymes of key metabolic importance (Vallee, 1959; O'Dell and Campbell, 1971), and is found in high concentration in the male reproductive tract, especially in the prostate gland, where it may have a more specific function (Gunn and Gould, 1970). The original discovery that an extremely high concentration of zinc occurred in the human prostate gland was made by Bertrand and Vladesco in 1921. The rat prostate gland also contains a high concentration of zinc (Mawson and Fischer, 1952b and 1953;

Fischer et al., 1955). The capacity to accumulate zinc is not a function of the complete rat gland, but occurs in the lateral portion of the dorso-lateral lobe (Gunn and Gould, 1956a; Rixon and Whitefield, 1959; Fischer et al., 1955; Miller et al., 1961; and Webb et al., 1973). Prostatic zinc concentrations are similar in dogs and men (Weitzel et al., 1956; Lo et al., 1960; Whitemore, 1963 and Webb et al., 1973). The prostate glands from bulls (Bertrand and Vladesco, 1921), cats (Aughey, 1970), rhesus monkeys (Kar and Chowdhury, 1966), baboons (Müntzing et al., 1974), and rabbits (Mawson and Fischer, 1952b) also contain high levels of zinc. The boar, however, concentrates zinc in the seminal vesicles and not in the prostate gland, (Boursnell et al., 1972). In other mammalian tissues such as muscle, liver, heart, intestine, pancreas, ovary and adrenals, much lower levels of zinc have been observed (Tipton and Cook, 1963).

Results from a number of analytical studies which confirm the existence of a high concentration of zinc in the human prostate gland are summarised in Table 1. To allow comparisons between these results, a conversion of values from µg zinc/g wet wt. to µg zinc/g dry wt. was carried out for the cases indicated, making use of the finding of Kerr et al. (1966) that moisture accounted for 80% of the wet weight of prostatic tissue.

It appears from the analytical results presented in Table 1 that prostatic zinc becomes depleted during adenocarcinoma. This finding has been verified by histological studies in which zinc was detected by specific staining techniques using dithizone and

Table 1. ZINC CONTENT OF THE HUMAN PROSTATE

Region of Gland	Normal Mean	Normal Range	Normal No	B.P.H. Mean	B.P.H. Range	B.P.H. No	Cancer Mean	Cancer Range	Cancer No	Method	Reference
Not reported	504	-	2	-	-	-	-	-	-	?	Bertrand and Vladesco (1921)
Not reported	359	598 - 1265	7	772	268 - 1806	20	190	65 - 399	5	Colorimetry	Mawson and Fischer (1952)
Not reported	744	206 - 2316	19	486	30 - 884	51	273	65 - 916	18	Colorimetry	Moore et al (1956)
	—	—	—	268	24 - 570	8†	229	140 - 481	8†		
Dorsal	576	-	-	-	-	-	-	-	-	* Colorimetry	Kerr et al (1960)
Right Lateral	784	-	?	-	-	-	580	-	1		
Left Lateral	627	-	?	-	-	-	219‡	-	1		
Not reported	520	130 - 1120	12	2300	1000 - 5180	9	285	160 - 460	10	* X-Ray Fluorescence	Schrodt et al (1964)
	-	-	-	-	-	-	170	60 - 260	3†		
Dorsal	635	590 - 660	?	-	-	-	-	-	-	* Atomic Absorption Spectrometry	Gyorgy et al (1967)
Lateral	1005	956 - 1154	?	-	-	-	-	-	-		
Anterior	420	407 - 434	?	-	-	-	-	-	-		
Interior	135	418 - 452	?	-	-	-	-	-	-		
Not reported	-	-	-	3800	3310 - 4290	?	230	227 - 233	?		
Interior	-	-	-	o 531	307 - 757	19	o 273	140 - 440	12	Atomic Absorption Spectrometry	Gonick et al (1968)
	-	-	-	o 274	106 - 442	25	o 206	136 - 276	10		
Not reported	2700	2700 - 2700	5	3732	3724 - 3739	57	841	817 - 865	13	* Polarography	Ohar et al (1973)

Values expressed as µg Zn/g dry wt. of tissue (see text)

B.P.H. = benign prostatic hyperplasia

* converted to µg Zn/g dry wt. (see text)
† estrogen treated
‡ cancer localised in left lateral lobe
o less than 60% stroma
o more than 60% stroma

silver sulphate (Gyorkey et al., 1967; Maquinay et al., 1963). A lowered prostatic uptake of zinc-65 has been observed during in vivo perfusions of this isotope into patients suffering from prostatic carcinoma (Prout et al., 1959; Rosoff and Spencer, 1965). Clincial studies have been performed to discover whether prostatic zinc-65 uptake can be related to the response of prostatic carcinoma to therapy (Boddy et al., 1970; Chisholm et al., 1974). The results of these studies, however, were rather inconclusive.

Maquinay et al. (1963) made the observation that in areas of glandular epithelium which proliferate prior to the de-differentiation of adenocarcinoma, the accumulation of prostatic secretion caused a rise in the zinc level. Subsequent loss of differentiation was accompanied by a fall in the zinc content of the tumour. This observation may explain the wide range of zinc levels found in prostatic adenocarcinomatous tissue. Gyorkey et al. (1967) showed that the decrease in zinc during adenocarcinoma occurred mainly in the cytoplasm, but zinc still remained detectable in the nucleus, particularly in the nucleolus.

The studies of Mawson and Fischer (1952a) and Hoare et al. (1956) showed that in hyperplastic samples the zinc content was lower than in normal prostatic tissue samples. This, however, was in contrast to the studies of Shrodt et al. (1964); Gyorkey et al. (1968); Gonick et al. (1969) and Dhar et al. (1973), and the histological findings of Voigt (1958) and Gyorkey et al. (1967). A possible explanation for these differences was offered in the careful study of Shrodt et al. (1964), who suggested that the

discrepancy may be due to variations in the loss of prostatic fluid from the specimen prior to the zinc determination. This fluid contains a high concentration of zinc (Mackenzie et al., 1962a). Shrodt et al., 1964 reported that if pieces of prostatic tissue were squeezed to remove prostatic fluid prior to analysis, the tissue zinc level was depressed. The mass of published evidence therefore suggests that zinc levels are slightly greater than normal in hyperplastic glands but substantially lower than normal in adenocarcinomatous glands. Dhar et al. (1973), found unusually high prostatic zinc levels in normal and diseased prostates when their results are compared to those from other studies (see Table 1). This may be explained by the fact that no attempt was made by Dhar et al. (1973) to protect against or monitor for zinc contamination.

Zinc is not distributed uniformly throughout different areas of the human prostate gland, and the variations which occur, summarised in Table 1 , do not correlate well with anatomical divisions of the gland (Kerr et al., 1960; Gyorkey et al., 1967). In addition, in some investigations no correlation has been shown between the prostatic tissue zinc level and the content of epithelium within the tissue (Kerr et al., 1960; Hoare et al., 1956). Gyorkey et al. (1967), however, showed that in the normal prostate the acini, epithelial cells and cell nuclei of various zones stained for zinc, whereas in hyperplastic tissue, intense staining occurred in the epithelial cells and secretions. These findings are similar to those of Voigt (1958), and of the radioautographic

study of Daniel et al. (1956).

The presence of cytoplasmic granules has been detected in the epithelial cells which stain for zinc (Maquinay et al., 1963). These granules occupy the basal and apical poles within the cells. The authors suggest that zinc complexes are secreted into the lumen from the apical region and that zinc is accumulated from the peripheral plasma into the basal region. It is interesting that zinc is also associated with granules in leucocytes (Thiers and Vallee, 1957).

A more biochemical approach to the problem concerning the histological distribution of prostatic zinc has been attempted by Müntzing et al. (1974). Zinc content was compared with β -glucuronidase since the concentration of this enzyme is related to the amount of epithelium in human prostatic tissue (Müntzing and Nilsson, 1972). A strong correlation was found between zinc and β -glucuronidase activity in non-cancerous, but not in cancerous tissue. High levels of zinc were also detected in epithelial cells and glandular lumen in the prostate glands of rat (Rixon and Whitefield, 1959; Logothetopoulus, 1960), cat (Aughey, 1970) and dog (Mackenzie et al., 1962b).

The prostate gland is known to be androgen dependent and there is considerable evidence that the accumulation of prostatic zinc along with other specialised functions is under androgenic control. Administration of suitable doses of androgen to castrated mature male rats prevented the striking fall in zinc-65 uptake by the dorso-lateral prostate, which should follow castration

(Gunn and Gould, 1956b and 1957; Mirand and Bender, 1956). Zinc accumulation in the rat testes is similar to that in the prostate and may be under hormonal control (Gunn and Gould, 1970). In the dog pre-pubertal zinc levels are low. A 10% decrease in prostatic zinc occurs following castration and a restoration of normal zinc levels in the prostates of castrated animals may be achieved by administration of testosterone (Whitmore, 1963). Zinc accumulation may also be under androgenic control in the baboon (Müntzing et al., 1974).

In man, although prostatic zinc concentration is probably under androgenic control, reliable evidence on this point is still lacking. It has, however, been shown that in the human hyperplastic prostate gland zinc and testosterone accumulation are carried out by the binding of these compounds to different proteins (Reed and Stitch, 1973). There is evidence that, in addition to a specialised role in the human prostate, zinc may play a more general role in male sexual development (Sandstead et al., 1967; Caggiano et al., 1969; Prasad et al., 1963).

Testosterone may not be the only endocrine factor controlling zinc uptake. Testosterone and prolactin have been shown to increase zinc uptake synergistically in the rat prostate (Gunn et al., 1965; Moger and Geschwind, 1972). Prolactin has also been shown to stimulate zinc uptake in the absence of testosterone (Moger and Geschwind, 1972).

In the female, zinc accumulation may also be under steroidal control but here, progesterone may be the controlling influence.

For example, in women (Hagenfeldt et al., 1973) and in female rabbits (Lutwak-Mann and McIntosh, 1969) endometrial zinc increases during the progestational phase of the menstrual cycle. Serious interference with reproductive performance has been detected in zinc deficient female rats (Mann, 1964).

The function of a high concentration of zinc in the prostate gland is uncertain, but cannot be accounted for by the incorporation of this cation into metalloenzymes (Fischer et al., 1955). In a variety of species much of the prostatic zinc is located within the epithelium and cellular lumen. Human sperm cells (Mawson and Fischer, 1953), seminal plasma (Mawson and Fischer, 1956) and especially prostatic secretion (Mackenzie et al., 1962a) contain high levels of zinc, implying a special, as yet undiscovered, physiological role in reproduction, perhaps in the female reproductive tract. In support of this hypothesis, it has been demonstrated that in the roe deer, the endometrial zinc concentration remains high after coitus and throughout delayed implantation (Aitken, 1974). Contrary evidence to this role in the case of the rat, however, was produced by Gunn and Gould (1958) who showed, by ligation of the dorso-lateral prostate, that prostatic fluid, presumably of high zinc concentration, is superfluous for rat fertility. In this species, however, zinc may be supplied to semen from the testes (Wetterdal, 1958; Birnbaum et al., 1961), leaving open the possibility of a role for high concentrations of zinc in fertility. It is interesting that in rat semen little of the zinc is extractable (Saito et al., 1968), and zinc is probably complexed to thiol groups

in the keratin-like structures of sperm tails (Calvin and Bleau, 1974). In man (Eliasson et al., 1971) and dog (Saito et al., 1967), on the other hand, a large proportion of zinc is extractable and is probably protein bound (Ekbom and Wetterdal, 1961; Johnson et al., 1969). Zinc binding in seminal plasma may be of some importance, since free zinc is toxic to spermatozoa (Lindholmer, 1974). This might explain the suppression of fertility caused by intra-uterine devices containing zinc (Zipper et al., 1969).

It has been suggested that one possible role of zinc in semen might be to inhibit sperm mobility, thus holding the energy system in check until the actual time that fertilization is to take place. If spermatozoa are washed in vitro, an increase in respiratory activity occurs and this may be associated with the concomitant loss of zinc (Eliasson et al., 1971). Conversely a decrease in sperm mobility may be related to an increase in the concentration of zinc in the epididymis following administration of monochlorhydrin, a male antifertility drug (Gunn and Gould, 1972). The fact that the sperm of some marine species do not become motile until the zinc concentration has become diminished suggests that an inactivating role for zinc may also occur in primitive forms with external means of fertilization (Fujii et al., 1955).

6. Outline of Research

The present study is concerned with some aspects of androgen metabolism in the human prostate gland. Initial findings

by Grant et al. (1971) suggested that the nuclear 5 α-reductase of the human hyperplastic prostate was modified by zinc. Since zinc is present in high concentration in the human prostate gland, interactions of this element with testosterone 5 α-reductase may have physiological implications. This study therefore set out to learn more about the general characteristics of the 5 α-reductase present in the human hyperplastic prostate and, more specifically, to study the effect of zinc on this enzyme.

B. MATERIALS AND METHODS

1. Chemicals

Non-labelled steroids were obtained from Koch-Light Laboratories Ltd., Colnsbrook, U.K. and Steroloids, Croydon, U.K. Bovine serum albumin, DNA from calf thymus type 1 (highly polymerised), glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from Torula yeast type X1), o-phenanthroline and nucleotides were from Sigma Chemical Co. Ltd., Kingston-upon-Thames, U.K. Zinc chloride (Specpure) and copper chloride (Specpure) were from Johnson and Matthey, London, U.K. All other chemicals were obtained from British Drug Houses Ltd., Poole, U.K. unless otherwise stated.

2. Solvents

Ethanol and methanol (Burroughs, A.R. Grade) were used without further purification. Benzene, obtained from British Drug Houses Ltd., was passed through a column containing silica gel and concentrated sulphuric acid, washed with water, dried and distilled twice before use. All other solvents were also obtained from British Drug Houses Ltd., unless otherwise stated, and were used without further treatment.

3. Distilled Deionised Water

All solutions were prepared with water which had been distilled and deionised in an Aquator 60E (Anderman and Co. Ltd., London, U.K.) water purifier.

4. Radioactive Steroids

[1,2-^{3}H] Testosterone (specific radioactivity 56 Ci/mmol) and [4-^{14}C] testosterone (specific radioactivity 58.2mCi/mmol) were purchased from the Radiochemical Centre, Amersham, U.K. [1,2-^{3}H] 3α-Androstanediol (specific radioactivity 44mCi/mmol) and dihydro [4-^{14}C] testosterone (specific radioactivity 56Ci/mmol) were the products of New England Nuclear, Frankfurt, Germany. Although these radioactive steroids should be pure on leaving the manufacturer, some decomposition may have occurred during transit and storage. They were therefore purified and radiochemical purity was established before they were used in experiments.

Purification was accomplished by paper chromatography in light petroleum (b.p.80°-100°C)-benzene-methanol-water (4:1:4:1 by vol.). The sample, [1,2-^{3}H] testosterone (25μCi), [1,2-^{3}H] 3α-androstanediol (25μCi), [4-^{14}C] testosterone (5μCi) or dihydro [4-^{14}C] testosterone (5μCi) was spotted on Whatman No. 1 paper (50cm x 5cm), equilibriated overnight in a tank saturated with the above solvent system, and then developed in a descending manner for 2½h in the supernatant ("mobile") phase, light petroleum (b.p.80°-100°C)-benzene (4:1 v/v). The radioactive steroids were detected on a radiochromatogram scanner (Model 2700; Packard Instrument Co. Ltd., Wembley, U.K.) and the areas located on the paper was cut into small squares (1cm x 1cm), placed in a flask (50ml) and eluted overnight with 95% aqueous ethanol (10ml). The supernatant was then decanted and the flask rinsed with ethanol (5ml). The pooled extracts were placed in a glass tube in a water bath (40°C) and the solvent evaporated under a stream of nitrogen. Benzene (4ml) was added and a sample (0.05ml) counted. This stock solution of purified

steroid was stored at $4^{0}C$. Benzene was used as storage solvent as suggested by Bayly and Evans (1968). In the case of [1,2-^{3}H] testosterone the specific radioactivity was decreased to 14Ci/mmol by the addition of cold steroid.

To provide proof of radiochemical homogeneity, carrier steroid (20mg) was added to a sample (0.05ml) of stock solution and the radioactive and carrier steroids in the mixture crystallised from acetone/hexane (5:1 v/v). If the specific radioactivity of both crystals and mother liquor in successive crystallisations were constant to within 5% of the specific radioactivity before crystallisation, then radiochemical purity had been achieved (Axelrod et al., 1965). In all cases the method just described produced radiochemically pure steroid.

5. Tissue

(a) Rat Ventral Prostatic Tissue

Sprague-Dawley rats were reared in an animal house on a recommended diet (Diet 41, Bruce and Parkes, 1949). Adult males weighing between 200g and 400g were killed by decapitation, and the ventral prostate gland, located at the base of the bladder, removed from each rat. The gland was separated from its surrounding capsule of connective tissue with forceps and placed in a plastic bag in crushed ice. Since the average weight of the rat ventral prostate gland is around 0.5g, six glands were used for each experiment. No longer than 20min elapsed between removal of the glands and the start of an experiment.

(b) Human Prostatic Tissue

Fresh human prostatic tissue was made available by the Urology Departments of three Glasgow hospitals - Glasgow Royal Infirmary, the Western Infirmary, and on occasions Stobhill General Hospital. Surgical removal of the prostate gland from elderly males suffering from symptoms of benign hyperplasia was carried out by either the retropubic or the suprapubic route (see Figure 2). The removed gland was transferred immediately to a plastic bag, placed on crushed ice, and taken without delay to the Pathology Department of Glasgow Royal Infirmary. There it was weighed, examined, and large sections cut for histological investigation. The remaining tissue, which varied in weight from 5g to 70g depending on the size of the benign growth, was taken to the laboratory. The period between removal of the gland and the start of an experiment was usually less than 30min. However, in some cases when the samples were obtained from hospitals other than the Royal Infirmary, this period may have been longer, but never exceeded 90min. In experiments using this fresh tissue, samples exceeding 10g were not required. Any remaining tissue was stored for subsequent experimentation by the method outlined in the following section.

(c) Freezing of Human Prostatic Tissue

Prostatic tissue (10g) was placed on a plastic dish in crushed ice, cut into small squares (0.5cm x 0.5cm approximately) with a razor blade, and dropped into liquid nitrogen ($-196^{o}C$). After 10min the pieces were transferred to a plastic bag by a wooden spoon and stored in a refrigerator at $-70^{o}C$. When required tissue was thawed rapidly to minimise ice crystal formation (Mazur et al., 1970). The frozen

tissue squares were placed on a plastic dish partially immersed in warm water maintained at 30°C. To increase the surface area of the tissue exposed to this heat it was continually chopped with a razor blade. Thawing was complete after 5min when, to prevent enzymatic degradation, the warm water surrounding the dish was replaced by ice. The chopping procedure was continued until a fine mince was obtained, after which the tissue was ready for homogenisation.

6. Fractionation of Human Prostatic Tissue

The method used was based on that of Kowarski et al. (1969), but considerable modification was required to accommodate the peculiarities of human benign hyperplastic prostatic tissue. A major problem was its extreme toughness, due to a high concentration of fibromuscular and connective tissue elements. All operations were carried out at or as near as possible to 0°C by keeping vessels and solutions in crushed ice. A cold room was not available.

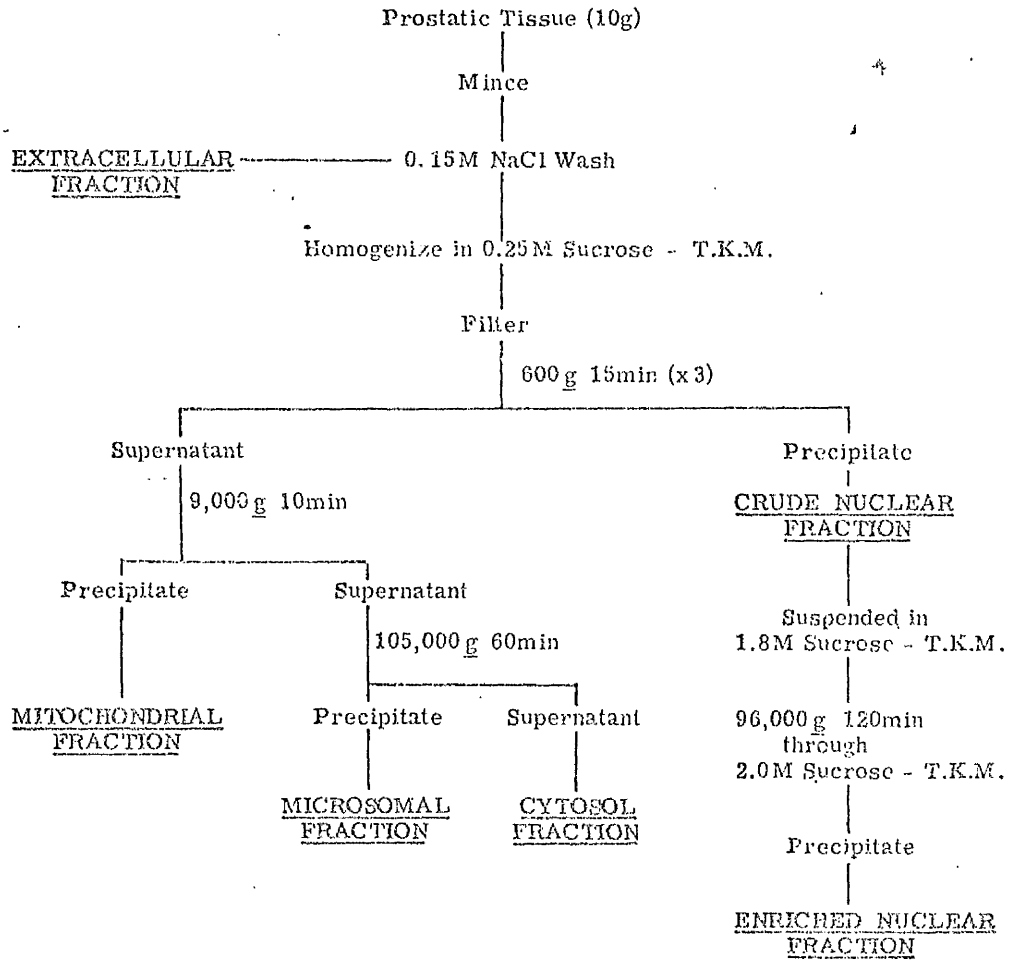
An outline of the method used is shown in Figure 4.

(a) Homogenisation

Prostatic tissue (10g) was placed on a plastic dish partially immersed in crushed ice and finely minced with a razor blade. To remove blood contamination, extracellular fluid and cell debris, the minced tissue was transferred to a flask (50ml), and mixed with a buffered 0.15M NaCl pH 7.0 solution for 10min at 0°C with a magnetic stirrer. The supernatant was decanted, fresh buffered 0.15M NaCl pH 7.0 solution (20ml) added, and the washing procedure repeated.

Figure 4.

SUBCELLULAR FRACTIONATION OF HUMAN HYPERPLASTIC PROSTATIC TISSUE



The pooled washes were filtered through nylon bolting cloth (Nybolt 25T 45μ pore; John Stanier and Co., Manchester, U.K.). The resulting filtrate was termed the extracellular fraction.

The washed minced tissue was separated into three equal batches and suspended in a solution (20ml) of 0.25M sucrose, 0.05M Tris-HCl (pH 7.0 at 20°C), 0.025M KCl and 5×10^{-3}M $MgCl_2$ (0.25M sucrose-T.K.M. solution). Each batch was placed in a polycarbonate tube (100ml). An Ultra-Turrax homogeniser (Janke and Kunkel, Breslov, Poland; U.K. agents The Scientific Instrument Centre Ltd., London), which uses steel blades, was found to be most effective for the further disintegration of this tough tissue, and was operated at setting No. 4 with three periods of 10s and intervals of 10s. Homogenisation was completed with a Thomas homogeniser (size C; A.H. Thomas Company, Philadelphia, U.S.A.). This is a Potter-type homogeniser with a teflon pestle and glass vessel and a clearance of 0.006in - 0.009in. The pestle was operated at 600 r.p.m. with two vertical movements of the vessel. During homogenisation with both types of apparatus the vessel containing the suspension was surrounded by crushed ice in order to minimise the effect of a rise in temperature due to friction. The homogenate was filtered through two layers of nylon bolting cloth (Nybolt 25T 45μ pore) and the filtrate volume made up to 70ml with 0.25M sucrose-T.K.M. solution. During this filtration some material was unable to pass through the 45μ pores of the nylon bolting cloth. Since this mesh size is about twice the diameter of whole cells, the retained material must be resistant to the extreme homogenisation procedure. Under the light microscope the filtrate was found to consist of many broken cells, occasional epithelial

whole cells, and clumps of stromal tissue; while the fraction held back by the filter contained a conglomerate of homogenisation resilient stromal and connective tissue. The filtrate thus constituted an enriched epithelial homogenate fraction.

(b) Isolation of the Nuclear Fraction

The method suggested by Blodel and Potter (1966) resulted in an extremely low nuclear yield when applied to human hyperplastic prostatic tissue. In order to effect some increase in this yield, the method was modified by isolating a crude nuclear pellet prior to further purification. The enriched epithelial homogenate fraction (see previous Section) was placed in M.S.E. polycarbonate tubes (100ml) which were fitted to the swing-out head of an M.S.E. Mistral 4L centrifuge (Measurement and Scientific Equipment Ltd., London, U.K.), operated at 600 g for 15min at 4°C. (All "g" values reported in this study were calculated from the average radius of rotation of the column of liquid in the rotor tubes). The pellet obtained was suspended in 0.25M sucrose-T.K.M. solution (10ml) and the centrifugation procedure repeated twice. Supernatants from the three precipitations were pooled and the final crude nuclear pellet was gently suspended in 1.8M sucrose-T.K.M. solution by repeated passage through the tip of a Pasteur pipette.

Volumes (3ml) of 2.0M sucrose-T.K.M. solution were added to three M.S.E. polycarbonate tubes (23ml). Crude nuclear suspension (20ml to each tube) was gently layered on top of the 2.0M sucrose-T.K.M. solution and the interphase between the two layers gently disturbed with a glass rod. The tubes were centrifuged for 120min at 96,000 g

($r_{av.}$ 3.70cm) in the 3 x 23ml aluminium swing-out rotor of an M.S.E. Superspeed 65 centrifuge. The temperature control was set at $5^{o}C$, since if centrifugation is carried out at below $4^{o}C$, the resulting change in the viscosity of concentrated sucrose produced poor nuclear yields (Jouan et al., 1973).

On completion of centrifugation a white slightly opalescent pellet in the shape of a thin disc was deposited at the bottom of the centrifuge tube. The supernatant sucrose solution was carefully removed by suction and the sides of the tube cleaned with Kleenex medical wipes. The pellet was resuspended in 0.25M sucrose-T.K.M. solution (15ml) and washed twice by precipitation for 10min at 600 g ($r_{av.}$ 15cm). The final nuclear pellet was carefully suspended in the solution used for incubation. This consisted of Tris buffer, pH 7.0 at $20^{o}C$ (0.01M.) containing $MgCl_2$ ($5 \times 10^{-3}M$), NaCl (0.05M), and, in specified instances, EDTA ($5 \times 10^{-5}M$) and dithiothreitol ($5 \times 10^{-4}M$). A smooth suspension was achieved by gentle stirring with a loose-fitting glass pestle followed by repeated passage through the tip of a Pasteur pipette. To remove any clumps of nuclear material which might remain, the suspension was finally filtered through nylon bolting cloth (Nybolt 25T 45μ). In most experiments this final "pure" nuclear fraction was made up to 15ml with incubation solution.

(c) Isolation of Other Cellular Constituents

Other cellular constituents were isolated in M.S.E. polycarbonate tubes (10ml) by centrifugation in the 10 x 10ml aluminium angle rotor of an M.S.E. Superspeed 50 centrifuge.

The pooled post-nuclear supernatant (80ml approximately) was centrifuged at 9,000 g ($r_{av.}$ 2.55cm) for 10min. The precipitated material was resuspended by agitation on a vortex mixer in 0.25M sucrose-T.K.M. solution (10ml) and again centrifuged at 9,000 g for 10min. The final pellet was termed the mitochondrial fraction. The supernatants were pooled and centrifuged at 105,000 g ($r_{av.}$ 2.55cm) for 60min. In some experiments this 105,000 g pellet was again suspended in 0.25M sucrose-T.K.M. solution (20ml) and centrifuged once more at 105,000 g for 30min. The final pellet and supernatant were termed the microsomal and cytosol fractions respectively. The mitochondrial and microsomal pellets were suspended in incubation solution by gentle stirring with a loose-fitting glass pestle followed by repeated passage through the tip of a Pasteur pipette. Incubation solution was added to make up a final volume of cytosol (100ml), microsomal (50ml) and mitochondrial (15ml) fractions.

7. Isolation of Nuclear Fraction from Rat Ventral Prostatic Tissue

In a few experiments nuclear fractions from rat ventral prostatic tissue were isolated using the method of Blodel and Potter (1966) without further modification. Prostatic tissue from six rats (total weight 2.5g - 3.5g) was used for each nuclear isolation. This tissue was washed with 0.25M NaCl in the manner described for human prostatic tissue. The washed mince was suspended in 0.25M sucrose-T.K.M. solution (20ml) and homogenised with the Thomas homogeniser already described in 6 (a). The homogenate was filtered through nylon bolting cloth (Nybolt 25T 45μ) and the filtrate volume made up to 20ml with

0.25M sucrose-T.K.M. solution. In the case of rat ventral prostatic tissue, little material was held back during filtration. In order to raise the sucrose concentration to 1.6M, 2.3M sucrose-T.K.M. solution (35ml) was added to the filtrate. Volumes (6ml) of 2.3M sucrose-T.K.M. solution were added to 3 x 23ml M.S.E. polycarbonate tubes. Homogenate (17ml to each tube) was then carefully layered on top of the 2.3M sucrose solution, the interphase between the two layers gently disturbed with a glass rod, and the tubes centrifuged for 90min at 96,000 g ($r_{av.}$ 3.70cm) in the 3 x 23ml aluminium swing-out rotor of an M.S.E. Superspeed 65 centrifuge. The nuclear fraction was suspended in incubation solution (15ml) in the manner described for human prostatic nuclear fractions.

