BIOCHEMICAL AND HISTOCHEMICAL INVESTIGATIONS
OF BENIGN PROSTATIC HYPERPLASIA

Being a thesis presented for the award of
a degree of Master of Philosophy at the
University of Surrey. February 1980

By D.C. Cannon FIMLS
Dept. of Pathology
St. Helier Hospital
Carshalton

in collaboration with
Faculty of Biochemistry
University of Surrey
SUMMARY

The thesis is presented as a series of chapters and with the exception of chapter one, each has a discussion section and is complete in itself.

The first chapter is an introduction to the human prostate covering its development and structure, and the nature of benign hyperplasia and its effects on the urinary system. Some aspects of research into the disease and their shortcomings are mentioned, together with modern surgical and other treatments.

The next chapter covers a histochemical study of human benign prostatic hyperplasia. In it levels and sites of hydroxysteroid dehydrogenases, aminopeptidase, acid phosphatase and cholesterol are examined and compared with those of normal tissue from young men. The third chapter is an extension of the histochemistry where the prostate is examined for endogenous steroids using immunofluorescence. For this purpose, steroid antisera were developed in guineapigs by injecting them with steroids that had been chemically bound to bovine serum albumin. In a controlled technique these antisera, when used in conjunction with fluorescently labelled anti-guineapig globulin, demonstrated androgen binding sites and sites of androgen production.

Chapter four describes an electrophoretic study of aminopeptidases and acid phosphatase extracted from normal and hyperplastic prostates. By this means six different
peptidases and over twenty bands of acid phosphatase were examined. The chapter also covers measurement of total and ester cholesterol in young, old but non-hyperplastic, and hyperplastic prostates. The following chapter covers the metabolism of testosterone in human prostate during six hour organ cultures and describes how eight metabolites were carefully separated and studied. Two different culture media are compared and varying degrees of tissue binding are shown by the metabolites.

Chapter six covers the changes that occur in organ cultures when cyproterone, cyproterone acetate and stilboestrol were included in small quantities. The final chapter is a discussion of all the investigations covered.
Acknowledgements

I would like to commence my acknowledgements with very grateful thanks to Professor D.V.Parke who first offered the opportunity for post graduate study and then guided and encouraged me through qualifying studies. Every post-graduate student knows the value of an academic advisor and I was particularly fortunate in having three. I am deeply indebted to Professor V. Marks and Doctor B. Gould from Surrey University for valuable criticism, advice and guidance and also to Doctor T.E.W. Goodier from St Helier Hospital who in addition arranged my interview with Professor Parke thus opening the way for this research. I wish to acknowledge Doctor L. Bernstock for his help during a staff shortage.

To attempt to mention all my scientific colleagues who have helped me would probably require a hundred names. Space allows only a corporate vote of thanks, but special mention must be given to Doctor Mary Moore and Doctor P. Lewis for invaluable help and co-operation, and also to Mr J. Lamberth and Mr P. Martin whose active and practical support over a long period lightened the burden of managing an effective department.

I wish to thank those who gave of time and service without the slightest obligation to help. In this respect my thanks go to Doctor S. Jeffcoate for a timely supply of steroid antisera, to Mrs Sandra Rapley and to Biorex of Canonbury for housing animals used in the study,
and to Panax of Mitcham for providing $\beta$-scans of some of the TLC plates.

My deepest thanks go to my wife who has showed patient understanding throughout the study and has untiringly typed and re-typed this thesis and all the research reports that were a preliminary to it. Finally, I shall ever be indebted to Doctor B. Gould for his very patient encouragement during the writing of this thesis, for without him I feel it would never have been completed.
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1.10 The Human Prostate Gland

The normal adult prostate gland is about 20g in weight and the size of a large horse-chestnut. It is a male accessory sex organ and it is found directly below the bladder and surrounding the urethra.