8. Incubation Procedure

[1,2-^{3}H] Testosterone in benzene was placed in a glass tube and the solvent evaporated in a water bath (40^{o}C) under a stream of nitrogen. N:N-dimethylacetamide (10µl) was added to aid solubilisation of the steroid. If the assay system contained large amounts of protein the steroid might bind to this, and the observed rate of reaction would then be due to the rate of dissociation of the steroid protein complex rather than to the rate of the enzyme catalysed reaction. This type of effect can be eliminated by the use of a solubilisation agent such as N:N-dimethylacetamide (Engel, 1969).

A known volume of incubation fluid containing nicotinamide (0.4M) and NADPH (2 x 10^{-3}M) was added to the solubilised [1,2-^{3}H] testosterone. In order to allow the final concentration of components

in the complete incubation (total volume, 1ml) to correspond to the values shown in Table 2, volumes (0.1ml) of this solution were added to each incubation. The final concentration of subcellular fractions in each incubation is shown in Table 3. To quantitate the amount of ^{3}H-testosterone added, duplicate samples (0.1ml) of ^{3}H-testosterone, with the addition of absolute alcohol (3ml) to aid evaporation, were placed in plastic scintillation vials (Packard Instrument Co.), evaporated to dryness under a stream of air, and counted at the same time as the purified radioactive metabolites. Incubations (1ml) were carried out in air in stoppered "Exelo" C-10 test tubes in a shaking water bath (37^{0}C) for 20min. The tubes were then transferred to an ice bath and the reaction terminated by the addition of chloroform : methanol 3:1; v/v (2ml) . Methanol (0.1ml) containing 2 x 10^{3}c.p.m. [4-^{14}C] testosterone, methanol (0.1ml) containing 2 x 10^{3}c.p.m. dihydro [4-^{14}C] testosterone, and methanol (0.1ml) containing 3 α-androstanediol (200μg) were added to permit determination of procedural losses in subsequent purifications. To quantitate the amount of ^{14}C steroids added, duplicate samples (0.1ml) of [4-^{14}C] testosterone and dihydro [4-^{14}C] testosterone were placed in plastic counting vials, evaporated to dryness under a stream of air, and counted at the same time as the purified radioactive metabolites.

Steroids were extracted after incubation by a method similar to that used by Folch et al. (1957) to isolate lipids from animal tissues. Incubation tubes were stoppered, shaken by hand, and the contents transferred to "Quickfit" 14/23 test tubes (15ml). The incubation tubes were rinsed with chloroform : methanol 3:1; v/v (5ml)

TABLE 2.

Final Concentration of Components of the 5α-Reductase Assay System

Component	Concentration
^{3}H-Testosterone	7×10^{-9}M (2ng/ml) (approx. 2×10^{5}d.p.m./ml)
$MgCl_2$	5×10^{-3}M
NaCl	0.05M
Nicotinamide	0.04M
NADPH	2×10^{-4}M
EDTA$^{\vdash}$	5×10^{-5}M
Dithiothreitol$^{\vdash}$	5×10^{-4}M

Buffer = Tris - HCl (0.01M) pH 7.0 at 20°C.

$^{\vdash}$ Only present in specified instances.

TABLE 3.

Final Concentration of Subcellular Fractions in the 5 α-Reductase Assay System

Subcellular Fraction *	Protein Concentration (µg/ml)
Homogenate	100
Nuclear	200
Mitochondrial	100
Microsomal	100
Cytosol	400

* Usually only one fraction per incubation.

to wash out any remaining steroid, and this wash added to the extraction tubes. The tubes were then fixed with "Terry" clips to a plate which was rotated by an electric motor in a vertical plane at 80 r.p.m. for 10min to extract steroids. Following extraction the tubes were centrifuged in an M.S.E. bench centrifuge operated at speed setting No. 6 for 10min. The upper aqueous phase was siphoned off and the lower solvent phase containing the extracted steroids placed in a water bath ($40^{o}C$) and evaporated to dryness under a stream of air.

9. Purification of the Radioactive Steroids from the Incubation

(a) Method A.

The steroids present in each incubation extract were purified by paper chromatography in light petroleum (b.p. 80^{o}-$100^{o}C$)-benzene-methanol-water (4:1:4:1 by vol). The sample was spotted on Whatman No. 1 paper (50cm x 5cm), equilibriated overnight in a tank saturated with the solvent system just described, and developed for 2½h in descending manner in the supernatant "mobile" phase light petroleum (b.p. 80^{o}-$100^{o}C$)-benzene (4:1: v/v) . The radioactive steroids were located by detection on a radiochromatogram scanner (Model 2700; Packard Instrument Co.). This system yielded an R_f of 0.39 for testosterone and 0.64 for dihydrotestosterone. The detected areas on the paper were cut into small squares (1cm x 1cm), and each area thus cut placed in a flask (50ml) and eluted overnight with 95% aqueous ethanol (10ml). The supernatant was decanted into a "Quickfit" 14/23 test tube (15ml), the flask rinsed with ethanol (5ml) and this wash added to the supernatant.

Volumes (200µg) of either testosterone or dihydrotestosterone were added to the supernatant, and the test tube placed in a water bath ($40^{o}C$) and evaporated to dryness under a stream of air.

The steroids were further purified on thin layers (0.4mm) of silica gel (Kiesel Gel $Hf_{254+366}$; E. Merck, Darmstadt, Germany). In order to solubilise any lipids present, acetone (10 drops) was added down the sides of the test tube containing the evaporated paper extract. The resulting solution was carefully spotted at the origin of the t.l.c. plate with a Pasteur pipette. An interval of several minutes was allowed to elapse between applications to permit evaporation of the solvent. This procedure was repeated with ether (10 drops). The t.l.c. plate was developed three times in acetone to a height of 4cm to concentrate the spotted extract into a thin line. Final development was carried out in ascending manner in a chloroform : acetone (7:1; v/v) mixture for 40 min. The steroids were detected by an ultra-violet lamp (Camlab Ltd., Cambridge, U.K.). Testosterone (R_f = 0.41) was detected at 254nm, and dihydrotestosterone (R_f = 0.50) at 350nm. The silica gel in the detected area was loosened with a razor blade and sucked into a shortened Pasteur pipette which was blocked at the narrow end with a cotton wool plug and attached to a water suction pump. The Pasteur pipette was then placed in a scintillation vial and the steroids adsorbed to the silica eluted into this vial by the addition of ether (2ml) into the top of the pipette. The vial was placed in a water bath ($40^{o}C$) and the solvent evaporated under a stream of air.

(b) Method B.

The steroids present in each incubation extract were purified by t.l.c. of acetylated derivatives. Testosterone (200µg) and dihydrotestosterone (200µg) were added to the incubation extract in a 'Quickfit' 14/23 test tube (15ml). The test tube was placed in a water bath (40°C) and the solvents evaporated to dryness under a stream of air. Pyridine (0.1ml) and acetic anhydride (0.1ml) were added down the sides of the tube, which was then stoppered and shaken briefly on a vortex mixer. Acetylation then proceeded in darkness either at 60°C for 1h or at room temperature overnight. Volumes (5ml) of chloroform : methanol 3:1; (v/v) and distilled deionised water (2ml) were then added; the water was present to facilitate partition. The steroids were extracted into the solvent layer and evaporated to dryness as described for the extraction of steroids after incubation (Section 8). The acetylated steroid extract was spotted onto thin layers (0.3mm) of alumina (Kiesel Gel $Hf_{254+366}$ Type 60) and developed in acetone to a height of 4cm in the manner described for Method A. Final development was carried out in ascending manner in a cyclohexane : ethyl acetate (4:1; v/v) mixture for 90min. The steroids were located under ultra-violet light. Testosterone acetate (R_f = 0.28) was detected at 254nm and both dihydrotestosterone acetate (R_f = 0.44) and 3α-androstanediacetate (R_f = 0.75) at 350nm. Elution from the alumina into plastic counting vials was carried out in the manner described for Method A.

In the case of 3α-androstanediacetate, only half the sample was transferred to the vial, the remainder being used to calculate

procedural losses of the steroid by gas liquid chromatography.

10. Gas Liquid Chromatography of 5α -Androstane-3α ,17β-Diol

A Series 104 gas chromatogram (Pye-Unicam; York St., Cambridge, U.K.) was fitted with a glass column (210cm) packed with 1% SE-30 on Gas-Chrom Q (Applied Sciences Inc., State College, Pennsylvania, U.S.A.) and maintained at a temperature of 230°C. Nitrogen (2.1Kg/cm^2) was used as carrier gas and, after passage over the stationary phase, the sample was detected by a flame ionisation detector. Hydrogen (1.0Kg/cm^2) was used as fuel, and air (0.7Kg/cm^2) as oxidant. An Autolab 6300 digital integrator with a 6300 Accessory module (Spectra-Physics, Harpendale, U.K.) monitored the detector output. This calculates, electronically, the peak area of the amplified signal from the flame ionisation detector.

Absolute alcohol (0.1ml) was added to dried samples containing 3α -androstanediacetate. A sample (2μl) of this solution was injected into the top of the gas liquid chromatogram with a graduated Hamilton syringe (10μl).

During each experiment the gas liquid chromatogram was calibrated by injection of 3α -androstanediacetate between each batch of five injections of unknown samples. This steroid had a retention time of 340s. The integration delay, which controls an adjustable delay period before the integrated output is recorded, was set at 200s so that the area of the solvent peak, which had a retention time of less than 200s, should not be calculated. The concentration of 3α -androstanediacetate in the unknown sample was then calculated by

comparison of its peak area with the peak area obtained from the known 3 α-androstanediacetate sample (2μg).

11. Counting of Radioactivity

Samples which had previously been dried in plastic vials were counted after addition of scintillation fluid [(10ml) N.E.233, Nuclear Enterprises Ltd., Edinburgh, U.K.]. This scintillation fluid contains highly purified toluene, 2,5 diphenyloxazole (P.P.O.) activator, naphthalene for reduction of chemical quenching, and 1,4 di [2-(5-phenyl-oxazolyl)]-benzene (P.O.P.O.P.) spectrum shifter. It was chosen for use in these experiments because the samples to be counted were completely soluble in toluene, counting efficiency was high, and minimal quenching occurred. Double isotope counting was performed in two channels (green and blue) of a Tri-Carb liquid scintillation spectrometer (Model 3380; Packard Instrument Co.). The width of the window in the green channel was adjusted to allow the detection of carbon-14 alone, while in the blue channel tritium and carbon-14 were counted. To obtain a sufficiently high efficiency of carbon-14 detection, about one-fifth of the counts due to this isotope in the green channel overlapped into the blue channel. The exact degree of overlap under the experimental counting conditions was determined by the inclusion of a carbon-14 standard in each set of samples. The carbon-14 overlap value, when subtracted from the total counts in the blue channel, produced the counts due to tritium in the blue channel. The channel selection controls were set as follows:-

Green C-D $C_{210} - D_{1000}$ and Blue E-F $E_{50} - F_{190}$.

This allowed a counting efficiency of 33%-35% for tritium in the blue channel, and 42%-45% for carbon-14 in the green channel. Counts were accumulated in each channel to give an S.E.M. of less than one percent, which in most samples required counting for 10min. The degree of quench in each sample was monitored by use of the automatic external standard (A.E.S.) ratio. Tritium counting efficiency was calculated by the inclusion with each set of samples of vials containing ^{3}H-toluene (Packard Instrument Co.) of known d.p.m. With this knowledge, ^{3}H-c.p.m. could be converted to d.p.m. All calculations were performed on a desk computer (Programma 101; British Olivetti, London, U.K.) by means of specially prepared programmes.

12. Analytical Methods

(a) Preparation of Protein, RNA and DNA Fractions

The method used was adapted from that of Schmidt and Thannhauser (1945), recommended by Munro and Fleck (1966) in a detailed review as the most satisfactory method of nucleic acid purification prior to estimation. Ice-cold 10% (w/v) trichloroacetic acid (5ml) was added to samples to be analysed. These usually consisted of homogenate (1ml) or nuclear suspension (1ml). The test tube (10ml) containing this solution was placed on ice for 10min prior to centrifugation. Nucleic acids and proteins were precipitated by centrifugation in an M.S.E. Mistral 4L centrifuge at 500 g ($r_{av.}$ 17cm) for 10min at 0^{o}C. The supernatant (the acid soluble fraction containing low molecular weight components) was discarded and the precipitate washed

with 5%(w/v) trichloroacetic acid (5ml). The precipitate was then dehydrated with 95% aqueous ethanol (3ml) and the lipids extracted by washing with ethanol : ether 3:1(v/v) (3ml). This procedure also helped to remove trichloroacetic acid, which may interfere with subsequent DNA determinations, from the precipitate. The remaining precipitate was dried under a stream of air and digested overnight in a slowly shaking water bath (37°C) after addition of 0.3M KOH(1ml). A sample (0.2ml) was taken for analysis of protein, and to the remaining digest ice-cold 1.2M perchloric acid (0.5ml) was added, and the mixture allowed to stand on ice for 10min. The supernatant (RNA fraction) was decanted and the precipitate washed with 0.2M perchloric acid (0.5ml). This wash was added to the RNA fraction which was then made up to 3ml with distilled water. The precipitate (DNA fraction) was made up to 4ml with 0.5M perchloric acid and maintained at 70°C for 30min to solubilise DNA.

(b) Protein Determination

The method used for protein determination was that of Lowry et al. (1951). Reagents used were:-

2%(w/v) Na_2CO_3 in 0.1M NaOH;

1%(w/v) $CuSO_4$ solution;

2%(w/v) Na tartrate;

normal Folin Ciocalteau reagent;

working alkaline Cu reagent; (This was prepared immediately prior to use by adding 1% $CuSO_4$ solution (1ml) and 2% Na tartrate (1ml) to the 2% Na_2CO_3 solution (98ml), and

standard bovine serum albumin (100µg/ml) in 0.03M KOH.

This alkaline digest sample was diluted to 2ml with distilled water. Aliquots (1ml) of this protein solution and aliquots (5ml) of the freshly prepared alkaline Cu reagent were placed in a test tube (10ml), mixed thoroughly and left for 10min. Folin Coicalteau reagent (0.5ml) was added and the solution mixed immediately and left to stand for exactly 10min at room temperature. The optical density was determined in a Uvichem S.P. 600 spectrophotometer at 625nm. A standard curve in a linear range (10µg - 100µg) of bovine serum albumin was prepared for every set of determinations.

(c) RNA Determination

The method used for RNA determination was a modification of the orcinol reaction used by Kerr and Seradiadarian (1945). This method depends on the conversion, in the presence of hot acid, of pentose to furfural, a product which reacts with orcinol to yield a green colour. Reagents used were:-

20%(w/v) orcinol in 95% aqueous ethanol; (This solution was prepared daily).

0.03%(w/v) $FeCl_3$ in concentrated HCl; and

standard RNA solution (50µg/ml) in 0.05M NaOH.

Aliquots of the RNA fraction [1.5ml; prepared as described in 12 (a)], 0.03%(w/v) $FeCl_3$ (1.4ml) and 20% orcinol (0.2ml) were pipetted into a test tube (10ml) and left in a vigorously boiling water bath for 30min. The solution was then cooled under a stream of cold water and the optical density determined in a Uvichem S.P. 600 spectrophotometer at 665nm. A standard curve in the linear range 10µg - 50µg was prepared for every set of determinations.

(d) DNA Determination

The method used for DNA determination was that of Burton (1956 and 1968), which depends on the reaction between deoxyribose and diphenylamine. Reagents used were:-

1.6%(w/v) aqueous acetaldehyde;

diphenylamine reagent; (This was prepared by dissolving diphenylamine (1.5g) in glacial CH_3 COOH (100ml), and adding concentrated H_2SO_4 (1.5ml) to this solution); and

standard DNA solution (100µg/ml) in 0.5M $HClO_4$; (This was heated at 70°C for 15min before use.)

To each sample (1ml) of the DNA fraction [prepared as described in 12 (a)] an aliquot (2ml) of freshly prepared acetaldehyde : diphenylamine reagent [1.6% aqueous acetaldehyde (0.1ml) : diphenylamine reagent (20ml)] was added. The solution was then mixed in a test tube (10ml) and left in a water bath (30°C) for 18h. The optical densities were then determined in a Uvichem S.P. 600 spectrophotometer at 665nm. A standard curve in the linear range 0µg - 100µg was prepared for every set of determinations.

(e) Preparation of Glassware for Zinc Determination

Since glass has a high affinity for zinc, all glassware used in zinc analysis was thoroughly washed to eliminate the risk of zinc contamination. Glassware was soaked overnight in a solution of Decon 90 (Decon Laboratories Ltd., Brighton, U.K.), rinsed in distilled deionised water, and immersed in 5M HCl for 24h. It was then rinsed four times in distilled deionised water and dried in an oven. During each experiment control samples were analysed to monitor for zinc

contamination.

(f) Zinc Determination

Zinc was determined by atomic absorption spectrophotometry on an absorption spectrophotometer (Model 403; Perkin-Elmer, Beaconsfield, U.K.) equipped with a "Boling" triple slot burner head which required acetylene as fuel and air as oxidant. The analysis was performed in a non-luminous flame at a wavelength of 213.8nm. The instrument was set to zero with a solution of 0.3M HCl and standardised against solutions of 7.69μM (50μg/100ml) and 15.38μM (100μg/100ml) $ZnCl_2$ in 0.3M HCl. The standard zinc solutions were prepared by diluting a reference solution of certified atomic absorption standard (Fisher Scientific Co., New Jersey, U.S.A.) containing 1.538×10^{-3}M(10^4μg Zn/100ml) $ZnCl_2$ with 0.3M HCl. The sample to be analysed was aspirated through a PTFE tube into the spectrophotometer flame and the zinc concentration determined by manual control on the 100 average scale. (The sample is counted 100 times and the calculated average appears on an electronic display). Samples were read in the range where absorbance bore a linear relationship to concentration. This occurred up to 18.5μM (120μg/100ml) for concentrations of zinc.

To eliminate cross-contamination of zinc between samples, the PTFE tube was washed with 0.3M HCl after each sample aspiration. The atomic absorption spectrophotometer was also restandardised after each batch of ten samples was determined to correct for minor electronic fluctuations.

Zinc determination on biological samples was performed after destruction of the organic material by dry oxidation. The samples,

together with suitable reagent controls, were placed in "Exelo" C-10 test tubes (1cm in diameter; 8cm in height) which were sealed with aluminium foil and placed in a stainless steel test tube rack. It was discovered that if samples which had been prepared in 0.25M sucrose-T.K.M. solution were heated rapidly to a high temperature (475°C), spitting of sucrose solution occurred causing loss of sample. These tubes were therefore placed in an oven at 110°C for 4h prior to the ashing process.

Duplicate prostatic tissue samples (approximately 50mg wet weight) were also pre-heated at 110°C for 4h so that the dry weight of the sample could be calculated. During this process the sample lost about 80% of its original weight through evaporation. To monitor for contamination zinc was also determined in blank tubes and the low value obtained subtracted from the zinc concentration measured in the tubes containing the prostatic sample. After pre-heating, samples to be analysed were transferred to a muffle furnace and heated at 475°C overnight or until only a white ash remained. The sample was then allowed to cool, 0.3M HCl (3ml) added, the tube stoppered, and the solution shaken for 10s on a vortex mixer. The 0.3M HCl solution containing the extracted zinc was aspirated into the atomic absorption spectrophotometer for zinc analysis. Samples were analysed initially on the 10 average scale to determine whether the zinc concentration was greater than 1.946μM (120μg/100ml), in which case the sample was diluted with 0.3M HCl and determined finally on the 100 average scale.

13. Solubilisation of p-Chloromercuribenzoate

Since this thiol group blocking reagent is insoluble at neutral pH, the method suggested by Gunsalus and Razzel (1957) was used to produce a soluble stock solution (10^{-3}M). p-Chloromercuribenzoate (35.6mg) was dissolved in 0.2M KOH (2.0ml) and diluted to 80ml with incubation solution. The pH was adjusted to 7.0 with 1.0M HCl, the sample transferred to a volumetric flask, and diluted to 100ml with incubation solution.

14. Preparation of 5α -Androstane-3α ,17 β-diacetate

3 α-androstanediacetate was required for use in some experiments. This steroid was unavailable commercially, and was produced chemically in the laboratory by acetylation of 3α -androstanediol. 3α -androstanediol (100mg), pyridine (0.5ml), and acetic anhydride (0.5ml) were placed in a test tube (10ml) and left overnight to acetylate in darkness. After acetylation, the sample was extracted and purified on thin layers of alumina (20mg/t.l.c. plate) and detected as described in 9 (b). The purified 3α -androstanediacetate fraction from five t.l.c. plates was pooled and crystallised from an absolute alcohol/water (20:1; v/v) mixture. The supernatant was discarded and the crystals dried under vacuum. During this procedure 60% of the initial 3 α-androstanediol was recovered as 3 α-androstanediacetate in the final crystals.

15. Determination of Glucose-6-Phosphate Dehydrogenase Activity

Glucose-6-phosphate dehydrogenase catalyses the conversion of glucose-6-phosphate to 6-phosophogluconate, reducing the coenzyme NADP

to NADPH in the process. The rate of production of NADPH thus provides a measurement of glucose-6-phosphate dehydrogenase activity. The production rate of NADPH at room temperature ($20^{o}C$) was measured by monitoring the increase in absorption at 360nm in a Uvichem S.P. 800 spectrophotometer. In the standard assay a sample (2ml) of a solution containing 2×10^{-3}M NADP and 0.01M glucose-6-phosphate was placed in a glass spectrophotometer cuvette (2.5ml; 1cm in diameter). Glucose-6-phosphate dehydrogenase (0.1ml containing 0.1 unit, where unit is defined as the enzyme concentration which can convert 1μmole glucose-6-phosphate to 6-phosphogluconate per min in NADP at pH 7.4 at $25^{o}C$) was added and the cuvette contents mixed and placed immediately in the light path of the spectrophotometer. The rate of production of NADPH was equivalent to the rate of increase in optical density at 360nm, this value being automatically traced out on a chart recorder attached to the spectrophotometer.

C. EXPERIMENTAL

1. Development of Analytical Methods

(a) Reliability and Practicability

(i) Reliability

Without reliable analytical methods, results are of little value. Certain criteria of reliability are now generally accepted. These are specificity, accuracy, precision and sensitivity, (Booth, 1952; Anastassidis and Common, 1968 and Lorraine and Bell, 1966). These criteria may be defined as follows:-

Specificity is the ability to determine a chemical compound to the exclusion of all others.

Accuracy provides a reference point against which analytical results can be reliably assessed. In a situation in which the true value cannot be determined, it represents the degree to which a given result approximates to the most probable or "true" value. For example, accuracy may be assessed by a comparison of determinations made on the material under assay both before and after the addition of known amounts of the substance under investigation.

Precision is the concurrence of a series of measurements of the same quantity, and is expressed as the standard deviation of a series of replicate determinations. Precision may also be determined by using the standard deviation calculated from the difference between duplicate results through a series of assays (Snedecor, 1952). That is, estimate of variance = $(\text{standard deviation})^2 = \frac{d^2}{2N}$, where d is the difference between two results in a duplicate determination and N

(which should be greater than 20) is the number of duplicate determinations. This method is useful because it takes into account the precision obtained in a number of samples at different levels of measurement over a period of time.

Sensitivity is the smallest value which can be accurately determined. Its numerical value is often taken to be that of the smallest result where the fiducial level (at the 95% confidence level) does not involve zero. The sensitivity of a given method is also approximately equal to twice the standard deviation of replicate determinations provided that the standard deviation is based on at least twenty such determinations.

(ii) Practicability

In any biochemical study the practicability of the methods used is of utmost importance. Such factors as speed, cost, skill, availability of equipment and, in this case, of tissue, must be borne in mind. Ultimately reliability will be related to practicability. For example, in the present study the activity of testosterone 5α-reductase is determined after chromatographic separation of radioactive substrate and product. By identification of purified radioactive steroids which co-crystallised with cold steroids (20mg) known to have similar R_f values, the method was found to be satisfactory. However, to show that the assay was 100% specific, recrystallisation would have to be carried out for individual incubations in all experiments. This is not practical, in a study of this type, and it must be assumed that since the conditions used were identical to those in the recrystallisation studies undertaken, the compounds were pure after

chromatographic separation in all samples.

(b) Evaluation of DNA, RNA and Protein Determinations

DNA, RNA and protein were determined by well-established spectrophotometric methods which depend on the production of coloured compounds by specific chemical reactions. These were assumed to be specific for the compounds under measurement. All methods gave a reproducible linear standard curve in the stated range, precision being determined in the following manner:-

A 10%(w/v) rat prostatic homogenate was prepared in 0.25M sucrose-T.K.M. solution. DNA, RNA and protein were determined on 0.5ml and 1.0ml volumes. The remainder of the homogenate was stored at $-20^{o}C$ for one week and the determinations repeated. Results are shown in Table 4. To ensure that the precision of analyses did not decrease as the limit of sensitivity was approached, no sample containing 5µg or less of DNA, RNA or protein were judged to give results sufficiently accurate for use in calculations.

(c) Evaluation of Zinc Determination

Zinc was determined by atomic absorption spectrophotometry, an extremely sensitive quantitative technique for the determination of elements. Analytical samples are heated to a high temperature, usually by burning in a flame which breaks up the chemical bonds between the molecules, enabling individual atoms to float freely in the sample area. In this condition the atoms can absorb ultra-violet or visible radiation of a specific wavelength. This phenomenon is quantitative, the degree of absorption being proportional to the mass of the element absorbing at that wavelength.

TABLE 4.

Evaluation of DNA, RNA and Protein Determinations

Sample	DNA *	RNA *	Protein *
Week 1			
0.5ml	1.72 ± 3.1% (10)	2.50 ± 7.4% (10)	92.03 ± 4.3% (7)
1.0ml	1.83 ± 3.8% (10)	2.72 ± 3.5% (9)	85.75 ± 3.1% (10)
Week 2			
0.5ml	1.80 ± 3.2% (10)	2.64 ± 3.2% (10)	88.03 ± 2.0% (10)
1.0ml	1.86 ± 2.0% (9)	2.70 ± 3.7% (10)	89.53 ± 4.2% (8)

* mean amount per sample (mg per g wet weight tissue)

± coefficient of variation

( ) number of determinations

A linear standard curve for zinc at a wavelength of 213.8nm was found in the range 0-18.46μM (0-120μg/100ml) (Figure 5). Since this curve was reproducible, the spectrophotometer was set against two standard zinc solutions, 7.69μM (50μg/100ml) and 15.38μM (100μg/100ml) during the standard assay. By means of a computer unit connected to the spectrophotometer, (Model 403; Perkin-Elmer) the zinc concentration of an unknown sample could be electronically calculated by comparison with these standards.

The complex organic matter in a biological sample may interfere with trace metal analysis during atomic absorption spectrophotometry. Some procedure, such as wet or dry oxidation, must be developed to eliminate or reduce this interference (Gorsuch, 1970). During wet oxidation, the sample is heated, usually to 100^{o}-200^{o}C, with a mixture of oxidising agents such as sulphuric acid, nitric acid, perchloric acid or hydrogen peroxide; whereas in dry oxidation, which requires rather higher temperatures, the organic material is oxidised by gaseous oxygen, usually by heating in air.

The atomic absorption method may be further complicated by a number of possible sources of error. A high concentration of inorganic ions, for example, are known to depress absorption (Dean and Rains, 1969). The extent of this phenomenon, termed matrix interference, was evaluated in the following manner:-

A standard curve for zinc was determined in both 0.3M HCl and in 0.05M Tris buffer containing 0.15M NaCl, 5×10^{-3}M $MgCl_2$ and 0.025M KCl. The concentration of components in the latter solution was similar to or in excess of what might be expected in the unknown biological samples.

Results obtained (Figure 5) indicate that matrix interference did not occur at these concentrations of salts and that absorbence bore a linear relationship to zinc concentration up to a concentration of 18.5μM Zn (120μg/100ml).

In the determination of zinc, sources of error include contamination of the sample by extraneous zinc, loss of zinc by volatilisation during the ashing process, and incomplete extraction of zinc from the tubes after ashing. In order to establish a satisfactory method for zinc analysis it was necessary to take these factors into consideration.

The measurement of zinc in prostatic samples was first attempted by a wet oxidation method. The sample was placed in a 5ml pyrex volumetric flask to which was added concentrated HNO_3 (1ml) and concentrated $HClO_4$ (1ml). The flask was transferred to a sand bath on a hot plate in a fume cupboard and maintained at 110°C for 3-4h. After cooling the sample was diluted to 5ml with distilled water and zinc measured by atomic absorption spectrophotometry. It was found that this method led to incomplete oxidation of prostatic tissue and unacceptably high levels of zinc contamination. In subsequent experiments, therefore, use was made of dry oxidation techniques.

The first method attempted involved ignition of samples in "Vitreosil" silica crucibles (10ml) (Gallenkamp&, London, U.K.) by heating overnight at 475°C. The sample was then extracted by addition of 0.3M HCl and the final extract made up to a known volume in 0.3M HCl and analysed for zinc by atomic absorption. This method proved to be unsatisfactory due to incomplete extraction of zinc from the crucibles,

FIGURE 5.

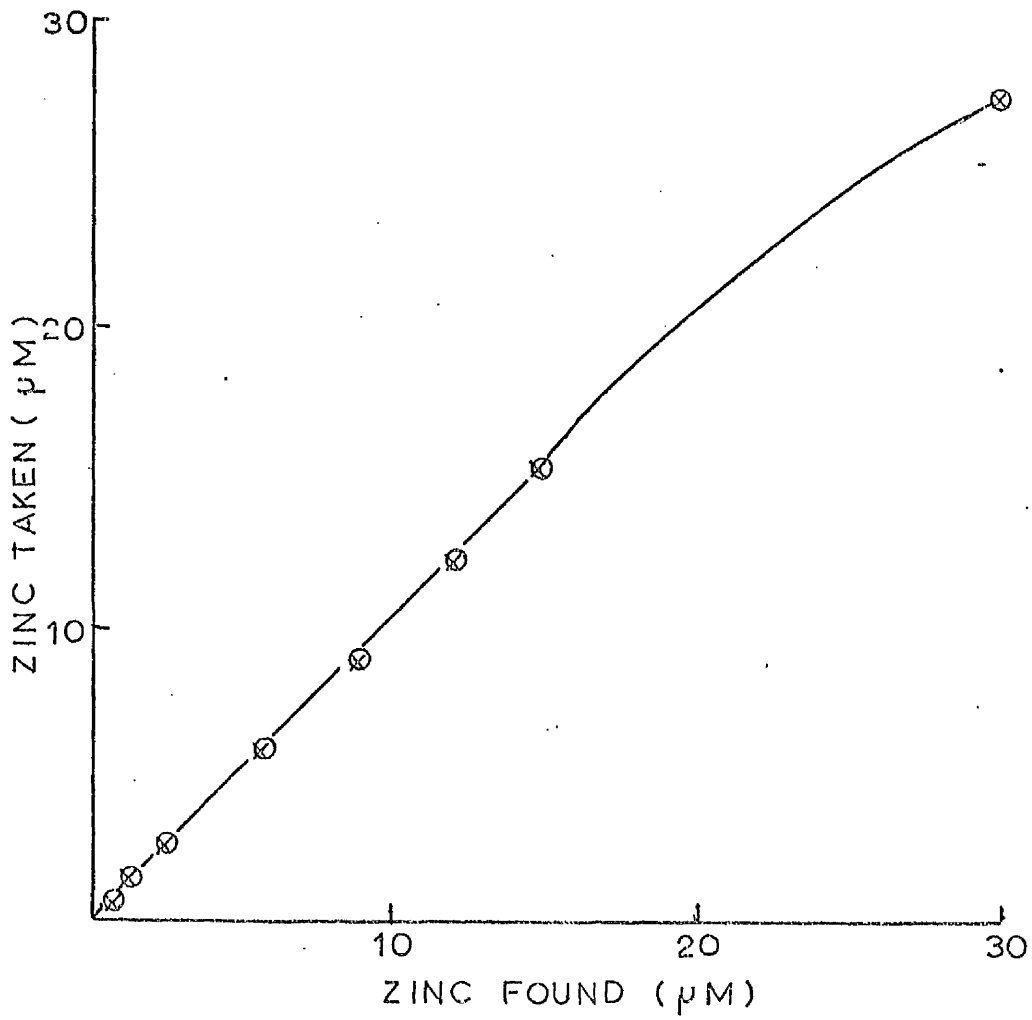
Standard Curve for Zinc Determination

A standard curve for zinc was determined in a solution of either 0.3M HCl or 0.05M Tris buffer containing 0.15M NaCl, 5×10^{-3}M $MgCl_2$ and 0.025M KCl.

○ in 0.3M HCl

× in 0.05M Tris buffer containing 0.15M NaCl, 5×10^{-3}M $MgCl_2$ and 0.025M KCl.

Figure 5. STANDARD CURVE FOR ZINC DETERMINATION.



leading to low recoveries and decreased precision. For example, in an experiment in which zinc (2µg) was added to each of 9 crucibles, the recovered zinc was 1.68µg $\pm$ 9.5%.

The method finally adopted involved dry oxidation of samples in borosilicate glass tubes and is described in detail in the methods Section B, 12 (e). It was evaluated in the following manner:-

(a) To monitor contamination, 10 blank tubes were ashed and any zinc present extracted with 0.3M HCl and zinc assayed by atomic absorption spectrophotometry.

(b) To assess accuracy, $ZnCl_2$ (Specpure) was dissolved in distilled deionised water at a concentration of 1µg/100ml (Zn Solution A). Samples (0.1ml) were pipetted into 10 tubes and assayed for zinc. Control samples (0.1ml) of distilled deionised water were pipetted into 5 blank tubes and assayed in the same manner. The results shown in Table 5 were corrected for these controls.

(c) Determinations on 10 x 1ml aliquots of 0.25M sucrose-T.K.M. solution were carried out to monitor zinc contamination.

(d) To establish whether 0.25M sucrose-T.K.M. solution interfered with the accuracy of the zinc determination, aliquots (1ml) of this solution and aliquots (0.1ml containing 1µgZn) of Zn Solution A were added to each of 10 tubes. Zinc analysis was then carried out and results were corrected in the light of the results from the control experiment (c) above.

(e) To assess precision, one 20%(w/v) human prostatic homogenate was prepared in 0.25M sucrose-T.K.M. solution and diluted 1 in 6 with this same solution. To ensure the concentration of zinc fell below

TABLE 5.

Validation of Method for Determining Zinc

Sample	Amount of Zinc Found *
a) Blank tubes	0.024 ± 7%
b) 1µg $ZnCl_2$	1.020 ± 4.3%
c) 1ml 0.25M sucrose-T.K.M. solution	0.035 ± 10%
d) 1µg $ZnCl_2$ in 1ml sucrose-T.K.M. solution	0.983 ± 3.1%
e) Week 1	
1ml prostatic homogenate	1.844 ± 6.3%
0.5ml prostatic homogenate	0.898 ± 6.0%
Week 2	
1ml prostatic homogenate	1.721 ± 5%
0.5ml prostatic homogenate	0.840 ± 3.9%
f) 1µg $ZnCl_2$ in 0.5ml prostatic homogenate	1.010 ± 3.4%

* mean amount per sample (µg)

± coefficient of variation.

Each sample was analysed ten times.

100µg/100ml, zinc was determined in samples (10 x 1ml and 10 x 0.5ml) of this homogenate solution. Control samples (5 x 1ml and 5 x 0.5ml) of 0.25M sucrose-T.K.M. solution were also determined, and the results presented have been corrected for these controls. The homogenate solution was then stored for one week at -20°C and the experiment repeated.

(f) To establish whether prostatic homogenate interfered with the accuracy of the zinc determination, samples (1ml) of this homogenate plus samples (0.1ml containing 1µg Zn) of Zn Solution A were pipetted into each of 10 tubes which were assayed for zinc. Results (Table 5) were corrected for prostatic homogenate zinc concentration and indicate that (a) the precision and accuracy of the method was acceptable at the required level of sensitivity; (b) zinc contamination was negligible; (c) the sucrose-T.K.M. solution did not "mask" or interfere with the determination; and (d) volatilisation of zinc did not occur to any appreciable extent. Since zinc chloride is the most volatile zinc salt, with a melting point of 210°C and a vapour pressure of 1mm Hg at 420°C (Corsuch, 1970), this lack of volatilisation suggests that either zinc chloride was converted to a less volatile compound, such as zinc oxide, or that volatilisation was prevented by the degree of pressure maintained within the muffle furnace during ashing.

(d) Radiochemical Assay for the Determination of 5α-reductase Activity

The low physiological substrate concentrations at which steroid metabolising enzymes operate may give rise to experimental difficulties.

The availability, within the last decade, of labelled steroid substrates of high specific activity has made it possible to assess the activity of these enzymes. However, when radioactive steroid metabolites are identified consideration must be given to the large number and low concentration of closely related compounds which may be present (Brooks et al., 1970). In order to use radiochemical determination to assess the activity of testosterone 5α-reductase it was felt important to show that the radioactivity of the label was due to a single chemical entity. Since the primary interest of this study was the activity of the 5α-reductase and not the identification of all ^{3}H-testosterone metabolites, only two of these, ^{3}H-dihydrotestosterone and ^{3}H-3α-androstanediol were considered. In order to determine whether any other major pathway of testosterone metabolism was involved the amount of ^{3}H-testosterone disappearing was compared with the amount of ^{3}H-dihydrotestosterone plus ^{3}H-3 α-androstanediol formed.

Two methods were used to purify steroids present in incubation extracts. Method A involved two chromatographic separation steps of ^{3}H-testosterone and ^{3}H-dihydrotestosterone, one on paper and the other on silica and was used initially. When it became necessary to monitor for other suspected products, method B, which involved separation of acetylated derivatives, was developed and used in all subsequent experiments. Hence this method was subjected to the more rigorous evaluation.

(i) Radioactive Counting Efficiency

It is important in the counting of radiochemical samples

to determine whether the efficiency of counting remains constant. Variation in counting efficiency between samples may be caused by either chemical or colour quenching and leads to artifactual results. The automatic external standard (A.E.S.) ratio as determined on the Packard Tri-Carb liquid scintillation spectrometer (Model 3380) is a measure of the degree of quench. Where no quenching occurs, the A.E.S. ratio is 1.0000; with infinite quenching the A.E.S. ratio is 0.0000.

In all samples counted the A.E.S. ratio ranged from 0.8100 to 0.9100. Using a quench curve, on which the degree of quench is plotted against the efficiency of radioactive counting of a range of varying quenched standards, these A.E.S. values corresponded to a counting efficiency in the range 33% - 35% for tritium and 42% - 45% for carbon-14. Within a given experiment the range of the A.E.S. ratio was considerably smaller. For example, in an experiment in which n = 34, the coefficient of variation for the A.E.S. ratio was 1.14%. In all samples counted, therefore, quenching was minimal, with little variation between samples.

(ii) Specificity

Definitive evidence for the purity of radioactive steroid metabolites was provided by recrystallisation studies on purified steroids from incubation extracts. Purity of ^{3}H-testosterone, ^{3}H-dihydrotestosterone and their acetates was monitored in relation to ^{14}C steroids of known purity, while that of ^{3}H-3α-androstanediacetate was monitored in relation to cold 3α-androstanediacetate. This latter steroid was unavailable commercially in the carbon-14 labelled form.

To evaluate the specificity of method A, recrystallisation studies were performed as follows:-

Metabolites formed after incubations of ^{3}H-testosterone with rat prostatic nuclei were purified by paper and thin layer chromatography. ^{14}C-Testosterone, ^{14}C-dihydrotestosterone, testosterone (20mg) and dihydrotestosterone (20mg) were added to fractions designated testosterone and dihydrotestosterone respectively according to their chromatographic mobilities. The solvent systems used for recrystallisation are presented, with results, in Table 6.

To evaluate the specificity of method B, metabolites formed after incubations of ^{3}H-testosterone with rat prostatic homogenates were purified by derivative formation and t.l.c. on alumina. ^{14}C-Testosterone acetate, ^{14}C-dihydrotestosterone acetate, testosterone acetate (20mg), dihydrotestosterone acetate (20mg) and 3 α-androstanediacetate (20mg) were added to fractions designated testosterone acetate, dihydrotestosterone acetate and 3 α-androstanediacetate respectively according to their chromatographic mobilities. The solvent systems used for recrystallisations are presented, with results, in Table 7.

Axelrod et al. (1956) have carried out a mathematical analysis of the errors involved in recrystallisation studies. They calculate that three successive crystallisations which yield values for specific activity (equivalent to isotopic ratio) within $\pm$ 5% of the average of these three values is definitive proof of purity. Applying this reasoning to the results in Tables 6 and 7, it can be seen that the steroids are essentially pure after chromatographic separation by both methods A and B.

TABLE 6.

Recrystallisation Study - Method A.

	$^{3}H/^{14}C$-Testosterone		$^{3}H/^{14}C$-Dihydrostestosterone	
	Crystals	Mother Liquor	Crystals	Mother Liquor
Before crystallisation	3.98	-	1.07	-
1. Acetone/hexane 5:1(v/v)	4.02	3.99	1.04	1.08
2. Acetone/hexane 5:1(v/v)	3.90	4.01	1.06	1.08
3. Acetone/hexane 5:1(v/v)	3.99	4.00	1.07	1.08

TABLE 7.

Recrystallisation Study - Method B.

	$^{3}H/^{14}C$- Testosterone Acetate		$^{3}H/^{14}C$- Dihydrotestosterone Acetate		* S.A. 3α- Androstanediacetate	
	Crystals	Mother Liquor	Crystals	Mother Liquor	Crystals	Mother Liquor
Before crystallisation	7.57	-	0.94	-	2325	-
1. Ethanol/ water 20:1(v/v)	7.39	7.83	0.90	1.01	2212	2326
2. Methanol/ water 20:1(v/v)	7.43	7.75	0.92	0.99	2451	2544
3. Acetone/ water 20:1(v/v)	7.74	7.67	0.90	0.88	2318	2428
4. Hexane	7.50	7.43	0.90	0.90	2232	2522

* S.A. Specific Activity (cpm/mg)

(iii) Precision

The overall precision of the assay system was determined by use of the Snedecor equation for the calculation of standard deviation (SD). Purification of metabolites after ^{3}H-testosterone incubation with rat prostatic homogenates by method A resulted in a SD of 6.09 (n = 22) for ^{3}H-testosterone and 8.1 (n = 23) for ^{3}H-dihydrotestosterone. Purification of metabolites after ^{3}H-testosterone incubation with human prostatic homogenate by method B resulted in a SD of 6.96 (n = 29) for ^{3}H-testosterone and 6.51 (n = 25) for ^{3}H-dihydrotestosterone. In experiments with human prostatic homogenates there was only very small conversion to 3 α-androstanediol. In incubations of human prostatic cytosol with ^{3}H-dihydrotestosterone, however, 3 α-androstanediol was formed and upon purification a SD of 7.01 (n = 22) was obtained.

(iv) Accuracy

(a) Method A

The accuracy of analysis of radioactive steroid metabolites by method A was determined by addition of ^{3}H-testosterone and ^{3}H-dihydrotestosterone to incubation solutions (1ml) at the concentration expected at the end of a rat prostatic nuclear incubation. The samples were purified and analysed by method A and the results are shown in Table 8.

(b) Method B

The accuracy of analysis of radioactive steroid metabolites by method B was more rigorously determined. Human prostatic homogenates were diluted with incubation solution to a concentration of 100µg protein

TABLE 8.

Evaluation of Accuracy - Method A

Sample	Testosterone*	Dihydrotestosterone*
^{3}H Theoretical	65418	11809
^{3}H Calculated After Method A	62625	11774
	63511	11591

* Values expressed as c.p.m.

TABLE 9.

Evaluation of Accuracy - Method B

ng ^{3}H Steroid Added to Incubation Tube	ng ^{3}H Steroid After Analysis by Method B		
	Testosterone	Dihydrotestosterone	3 α-Androstanediol
2.000	1.972	2.174	2.105
	2.014	1.934	1.940
1.000	0.975	1.070	0.936
	0.964	1.050	1.034
0.500	0.483	0.489	0.524
	0.479	0.488	0.507
0.250	0.247	0.250	0.256
	0.230	0.241	0.249
0.100	0.099	0.095	0.112
	0.094	0.088	0.102
0.050	0.052	0.049	0.053
	0.045	0.046	0.068
0.010	0.010	0.010	0.018
	0.009	~~0.092~~ 0.009	0.023
0.000	0.000	0.000	0.000
	0.000	0.000	0.000

in a total volume of 1ml, and placed in incubation tubes in crushed ice. These tubes already contained known amounts of ^{3}H-testosterone, ^{3}H-dihydrotestosterone and ^{3}H-3 α-androstanediol in a range similar to that which might be expected after incubation with ^{3}H-testosterone. The samples were purified and analysed by method B and the results are shown in Table 9.

The accuracy of the g.l.c. determination, which was used in method B to determine recoveries of 3 α-androstanediol, was assessed by analysis of known concentrations of 3 α-androstanediacetate. Results for a range of 3α -androstanediacetate concentrations similar to those which might be expected in assessment of procedural losses of 3 α-androstanediol in method B are shown in Figure 6. These indicate that 3 α-androstanediacetate concentrations measured were similar to the theoretical concentrations.

It may be assumed, therefore, that both methods A and B were adequately accurate for the analysis of steroid metabolites present in incubation extracts.

2. Addition of Cofactors to Incubations

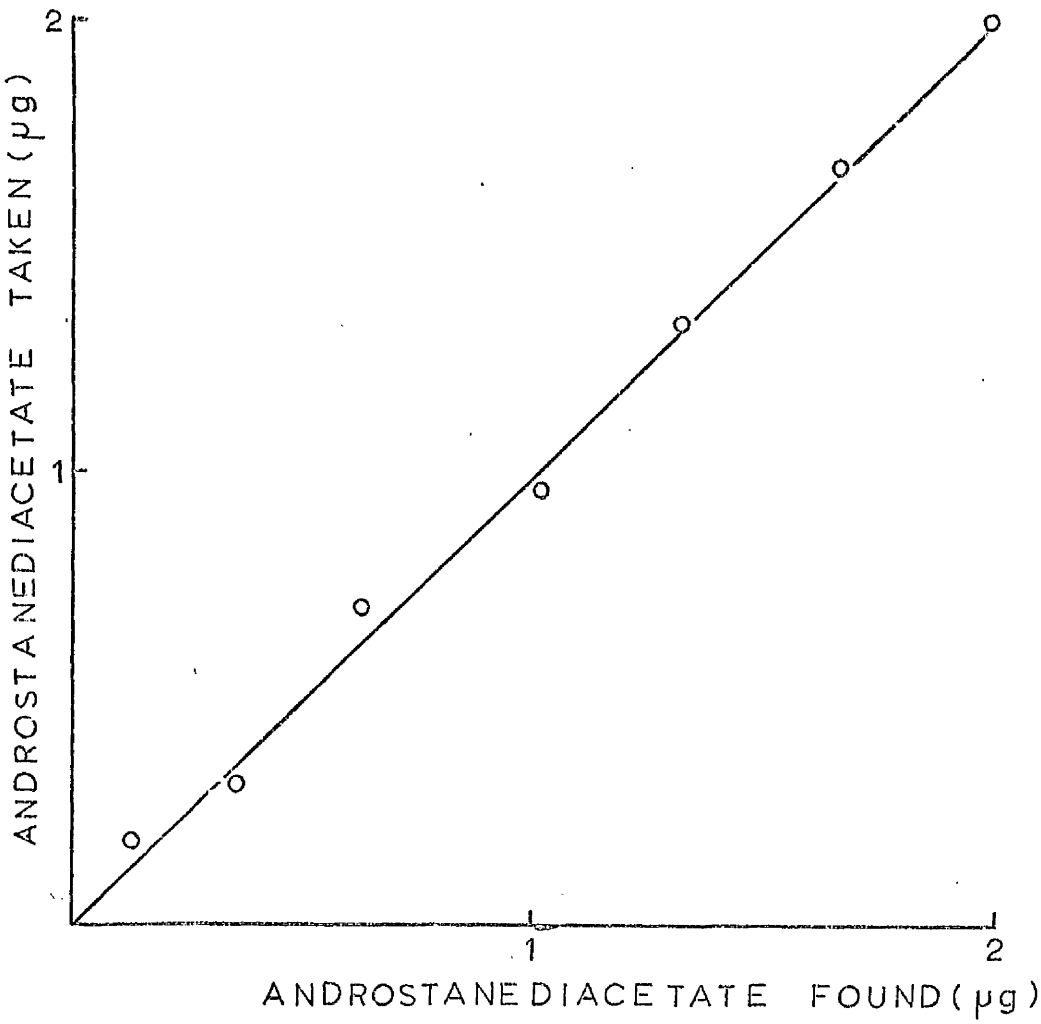
Human hyperplastic prostatic testosterone 5 α-reductase, like similar enzymes present in many other androgen dependent tissues (King and Mainwaring, 1974) has a cofactor requirement for NADPH (Farnsworth and Brown, 1963). Since the ability to produce sufficient NADPH is diminished during cell-free incubations, it is imperative, in a study in which the only limiting factor should be enzyme activity, that NADPH should be artificially maintained at

FIGURE 6.

Accuracy of g.l.c. Determination for
5α-Androstane-3α, 17β-Diacetate

The accuracy of the g.l.c. determination, which was used in method B to determine recoveries of 3α-androstanediol, was assessed by analysis of known concentrations of 3α-androstanediacetate.

Figure 6. ACCURACY OF g.l.c. DETERMINATION.



saturation level. This may be done by adding to the 5α-reductase incubation NADP, glucose-6-phosphate dehydrogenase or some other enzyme known to reduce NADP to NADPH, and a suitable substrate. This system has the advantage of reproducing *in vitro* the probable situation *in vivo* where oxidised NADPH may be continually reduced.

A generating system based on glucose-6-phosphate dehydrogenase has been used both in the study of rat prostatic 5α-reductase (Bruchovsky and Wilson, 1968) and the 5α-reductase of human prostatic nuclei (Grant *et al.*, 1971). Attempts to utilise such a system in the present study raised the possibility that factors modifying the 5α-reductase activity may do so as an effect secondary to the primary effect on the glucose-6-phosphate dehydrogenase activity. Since divalent cations are known to modify the activities of many enzymes, it was important to determine whether the divalent cations with which part of this study is concerned modified the production of NADPH within this generating system.

Results for zinc inhibition, shown in a double reciprocal plot in Figure 7 indicate that zinc, at a low concentration, inhibits glucose-6-phosphate dehydrogenase in a non-competitive manner with respect to NADPH concentration. (A full outline of the double reciprocal method used to analyse the type of inhibition exerted by an enzyme inhibitor is given in Section 4, d.) Further studies indicated that a range of divalent cations inhibit the enzyme activity in the following order of effectiveness:-

$$Hg^{2+} = Cu^{2+} > Cd^{2+} > Zn^{2+} \gg Mn^{2+}$$

To avoid modification of the 5α-reductase activity by secondary

FIGURE 7.

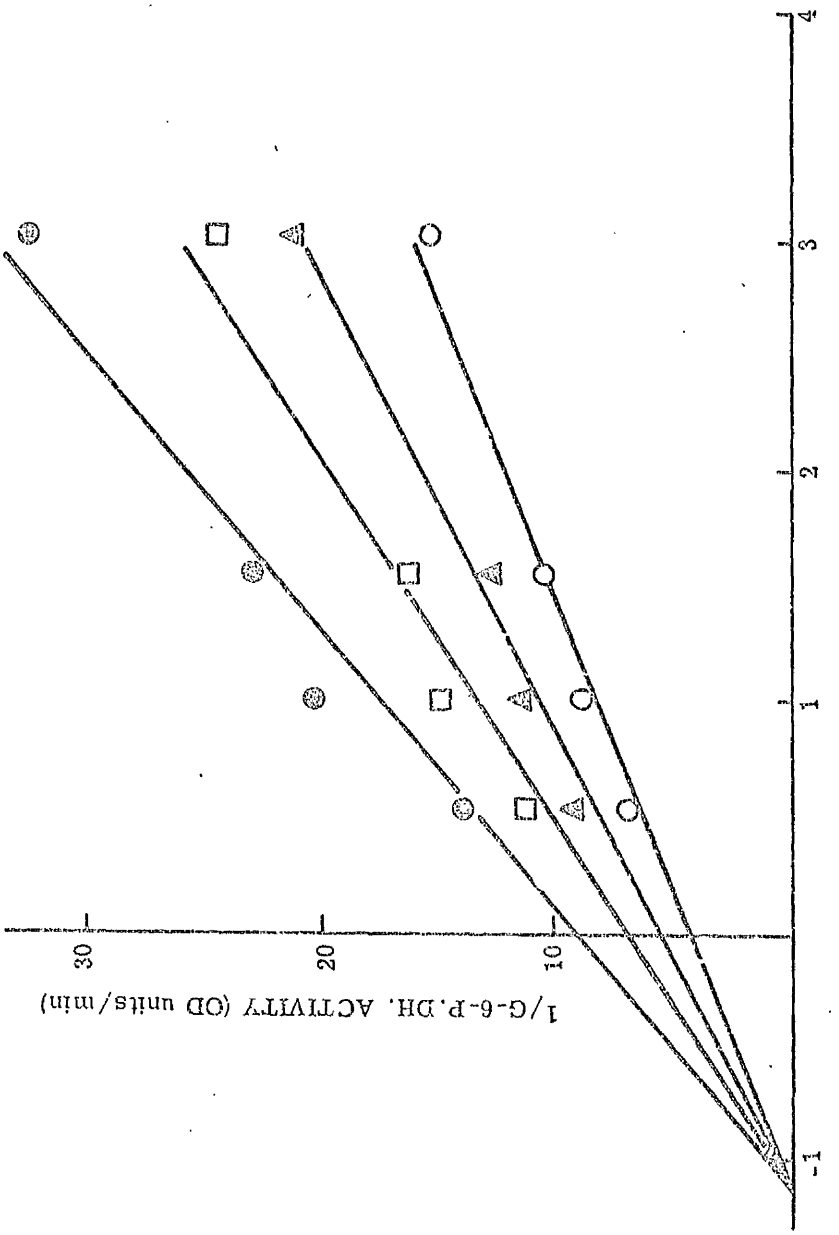
Double-Reciprocal Plot of Inhibition of Glucose-6-Phosphate Dehydrogenase by Zinc

Low concentrations of zinc were added to incubations of glucose-6-phosphate and NADPH with glucose-6-phosphate dehydrogenase (G-6-P.DH). G-6-P.DH activity was monitored by measurement of the rate of NADPH formation.

- ◍ Control $+10^{-3}$M $ZnCl_2$
- □ Control $+10^{-4}$M $ZnCl_2$
- △ Control $+10^{-5}$M $ZnCl_2$
- ○ Control

Figure 7.

DOUBLE-RECIPROCAL PLOT OF INHIBITION OF G-6-P.DH BY ZINC



effects on glucose-6-phosphate dehydrogenase, the generating system was omitted from incubations. In its place NADPH was added at saturation level (2×10^{-4}M) and any possible enzymatic degradation prevented by addition of nicotinamide (0.04M) (Handler and Klein, 1942).

3. Chemical Composition of Nuclear Fraction

The cytological homogeneity, subsequently loosely referred to as the purity of the nuclear fraction isolated from human hyperplastic prostatic tissue, was determined by comparison of its chemical composition with the same fraction isolated from rat prostatic tissue. A comparison (Table 10) was also made with published values obtained from guinea pig liver nuclear fraction (Maggio et al., 1963).

An explanation is required for the extremely low DNA content of human prostatic samples when this is expressed as mg/g wet weight equivalent of filtered homogenate. In the preparation of homogenate fractions from human prostatic tissue a large amount of material was held back, probably due to the presence of a high proportion of homogenisation resistent stromal and connective tissue. Therefore, although for guinea pig liver and rat prostatic tissue fractions the DNA content expressed as mg/g wet weight equivalent of filtered homogenate was approximately the same as mg DNA/g wet weight of tissue (after correction for nuclear recovery) this was not the case for human tissue.

The purity of the nuclear fraction isolated from rat prostatic tissue compared very favourably with that from guinea pig liver

TABLE 10.

Chemical Composition of Nuclear Fraction

	Rat Prostate	Human Prostate	†Guinea Pig Liver
No. of Experiments	3	3	7
% Recovery *	44	14.3	25
DNA mg/g	0.539	0.092	0.468
% DNA ✣	37.9	7.7	22.3
RNA mg/g	0.102	0.033	0.100
% RNA ✣	7.2	2.76	4.7
Protein mg/g	0.78	1.069	1.22
% Protein ✣	55	89.5	73.6
DNA/RNA	5.27	2.8	4.7

† – Maggio et al. (1963)

* % Recovery = % of homogenate DNA recovered in nuclear fraction

mg/g = mg/g wet weight equivalent of filtered homogenate – see text

✣ where DNA + RNA + Protein = 100%

tissue in that it contained less protein and a higher DNA/RNA ratio. The DNA/RNA ratio is a useful index of nuclear purity since, within limits, the proportion of RNA in the nuclear fraction will correspond to cytoplasmic contamination, the bulk of the cellular RNA being present in the cytoplasm.

Nuclear fraction isolated from human hyperplastic prostatic tissue had a lower yield, less DNA, and considerably more protein than rat prostatic nuclear fraction. The DNA/RNA ratio was lower, indicating that cytoplasmic contamination was greater. Examination under the light microscope indicated that the nuclear fraction prepared from rat prostatic tissue contained nuclei with little cytoplasmic contamination and few cytoplasmic attachments. The corresponding fraction prepared from human tissue contained clumps of non-nuclear material and occasional cytoplasmic attachments.

In initial experiments this non-nuclear material predominated; filtration of the homogenate prior to subcellular fractionation improved the situation. Attempts to isolate a purer nuclear fraction from human hyperplastic prostatic tissue by centrifugation through 2.3M and 2.2M sucrose resulted in even lower recoveries. The results obtained highlight the problems involved in the isolation of nuclei from human hyperplastic prostatic tissue.

4. Testosterone Reduction by Human Prostate

(a) Effect of Freezing on 5 α-Reductase Activity

The irregular supply of human prostate glands, particularly during summer months, made the planning of experiments extremely

difficult. To overcome this practical problem, a method was developed to store tissue without altering 5 α-reductase activity.

Freezing of biological samples has become a viable proposition in many branches of biochemistry. Its successful use in the storage of mammalian sperm was first outlined by Polge et al. (1949) and has greatly aided the technique of artificial insemination. Many authors have suggested that agents such as dimethylsulphoxide and glycerol greatly reduce tissue damage during freezing (Meryman, 1966). Lasnitzki (1955), however, has shown that Ehrlich ascites tumour cell suspensions will survive unprotected freezing at -79^{o}C sufficiently well to produce takes in transplantation. Freezing injury in rapidly cooled cells may be decreased by quick thawing (Mazur et al., 1970).

In the development of a method for storage of human prostatic tissue, factors such as those outlined above were taken into consideration. Two freezing methods were investigated. Method A involved freezing small squares (0.5cm x 0.5cm) of fresh prostatic tissue by placing them in a glass tube and immersing in a mixture of solid CO_2/acetone. Subsequent storage was carried out at -20^{o}C. In method B tissue squares were immersed directly into liquid nitrogen (-196^{o}C) and stored at -70^{o}C. Method B is described in full in the methods section B, 5 (c).

Experiments were performed to evaluate the effect of freezing, storing and thawing on 5α-reductase activity. Fresh prostatic tissue was cut into small squares (0.5cm x 0.5cm) which were thoroughly mixed prior to freezing and storage by methods A and B.

In both methods samples (1g) were thawed and homogenised prior to and at different periods after freezing. The tissue was homogenised in 0.25M sucrose-T.K.M. solution (10ml). Reductase activity was determined in these homogenates and the results are shown in Table 11.