It is usual to speak of the organ in the human as if it were one gland, but this is probably only justified from its anatomical appearance. The prostate consists of gland tissue enclosed by trabeculae of plain muscle and collagenous connective tissue. The ducts of the glands open into the prostatic urethra. It is traversed by the ejaculatory ducts which pass through the posterior part of the gland and also open into the prostatic urethra. The gland secretes prostatic fluid which is stored in the gland ducts. During ejaculation the muscle contracts and the secretions are squeezed out and mix with the suspension of spermatozoa in seminal fluid coming from the ejaculatory ducts. The main constituents of the prostatic fluid are known, but their role remains uncertain.

Embryologically the prostate starts as five groups of buds from the posterior and lateral walls of the urethra (Lowsley 1912). The desire to follow the development of the five groups and other embryonic features to the adult stage, by relating their position to the prostatic urethra
and ejaculatory ducts, has lead to conflicting statements in the literature, and although some arise from different terminology the rest cannot be reconciled. The following together with Fig.1.01 is a description of the fully developed prostate using 'lobe' terminology and omitting the more controversial aspects. The inference that a lobular pattern can be seen is unjustified however for in the adult prostate the original groups have merged together with generally no lines of demarcation. Since the division into lobes is in part subjective, this reduces the value of the use of this terminology. This point should be borne in mind when studying Fig.1.01, in case the figure be interpreted as implying well defined lobes. However, Fig.1.01 does give some indication of the size and shape of the lobar division in common use.

According to many authorities the anterior prostate undergoes atrophy before puberty, its place being largely occupied by fibro-muscular tissue and consequently it is not involved in any later glandular hyperplasia. It may be therefore that to comply with this opinion the anterior prostate in the figure should be shown smaller and the lateral lobes enlarged in proportion.

It is the different names given to what is shown as the middle lobe that has caused much of the confusion found in the literature. Tissue in this area has been called the gland of Albaran and the subcervical lobe (Lowsley 1912 and Hutch 1972).
Fig. 1.01 Division of the prostate into lobes at three levels (according to anatomical theory). Age about 20 years.
It can be seen from Fig. 1.01 that the lateral lobes rather 'fill in' the remaining spaces after the other lobes have been defined.

The highest point of the posterior lobe is at the level at which the ejaculatory ducts enter the prostate. The posterior lobe is like a shallow cup fitted to the back and lower rear of the prostate gland.

The prostate was studied by Loeschke (1920) and Adrian (1922) who divided the organ according to its glandular structures. The gland was reviewed by Franks (1954) and Fig. 1.02 shows the type of diagram now to be found in most works involving the micro-anatomy of the prostate as a result of these studies. The main gland type is found in the posterior lobe and the ducts from them enter the lower urethra from the level of the verumontanum (colliculus seminalis) downwards. The other four lobes are made up of the periurethral (or mucosal) and submucosal type. The ducts from these lobes enter the upper urethra and urethral sinuses on either side of the verumontanum. The verumontanum is a somewhat ovoid projection, some 3 mm long, of the posterior wall of the prostatic urethra at about the mid-point and containing the prostatic utricle (Fig. 1.01).

Loeschke (1920) and Adrian (1922) both describe a definite capsule between the main gland area and the rest of the gland. Jacoby (1923) confirmed these findings but maintained that the capsule was not always recognisable.
Fig. 1.02  The arrangement of acinar glands and ducts within the human prostate gland.

When hyperplasia is present the capsule is often more obvious and provides a cleavage plane permitting the surgeon to remove the hyperplastic area and leave the flattened posterior lobe as part of the gland capsule. The fact that benign prostatic hyperplasia arises in the innermost (mucosal or peri-urethral) glands is probably beyond dispute. The position of the submucosal glands (Fig. 1.02) and their relationship to the inner capsule is not so clear. Hutch (1972) describes how the anterior, middle and two lateral lobes develop within the urethral wall and that the outer muscle layer of the urethra collectively surrounds these lobes of the prostate. The posterior lobe is external to the urethra although fused to the rest of the gland. Thus it appears that it is this muscle layer which is the basis of the inner capsule, described by anatomists and used by surgeons, separating the part exhibiting benign hyperplasia from the posterior or true prostate which seldom becomes hyperplastic without malignancy.