Method A resulted in a 55% loss of 5 α-reductase activity during freezing and a subsequent 50% reduction after storage at -20°C for 16 days. Method B resulted in no loss of 5 α-reductase activity during freezing but an approximate reduction of 30% occurred after the first few days of storage at -70°C, followed by a much more gradual decline until day 30, after which the activity remained constant. Method B was, therefore, used in subsequent experiments.

It is possible that slight improvements in Method B could have resulted from the use of protective agents such as dimethylsulphoxide. Such a procedure, however, introduces new parameters which might modify 5 α-reductase activity, and it was therefore felt that the complications involved would outweigh the improvements gained.

(b) Subcellular Distribution of 5 α-Reductase Activity

Human prostatic tissue was minced and homogenised, and subcellular fractions isolated. Protein and 5 α-reductase activity were determined, in duplicate, on the homogenate, nuclear, mitochondrial and cytosol fractions. To provide an indication of nuclear recovery DNA was also analysed, in duplicate, on both the nuclear and homogenate fractions. On the assumption that all the cellular

TABLE 11.

Effect of Freezing on the 5 α-Reductase Activity of Human Prostatic Homogenates

Treatment	Method A S.A.	% *	Method B S.A.	% *
Before Freezing	5.42	100	15.43	100
After Freezing	2.45	45.2	15.59	101
After 2 days	-	-	10.79	70
After 4 days	-	-	11.64	75
After 8 days	-	-	11.48	75
After 16 days	1.43	26.4	10.27	67
After 32 days	-	-	9.28	60
After 64 days	-	-	9.23	60

S.A. = Specific Activity = pmol/h per mg protein.

% * = Percentage of 5 α-reductase activity in samples before freezing.

DNA is located in the nucleus, the percentage of homogenate DNA in the nuclear fraction should be approximately equivalent to the nuclear recovery. Mitochondrial DNA, which represents a very small proportion of cellular DNA, was ignored in this calculation. (Goodenough and Levine, 1970).

The standard 5α-reductase assay was performed with the addition of dithiothreitol (5×10^{-4}M) and EDTA (5×10^{-5}M). These reagents were present to counteract any divalent cation inhibition so that the maximum 5α-reductase activity should be measured. The experiment was carried out three times, once with fresh and twice with frozen tissue. Results are shown in Table 12 and Figure 8.

In order to reduce the errors introduced by defective recoveries of cellular constituents, the results represented in Table 12 were recalculated on the basis of a 100% overall recovery. In Figure 8 the relative specific activity (percentage of homogenate activity in a specific fraction/percentage of homogenate protein in that fraction) is plotted against the percentage protein in the fraction. The area of each block is proportional to the percentage of the total activity recovered in the corresponding fraction, and its height proportional to the degree of purification achieved over the homogenate. This method of representation was first suggested by deDuve (1955).

Further purification of the nuclear and microsomal fractions (those containing the bulk of the 5α-reductase activity) were carried out in the following manner. The 600 g ($r_{av.}$ 17cm)

TABLE 12.

Subcellular Distribution of 5α-Reductase Activity

Fraction	5α-Reductase	Protein	Relative Specific Activity†
Total Activity in Filtered Homogenate	1502 pmol/h	165 mg	
600 g Pellet Suspension* (Nuclear)	47.7	31.7	1.5
9,000 g Pellet Suspension (Mitochondrial)	13.4	4.3	2.7
105,000 g Pellet Suspension (Microsomal)	26.2	10.8	2.3
105,000 g Supernatant (Cytosol)	1.9	45	0.05
Total % Recovery in Subcellular Fractions	89.2	91.8	-

Results expressed as % of homogenate activity unless otherwise stated.

Results are the average of three experiments.

* - corrected for DNA recovery (on average 70% of homogenate DNA was recovered in the 600 g pellet).

† - relative specific activity = % of the homogenate activity in a specific fraction/% of homogenate protein in that fraction.

FIGURE 8.

Subcellular Distribution of 5 α-Reductase

Results presented are recalculated from Table 12 on the basis of a 100% overall recovery.

Relative Specific Activity:- percentage of homogenate activity in a specific fraction/ percentage of homogenate protein in that fraction.

Figure 8.

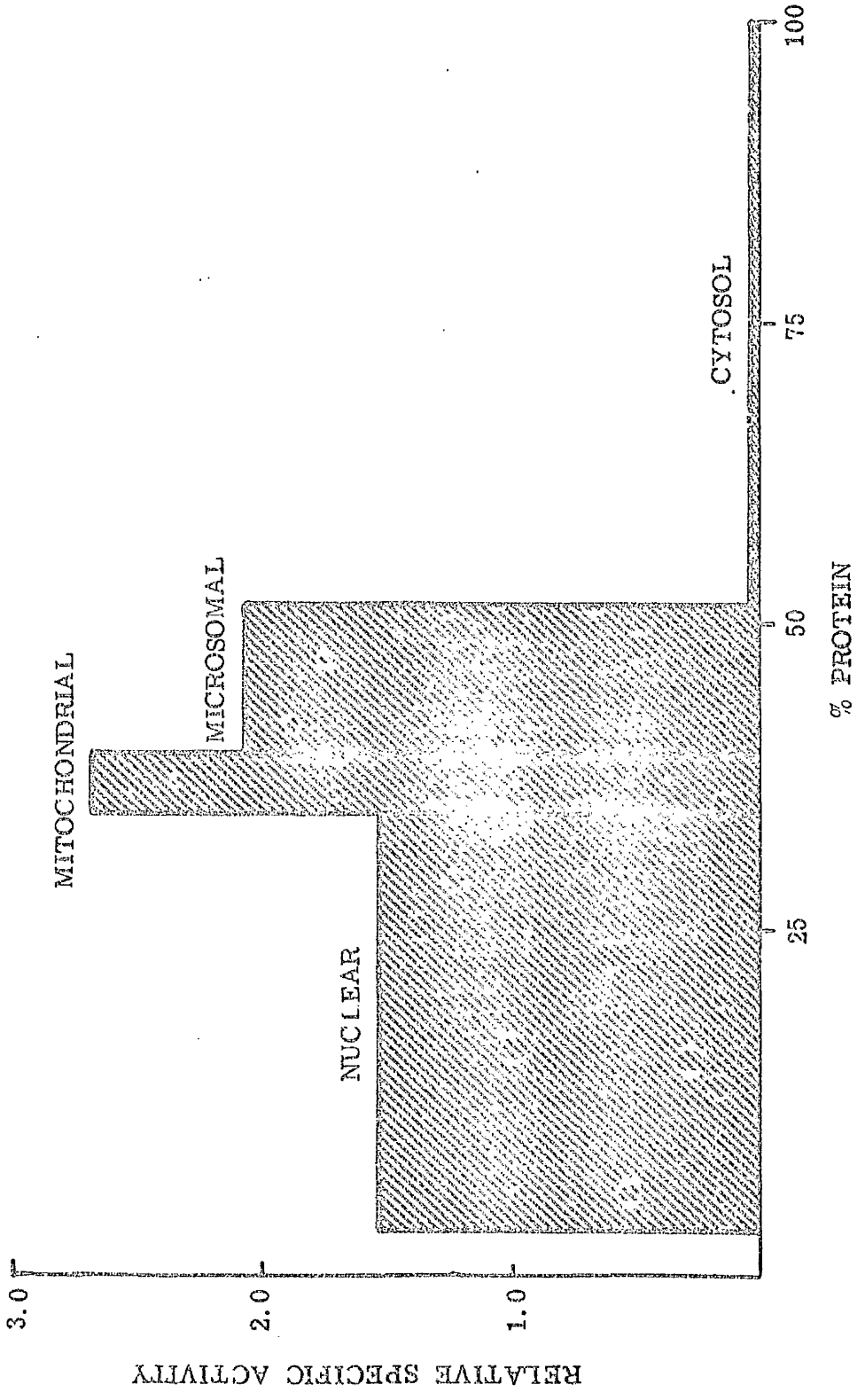

SUBCELLULAR DISTRIBUTION OF 5α-REDUCTASE
MITOCHONDRIAL
MICROSOMAL
NUCLEAR
CYTOSOL
RELATIVE SPECIFIC ACTIVITY
3.0
2.0
1.0
25
50
75
100
% PROTEIN

TABLE 13.

Activity of the 5α -Reductase in Nuclear and Microsomal Fractions after Further Purification

Nuclear Fraction

600 g Pellet Suspension		96,000 g Pellet Suspension		Purification Factor
Specific Activity	% DNA Recovery	Specific Activity	% DNA Recovery	
14.4	70	27	15	1.87
13.02	65	16.26	13	1.24

Microsomal Fraction

105,000 g Pellet Suspension	105,000 g Wash Pellet Suspension	Purification Factor
13.2	22.6	1.72
22.0	26.0	1.18

- % DNA recovery = % of homogenate DNA present in nuclear fraction.

- Specific Activity = pmol/h per mg protein.

- Purification Factor = Specific Activity in fraction after further purification/specific activity prior to further purification.

nuclear pellet suspension was centrifuged through a layer of 2.0M sucrose-T.K.M. solution at 96,000 g ($r_{av.}$ 3.692cm) for 2h. The microsomal pellet was resuspended in 0.25M sucrose-T.K.M. solution and re-centrifuged at 105,000 g ($r_{av.}$ 2.55cm) for 30min. Results are shown in Table 13. The purification factor, shown in this table, is defined as the figure obtained by dividing the specific enzyme activity in a fraction after further purification by the specific enzyme activity prior to further purification. This factor allows comparison of results from different experiments. Further purification of both the nuclear and microsomal fractions resulted in an increase in the specific enzyme activity.

In subsequent experiments further purification of the nuclear and microsomal fractions was performed prior to analysis of 5 α-reductase activity.

(c) Cofactor Requirement for Reduction

A study was performed to assess the cofactor requirement of human prostatic 5 α-reductase under the present experimental conditions. The standard 5 α-reductase assay was used with the addition of dithiothreitol (5×10^{-4}M) and EDTA (5×10^{-5}M). The effects of increasing the concentration of NADPH and NADH on testosterone reduction by nuclear and microsomal fractions are illustrated in Figure 9 and results for the homogenate fraction are illustrated in Figure 10. In all three cases reduction of testosterone was completely dependent on NADPH; NADH was incapable of supplying the reducing equivalents. In nuclear and microsomal

FIGURES 9 AND 10.

Addition of Cofactors to Nuclear, Microsomal and Homogenate Fraction 5 α-Reductase Incubations

Cellular fractions were assayed for 5 α-reductase activity in the presence of increasing concentrations of NADPH and NADH.

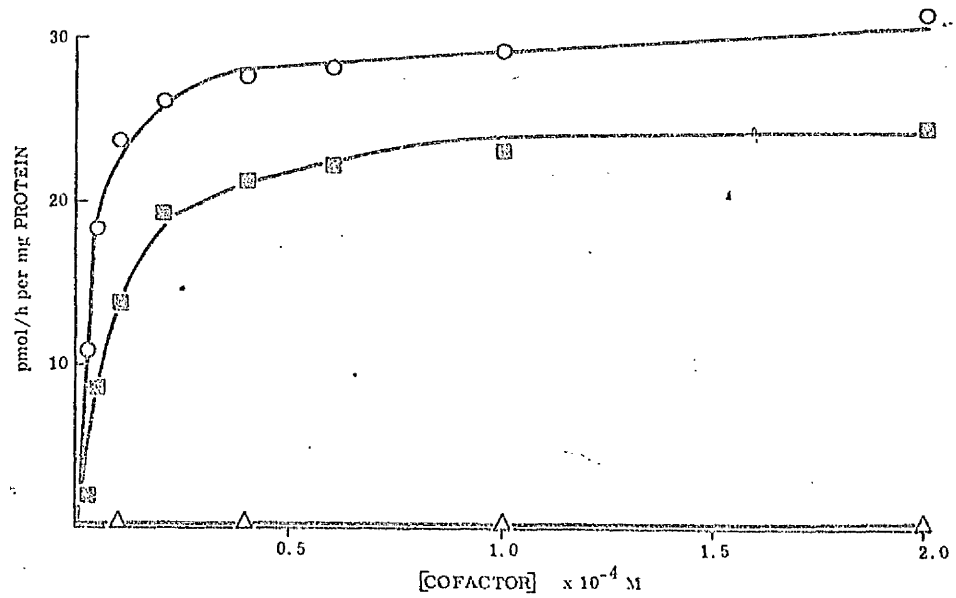
○ NADPH (supplied by NADPH generating system).

▩ NADPH (direct addition).

△ NADH (direct addition).

Figure 9.

ADDITION OF COFACTORS TO NUCLEAR 5α-REDUCTASE



ADDITION OF COFACTORS TO MICROSOMAL 5α-REDUCTASE

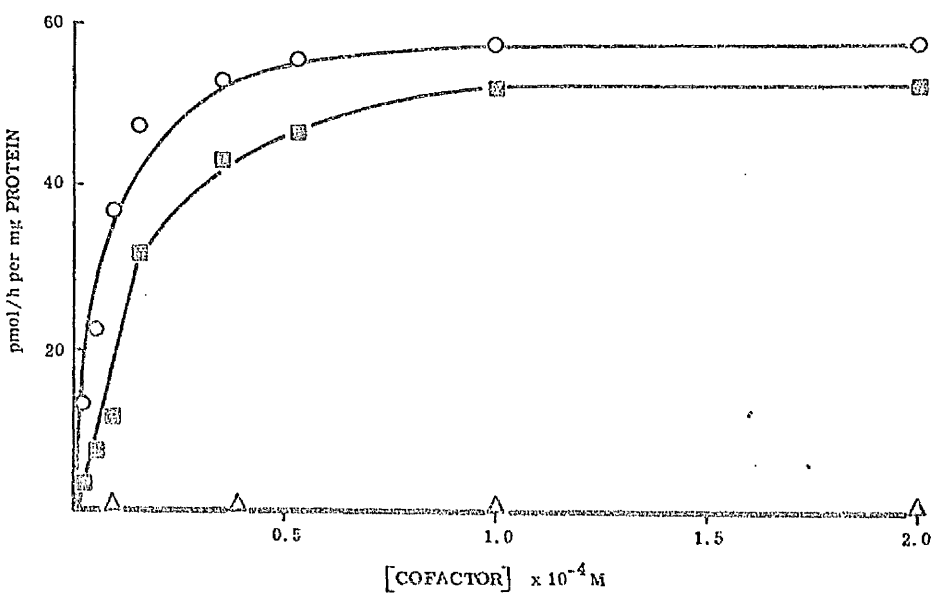
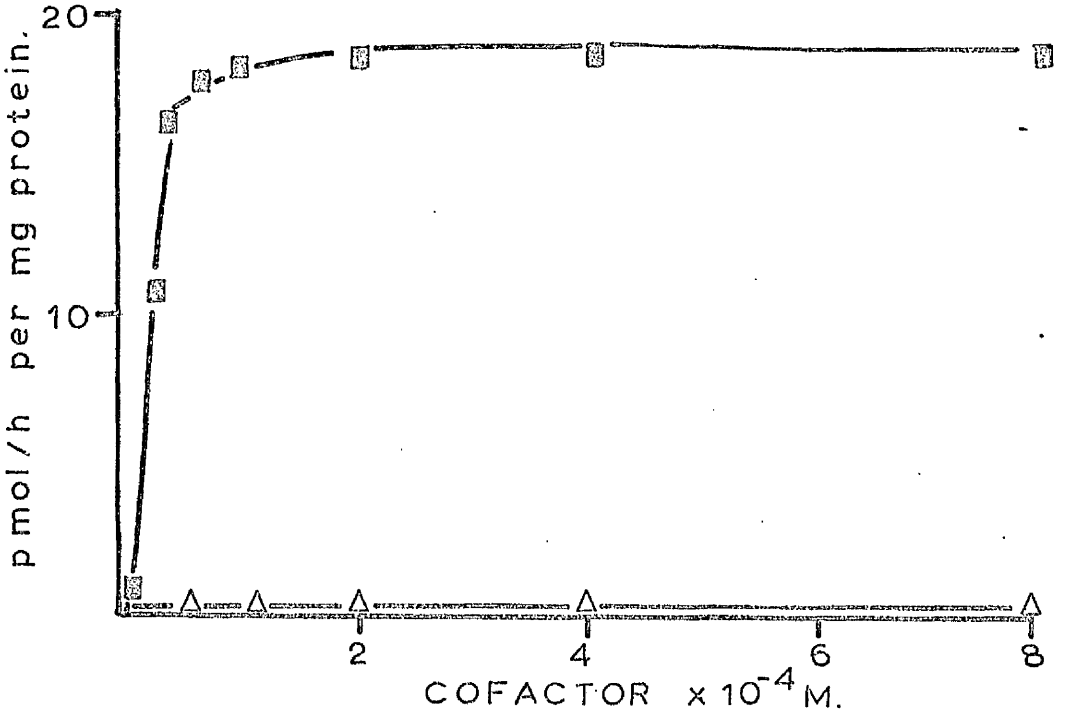


Figure 10.

ADDITION OF COFACTORS TO HOMOGENATE INCUBATIONS



incubations, NADPH was supplied either by a generating system, in which NADP is continually reduced to NADPH by glucose-6-phosphate dehydrogenase, or by direct addition. When a generating system was used reduction proceeded at a slightly higher rate, probably because a relatively higher concentration of NADPH is maintained by continued regeneration of the NADP produced during testosterone reduction. In all three fractions NADPH limits reduction if it is present in concentrations lower than 5×10^{-5}M. The initial hypothesis that in the standard 5α-reductase assay a concentration of 2×10^{-4}M NADPH should not limit reduction was therefore proved correct.

After all incubations the sum of ^{3}H-testosterone and ^{3}H-dihydrotestosterone accounted for over 95% of all the radioactivity. No significant production of 3α-androstanediol was detected.

(d) Time Course of Reduction and the Effect of Trypsin Inhibitor.

Incubations of nuclear, microsomal and homogenate fractions, with ^{3}H-testosterone, dithiothreitol (5×10^{-4}M) and EDTA (5×10^{-5}M) were performed for varying time periods with and without soya bean trypsin inhibitor (0.02%; w/v). Results for nuclear and microsomal incubation are shown in Figure 11 and for homogenate incubation in Figure 12. In all experiments ^{3}H-testosterone and ^{3}H-dihydrotestosterone accounted for over 95% of the radioactivity present at the end of the incubation. No ^{3}H-3α androstanediol was detected.

In all three fractions the 5α-reductase activity increased linearly with time for 40min when a decrease occurred. The enzyme

FIGURES 11 AND 12.

Time Course of Nuclear, Microsomal and Homogenate Fraction 5 α-Reductase.

Incubations were performed for different time periods in the presence and absence of 0.02% trypsin inhibitor.

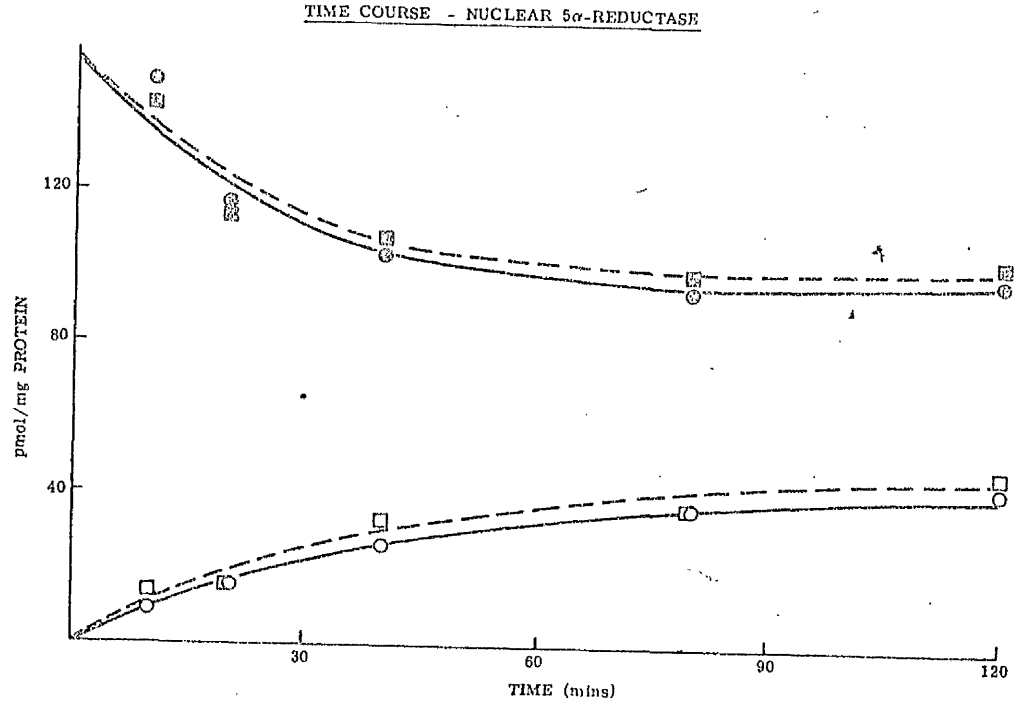
● ^{3}H-testosterone control.

▣ ^{3}H-testosterone + 0.02% trypsin inhibitor.

○ ^{3}H-dihydrotestosterone control.

□ ^{3}H-dihydrotestosterone+ 0.02% trypsin inhibitor.

Figure 11.


TIME COURSE - NUCLEAR 5α-REDUCTASE
pmol/mg PROTEIN
120
80
40
30
60
90
120
TIME (mins)

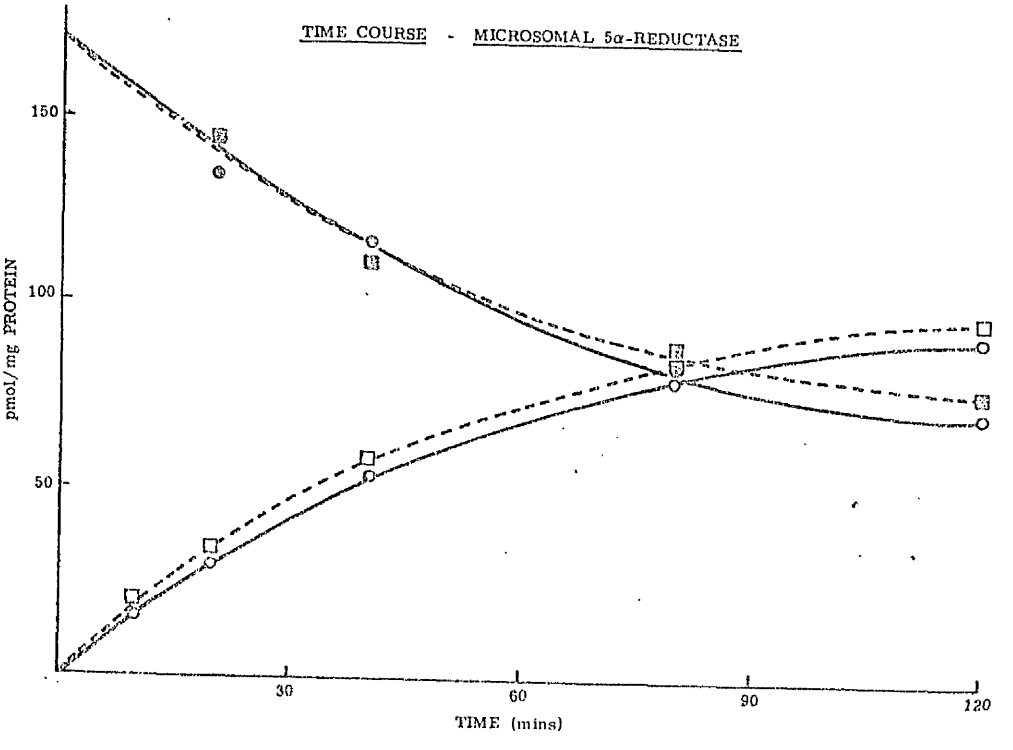
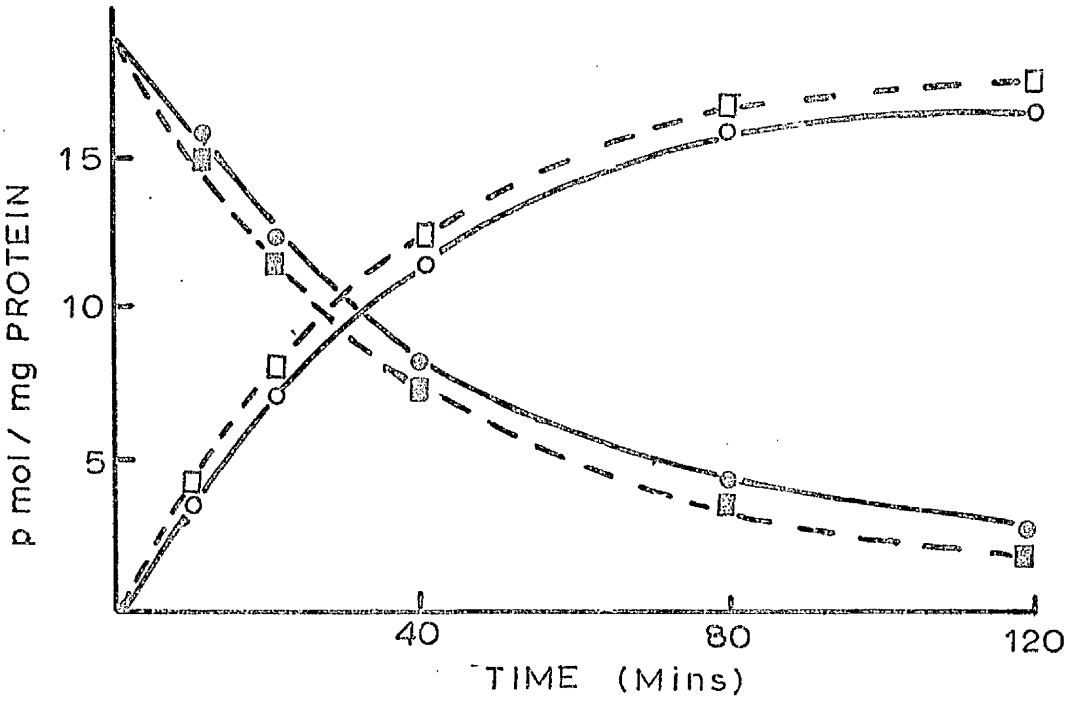

TIME COURSE - MICROSOMAL 5α-REDUCTASE
pmol/mg PROTEIN
150
100
50
30
60
90
120
TIME (mins)

Figure 12. TIME COURSE - HOMOGENATE 5α-REDUCTASE.



was totally inactive after 80min. It was found that addition of extra NADPH (2×10^{-4}M) to a microsomal incubation after 80min did not stimulate reduction. These results provide evidence that in the standard 5α-reductase assay, which was carried out for 20min, velocities similar to initial reaction velocities were being measured. The inclusion of trypsin inhibitor in incubations did not alter the rate of testosterone reduction.

(e) Effect of Divalent Cations

(i) Effect of a Range of Divalent Cations on 5α-Reductase Activity.

The 5α-reductase activity of rat prostatic nuclear fraction is sensitive to a range of divalent cations (Frederiksen and Wilson, 1971). Modification of human prostatic nuclear fraction 5α-reductase activity by zinc has also been reported (Grant et al., 1971). An investigation was carried out on the effect of a range of divalent cations on 5α-reductase activity from both nuclear and microsomal fractions isolated from human hyperplastic prostatic tissue. The standard 5α-reductase assay was carried out without the addition of dithiothreitol (5×10^{-4}M) or EDTA (5×10^{-5}M) since these agents may interfere with certain divalent cation effects.

To allow direct comparisons between the effects of different cations, all incubations were performed simultaneously. To provide sufficient nuclear fraction for the large number of incubations involved, pooled nuclear fractions from two isolations were necessary. A single microsomal isolation, however, provided

ample microsomal fraction for the experiment.

Results for nuclear and microsomal fractions are shown in Figures 13 and 14 respectively. In all incubations ^{3}H-testosterone and ^{3}H-dihydrotestosterone accounted for 95% of the radioactivity. No ^{3}H-3 α-androstanediol was detected. The results were similar for both fractions in that 5α-reduction was inhibited by low concentrations (approximately 10^{-5}M) of divalent cations in the following order of effectiveness:-

$$Cu^{2+} = Hg^{2+} > Zn^{2+} = Cd^{2+} \gg Mn^{2+}.$$

(ii) Effect of Zinc on Testosterone Reduction by Nuclear Fraction Isolated From Human and Rat Prostatic Tissue

In the previous experiment, although zinc caused a marked inhibition of 5α-reductase activity above a concentration of 10^{-5}M, no stimulation was observed at lower concentrations. One of the initial objectives of this study was to confirm that the 5α-reductase activity of human prostatic nuclear fraction was stimulated by zinc at a concentration of 5×10^{-4}M as shown by Grant et al. (1971). The experiment in which zinc was added to nuclear fraction 5α-reductase incubations was therefore repeated. Similar experiments using rat prostatic nuclear fraction were also performed.

The results, shown in Figure 15, confirm that zinc inhibits 5α-reductase activity at concentrations above 10^{-6}M, while no stimulation of activity was observed at lower levels. Testosterone 5α-reductase was slightly more sensitive to zinc in rat than in human prostatic nuclear fraction. The activity of nuclear

FIGURE 13.

Effect of Divalent Cations on Nuclear Fraction 5α -Reductase Activity

Incubations were performed in the presence of different concentrations of a range of divalent cations. Two nuclear isolations were pooled to provide sufficient nuclear fraction for the experiment.

○ Mn^{2+}

▩ Cd^{2+}

△ Zn^{2+}

◒ Hg^{2+}

□ Cu^{2+}

Figure 13.

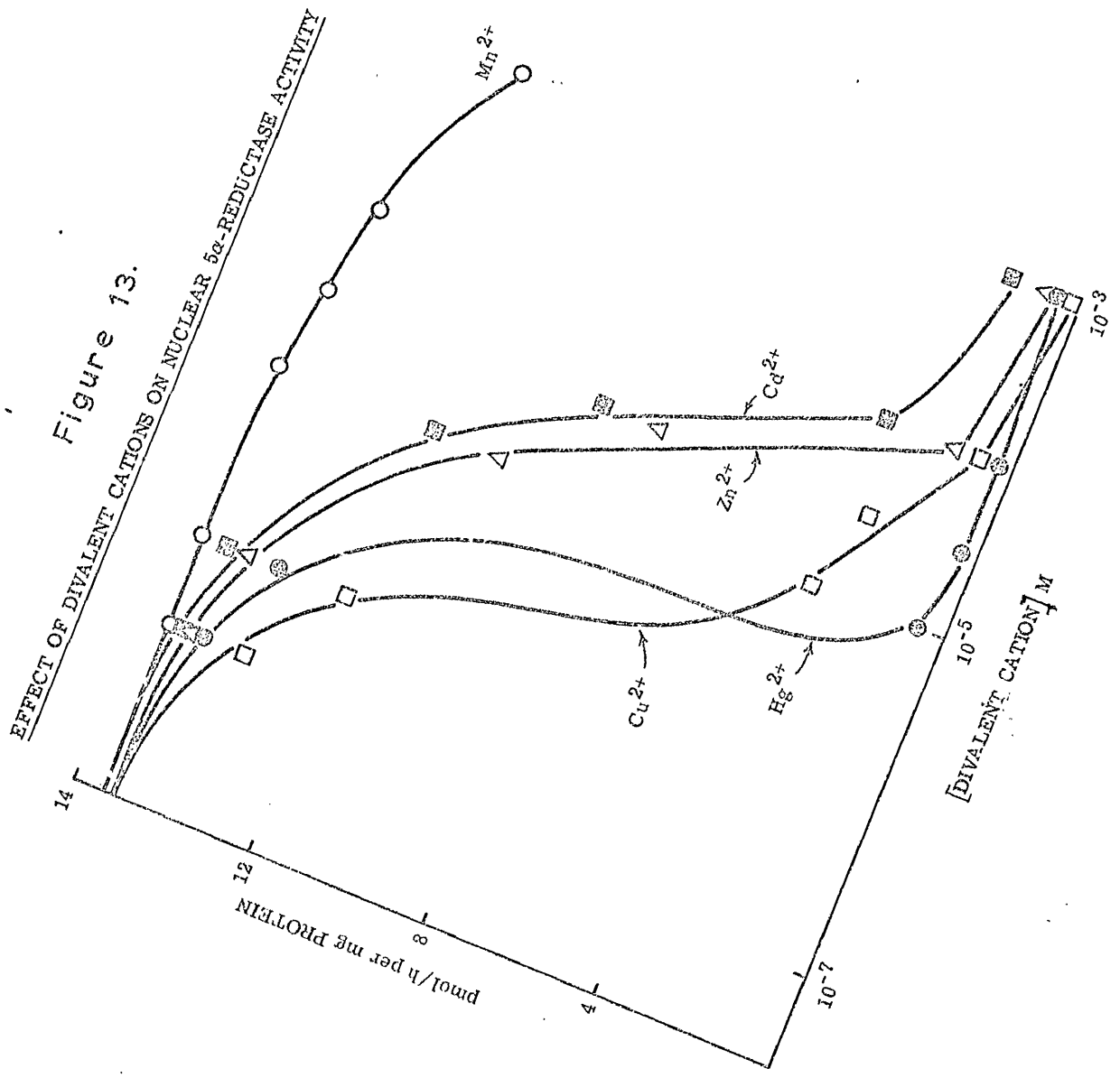

EFFECT OF DIVALENT CATIONS ON NUCLEAR 5α-REDUCTASE ACTIVITY
Mn 2+
Cd2+
Zn2+
Cu2+
Hg2+
14
12
8
4
pmol/h per mg PROTEIN
10-7
10-5
10-3
[DIVALENT CATION] M

FIGURE 14.

Effect of Divalent Cations on Microsomal Fraction 5 α-Reductase Activity

Incubations were performed in the presence of different concentrations of a range of divalent cations.

○ Mn^{2+}

▩ Cd^{2+}

△ Zn^{2+}

● Hg^{2+}

□ Cu^{2+}

Figure 14.

EFFECT OF DIVALENT CATIONS ON MICROSOMAL 5α-REDUCTASE ACTIVITY

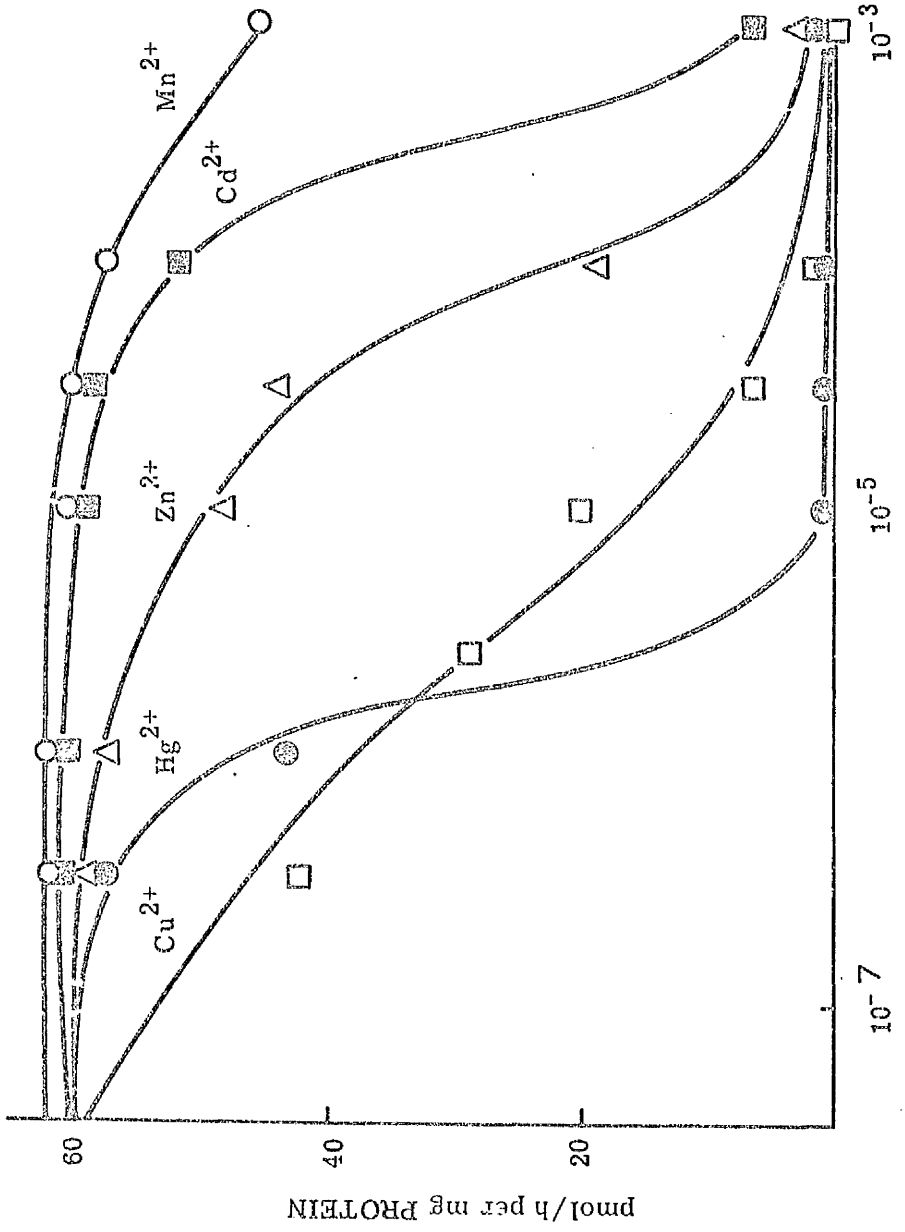
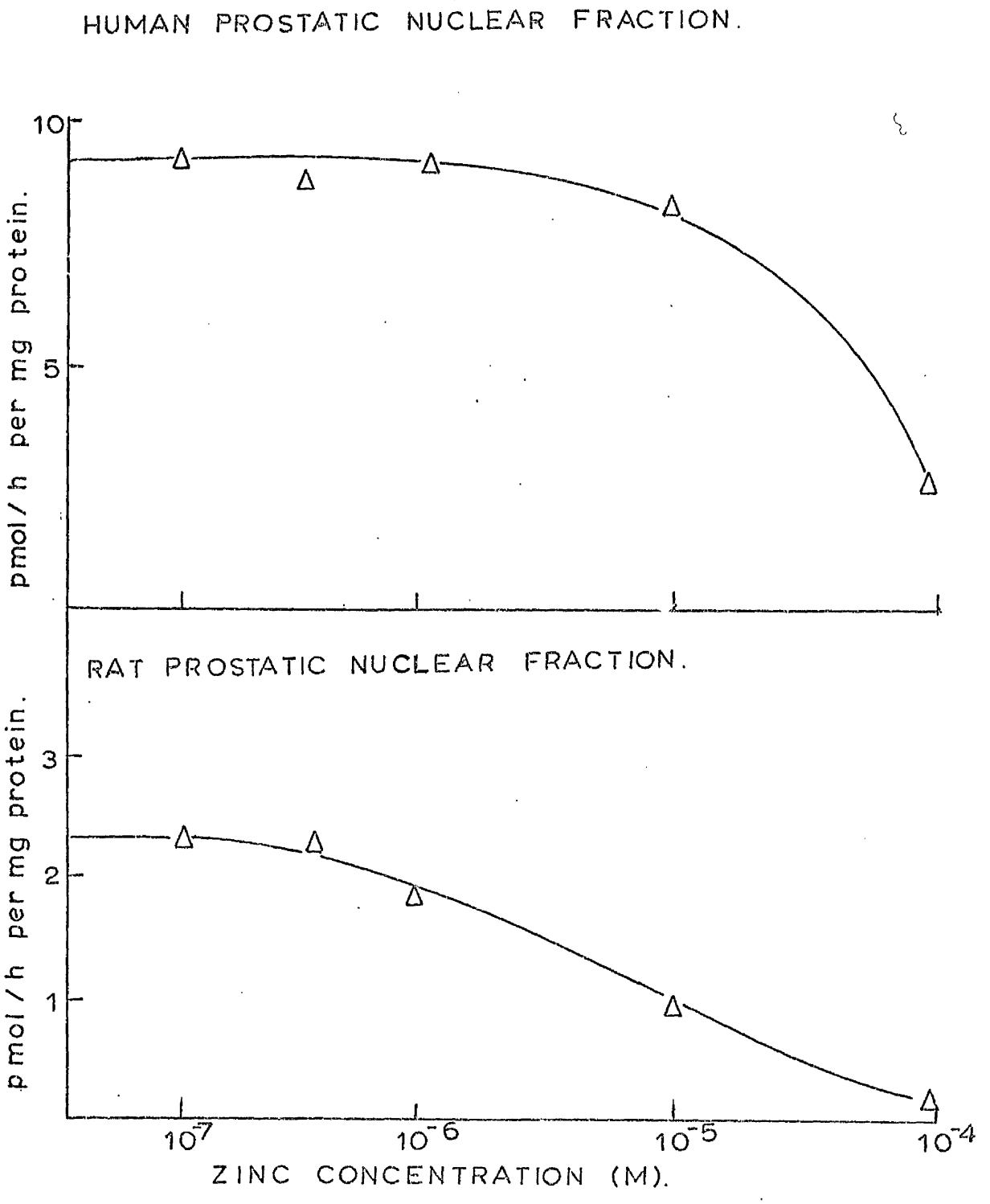


FIGURE 15.

Effect of Added Zinc on 5α-Reductase Activity

Incubations of nuclear fraction isolated from human hyperplastic and rat prostatic tissue were performed with different concentrations of zinc.

Figure 15. EFFECT OF ADDED ZINC ON 5α-REDUCTASE ACTIVITY.



5 α-reductase in rat prostatic tissue, however, was considerably lower, measured on a protein basis, than that in human prostatic tissue.

(iii) Effect of Zinc on 5 α-Reductase Activity of Nuclear Fractions Washed with Triton X-100

Comparison of the methods used in the present study and in the study carried out by Grant et al. (1971) revealed the difference that in the latter, but not the former, Triton X-100 had been used to wash nuclei. Since this difference may explain the inability, in previous experiments, to detect a stimulation of 5 α-reductase activity in the presence of 5×10^{-7}M zinc, the experiment was repeated using Triton X-100 washed nuclei.

A nuclear fraction was isolated from fresh human hyperplastic prostatic tissue and divided into three equal batches. Two of these batches were washed by suspension in 0.25M sucrose-T.K.M. Triton X-100 solutions containing Triton X-100 at a concentration of either 0.1%(w/v) or 0.001%(w/v). The suspension was centrifuged at 600 g ($r_{av.}$ 17cm) for 10min. The pellet was then rewashed with 0.25M sucrose-T.K.M. solution to remove traces of detergent.

Incubations of all three fractions (unwashed 0.1%(w/v) Triton X-100 washed and 0.001%(w/v) Triton X-100 washed) were performed by the standard procedure with and without 5×10^{-7}M zinc. The experiment was repeated with a nuclear fraction from a different batch of prostatic tissue.

Results obtained are shown in Table 14. To allow comparison of results between the two experiments, 5α-reductase activity is represented as the percentage of the activity present in the unwashed control to which no zinc had been added.

TABLE 14.

Effect of Zinc on Nuclear Fraction

5α -Reductase Activity after Washing with Triton X-100

Sample	Control	0.1% Triton	0.001% Triton
No Zinc	100	54.8	92.6
	100	56.9	91.8
+ 5 x 10^{-7}M Zinc	102	54.7	96.8
	104	55.0	93.2

Results are expressed as % of the 5α -reductase activity present in the unwashed nuclei.

5. Nature of Divalent Cation Inhibition of 5α-Reductase Activity

(a) Addition of Thiol Blocking Reagents and Thiol Groups

There are many chemical compounds capable of forming a covalent complex with thiol groups. In certain instances these reagents cause inhibition of enzymes requiring free thiol groups for activity. It was considered that a study of the effects of a range of such compounds on enzyme activity could provide valuable information concerning the role of thiol groups in catalysis. Iodoacetamide, iodoacetic acid, N-ethylmaleimide and p-chloromercuribenzoate were used as thiol complexing reagents. These reagents complex with thiol groups in the following manner. Iodoacetamide and iodoacetic acid react relatively specifically with thiol groups by alkyl substitution. The univalent organic mercurial, p-chloromercuribenzoate, reacts stoichometrically and also extremely specifically with thiol groups to form a mercaptide, while N-ethylmaleimide forms a complex derivative with thiol groups. These reagents were added to incubation tubes containing nuclear and microsomal fractions. The tubes were shaken, placed in an ice-bath for 5min followed by addition of ^{3}H-testosterone and NADPH. The 5α-reductase assay was then performed in the usual manner at 37^{o}C. The concentration of thiol group blocking reagents in the final incubation was either 10^{-2}M or 10^{-4}M. To some incubations a freshly prepared solution of dithiothreitol was added to a final concentration of 10^{-2}M. This water soluble compound contains many free thiol groups which enable it to reduce disulphide groups.

The results of these incubations are shown in Table 15. These are expressed as the percentage of ^{3}H-dihydrotestosterone produced in a control incubation where no additive was present. Each value is the average of at least two experiments. In one series of experiments fractions were isolated from frozen tissue. Results were obtained identical to those from fresh tissue. In all cases the increase in ^{3}H-dihydrotestosterone was equivalent to a decrease in ^{3}H-testosterone. No ^{3}H-3 α-androstanediol was detected.

The results indicate that the 5 α-reductase activity in both nuclear and microsomal fractions is sensitive to compounds known to form complexes with thiol groups. The most effective agent was p-chloromercuribenzoate which inhibited completely at a concentration of 5×10^{-5}M. Iodoacetamide caused marked inhibition at 10^{-2}M, but not at 10^{-4}M. Iodoacetic acid and N-ethylmaleimide were the least effective agents, causing approximately 50% inhibition at a concentration of 10^{-2}M. Dithiothreitol at the relatively high concentration of 10^{-2}M caused slight stimulation of reduction. Results obtained in this series of experiments were similar for incubations with nuclear and microsomal fractions.

(b) Effect of Chelating Agents

Testosterone reduction has been shown to be sensitive to divalent cations in Section 2, (e). It was, therefore, of interest to study the effect of chelating agents on the 5 α-reductase activity in nuclear and microsomal fractions isolated from human hyperplastic

TABLE 15.

Effect of Thiol Group Blocking Reagents and Thiol Groups on Testosterone Reduction

Reagent	No. of Experiments	Nuclear Fraction 5 α-Reductase *	Microsomal Fraction 5 α-Reductase *
Iodoacetamide			
10^{-2}M	2	13.2	6.1
10^{-4}M	2	102.0	99.6
Iodoacetic Acid			
10^{-2}M	2	57.9	39.7
10^{-4}M	2	97.8	102.4
N-ethylmaleimide			
10^{-2}M	2	58.3	52.4
10^{-4}M	2	86.6	85.0
p-chloromercuri-benzoate			
5×10^{-5}M	5	0.8	0.7
Dithiothreitol			
10^{-2}M	6	116.0	123.0

* Results expressed as percentage of ^{3}H-dihydrotestosterone produced in a control incubation where no additive was present.

prostatic tissue. Three chelating agents, EDTA, o-phenanthroline and citrate were used. The results are shown in Table 16. Each value is the average of at least two experiments. Results obtained from fractions isolated from frozen prostatic tissue were again identical to those for fresh tissue. Results are expressed as the percentage of ^{3}H-dihydrotestosterone produced in a control incubation where no additive was present. In all cases the increase in ^{3}H-dihydrotestosterone was equivalent to a decrease in ^{3}H-testosterone. No ^{3}H-3 α-androstanediol was detected.

The results show that in both nuclear and microsomal fractions EDTA produced a slight stimulation, o-phenanthroline a slight inhibition, and citrate no effect on 5α -reductase activity.

(c) Reversal of Inhibition

Inhibition of the activity of an enzyme may be either reversible or irreversible. If inhibition is reversible, the enzyme activity will be restored upon elimination of the inhibitor; for irreversible inhibition to occur, a fundamental change within the protein structure of the enzyme (i.e. a denaturation) may have taken place. Experiments were performed to determine whether inhibition of the 5α -reductase activity by zinc and p-chloromercuribenzoate could be reversed.

The experiments were designed in such a manner that the reversal agent was added 10min after the beginning of the incubation. At this point, therefore, the enzyme was known to be under inhibition, ensuring that a true reversal was being observed. If inhibition

TABLE 16.

Effect of Chelating Agents on Testosterone Reduction

Chelating Agent	No. of Experiments	Nuclear Fraction 5 α-Reductase *	Microsomal Fraction 5 α-Reductase *
EDTA 10^{-3}M	5	117	125
o-phenanthroline 10^{-3}M	4	86	90
Citrate 10^{-2}M	2	94	101
10^{-3}M	4	100	103

* Results expressed as percentage of ^{3}H-dihydrotestosterone in a control incubation where no additive was present.

and reversal agents were added at the same time it would be impossible to determine whether the observed effect was due to true reversal or to deactivation of the inhibitor. Incubations were terminated 15min after the addition of the reversal agent.

To quantitate the effect that a reversal agent had on inhibited enzyme activity the percentage release of inhibition was determined. This value was calculated by subtraction of the ^{3}H-dihydrotestosterone produced in a 25min incubation with inhibitor from the ^{3}H-dihydrotestosterone produced in a 25min incubation to which a reversal agent was added after 10min. The mathematical basis for this calculation is outlined as follows:-

^{3}H-dihydrotestosterone produced in the inhibited enzyme after a 25min incubation = y ng.

^{3}H-dihydrotestosterone produced by the inhibited enzyme during the first 10min of incubation = x ng.

^{3}H-dihydrotestosterone produced by the inhibited enzyme after a 25min incubation to which a releasing agent had been added after the first 10min of the incubation = z ng.

The ^{3}H-dihydrotestosterone produced by release of inhibition in the last 15min of incubation = $(z-x)-(y-x)$ ng. = $z - y$ ng.

This value was divided by the enzyme activity of a control incubation containing releasing agent alone and then multiplied by 100 to give the percentage release of inhibition.

Since previous results indicate that 5α-reductase inhibition involves thiol groups it is possible that if these groups were added

in excess to an inhibited enzyme this may cause some reversal of inhibition. Experiments were therefore performed in which dithiothreitol (10^{-2}M) was added to the nuclear and microsomal fraction incubations under inhibition by p-chloromercuribenzoate (5×10^{-5}M) or by zinc (10^{-4}M). The results obtained are shown in Table 17 and Figure 16.

Two experiments were performed with fractions from fresh prostatic tissue while in the others fractions were isolated from frozen tissue. In all incubations the only detected metabolite of ^{3}H-testosterone was ^{3}H-dihydrotestosterone.

Results show that reversal of zinc or p-chloromercuribenzoate inhibition occurred if dithiothreitol was added 10min after the beginning of the incubation. Dithiothreitol was most effective in reversing inhibition by p-chloromercuribenzoate. The extent of reversal was approximately the same in nuclear and microsomal fractions.

It is possible that chelating agents with a high affinity for zinc may reverse zinc inhibition. Reversal experiments were therefore performed with two such chelating agents, EDTA and o-phenanthroline and also the naturally occurring chelating agent citrate. The procedure was exactly similar to that used in the previous experiment. Incubations were performed in duplicate and the only detected metabolite of ^{3}H-testosterone was ^{3}H-dihydrotestosterone. The results, shown in Table 18 and Figure 16, indicate that inhibition of 5α-reductase activity by 10^{-4}M zinc can be reversed by EDTA and o-phenanthroline. No significant difference could be

TABLE 17.

Reversal of p-chloromercuribenzoate and Zinc Inhibition by Dithiothreitol (10^{-2}M)

Inhibitor	No. of Experiments	Nuclear Fraction *	Microsomal Fraction *
p-chloromercuri-benzoate 5×10^{-5}M	5	51	64
Zinc 10^{-4}M	4	34	23

* Results expressed as percentage release of inhibition - see text.

TABLE 18.

Reversal of Zinc Inhibition by Chelating Agents

Chelating Agent	No. of Experiments	Nuclear Fraction *	Microsomal Fraction *
EDTA 10^{-3}M	2	39	47
o-phenanthroline 10^{-3}M	2	42	30
Citrate 10^{-2}M	2	25	9
10^{-3}M	2	1.9	1.2

* Results expressed as percentage release of inhibition - see text.

FIGURE 16.

Reversal of 5 α-Reductase Inhibition

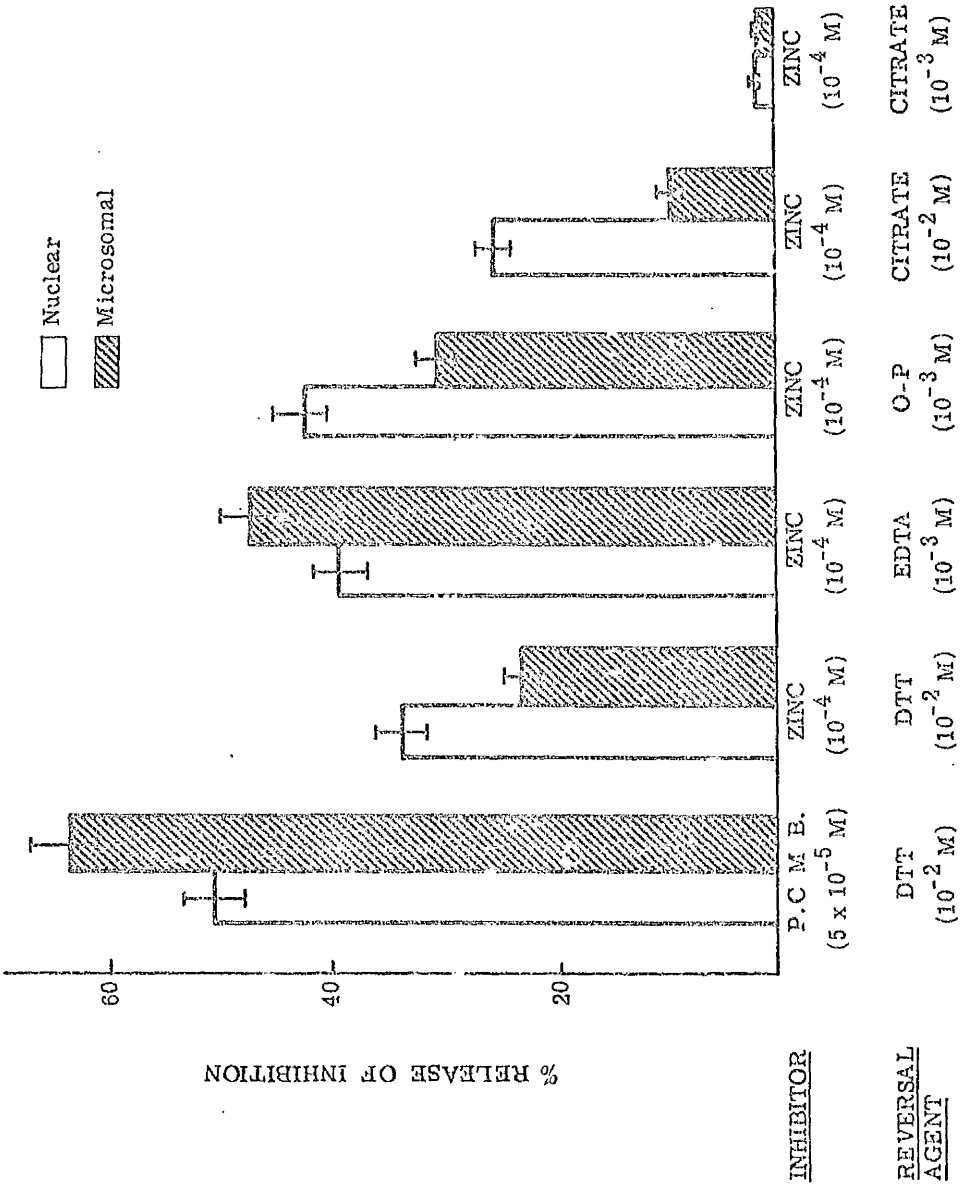
Nuclear and microsomal fractions were incubated with inhibitor for 10mins before addition of reversal agent. Incubations were terminated 15min after the addition of the reversal agent.

% Release of inhibition is an index of the increase of 5 α-reductase activity after the addition of the reversal agent. The method for calculation of the % release of inhibition is outlined in the text.

	Nuclear Fraction
	Microsomal Fraction.
P.CMB	p-chloromercuribenzoate
DTT	Dithiothreitol
O-P	o-phenanthroline

Figure 16.

REVERSAL OF 5α-REDUCTASE INHIBITION



detected between nuclear and microsomal fractions in this respect. High concentrations of citrate (10^{-2}M) also caused reversal which was most marked in the nuclear fraction.

(d) Kinetic Study of Zinc Inhibition

Since the inhibition of 5 α-reductase activity by zinc was found to be reversible, information concerning the mechanism of inhibition could be gained by a Michaelis-Menten type analysis (Michaelis and Menten, 1913).

All enzyme activities increase linearly with time as the concentration of substrate (or cofactor) is increased up to saturating level. The velocity of the enzyme reaction at saturation is termed Vmax and the substrate concentration when the enzyme activity is half of that at saturation is termed K_m. The relationship between the enzyme's initial reaction rate (v), the substrate concentration $[S]$, Vmax and K_m is given by the Michaelis-Menten equation:-

$$v = \frac{Vmax \quad [S]}{K_m + [S]}$$

The concepts Vmax and K_m, are dependent on the affinity of the substrate (or cofactor) for the enzyme and the efficiency of catalysis. They are useful parameters for the comparison of enzyme activities. A plot of v against $[S]$ gives a hyperbolic curve from which Vmax and K_m can be determined. Since this plot is an asymptote it is extremely difficult to determine the exact point at which the reaction has reached saturation. To determine Vmax and K_m more precisely the Michaelis-Menten equation can be rearranged to the form:-

$$\frac{1}{v} = \frac{K_m}{Vmax} \frac{1}{[S]} + \frac{1}{Vmax}$$

This is called the Lineweaver-Burk equation (Lineweaver and Burk, 1934).

If 1/v is plotted against 1/[S] (a double reciprocal plot), a straight line should result, the intercept with the ordinate being equal to 1/Vmax while that with the abscissa being equal to - 1/Km.

If an inhibitor combines at or near to the substrate (or cofactor) binding site, or is structurally sufficiently similar to the substrate (or cofactor) to compete with it for its binding site the effect will be reflected by an increase in the substrate (or cofactor) concentration required to attain half the maximum velocity. The maximum velocity, however, should not be altered. (K_m is increased and now termed K_{mi}, but Vmax is unaltered). This type of inhibition is called competitive inhibition.

If, on the other hand, an inhibitor combines with a site far removed from the substrate (or cofactor) binding site the effect will be reflected by a decrease in the maximum velocity of the reaction. The substrate concentration at which half the maximum velocity is attained should be the same in the inhibited and uninhibited enzymes (Vmax is decreased and now termed Vmaxi, K_m is unaltered). This type of inhibition is called non-competitive inhibition.

The present series of experiments were designed to determine what type of inhibition was exerted by zinc on the 5 α-reductase activity present in the nuclear and microsomal fractions of the human hyperplastic prostate. The results obtained were analysed by

double reciprocal plots. If the inhibition is competitive such a plot is characterised by straight lines of differing slopes intersecting at a common intercept on the 1/v axis, if it is non-competitive the lines differ in slope but do not share a common intercept on the 1/v axis.

In the first series of experiments of this type nuclear and microsomal fractions were incubated with increasing levels of testosterone with and without 10^{-4}M zinc. The cofactor (NADPH) concentration was kept constant (2×10^{-4}M). It was found in a preliminary experiment that as the substrate concentration increased, the percentage conversion of ^{3}H-testosterone to ^{3}H-dihydrotestosterone formed during incubations decreased. The level of ^{3}H-testosterone was therefore increased from 2×10^{5} d.p.m. (the level in the standard 5α-reductase assay) to 8×10^{5} d.p.m. in these experiments. This allowed a more precise detection of ^{3}H-dihydrotestosterone formed during these incubations.