Hutch (1972) also described a median (or posterior commisural) lobe in one of his dissections from a young man, which was above the ejaculatory ducts between the middle lobe of Fig. 1.01 and the posterior edge of the gland. This lobe like the posterior lobe he considered external to the urethral wall. The median lobe was not found by him in three other dissections on older men.

Price (1963) states that the ventral prostate in rats relates to the anterior lobe in humans. Further
mention will be made of this because the ventral prostate from rats is often used by researchers indiscriminately, probably owing to its ease of dissection.

1.20 Benign Prostatic Hyperplasia

Human benign prostatic hyperplasia is a fibromuscular glandular hyperplasia that occurs in the prostate gland as the human male ages. In at least 80% of men of the white races the periurethral area of the prostate gland containing the mucosal and submucosal glands starts to grow with advancing years. It is possible for the enlargement to be noticeable before 30 years of age but symptoms tend to present after the age of 50. As the centre of the prostate swells it affects the urethra and the base of the bladder. There develops a progressive obstruction to the flow of urine. If this did not occur prostatic hyperplasia would rarely cause a medical problem.

The restrictions to urine flow are multiple and one or more of them apply in each case. The most obvious result of the enlargement is a tortuous stretching and compression of the prostatic urethra. As nodules of hyperplasia arise in the gland the geometry of the compression, can by chance, maintain urine flow and even improve flow where it has previously been restricted; so that a large gland may give no trouble whereas a comparatively small gland can cause retention.

With enlargement, especially in the middle lobe, the
gland pushes up the base of the bladder. The internal urethral sphincter muscle becomes disorganised and progressively less able both to release the urine and to prevent overflow. The sequence of events is as follows.

(1) Hyperplasia and thickening of bladder wall.
   Bladder emptying may still be complete.

(2) Neuromuscular changes.
   Smaller bladder volumes excite emptying and there is reduced inhibition from higher centres.

(3) Bladder dilation.
   Incomplete bladder emptying.

The musculature of the bladder is so arranged that as the muscle hypertrophies the wall becomes trabeculated. If the condition continues unresolved, the muscle becomes replaced with fibrous tissue and the bladder is turned into an inert bag. The constant pressure within the bladder often causes the wall between the muscle trabeculae to bulge outwards forming sacs or diverticulae. A bladder that is never completely emptied encourages bladder infection. In time this infection will work its way up the ureters and involve the kidneys. Another factor affecting kidney function is ureteric obstruction. The ureters have to pass through the muscle coat of the bladder and when the bladder muscle hypertrophies, the mouths of the ureters often become involved and urine flow restricted. The effect of age and urine retention often mean that prostatectomy patients have blood ureas of over 16mmol/l. If the urea cannot be reduced and held at this figure or
below it before the operation the chances of survival may be reduced.

At the present time the most successful treatment is the surgical removal of the hyperplastic tissue. Three forms of open surgery are used.

1. Suprapubic. The incision is through the lower abdomen and into the bladder and then down through the bladder floor to gain access to the gland.

2. Retropubic. The incision is again in the lower abdomen but then behind the pubic bone and into the anterior aspect of the gland.

3. Perineal. The approach is upwards via the perineum in this operation.

The first is the older method and has been modified by different surgeons. The second is the most popular method in Great Britain and is generally the favoured approach for very large glands. The third is more popular in the U.S.A. but it often renders the patient impotent. All these approaches can be successfully employed by a competent general surgeon although the statistics show the patient is much better off in a specialised urology department (Blandy 1974).

The best results in most cases are obtained by transurethral resection of the hyperplastic tissue. This method demands a long apprenticeship with the resectoscope and remains time consuming and tedious. In at least one in
ten cases the gland is too large for transurethral resection and open surgery is necessary. Inadequate resection may lead to retention years later from continuing hyperplasia of the remaining tissue.

Alternatives to prostatectomy do exist. Talwar and Pande (1966) revived a method of injecting a phenol, glycerol and acetic acid mixture to sclerose and shrink the hyperplastic tissue. Several injections are necessary and the patient has to be maintained on a catheter for some weeks with the accompanying danger of infection. Not all cases respond but the method has been favourably reported by Shipman and Akile (1967) Angell (1969) and Milroy (1968).