The results (Figure 17) indicate that, in both nuclear and microsomal fractions, zinc does not compete with testosterone during inhibition of the 5α-reductase. The following parameters were calculated from the double reciprocal plots:-

Fraction	app K_m x	Vmax	Vmaxi
Nuclear	105	12.5	1.94
Microsomal	66.7	208.3	100

x = nM = pmol/h per mg protein

In the next series of experiments nuclear and microsomal

FIGURE 17.

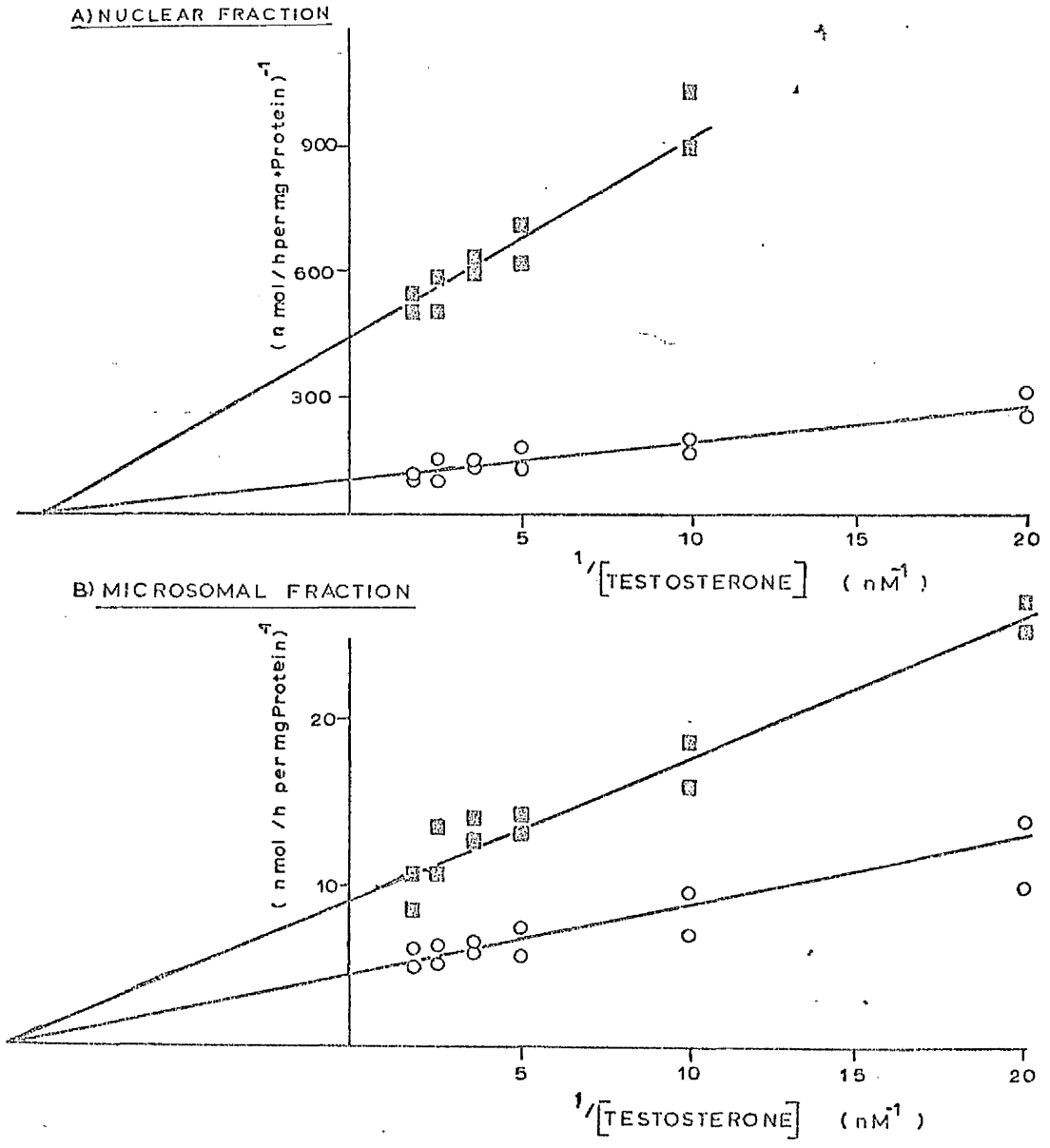
Plot of 1/v Versus 1/[Substrate] in the Presence and Absence of Zinc

Nuclear and Microsomal fractions were incubated with increasing levels of testosterone with and without 10^{-4}M $ZnCl_2$. The cofactor (NADPH) concentration was kept constant.

○ Control

▩ Control + 10^{-4}M $ZnCl_2$

Figure 17. PLOT OF 1/v VERSUS 1/[SUBSTRATE] IN THE PRESENCE AND ABSENCE OF ZINC.



fractions were incubated with increasing levels of NADPH with and without 10^{-4}M zinc. The concentration of substrate (^{3}H-testosterone) was kept constant (2×10^{5} d.p.m.). The results (Figure 18) indicate that in both nuclear and microsomal fractions 5 α-reductase activity is inhibited in a competitive manner with respect to cofactor. The following parameters were calculated from the double reciprocal plots:-

Fraction	Vmax ^{x}	app K_m	app K_{mi}
Nuclear	18.18	0.42	8
Microsomal	66.8	0.45	11.1

x = pmol/h per mg protein = $x10^{-4}$M NADPH

The later series of experiments give a positive result which indicated that zinc may bind at or near the cofactor binding site. The experiment was therefore repeated, with microsomal fraction and low concentrations of zinc (10^{-5}M and 10^{-4}M) to seek added confirmation of this effect. The results, presented on a double reciprocal plot, are shown in Figure 19. The following parameters were calculated from the graph:-

Vmax = 71.4 pmol/h per mg protein

app K_m = 0.3 x 10^{-4}M NADPH

app K_{mi} (10^{-5}M Zn) = 0.57 x 10^{-4}M NADPH

app K_{mi} (10^{-4}M Zn) = 3.2 x 10^{-4}M NADPH

FIGURE 18.

Plot of 1/v Versus 1/[Cofactor] in the Presence and Absence of Zinc

Nuclear and Microsomal fractions were incubated with increasing levels of NADPH. The substrate (testosterone) concentration was kept constant.

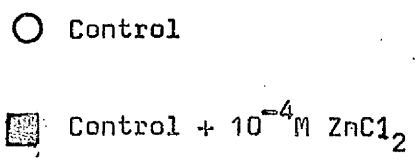


Figure 18 PLOT OF 1/v VERSUS 1/[COFACTOR] IN THE PRESENCE AND ABSENCE OF ZINC.

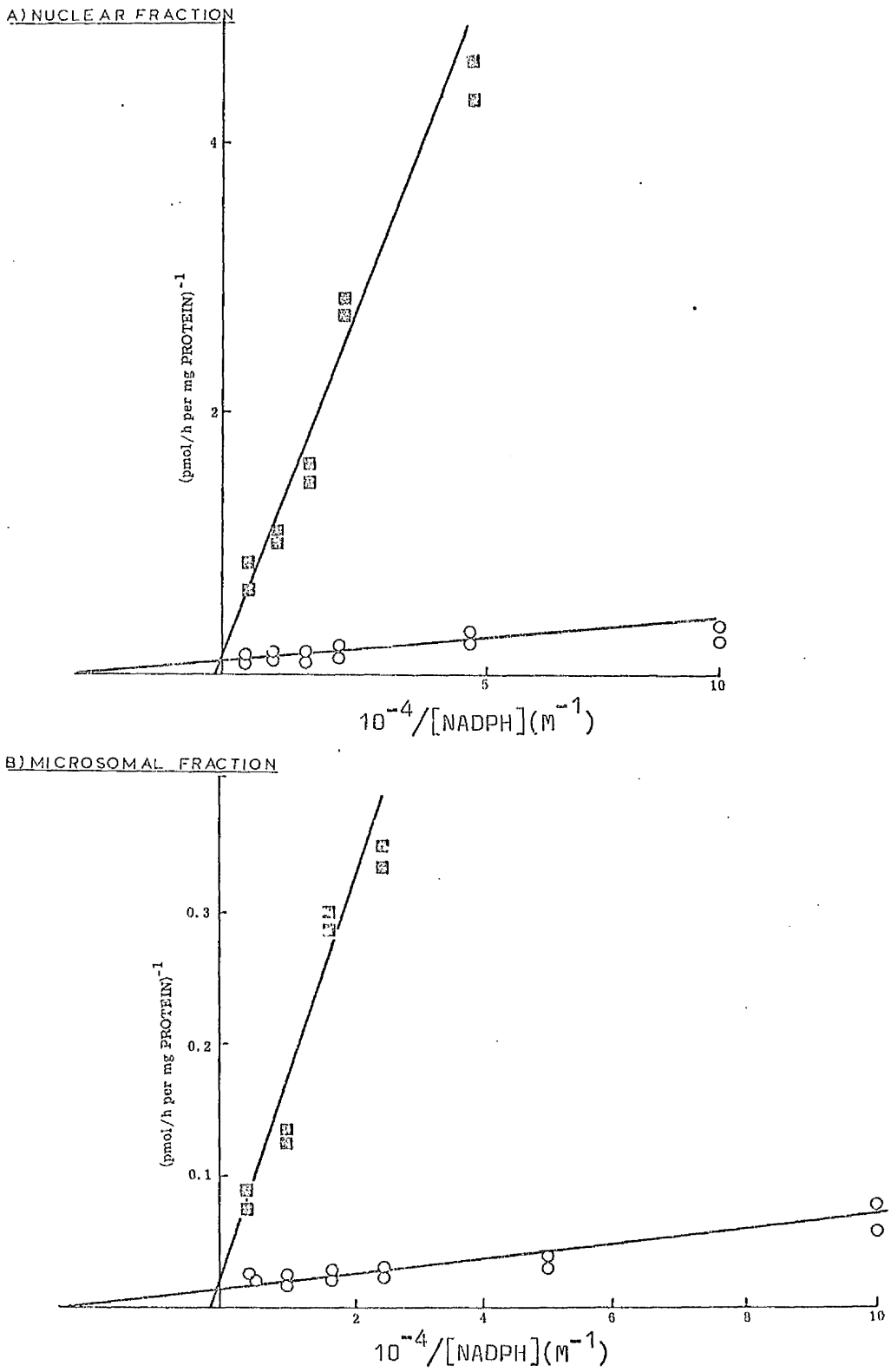


FIGURE 19.

Plot of 1/v Versus 1/[Cofactor] in The Presence and Absence of Zinc

Microsomal fraction was incubated with increasing levels of NADPH. The substrate (testosterone) concentration was kept constant.

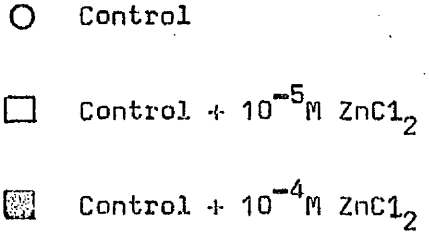
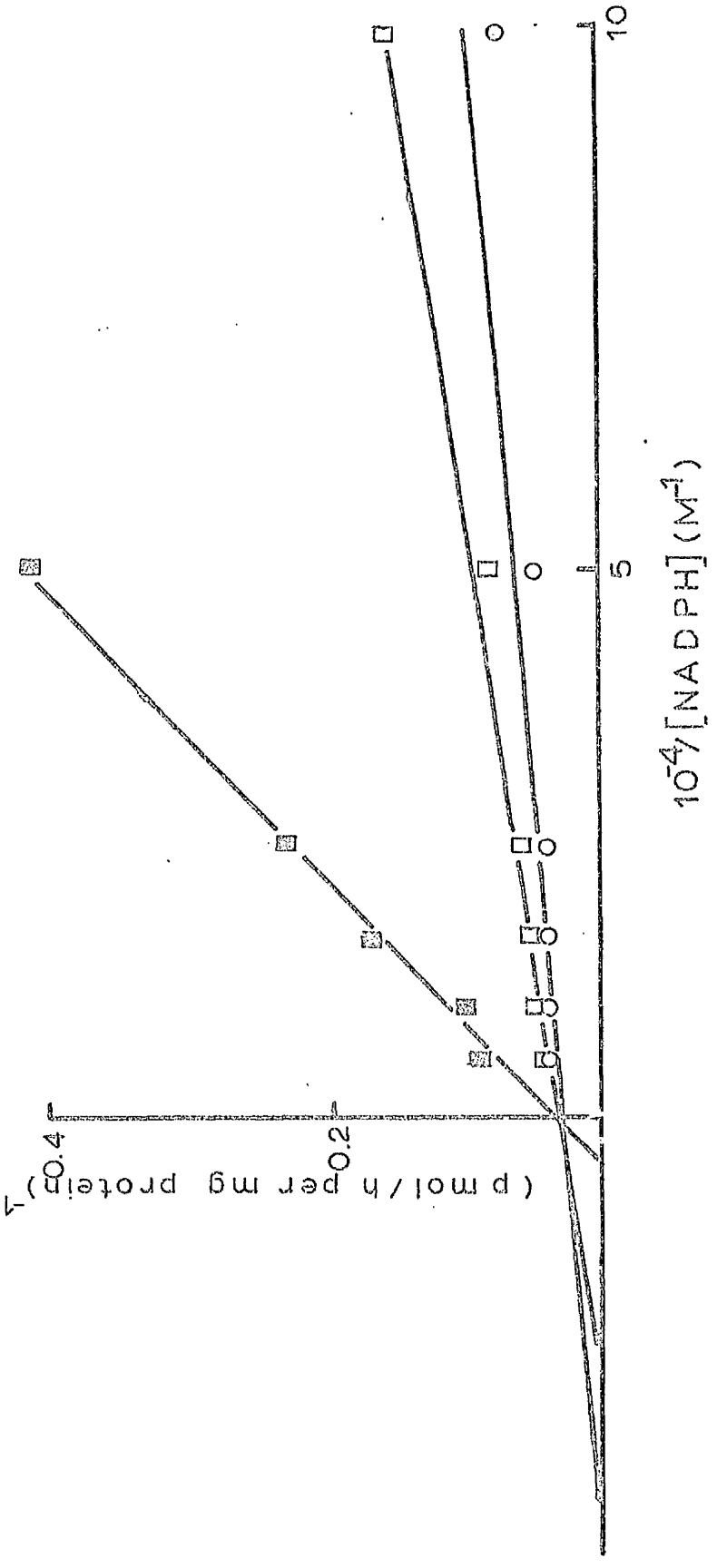


Figure 19. PLOT OF 1/v VERSUS 1/[COFACTOR] IN THE PRESENCE AND ABSENCE OF ZINC.



6. Relationship between Zinc Content and 5α-Reductase Activity

In the previous experiment zinc (10^{-4}M) was found to inhibit the reduction of ^{3}H-testosterone in nuclear and microsomal fractions isolated from human hyperplastic prostatic tissue. This reversible inhibition was competitive with respect to cofactor and may have involved binding of zinc to a thiol group. Although many enzymes are known to be sensitive to divalent cations the above observation may be of more physiological importance because of the high zinc content of human prostatic tissue. The objectives of these final experiments was to confirm that the prostate has a high zinc content, to discover where this zinc is located, and to search for evidence which might indicate that zinc controls 5α-reductase activity in vivo.

(a) Zinc Content of Hyperplastic and Adenocarcinomatous Prostatic Tissue

A study was performed to determine the zinc content of human prostatic tissue. The results obtained for the zinc content of tissue samples from two distinct areas of thirteen hyperplastic prostate glands are shown in Table 19. Results for three cases of adenocarcinoma are presented in Table 20.

The average zinc content of hyperplastic prostatic tissue was 1210 $\pm$ 901 (S.D.) µg/g dry wt. (n = 26) whereas the much lower value of 431.6 $\pm$ 238 (S.D.) µg/g dry wt. (n = 6) was determined in adenocarcinomatous tissue. There was an extremely large variation in the zinc content in both hyperplastic (450 - 4216 µg/g dry wt.)

TABLE 19.

Zinc Content of Human Hyperplastic Prostatic Tissue

Patients Initials	Age (Years)	Total Sample Wet wt. (g)	Zinc Content * of Sample A	Zinc Content * of Sample B
N.G.	67	51	971	1411
G.Mc.	60	77	449	564
W.Mc.	73	130	4215	2124
A.McG.	92	81	955	833
H.Mc.	51	60	1030	805
T.S.	73	85	772	726
D.L.	66	59	3462	612
J.R.	73	39	1074	1285
J.N.	67	83	675	905
A.G.	69	16	923	719
J.C.	72	22	988	1201
H.R.	70	50	2494	701
R.M.	68	56	682	882

* Expressed as µg Zn/g dry weight

Mean = 1210 $\pm$ 901 (S.D.) µg Zn/g dry weight (n = 26)

Range = 450 - 4216 µg Zn/g dry weight

TABLE 20.

Zinc Content of Adenocarcinomatous Prostatic Tissue

Patient's Initials	Age (Years)	Total Sample Wet wt. (g)	Histology	Zinc Content * of Samples from two distinct sites
J.Q.	66	30	Moderately well differentiated	328.2 244.4
S.F.	63	28	Well differentiated	769.7 281.9
R.S.	72	35	Well differentiated plus benign hyperplasia	701.6 263.5

* Expressed as µg Zn/g dry weight

Mean = 431.3 $\pm$ 238 (S.D.) µg Zn/g dry weight (n = 6)

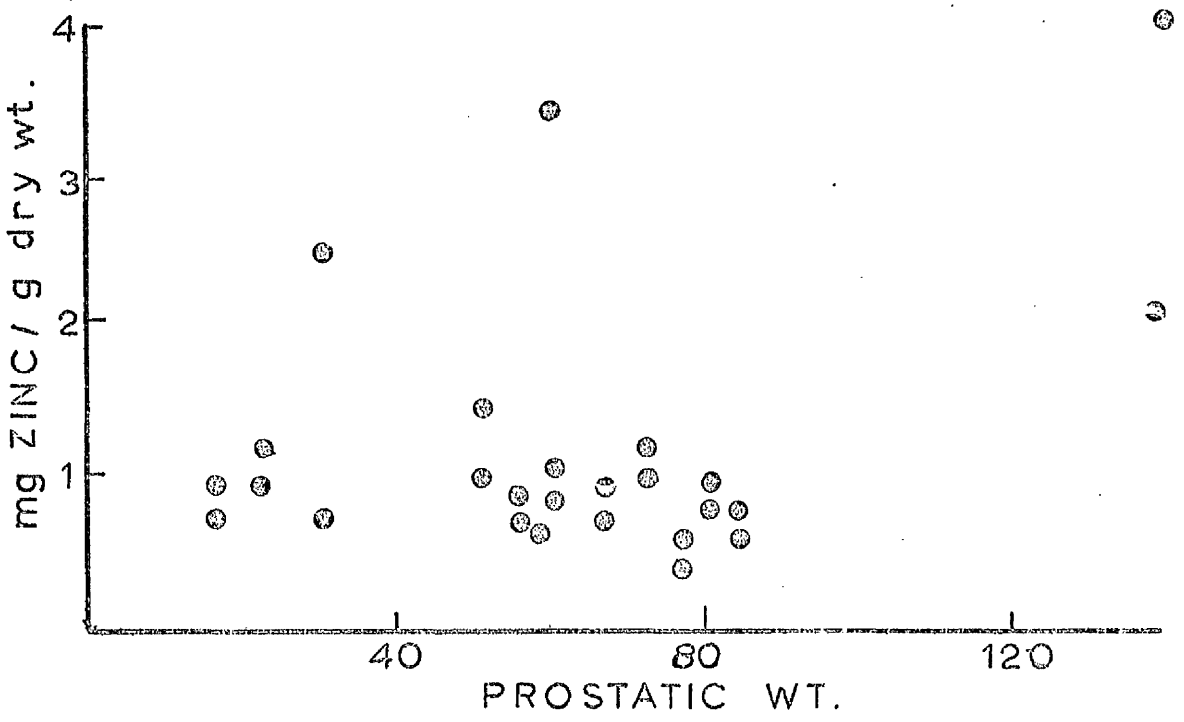
Range = 244.4 - 769.7 µg Zn/g dry weight

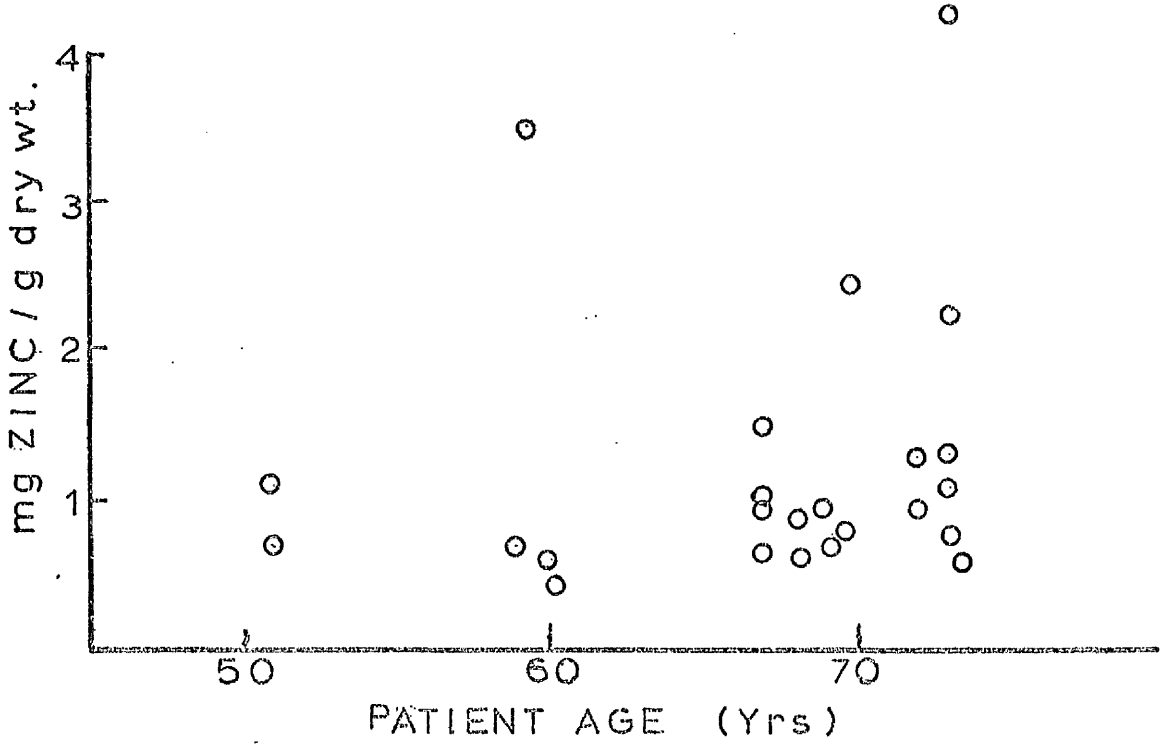
FIGURE 20.

Prostatic Zinc Content in Relation to Prostatic Weight and Patient Age

Zinc was determined in two samples from each of thirteen hyperplastic prostate glands and related to prostatic weight and patient age.

Figure 20. PROSTATIC ZINC CONTENT IN RELATION TO PROSTATIC WT. AND PATIENT AGE.





and adenocarcinomatous tissue (244 - 769 µg/g dry wt.). Within individual prostate glands there was also marked variation in the level of zinc in samples taken from two distinct sites. In hyperplasia this variation was most extreme in patient D.L. where there was a difference of 2850 µg zinc/g dry wt. between two sites. In adenocarcinomatous tissue a smaller difference of 538 µg zinc/g dry wt. was observed in patient R.S. It is important to point out that the carcinoma patients were not under estrogen treatment. The disease had been discovered only after histological examination of tissue removed for suspected benign hyperplasia.

Figure 20 shows that no relationship is apparent between the zinc content of hyperplastic prostatic samples and patient age or prostatic weight.

(b) Distribution of Zinc in Hyperplastic Prostatic Tissue

Since zinc is known to be a component of human prostatic secretion (Mackenzie et al., 1962) an initial experiment was conducted to determine whether any zinc was removed during the 0.15M NaCl washing procedure. Samples (5 x 1g) were taken from different areas of two prostate glands. Each sample was minced and washed with 0.15M NaCl as described in the methods section. The washed tissue was homogenised in 0.25M sucrose-T.K.M. solution and duplicate zinc and protein analyses were performed on both the NaCl wash and homogenate fractions. To correct for zinc contamination during the zinc determination control samples of 0.25M sucrose-T.K.M.

solution and 0.15M NaCl were also analysed and the value obtained subtracted from the relevant homogenate or NaCl wash sample values. Results are presented in Table 21.

The proportions of the total zinc and protein found in the extracellular fraction are relatively high. In prostatic tissue for patient P.A. 34% of the total protein and 39% of the total zinc was located in the extracellular fraction, while in the case of patient A.Mc. 21% of the total protein and 18% of the total zinc was present in the extracellular fraction. The complete distribution of zinc within hyperplastic prostatic tissue was studied, taking into account the large proportion washed out of the mince with 0.15M NaCl. Three samples (10g) of frozen hyperplastic prostatic tissue were used for this purpose. Cellular fractions were isolated as described in the methods section. Duplicate zinc and protein analyses were performed on each fraction. To correct for possible zinc contamination in the determination, zinc was also determined in suitable reagent controls and the value obtained subtracted from the sample value. Results (Table 22 and Figure 21) are represented according to the method suggested by de Duve (1955). In addition, values for the extracellular fraction are also included in this presentation. It can be seen that most of the prostatic zinc was located in the extracellular, nuclear and supernatant fractions (51.8%, 24.4% and 18% respectively).

(c) Relationship Between Zinc and 5α -Reductase Activity In Prostatic Homogenates

Tissue homogenates, prepared from fresh prostatic tissue,

TABLE 21.

Zinc and Protein Content of Extracellular Fraction

Concentration in Extracellular Fraction	P.A. Age 75 yrs. Prostatic wt. = 65g	A.Mc. Age 65 yrs. Prostatic wt. = 33g
mg Protein/g original tissue	17.7	11.2
% Total protein	33.9	22.1
µg Zn/g original tissue	40.3	5.1
% Total zinc	39.0	18.7
µg Zn/mg Protein	2.3	0.5
% Total Zn/% total protein	1.15	0.9

Results presented for each patient are the average of five determinations from distinct portions (1g) of tissue from different areas of the prostate.

TABLE 22.

Distribution of Zinc in Human Hyperplastic Prostatic Tissue

Fraction	Zinc	Protein	Relative Specific Activity †
Absolute Value	0.864mg	256.4mg	-
Extracellular	51.80	33.50	1.58
Intracellular	46.53	69.70	1.50
600 g Pellet Suspension ⁂ (Nuclear)	24.43	19.00	1.46
9,000 g Pellet Suspension (Mitochondrial)	0.70	3.13	0.21
105,000 g Pellet Suspension (Microsomal)	1.94	7.73	0.23
105,000 g Supernatant (Cytosol)	18.05	39.00	0.48
Total % Recovery in fractions	96.90	95.97	-

Results expressed as percentage of absolute value.

Results are the average of three experiments.

† = % Zinc in a specific fraction/% protein in that fraction.

⁂ corrected for DNA recovery (on average 70% of homogenate DNA was recovered in the 600 g pellet).

FIGURE 21.

Distribution of Prostatic Zinc

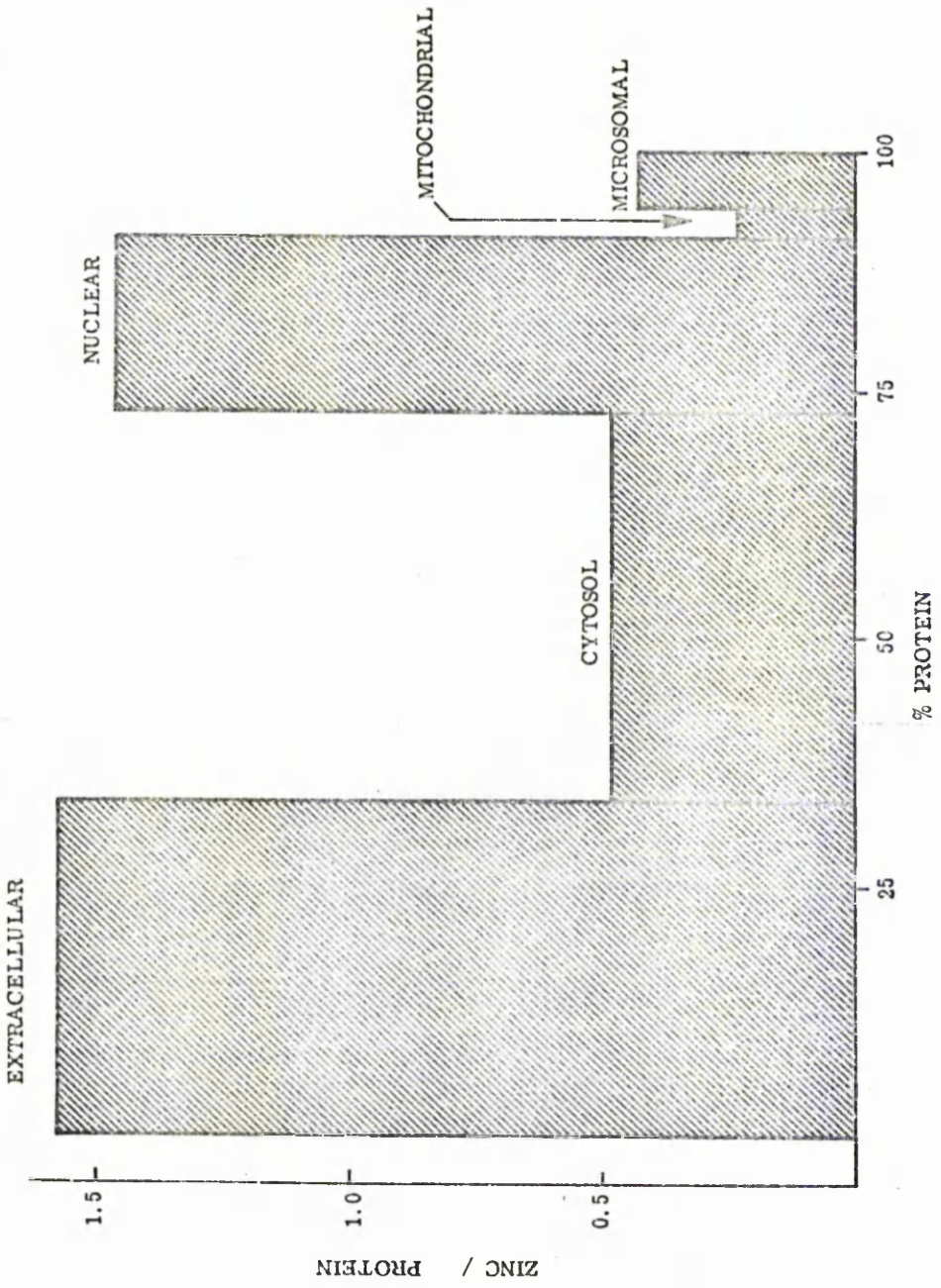
Results presented are recalculated from Table 22 on the basis of a 100% overall recovery.

Results are the average of three experiments.

Zinc/protein = % Zinc in a specific fraction/% Protein in a specific fraction.

Figure 21.

DISTRIBUTION OF PROSTATIC ZINC



were analysed for zinc and 5α-reductase activity. Samples (5 x 1g) were taken from each prostate gland. These samples were minced, washed, filtered and homogenised as described in the methods section. The final filtrate was made up to a volume of 10ml with 0.25M sucrose-T.K.M. solution and duplicate samples analysed for zinc and 5 α-reductase activity. To monitor for zinc contamination during the zinc determination control samples of 0.25M sucrose-T.K.M. solution were also analysed for zinc and the value obtained subtracted from the homogenate sample value. Results are shown in Table 23 and Figure 22.

Histological examination confirmed that four of these glands were hyperplastic. In the case of patient R.Mc., however, anaplastic malignant infiltration of lymphoid origin was diagnosed. This patient was subsequently found to be suffering from leukemia. The hyperplastic tissue results produce a coefficient of linear correlation of - 0.54. The best straight line to fit this data is shown in Figure ~~20~~ 22. The student-t value was 3.099 and with a two tail probability table the P value of <0.01 was obtained ($n = 19$). These results show that a significant negative relationship exists between the zinc concentration and 5α-reductase activity of human hyperplastic prostatic homogenates.

(d) Effect of Added Zinc on 5α-Reductase Activity of Prostatic Homogenates

An experiment was performed to determine whether zinc added to prostatic homogenates modified 5α-reductase activity. An homogenate was prepared from fresh hyperplastic prostatic tissue

TABLE 23.

Relationship between Prostatic Zinc and 5α -Reductase Activity

Patient and Details	µg Zn/mg Protein	S.A. * 5α -Reductase
J.N. Age 67	4.56	17.98
P.wt. 67g	2.20	19.60
B.P.H.	2.04	21.27
	3.94	16.31
	1.52	31.45
G.C. Age 86	3.84	16.06
P.wt. 135g	1.89	11.12
B.P.H.	2.55	71.85
	3.62	16.42
D.P. Age 73	1.41	30.39
P.wt. 62g	1.34	36.25
B.P.H.	1.69	34.22
	1.08	36.82
	2.99	33.98
D.B. Age 62	1.66	53.35
P.wt. 29g	1.62	55.59
B.P.H.	1.17	52.60
	2.15	31.60
	1.38	42.15
R.Mc. Age 76	0.68	17.91
P.wt. 44g	0.22	4.13
B.P.H.	0.50	41.55
	0.19	14.07
	0.24	6.54

* S.A. = Specific Activity = pmol/h per mg protein.

B.P.H. = Benign Prostatic Hyperplasia.

P.wt. = Prostatic Weight.

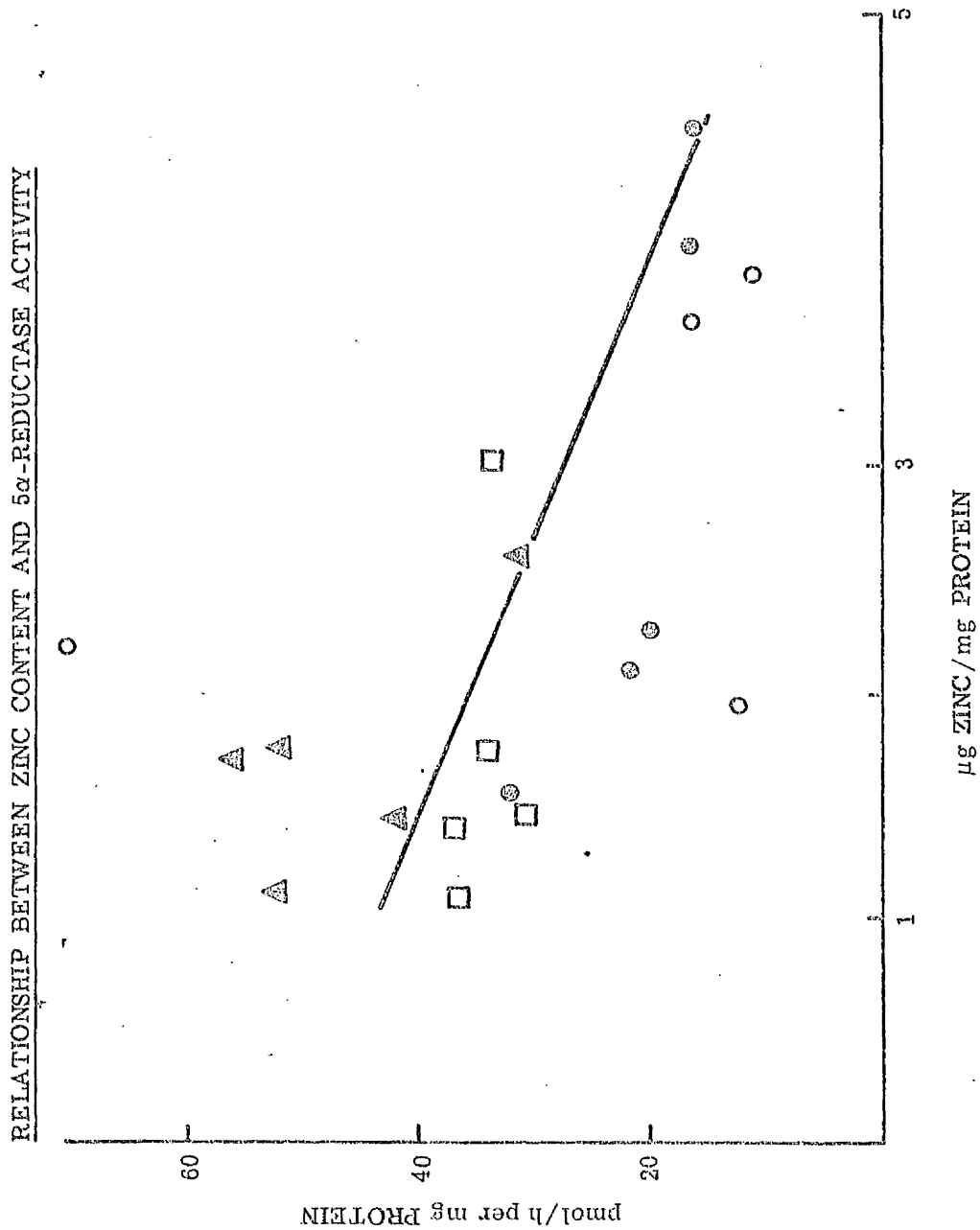
FIGURE 22.

Relationship between Zinc Content and 5α-Reductase Activity

Homogenates were prepared from samples(5 x 1g)from each of four hyperplastic prostates. These homogenates were analysed for both zinc and 5α-reductase.

	Patient's Initials
△	D.B.
□	D.P.
⊗	J.N.
○	G.C.

Figure 22.


RELATIONSHIP BETWEEN ZINC CONTENT AND 5α-REDUCTASE ACTIVITY
pmol/h per mg PROTEIN
60
40
20
1
3
5
µg ZINC/mg PROTEIN

as described in the methods section. Incubations were conducted, in duplicate, with increasing levels of added zinc. After all incubations the sum of ^{3}H-testosterone and ^{3}H-dihydrotestosterone accounted for at least 95% of the radioactivity. No ^{3}H-3α-androstanediol was detected.

The results (Figure 23) indicate that zinc caused inhibition of 5α-reductase activity of prostatic homogenates. Inhibition began at a zinc concentration of 10^{-6}M and was complete at 10^{-3}M.

(e) Effect of Chelating Agents and Dithiothreitol on 5 α-Reductase Activity in Prostatic Homogenates

Reversal of 5 α-reductase inhibition by zinc has previously been demonstrated in nuclear and microsomal fraction incubations by the addition of the thiol group containing reagent dithiothreitol, or the chelating agents EDTA or o-phenanthroline (see Section 3 a.). Since the concentration of zinc in prostatic homogenates bears an inverse relationship to 5 α-reductase activity, suggesting that the enzyme may in certain situations be under zinc inhibition, it was of interest to discover if these reagents modified 5 α-reductase activity in homogenate fractions.

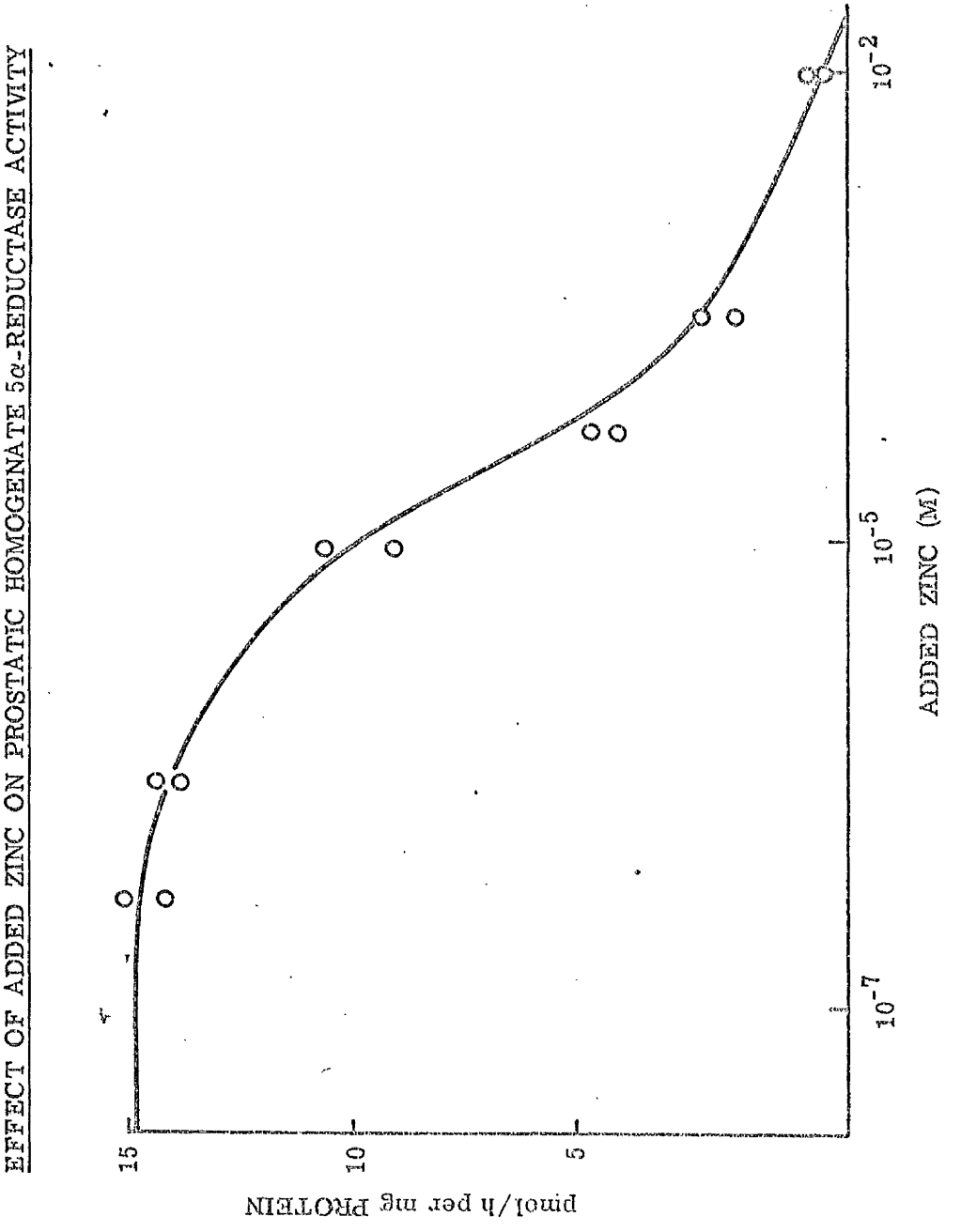
Homogenates were prepared from fresh hyperplastic prostatic tissue as described in the methods section. Incubations with ^{3}H-testosterone were performed, in duplicate, with the addition of dithiothreitol (10^{-2}M), EDTA (10^{-3}M) and o-phenanthroline (10^{-3}M). After all these incubations the sum of ^{3}H-testosterone and ^{3}H-dihydrotestosterone accounted for at least 95% of the radioactivity.

FIGURE 23.

Effect of Added Zinc on
Prostatic Homogenate 5 α-Reductase Activity

Homogenate incubations were performed in the presence of increasing concentrations of zinc.

Figure 23.


EFFECT OF ADDED ZINC ON PROSTATIC HOMOGENATE 5α-REDUCTASE ACTIVITY
pmol/h per mg PROTEIN
15
10
5
ADDED ZINC (M)
10^{-2}
10^{-5}
10^{-7}

No ^{3}H-3α-androstanediol was detected.

Results obtained (Table 24) are expressed as percentage of ^{3}H-dihydrotestosterone found in control incubations. Dithiothreitol had no effect, EDTA caused stimulation, and o-phenanthroline caused slight inhibition of prostatic homogenate 5α-reductase activity. The modifications of 5α-reductase activity by EDTA and o-phenanthroline are, therefore, very similar to those seen when these reagents were added to nuclear and microsomal fraction incubations. Although dithiothreitol caused stimulation of nuclear and microsomal fraction 5 α-reductase activity, in the present tissue homogenate incubations no stimulation occurred.

(f) Effect of Cytosol or Heated Cytosol on Microsomal 5α-Reductase Activity

The following experiments were designed to determine whether the cytosol fraction, which contains much of the intracellular zinc, could inhibit 5 α-reduction when it was added to microsomal fraction incubations. In the first experiment, microsomal and cytosol fractions were isolated from fresh hyperplastic prostatic tissue (5g). The cytosol fraction (50ml) was divided into two 25ml portions and one of these portions was heated in a boiling water bath for 20min, cooled in an ice bath and centrifuged at 600 g ($r_{av.}$ 17cm) for 10min to precipitate proteins. The supernatant fraction was decanted and termed the heated cytosol fraction. Duplicate incubations were performed of microsomes, cytosol, microsomes plus cytosol and microsomes plus heated cytosol

TABLE 24.

Effect of Dithiothreitol and Chelating Agents on Prostatic Homogenate 5α-Reductase Activity

Agent	% Control Activity	Average
Dithiothreitol (10^{-2}M)	99.2 102.5 101.6	101.6
EDTA (10^{-3}M)	115.8 126.3 130.8	124.3
o-phenanthroline (10^{-3}M)	95.2 85.4 81.7	87.5

with ^{3}H-testosterone. The activity of the 5α -reductase was determined.

The 5α -reductase activity detected in the cytosol fraction incubations was subtracted from the value obtained in incubations of microsomal fraction plus cytosol fraction, so that in these incubations the activity of the microsomal fraction alone could be seen. In microsomal plus cytosol incubations there was also a small conversion to ^{3}H-3α -androstanediol and this product was taken into consideration in the calculation of 5α -reductase activity. Results of this experiment (Table 25) show that the addition of cytosol to microsomal incubations caused stimulation, whereas addition of heated cytosol caused inhibition of 5α -reductase activity.

Further experiments were developed to investigate these effects. Microsomal and cytosol fractions were isolated from fresh hyperplastic prostatic tissue (10g). The cytosol fraction (100ml) was separated into two portions (60ml and 40ml). The first portion (60ml) was boiled and protein precipitated as previously described. A portion (30ml) of heated cytosol fraction thus formed was passed down a cation exchange column prepared as follows:- A glass column (0.5cm in diameter) was filled with washed Amberlite resin IR-120 (H) , which was suspended in distilled water to a height of 6cm. Amberlite was washed before use so as to remove fines. The resin was magnetically stirred in distilled water for 2min, allowed to sediment, and the supernatant containing fines was decanted by suction. Heated cytosol fraction

TABLE 25.

Addition of Cytosol Fraction to Microsomal 5 α-Reductase Incubations - Initial Experiment

Incubation	S. Activity *	% Microsomal Control
Control	40.3	100
+ Cytosol †	50.7	125.6
+ Heated Cytosol	26.7	66.1

* S. Activity = Specific Enzyme Activity = pmol/h per mg protein.

Results presented correspond to the activity of the microsomal 5α -reductase.

† in this case 5α -reductase and 3 α-hydroxysteroid dehydrogenase activity of the cytosol fraction were subtracted from the total activity.

was passed through the cation exchange column at a flow rate of approximately 3ml/min. To avoid dilution of the sample, the first 10ml of heated cytosol passed down the column was discarded. The following 20ml was collected and the pH carefully adjusted to 7.0, with 1M NaOH. This was necessary since after passage of the cytosol fraction through the column the pH was acid because cations had been exchanged for hydrogen ions. This fraction was then diluted to 30ml with 0.25M sucrose-T.K.M. solution and called the Amberlite heated cytosol fraction. Cytosol and heated cytosol fractions (20ml of each) were also diluted to 30ml before being used in incubations to ensure all fractions were at the same concentration. During passage through the cation exchange column the heated cytosol fraction lost 96% of its zinc and 70% of its protein.

Incubations of microsomal fraction with ^{3}H-testosterone plus the fractions shown in Table 26 were performed. As before the results presented correspond to the activity of the microsomal 5α-reductase after subtraction of the 5α-reductase and 3α-ketosteroid dehydrogenase activities of added fractions. Results for two experiments are shown in Table 26. These are in agreement with the previous experiment in so far as the heated cytosol fraction added to microsomal incubations caused about 30% inhibition. The pH of the heated cytosol fraction was checked and found to be unchanged at 7.0. This inhibition was, therefore, not due to a pH change.

A small activation of heated cytosol inhibition was achieved by the inclusion of EDTA (10^{-3}M) with these incubations.

TABLE 26.

Addition of Cytosol Fraction to
Microsomal 5 α-Reductase Incubations

Incubation	S. Activity *	% Microsomal Control
Control	28.9	100
	38.4	100
+ Cytosol †	34.3	119
	38.5	100
+ Heated Cytosol	20.2	69
	28.2	73
+ Amberlite Heated Cytosol	25.5	88
	39.2	102
+ Heated Cytosol and 10^{-3}M EDTA	25	85
	31	81

* S. Activity = Specific Enzyme Activity = pmol/h per mg protein.

Results presented correspond to the activity of the microsomal 5 α-reductase after subtraction of the 5 α-reductase activity of added fractions.

† in this case 3 α-hydroxysteroid dehydrogenase activity was also subtracted.

This activation is no greater than the stimulation of microsomal 5 α-reductase activity by EDTA (Table 16) and therefore does not constitute a reversal of heated cytosol inhibition. Passage of the heated cytosol fraction through a cation exchange column almost completely reversed the heated cytosol inhibition of microsomal 5α-reductase (Table 26). In experiments where cytosol was added to microsomal fraction a stimulation of approximately 20% of the 5 α-reductase activity occurred (Tables 25 and 26). This may be due to a pull exerted by a cytoplasmic 3α-ketosteroid dehydrogenase present in these incubations.

Zinc was determined in the cytosol and heated cytosol fractions and the concentration of this cation in incubations to which these fractions had been added was calculated. The effect that this level of added zinc would have had if added to 5α-reductase incubations was also calculated with reference to earlier results (Figures 13 , 14 and 15). The calculated value was compared to the actual inhibition by these fractions (Table 27). The calculations show that, in the case of heated cytosol, the inhibition of microsomal 5α-reductase activity is similar to that expected from the concentration of cytoplasmic zinc in the incubations. This is not the case for unheated cytosol incubations. These results indicate that in cases where the protein is denatured by heat cytoplasmic zinc may become free to inhibit 5α-reductase. In normal cytosol fractions zinc may be largely protein bound and unable to inhibit 5α-reductase activity.

TABLE 27.

Zinc Content of Cytosol and Heated Cytosol Added to Microsomal Incubations

	Cytosol	Heated Cytosol
Zinc Content	4.2×10^{-5}M	0.98×10^{-6}M
	2.7×10^{-5}M	0.90×10^{-6}M
Approx. Theoretical		
5α-Reductase	30 - 50%	16 - 31%
Inhibition	20 - 40%	15 - 30%
Actual 5 α-Reductase	(19% Stimulation)	31%
Inhibition	nil	27%

D. DISCUSSION

1. General Characteristics of Testosterone Reduction by Human Hyperplastic Prostatic Tissue

Certain characteristics of testosterone 5 α-reductase of the human hyperplastic prostate gland were found to be similar to those of rat ventral prostate as described by Frederiksen and Wilson (1971). In both species the enzyme is located in nuclear and microsomal fractions, in both NADPH but not NADH is required for activity, and in both the enzyme is unstable upon heating for long periods at 37°C. In addition the enzyme from each species has a similar K_m, and is inhibited to the same extent by certain divalent cations. No major difference was detected between nuclear and microsomal fraction 5α-reductase in the many experiments carried out in the present study. It is therefore likely that, as in the rat prostate, this activity in the human hyperplastic prostate belongs to a single enzyme entity.

It was originally intended to carry out a comparison of the 5α-reductase activity in normal and hyperplastic prostatic tissue, but this had to be abandoned because of the unavailability of fresh normal human prostatic tissue. Siiteri and Wilson (1970), however, in an as yet unconfirmed report, found that, although dihydrotestosterone was increased during hyperplasia, the 5α-reductase activity of normal and hyperplastic prostatic tissues was similar.

(a) Time Course of Reduction and the Effect of Trypsin Inhibitor

Time course experiments (Figures 11 and 12) revealed that under the present experimental conditions, activity of the human hyperplastic prostatic 5α-reductase increased linearly with time for 40min. Thereafter the enzyme progressively lost its activity and was inactive within 80min. This inactivation could not be explained by lack of cofactor or substrate. It is known that upon homogenisation of certain exocrine organs, stored secretory proteases and nucleases may be released, causing inactivation of cell organelles and constituents (Robinovitch et al., 1969). Since the prostate gland secretes proteolytic enzymes required for the liquefaction of coagulated semen (Mann, 1964) the presence of these enzymes in a cell-free system might cause enzymatic degradation of 5α-reductase. In time course experiments, however, no difference was observed between control incubations and incubations carried out in the presence of a known proteolytic enzyme inhibitor (soya bean trypsin inhibitor). This inhibitor eliminates some proteolytic enzymes as factors causing a time-related inhibition of 5α-reductase activity. It is of interest to note that the 11β-hydroxysteroid dehydrogenase studied in homogenates of rat submandibular salivary glands is degraded by certain proteolytic enzymes, an effect which may be overcome by the addition of soya bean trypsin inhibitor (McPhee, 1973).

(b) Activity of 3α-Hydroxysteroid Dehydrogenase in Homogenate Incubations

A cytoplasmic 3α-hydroxysteroid dehydrogenase has been

found in the rat ventral prostate gland (Bruchovsky and Wilson, 1968; Nozu and Tamaoki, 1973). In the human hyperplastic prostate gland, 3α -hydroxysteroid dehydrogenase activity has been shown in incubations with prostatic slices (Farnsworth and Brown, 1963; Siiteri and Wilson, 1970), minces (Acevedo and Goldzieher, 1963) and homogenates (Ofner, 1970). The 3 α-hydroxysteroid dehydrogenase is located in the cytoplasmic fraction isolated from human prostatic tissue (Chamberlain et al., 1966) as it is in the rat ventral prostate.

The prostatic homogenates in the present study appeared devoid of 3α -hydroxysteroid dehydrogenase activity, even when the concentration of cofactor or the incubation time was increased (Figures 10 and 12.). Lack of 3 α-hydroxysteroid dehydrogenase activity may be due to sensitivity of this enzyme to the rather extreme homogenisation procedure used in these experiments. Detectable 3 α-hydroxysteroid dehydrogenase activity, however, was found in human prostatic cytosol incubations, which makes sensitivity to homogenisation a rather unlikely possibility. It is more likely that the dilution of the homogenate during the assay procedure decreases the content of the 3 α-hydroxysteroid dehydrogenase, but not the 5 α-reductase, to such an extent that enzyme activity cannot be detected. In support of this possibility is the finding that a high ratio of 5α -reductase to 3α -hydroxysteroid dehydrogenase would be expected in human prostatic tissue (Jenkins and McCaffery, 1974). It is rather surprising that the 3 α-hydroxysteroid dehydrogenase of the human

hyperplastic prostate has not been more extensively investigated since it is possible that a decrease in the activity of this enzyme may account for the over-accumulation of dihydrotestosterone during prostatic hyperplasia (Siiteri and Wilson, 1970).

(c) Subcellular Distribution of 5 α-Reductase Activity

Chamberlain et al. (1966) have found that in the human hyperplastic prostate much of the 5α-reductase activity is located in the cytosol. Ofner et al. (1970), however, were unable to confirm this finding. They suggested that microsomal 5 α-reductase may be present on slowly sedimenting particles which were not completely precipitated from the cytosol fraction in the earlier study. Evidence from the present study would seem to confirm this suggestion, since in experiments in which microsomes were separated from the cytosol fraction by centrifugation at a high g force (105,000 g; $r_{av.}$ 2.55cm) for 60min, little 5 α-reductase activity was located in the cytosol.

The intramicrosomal site of the 5 α-reductase in human prostatic microsomes remains unknown. The intramicrosomal distribution of rat ventral prostatic 5 α-reductase is known to be similar to that of human placental enzymes related to steroidogenesis (Isurugi et al., 1971). In the rat ventral prostate, Nozu and Tamaoki (1974b) have shown an age-dependent shift of 5 α-reductase from the rough surfaced to the smooth surfaced microsomal fraction, and an age-dependent decrease of microsomal 5 α-reductase activity. The physiological explanation

for these findings remains uncertain. It is, however, known that microsomal enzymes are synthesized on the rough endoplasmic reticulum and transferred to the smooth endoplasmic reticulum where membrane catabolism takes place. The age-dependent shift of activity from rough to smooth microsomes may therefore be caused by a decrease in the degradation of the smooth membrane. The intramicrosomal distribution of enzymes related to steroidogenesis are discussed in a review by Tamaoki (1973).

The mitochondrial fraction isolated from human prostatic tissue was found to contain a small proportion of 5α-reductase activity. A similar situation has been found in the rat ventral prostate (Frederiksen and Wilson, 1972; Nozu and Tamaoki, 1973). It is possible that this activity is due to contamination of the mitochondrial fraction by endoplasmic reticulum. Further purification of the mitochondrial fraction by zonal centrifugation linked to studies of the distribution of microsomal enzyme markers as performed on adrenal mitochondria (Cowan et al., 1971) may prove useful in confirming this hypothesis.

A major problem in subcellular fractionation of human hyperplastic prostatic tissue is the isolation of pure nuclei. Extremely low yields of DNA are obtained, and nuclear purity, as judged by DNA/RNA ratio (Table 10) and light microscopy, does not compare favourably with rat prostatic nuclear fraction, or with the nuclear fraction isolated from guinea pig liver (Maggio et al., 1963). Mainwaring and Milroy (1973) described similar

problems in nuclear isolation from human hyperplastic prostatic tissue but Hansson and Tueter (1971) and Davies and Griffiths (1973) make no reference to such difficulties in their purification procedure.

As a result of cytoplasmic contamination, the amount of 5 α-reductase activity found in the nuclear fraction in the present study may be overestimated. In a careful study by Verhoeven et al. (1974) it was shown that, within limits, the quantity of the 5 α-reductase present in the nuclear fraction isolated from various rat organs was proportional to cytoplasmic contamination. Upon further purification of human hyperplastic prostatic nuclear fraction, an increase in specific activity of 5 α-reductase occurred (Table 13). This finding suggests a proportion of the nuclear fraction 5 α-reductase was not present as cytoplasmic contamination. Similar results were observed upon further purification of the microsomal fraction (Table 13).

The problemsinvolved in the isolation of prostatic nuclei may be related to the high content of fibromuscular, stromal and connective tissue in the human hyperplastic prostate gland (Franks, 1954). Filtration of the homogenate prior to subcellular fractionation removed much of this homogenisation resistent material, and resulted in higher nuclear yields. During centrifugation of the crude nuclear pellet through 2.0M sucrose some of the stromal connective tissue components still remaining were able to penetrate this dense layer. Similar problems are associated with nuclear isolation from muscle tissue

(de Duve, 1971). Purer nuclear fractions may be obtained by the use of organic solvents (Behrens, 1932) or citric acid (Higashi et al., 1966). These methods were avoided in the present study since the presence of such agents may damage membrane structures (Kier et al., 1967; Gurr et al., 1963).

(d) Kinetics of 5α -Reductase Activity

The apparent K_m values, determined by Lineweaver - Burk analysis, for the nuclear and microsomal 5α -reductase activity from human hyperplastic prostatic tissue were 0.105×10^{-6}M and 0.667×10^{-6}M respectively (Figure 17). These values are of the same order as those obtained for 5α -reductase from both rat prostatic nuclei and microsomes, and human skin microsomes, but are considerably lower than those found in rat liver microsomes (Table 28). The 5 α-reductase would thus seem to be much more active in liver, where its function is to deactivate circulating testosterone. In prostatic and skin tissue, where activity is lower, the function of the 5 α-reductase may be to activate circulating testosterone. The apparent K_m of the 5 α-reductase for testosterone in human hyperplastic prostatic tissue is at least ten times that of the testosterone concentration in the same tissue (Siiteri and Wilson, 1971). It is possible, therefore, that as soon as this steroid enters the prostate in an unbound form it will be converted to dihydrotestosterone.