Another form of treatment that has not sustained its early promise is cryosurgery. A cooling probe is inserted into the gland and the tissue partially frozen. The damage that occurs provokes an immune response and the tissue shrinks under the antibody attack. Unfortunately the freezing needs to be repeated and it probably needs critical control to achieve the best results. Dangerous failures have occurred where the once frozen tissue has detached from the remains of the gland and entered the bladder from whence it has had to be removed by open surgery (Blandy 1974).

A third alternative to surgery has been called 'pills for the prostate'. Natural and synthetic androgens oestrogens and progestogens have all been used without any
conclusive results. Any sort of trial on the prostate is
difficult to assess and it may be worth looking at some of
the problems here. The objective is permanently to restore
urine flow without the patient becoming incontinent or
losing his sexual potency. In the first place the
retention will not have been caused by the actual size
of the hyperplastic enlargement but rather because the
urethra has become tortuous and pinched and because the
normal actions of the sphincter, trigone and detrusor
muscles, (which are important to the emptying of the bladder,)
have been affected by the enlargement. Therefore it is
the functional geometry of the enlargement that counts
rather than the actual size. Any treatment that aims at
causing the prostate to shrink will fail to restore urine
flow in some cases even if shrinkage always occurs.
Furthermore in some patients the secondary damage to the
bladder, ureter and kidneys is beyond recovery so that
even surgery would not be completely successful. Patients
often get a remission of the symptoms of retention without
any treatment whatsoever (Blandy 1974, Clarke 1937). The
patient himself can influence the clinical assessment.
He may exaggerate the effects of the treatment if he fears
surgery or play it down if he is anxious to get the operation
over.

Where pills are being used a blind or double blind trial
should be instituted but the numbers need to be large to
allow the statistical analysis to overcome the influencing
factors mentioned. The treatments may need to be continued
for months and the patient's condition can become acute during this time. The most serious problem is that those patients on a placebo cannot get better and may seriously deteriorate while on the trial (Theodorides et al. 1972).

It seems to me that although it may be possible to find a simple treatment that will shrink the prostate without serious side effects, the list of failures suggests that this expectation is unrealistic. The greater area of promise lies in finding the root cause of hyperplasia. The prevention may then be much easier than the cure.

The greatest setback in research is that there is no suitable animal model. Only dogs and mastomyx are known to develop any form of spontaneous benign prostatic hyperplasia and this differs from the human condition. The prostate in rats has been extensively studied. In this animal the lobes are anatomically discrete and although the lateral lobes are possibly the nearest tissue to that which undergoes hyperplasia in man (Price 1963) the ventral prostate has received nearly all the attention. The ventral prostate is more analogous to the posterior prostate in man and hence relevant to studies on malignant change. Too often the scientific papers make no attempt to relate the findings in rats to human disease so that the metabolism and functions of the rat ventral prostate becomes an area of scientific study per se. New drugs for the prostate are tested on animals. This
of course gives some protection against dangerous side effects but often little information on how they will work on humans.

Nevertheless the literature now contains a wealth of information on the human prostate and yet the cause of benign hyperplasia remains unknown. Certain facts are well established and perhaps should be mentioned here.

It has been believed since 1936 (Chang and Char) that Chinese races (Korean, Japanese) and Indians rarely develop prostatic hyperplasia. This is an important observation and it is a pity that there seems to be no recent study confirming the claim.

1.30 Research on the Cause of Benign Prostatic Hyperplasia

Probably all the recent research on prostatic hyperplasia has originated from the knowledge that eunuchs do not develop the condition. According to Grayhack (1972) the number of observation documenting the fact are few but it is reinforced by the absence of any reported exception. The overwhelming evidence for the cause of benign prostatic hyperplasia indicates that it is due to an endocrine imbalance.

In view of the anabolic effects of androgens on the prostate it would be encouraging if their circulating levels increased with age. In fact androgens decrease with age although testosterone is least affected. Sitteri
and Wilson (1970) found that prostatic tissue levels of dihydrotestosterone are increased in hyperplasia, a fact verified by others. This is important as it is felt that testosterone mainly exerts its effect after conversion to dihydrotestosterone (and other metabolites) and therefore testosterone has been called a pre-hormone (Baird et al. 1968).

Although castration before puberty prevents the disease, once it is established castration is of doubtful value. The prostate may shrink but quite a high proportion of those undergoing the operation do not benefit from this rather severe treatment.

Farnsworth (1975) drew attention to the large amounts of dehydroepiandrosterone sulphate of adrenal origin in the human circulation. He suggested that the effects of castration are offset to a varying degree by the dehydroepiandrosterone sulphatase activity in the prostate and the conversion of the free steroid into the other androgens normally derived from testosterone.

The mass of research on steroid metabolism in the prostate has so far failed to suggest why prostatic hyperplasia develops. Some researchers therefore have included the pituitary and its hormones in their study. Haugen (1973) in a post mortem study found that where prostatic hyperplasia was present there was a significant increase in the number of periodic acid schiff (PAS) positive basophil cells in the anterior pituitary.
Boyns et al. (1975) have shown that prolactin will increase the uptake of testosterone and dihydrotestosterone in organ cultures of human benign hyperplastic prostate and all three lobes of rat prostate. The PAS positive cells of the pituitary are known to produce luteinising hormone, follicular stimulating hormone and thyroid stimulating hormone but not prolactin.

It is the epithelial cells of the prostatic acini that seem to be most involved in steroid metabolism. Consequently there are a number of publications covering the effects of androgens on the growth of prostatic epithelium and mechanisms of transport, binding and protein synthesis. Changes and effect of androgens on the fibro-muscular stroma are not easily demonstrated and very little work has been published on the subject; yet Franks et al. (1970) have clearly shown that the epithelial tissue is dependant on the stroma and that its integrity is important for glandular growth and metabolism.

It may be that the fibro-muscular stroma of the human prostate proliferates before the glandular acini penetrate it (Deming and Wolf, 1939; Anderson, 1961; Cameron, 1974). This has never been proved conclusively but it is a widely held opinion based on microscopic appearances and in some cases the results of needle biopsies taken over many months. Franks (1969) has described the differing microscopic appearances of benign human prostatic hyperplasia. Very few researchers have used any histological grouping of their material and it may be unreasonable to expect tissue
in all of Franks' five groups to react in a similar way. It is perhaps fortunate that the majority of cases of benign prostatic hyperplasia fall into one group (5), which means that by chance most workers will have used histologically similar material. A summary of the Franks grouping is as follows:

1. The stromal (fibrous or fibrovascular) nodule.
2. The fibromuscular nodule.
3. The muscular nodule ("leiomyoma").
4. The fibroadenomatous nodule.
5. The fibromyoadenomatous nodule.

Classification of benign prostatic tumours can be arbitrary as in practice examples are found that come between groups and the microscopic appearances can vary considerably from place to place within the same specimen. Only the last two of Franks' groups show epithelial hyperplasia, but whereas in group five many of the cells are tall and secretory, in group four they are low and inactive.

All the human prostatic tissue used in this research was examined histologically and where it could not be classified as Franks group five, the results were not included. In practice the number of specimens rejected was few and most of these were never actually studied microscopically, but were eliminated because of their obviously fibrous appearance.

When research literature on the non cancerous prostate
is examined it appears that although some aspects have been studied in great depth others have not. It seems to me that a lot of study has been given to the rat ventral prostate and little to other prostatic glands in the rat, and that a moderate amount has been given to human hyperplastic tissue but very little to normal tissue from old men or (perhaps less surprisingly) from young men. At the start of this research it was established that normal tissue from young men could be obtained post mortem. Obviously for some studies the tissue would need to be removed very shortly after death and a surgeon collecting cadaver kidneys for transplanting gave assurance that he would provide normal prostate for the research. For other studies a few hours delay was not so important and a hospital providing an autopsy service for the coroner was able to help here. Retropubic prostatectomy operations to treat benign prostatic enlargement were frequently performed at St. Helier Hospital and once the fresh specimen had been sampled for routine histological examination the rest was available for research. These sources of material provided an almost unique opportunity to study comparatively normal and hyperplastic human prostates.