TABLE 28.

Apparent K_m of the 5α -Reductase for Testosterone in Certain Tissues

Tissue	Subcellular Fraction	Apparent K_m x 10^{-6}M	Reference
Rat Prostate	Nuclear	0.62	Frederiksen and Wilson (1971)
Rat Prostate	Nuclear	0.32	Shimazaki et al. (1971)
Rat Prostate	Nuclear Microsomal	1.05 0.90	Nozu and Tamaoki (1973)
Rat Prostate	Microsomal	2.50	Roy (1971)
Human Skin	Microsomal	1.10	Voigt et al. (1970)
Rat Liver	Nuclear	20.30	Gustafsson and Pousette (1974)
Rat Liver	Microsomal	100	McGuire et al. (1960)

(e) Divalent Cation Inhibition of 5α -Reductase Activity

A number of enzymes are known which are both activated and inhibited by certain metal cations. For such enzymes inhibition usually occurs by cation concentrations greater than the optimum concentration for maximum activity (Hewitt and Nicholas, 1963). The findings of Grant et al. (1971) suggested that the nuclear 5α -reductase of human hyperplastic prostatic tissue was an enzyme of this type which was activated by low concentrations and inhibited by high concentrations of zinc. This hypothesis has not been confirmed. Results presented show that zinc inhibits nuclear 5 α-reductase activity at concentrations higher than 10^{-6}M but does not cause stimulation at lower concentrations (Figure 13). Microsomal 5α -reductase of human hyperplastic prostatic tissue and nuclear 5α -reductase of rat prostate showed similar results (Figures 14 and 15).

In an attempt to explain the difference between the observations of Grant et al. (1971) and those of the present study the experiment was repeated using nuclei washed with Triton X-100. This detergent was used by Grant et al. (1971) but avoided in the present study since it is a known inhibitor of 5α -reductase (Liao, 1974). The inhibition of nuclear 5 α-reductase by Triton X-100 is probably caused by disruption of the outer nuclear membrane on which the enzyme may be located (Moore and Wilson, 1972). The possibility was considered that a low concentration of zinc may, in some way, prevent membrane

disruption by Triton X-100. A finding adding strength to this hypothesis is that zinc has been implicated in membrane stability (Reynolds 1972; Chvapil 1973). Unfortunately, results presented provide no evidence for this hypothesis; zinc was unable to reverse Triton X-100 inhibition of nuclear 5 α-reductase (Table 14). No further explanation for zinc stimulation of 5α -reductase observed by Grant et al. (1971) can be offered.

Inhibition of both nuclear and microsomal fraction 5 α-reductase activity of human hyperplastic prostatic tissue was achieved by a range of divalent cations with the following order of effectiveness:-

$$Hg^{2+} = Cu^{2+} > Zn^{2+} = Cd^{2+} \gg Mn^{2+}.$$

Frederiksen and Wilson (1971) observed similar results for the 5 α-reductase of rat prostatic nuclear fraction.

Kinetics of zinc inhibition of the 5 α-reductase suggest that in both nuclear and microsomal fractions the inhibition may be non-competitive with respect to substrate (Figure 17) and competitive with respect to cofactor (Figures 18 and 19). It is, therefore, possible that zinc binds to a site at or near to the cofactor binding site.

A slight stimulation of human hyperplastic prostatic 5 α-reductase occurred in the presence of EDTA (Table 16). This may be due to the removal of endogenous or contaminating divalent cations. If this chelating agent is added to a zinc inhibited enzyme, reversal of inhibition occurred (Table 18 and Figure 16).

A much smaller reversal is achieved if a high concentration of citrate is substituted for EDTA. This phenomenon may be worthy of more thorough investigation since citrate, like zinc, is a component of seminal plasma and is found in high concentrations in the human prostate gland (Lutwak-Mann, 1963; Marberger et al., 1962).

(f) Thiol Group Involvement in 5α -Reductase Activity

Evidence presented in Section C.4. suggests that the 5α -reductase of human hyperplastic prostate may require an active thiol group for activity. The 5α -reductase of this tissue was extremely sensitive to the thiol group blocking reagent p-chloromercuribenzoate and was partially inhibited by high concentrations of iodoacetamide, iodoacetic acid and N-ethylmaleimide (Table 15). Reversal of p-chloromercuribenzoate inhibition was obtained by subsequent addition of dithiothreitol (Table 17 and Figure 16). High concentrations of dithiothreitol itself caused slight stimulation of 5α -reductase activity (Table 17).

Many enzymes which depend upon thiol groups for their activity are sensitive to low concentrations of some divalent cations (Hewitt and Nicholas, 1963). Moreover, Klotz (1954) suggests that where free thiol groups are binding sites for metals, the order of affinity for this binding is reflected by the relative magnitude of the solubility products of the metal sulphides formed. On the other hand, if chelating reactions occur the stability constants of the metal chelates formed

determine the relative affinities. The solubility products of metal sulphides in decreasing order are as follows:-

$$Hg = Ag \gg Cu \gg Pb = Cd > Zn > Co = Ni > Mn.$$

This is similar to the order of inhibition effected by some divalent cations on 5 α-reductase, suggesting that these cations inhibit by binding to a thiol group. Furthermore, inhibition of 5 α-reductase can be reversed by subsequent addition of dithiothreitol (Table 17 and Figure 15). Since zinc may bind to a site at or near to the cofactor binding site it is concluded that an active thiol group may be present at this site.

It is possible that a competitive inhibition with respect to cofactor would be observed if zinc bound to NADPH thereby reducing the effective concentration of the cofactor. There are several sites on the cofactor where zinc could bind, for example, at the adenine group (Wallenfels and Sund, 1957) or at the pyrophosphate group (Kosower, 1962). It was shown earlier that the 5 α-reductase requires an active thiol group. The order of divalent cation inhibition and the reversal of zinc inhibition by dithiothreitol suggested that these cations bind at a thiol group. It is, therefore, much more likely that zinc binds to a thiol group at, or near to, the cofactor binding site than to the NADPH itself. Many oxidoreductase enzymes are known to possess thiol groups which may play a role in catalysis (Jocelyn, 1972). Results presented in Section C.3. and C.4. indicate that human prostatic 5α -reductase may be an enzyme of

this type. Frederiksen and Wilson (1971) present an order of inhibition by divalent cations of the rat prostatic nuclear fraction 5α -reductase consistent with that expected if these cations inhibit by mercaptide formation with thiol groups. Roy (1971) found that rat prostatic microsomal 5α -reductase was strongly inhibited by 10^{-4}M p-chloromercuribenzoate, but not by 10^{-3}M iodoacetate or N-ethylmaleimide. Rat liver 5 α-reductase is also strongly inhibited by p-chloromercuribenzoate and this inhibition can be reversed by glutathione (5×10^{-3}M) [McGuire et al., 1960].

It is of interest to note that 5 α-reductase activity is slightly inhibited by o-phenanthroline (Table 16), a reagent which can also reverse zinc inhibition effected on this enzyme (Table 18 and Figure 16). This agent forms complexes with metals and is widely used as a chelating agent, particularly for zinc and ferrous ions (Vallee, 1960). Rabbit muscle fructose-1, 6-diphosphate aldolase is inhibited by this chelating agent (Kobashi and Horecker, 1967). Fructose-1,6-diphosphate aldolase from rabbit muscle, however, is not considered to be a metallo-enzyme (Rutter, 1964). Kobashi and Horecker (1967) consider that the inhibition of fructose-1,6-diphosphate aldolase by o-phenanthroline is due to the oxidation of thiol groups to disulphide groups by atmospheric oxygen, catalyzed by a metal - o-phenanthroline complex.

Since the 5 α-reductase may require an active thiol

group for activity, the oxidation of this group to a disulphide by atmospheric oxygen, catalyzed by o-phenanthroline, may explain why the 5 α-reductase is slightly innibited by this chelating agent. Furthermore, since the thiol group may be located at or near the NADPH binding site of the enzyme, it could participate in reduction by transferring hydrogen from NADPH to testosterone.

A possible outline of the process is shown in Figure 24. Glutathione reductase (Asnis, 1955), lipoamide dehydrogenase (Massey et al., 1960) and thioredoxin reductase (Moore et al., 1964) are three enzymes in which proof of this type of thiol group involvement, with transfer of hydrogen from the cofactor to the substrate, has been presented. Conclusive proof of a mechanism of this type for 5α -reductase, however, would require the use of a purified enzyme preparation, and was considered to be beyond the scope of the present study.

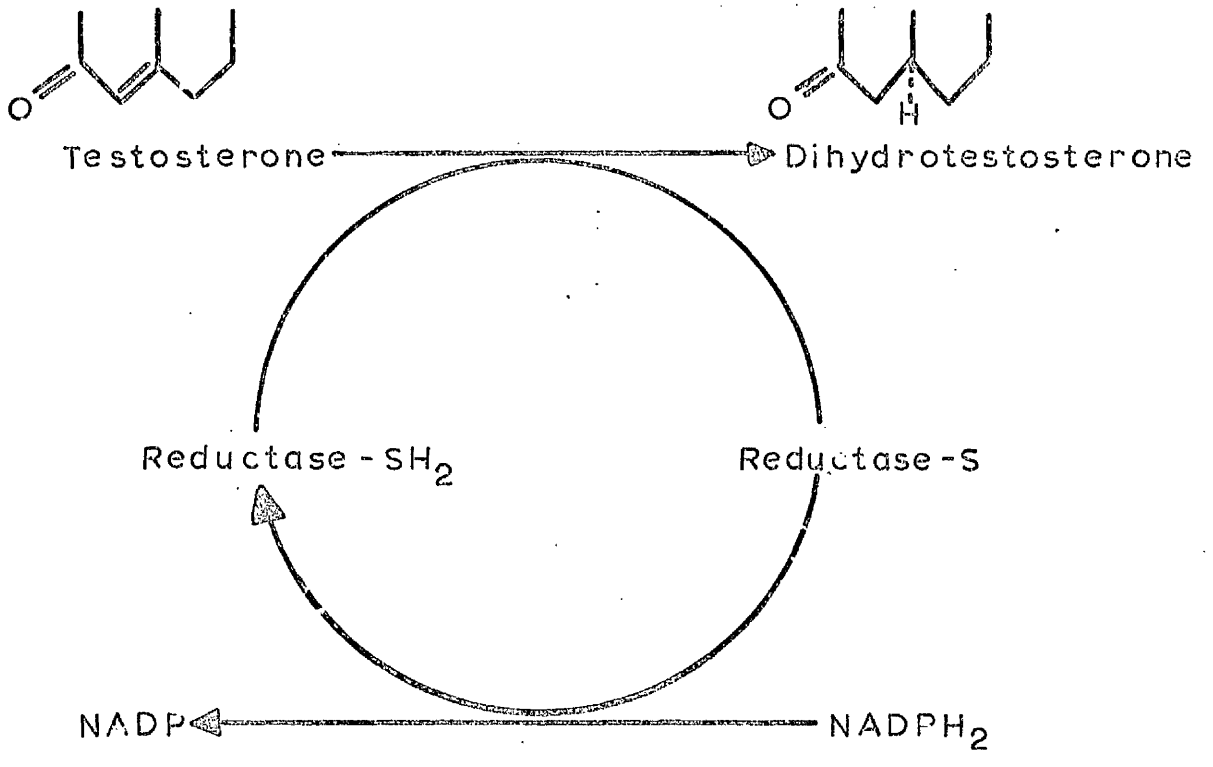
2. Zinc and Its Relationship to the 5 α-Reductase Activity in the Human Prostate

(a) Zinc Content of Human Prostatic Tissue

Many reports suggest that human hyperplastic tissue has a high zinc content while adenocarcinomatous prostatic tissue has a greatly reduced zinc level (see Table 1). Results presented confirm these observations (Table 11 and 20).

Unfortunately it proved impossible to include a comparison between the zinc content of normal and hyperplastic human prostates

Figure 24. POSSIBLE INVOLVEMENT OF THIOL GROUPS IN 5α-REDUCTASE ACTIVITY.



in this study because of unavailability of normal tissue. Although there are conflicting reports in the literature (Mawson and Fischer, 1952; Hoare et al., 1956) it would appear that the level of zinc, expressed as µg of zinc per mg dry weight of tissue, in the hyperplastic prostate is slightly greater than the level in the normal gland (Schrodt et al., 1964; Gyorkey et al., 1967; Gonick et al., 1969; Dhar et al., 1973). Since prostatic weight increases dramatically during hyperplasia a much more apparent increase will occur in the total zinc content of hyperplastic compared to normal prostate glands. The accumulation of such a high amount of zinc in the diseased organ may cause marked changes in the overall metabolism of zinc within the body.

A marked variation in the zinc content detected in both benign hyperplastic and carcinomatous glands in the present study was not due to lack of reliability in the experimental method used for measuring zinc. The reliability of this method is described in section C.1.(c). The variation in zinc content is more likely to be due to different amounts of zinc rich prostatic fluid stored in the samples analysed. The variations detected are in accordance with results from many other studies (see Table 1).

Since higher levels of zinc are found in hyperplastic than in normal prostate glands, a relationship may exist between zinc concentration and the degree of hyperplasia. No relationship,

however, was found between zinc concentration and hyperplastic prostate weight (Figure 20). However, since the gland is rarely removed completely during surgery prostatic weight may not be an accurate indicator of the extent of hyperplasia. Likewise, patient age did not bear any relationship to the zinc content of the removed prostate glands.

(b) Distribution of Prostatic Zinc

While studying zinc distribution within the human hyperplastic prostate consideration was given to the extracellular fraction. This was considered important since it is likely that high concentrations of zinc are stored in the extracellular compartment (i.e. lumen) of prostatic epithelium (Maquinay et al., 1963). To remove this compartment the prostatic mince was thoroughly washed with physiological saline prior to subcellular fractionation. This washing procedure may also remove small amounts of cell debris, blood contamination and intracellular fluid from damaged cells. The prostate gland, however, is rich in stored secretion. If a sample of surgically removed prostate is squeezed a relatively large volume of fluid can be forced out of the tissue. It is therefore felt that a large proportion of the fluid removed during the saline wash will be of extracellular origin. The saline wash also serves to prevent artifactual results obtained by the possible re-distribution of extracellular zinc during subcellular fractionation.

Investigation of the distribution of prostatic zinc within cellular fractions revealed that zinc is mainly located

within the soluble portion (extracellular plus cytosol fraction) and the crude nuclear fraction. These findings are in partial agreement with the distribution of zinc-65 in subcellular fractions isolated from human hyperplastic prostatic tissue after incubation of tissue homogenates with zinc-65 (Reed and Stitch, 1973). Dhar et al. (1973), however, obtained much lower values for the proportion of endogenous zinc in the cytosol fraction isolated from human hyperplastic prostatic tissue. No explanation can be offered for the different zinc distribution obtained in the latter study. Endogenous zinc levels measured in subcellular fractions isolated from the rat (dorso-lateral), monkey and dog prostates (Kar and Chowdhury, 1966; Webb et al., 1973) are also consistent with the zinc distribution reported in the present study. No allowance was made for zinc content of the extracellular fraction in any but the present study.

It is to be expected that zinc, a known component of prostatic secretion, should be present in the soluble portion of prostatic tissue. The high zinc content of prostatic nuclear fraction, however, requires some comment. Again it should be emphasised that zinc was not determined on pure nuclear preparations. Interpretation of results must, therefore, be approached with caution. Histological studies of this tissue have revealed that nuclei stain intensely for zinc (Gyorkey et al., 1967). Another non-destructive technique, using an electron microscope microanalyser, has confirmed that a

large portion of rat prostatic zinc is found within the nucleus (Chandler et al., 1974). It is concluded, therefore, that although the proportion of zinc found in the nuclear fraction in the present study may be an overestimate, it is probable that zinc is a component of hyperplastic prostatic nuclei.

Nuclear fractions of other male reproductive tissues are also capable of concentrating divalent cations similar to zinc. In two studies where cadmium-109 was injected into rats, approximately 40% of the testicular radioactivity was localised in the nuclear fraction (Chen et al., 1974; Johnson et al., 1970). In tissues such as liver, which contain a much lower level of zinc than that found in the hyperplastic prostate, approximately similar subcellular patterns were found with a high proportion of cellular zinc detected in the nuclear fraction (Thiers and Vallee, 1957). There are several important zinc metalloenzymes present in the cell nucleus such as DNA polymerase 1 (Springgate et al., 1973), but it is unlikely that these contain much of the nuclear zinc.

The high zinc level in prostatic nuclei may be a consequence of the high concentration of zinc found in the cytoplasm. The accumulation of nuclear zinc may occur as a result of the uptake of zinc from a zinc saturated cytoplasm due to binding of zinc to non-specific sites. Studies, similar to those of Bryan et al. (1974) on the intranuclear binding of Hg^{2+} in rat liver could help to elucidate whether nuclear zinc plays a functional role in the

biochemistry of the prostatic nucleus.

(c) Relationship Between Prostatic Zinc and 5α-Reductase Activity

As shown in Figure 22 a significant inverse relationship exists between the zinc content of fresh hyperplastic prostatic homogenates and 5α-reductase activity. This relationship is also apparent within different areas of three out of the four hyperplastic prostate glands studied, although statistical analysis would be invalid due to the small number of experiments performed on individual glands. In one prostatic sample, a negative relationship between zinc content and 5α-reductase activity was not found. Histological examination revealed that this gland was infiltrated with an anaplastic malignancy. This is an extremely rare condition to be discovered in surgically removed prostatic tissue. A malignancy of this type is associated with leukemia and not with prostatic disease. Properties of this tissue would not be expected to bear any similarity to normal, hyperplastic or adenocarcinomatous prostatic tissue.

It is rather unfortunate that normal or adenocarcinomatous tissue was not available for a study of the relationship between zinc and 5α-reductase activity. In the latter case, however, results similar to those described in the case of anaplastic malignancy may be expected due to the lower zinc levels (Table 1) and decreased 5α-reductase activity (Giorgi et al., 1973) in adenocarcinomatous tissue. It is regretted that, although gross histology was performed on prostatic samples, no information was

available as to the precise histological nature of the tissue sections removed for homogenisation. A more careful histological analysis may have revealed more information such as relationship between zinc and 5α -reductase activity and epithelial content of the tissue under examination.

Considering the high zinc concentration present in the prostate it must be assumed that this tissue has a high affinity for zinc. When zinc was added to prostatic homogenates the resulting inhibition of 5α -reductase activity (Figure 23) occurred in the same range of zinc concentrations as that seen for nuclear and microsomal incubations (Figures 13 and 14). This implies that any binding components which may be present in the homogenate are saturated with zinc. If this was not the case zinc added to homogenate incubations should immediately be bound to non-saturated zinc binding sites present and hence probably be unable to inhibit the 5α -reductase. In this situation zinc added to homogenate incubations should inhibit the 5α -reductase at a higher concentration than zinc added to nuclear and microsomal incubations which are unlikely to contain the zinc binding component responsible for the accumulation of zinc within the prostatic cell. This explanation for the experimental results reported assumes that bound zinc is unable to inhibit the 5α -reductase. Although this is probable no further evidence is available to confirm this hypothesis.

(d) Hypothetical Feedback Control Effected by Zinc on 5 α-Reductase Activity

Results of *in vitro* experiments carried out in the present study show that (a) an inverse relationship occurs between zinc content and 5 α-reductase activity, (b) prostatic homogenates may be supersaturated with zinc and (c) prostatic 5 α-reductase is a zinc sensitive enzyme, probably by virtue of an active thiol group at or near the NADPH binding site. This information leads to the suggestion that, *in vivo*, prostatic 5 α-reductase activity may be controlled by the concentration of zinc within the tissue. The results of an independent study by Habib *et al.* (1975) are also consistent with this proposal. These workers showed an inverse relationship between prostatic zinc and the level of dihydrotestosterone, the product of the 5α -reductase enzyme, in human prostatic tissue.

In a gland, such as the prostate, where secretory material is both synthesized and stored in preparation for episodic release some type of feedback control must exist. This feedback system must maintain secretory stores within narrow limits and must permit rapid accumulation of stores to follow release. A hypothesis involving zinc in a feedback control of prostatic secretion is postulated and illustrated in Figure 25. Upon entry into prostatic epithelial cells testosterone is rapidly converted, by the 5α -reductase, to dihydrotestosterone. Dihydrotestosterone, bound to a specific receptor, is transferred to the nucleus where it stimulates the synthesis of a specific messenger RNA. The dihydrotestosterone initiated message is then transferred back to the cytoplasm where

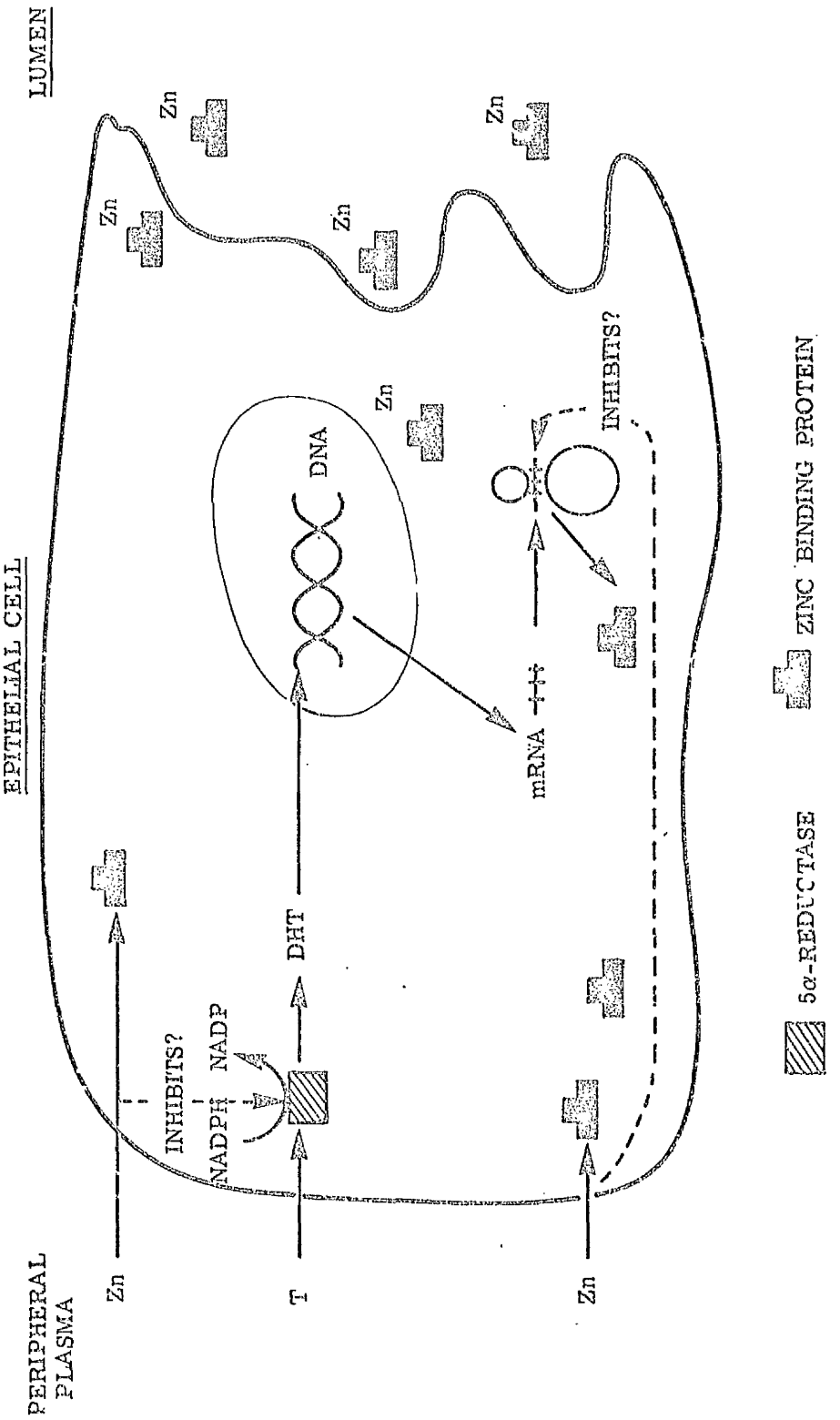


Figure 25. HYPOTHETICAL FEEDBACK CONTROL OF PROSTATIC SECRETION

it is translated into a protein which has a high affinity for zinc. Reed and Stitch (1973) have isolated and purified such a protein from human hyperplastic prostatic tissue. The zinc binding protein may be responsible for the accumulation of zinc from the peripheral plasma into the epithelial cells against a concentration gradient. As the epithelial cells and lumen become saturated with zinc the 5α-reductase will be inhibited by zinc, and this in turn will reduce the amount of zinc binding protein synthesized and reduce zinc uptake. Upon ejaculation the prostatic secretion containing much of this zinc is removed and testosterone is again able to be reduced to dihydrotestosterone. This prostatic system may be somewhat analagous to that suggested by Larkins et al. (1974) who suggest that as calcium accumulates in kidney tubule cells, it inhibits the hydroxylation of 25-hydroxycholecalciferol to the active 1,25-dihydroxycholecalciferol. The latter hormone is involved in the synthesis of a calcium binding protein by the tubule cells.

(e) Further Considerations

Although the hypothesis presented in Figure 25 provides a rather attractive explanation for the way in which prostatic secretion is controlled there are several considerations which cannot be ignored. For instance, the prostatic tissue used in this study was hyperplastic and not normal. Although benign hyperplasia is a disease which affects the size and number of prostatic epithelial cells and in some cases fibromuscular

elements, the secretory processes of the gland are not markedly impaired. It is therefore possible that the system involved in the control of prostatic secretion is similar in both normal and hyperplastic glands.

Artifacts may be produced by the use of tissue homogenates. During homogenisation considerable disruption of the prostatic cells occur which result in redistribution of cellular components. In this disrupted state prostatic zinc may be capable of inhibiting 5α -reductase activity. There is no conclusive evidence, however, that *in vivo*, where there is no disruption or redistribution such an inhibition would take place.

Methods which might partially overcome this problem could involve incubations of ^{3}H-testosterone with prostatic preparations which had not been as extensively disrupted such as mince or slice incubations. Because of the heterogeneity of human prostatic tissue, however, it would be extremely difficult to relate 5α-reductase activity to zinc content. In homogenate incubations this problem is partially overcome since a homogeneous preparation is obtained. Much of the stromal and connective tissue is also removed from this preparation by filtration.

Addition of reagents (EDTA, o-phenanthroline and dithiothreitol) which are known to reverse zinc inhibition of the 5α-reductase in nuclear and microsomal incubations, did not markedly stimulate reduction in prostatic homogenates. It is

possible that either these agents are deactivated during homogenate incubations before being able to act on the 5 α-reductase or that some other agent (the zinc binding protein?), which has a relationship with prostatic zinc, can inhibit the 5α-reductase. Citrate, which is related to zinc in the human prostate (both are components of prostatic secretion) has been eliminated as a compound able to inhibit 5α-reductase (Table 16).

Prostatic cytoplasm, which is known to contain a high proportion of the intracellular zinc (Figure 21) did not inhibit 5α-reduction when added to microsomal incubations. Heated cytosol fraction, however, was capable of inhibiting 5α-reductase activity (Table 25 and 26). The inhibitory factor present in heated cytosol could be removed by passage through a cation exchange column, a process known to remove zinc.

(f) Concluding Remarks

Much future research is necessary to clarify the feedback control hypothesis outlined in Figure 25 and to explain the contradictory observations outlined in Section (e) above. It is assumed that in the human prostate the zinc binding protein is under androgenic control via the nucleus. There is no experimental evidence presented for this, however, and other possibilities exist. Dihydrotestosterone could cause the accumulation of zinc by causing the conversion of pre-zinc binding protein to an active zinc binding protein.

The inhibition of the 5 α-reductase by zinc may be of a more complex nature. Calcium inhibition of kidney 25,hydroxy-cholecalciferol-1-hydroxylase, for example, may be associated with calcium influx into renal cells (Larkins et al., 1974). Depression of 5α -reductase activity by zinc may not be due to a direct effect on the 5α -reductase activity but may result from decreased synthesis of the enzyme. In this context Webb et al. (1973) have shown that protein synthesis by polyribosomes isolated from dog and rat (dorso-lateral) prostates may be modified by zinc.

An experiment that might provide useful information concerning the feedback control of zinc on 5α -reductase activity could be performed on the dog. It is known that pilocarpine hydrochloride will cause stimulation of prostatic secretion if injected into male dogs. For example, one hour after administration as much as 60ml fluid has been shown to be discharged from the gland (Huggins, 1945 and 1947). In this situation 5 α-reductase activity should be greatly increased within the gland due to a reduced zinc level.

Perhaps some questions concerning the androgenic control of prostatic secretion in the human prostate gland will remain unanswered until a more convenient system is developed for the biochemical study of human prostatic tissue. Surgically removed hyperplastic prostatic tissue, as used in the present study, is extremely heterogeneous and contains varying amounts of epithelial and stromal components. This leads to problems

when comparing and interpreting results from different experiments.

Another problem is that most research carried out on human hyperplastic prostate is hampered by lack of knowledge as to whether the results obtained apply to normal or only to diseased tissue. Difficulties occur in comparing results with experiments with fresh normal prostatic tissue which is extremely difficult to obtain. The great diversity in reproductive biochemistry may make comparison with normal prostatic tissue from other mammals unhelpful. The best comparison, however, could be with normal dog prostatic tissue since the dog is the only other species known with a high incidence of prostatic hyperplasia. If biochemical information is not available from normal human prostates results of experiments with hyperplastic tissue, although of importance in themselves, are unlikely to shed much light on the nature of prostatic hyperplasia.

Further difficulties arise from variations in parameters such as age, general health, prostatic weight, nutritional state, drug therapy and environmental status of patients. Reynolds and Dingle (1970) have used an organ culture system to study the effects of hormones and other agents on the movement of calcium into and out of bone. A similar system has been developed for the rat prostate (Lasnitzki and Franklin, 1972). It may be that the use of an organ or cell culture technique, which would by-pass many of the practical

problems inherent in the present approach, would facilitate further investigations into the relationship between zinc and androgen metabolism in the human prostate gland.

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