The tissue could be studied in three ways. The first was histologically, the second following extraction, and the third metabolically through organ culture.

Acid phosphatase, peptidase (Muntzing and Nilsson 1972) and cholesterol (unpublished information) were all
reputed to be increased in prostatic hyperplasia. It was
decided to examine these substances histochemically for
distribution in the gland and position within the cell,
and to extract them from the tissue and fractionate the
enzymes by electrophoresis and the cholesterol into ester
and total cholesterol, to see what differences could be
found between normal and hyperplastic prostatic tissue.
Endogenous steroids have never previously been demonstrated
histologically but an immunological technique was
developed that made it possible to demonstrate androgens
within the cell. A list of the androgens named in the
literature as being produced from testosterone by the
prostate was made. Also with the help of the literature
these were arranged in a probable metabolic pathway and
it became apparent that eight steroids needed simultaneous
examination if the fullest study was to be made. The
papers published on steroid metabolism in organ culture
of the prostate rarely considered more than three metabolites
of testosterone and this probably reflected the difficulty
of separating the different steroids. A thin layer
chromatographic system was developed that permitted the eight
steroids to be separated and then the metabolism of ($^3$H)
testosterone by the human prostate in organ culture was
studied. Another method of demonstrating differences in
steroid metabolism between normal and hyperplastic tissue
was to introduce oestrogens and drugs claimed to be anti-
androgens into the organ culture.

The main objective of this research therefore, was to
demonstrate biochemical differences between the normal and hyperplastic human prostate in the hope that this would point towards the aetiology of benign nodular hyperplasia of the prostate.
2.10 Introduction

The histochemical demonstration of enzymes differs considerably from the methods employed for the in vitro assay of enzymes in biochemistry. These differences impede a satisfactory comparison of results. The wide use of artificial substrates in both fields of study, especially in the case of hydrolases, has often resulted in two or more enzymes being demonstrated together when there is no biological association between them. In the case of peptidases Pearce (1972) said "Because there is virtually no correspondence between any histochemical peptidase and any biochemical enzyme possessing an E.C. number, it is probably best for the time being to use acceptable histochemical trivial names." Histochemical demonstration requires the enzyme to be insoluble when in fact the opposite is often the case. A mild fixation can reduce or prevent this solubility but to uncertain cost. The enzyme is inevitably altered structurally by the treatment and this could lead to a change in its activity and/or specificity.

Nevertheless with careful interpretation some comparison is usually possible. Aminopeptidase and acid phosphatase are studied here histochemically and in
chapter 3 after tissue extraction. A third group of enzymes the hydroxysteroid dehydrogenases are only examined histochemically. Cholesterol is also examined in sections and after tissue extraction.

Enzymes and cholesterol were studied to see if the results could in any way be associated with the amount of gland enlargement, or with the patient's age or body weight, or with the histological appearances.

2.11 Hydroxysteroid dehydrogenases (HSD)

Many tissues are able to metabolise steroids because hydroxysteroid dehydrogenases are widely distributed throughout the body. However substrate specificity varies from tissue to tissue and a 3α hydroxysteroid metabolised by one tissue will not necessarily be changed by another despite the presence of a 3α HSD in both tissues.

When selecting steroids as possible substrates two important considerations are apparent. (1) A steroid with more than one hydroxyl group cannot be used to identify a specific HSD. (2) If looking for a HSD in a tissue known to be involved in a particular pathway of steroid metabolism, selecting a steroid from that pathway as a substrate is more likely to give results than one selected from another pathway.

Eleven steroids (fig. 2.01) were selected as possible substrates for identifying HSDs in tissue from hyperplastic
Fig. 2.01 The steroids used as substrates in the hydroxysteroid dehydrogenase demonstrations.
prostates. The 4-pregnen 3,20β-diol and the 1,3,5 (10)oestratriene 3,17 diol are in conflict with (1) above, but were included because of their importance in steroid function and metabolism. Any reaction with them would have left some doubt about the enzyme(s) involved. Satisfactory methods and reliable techniques were established by first using the steroids on the testes and adrenals from rats between one and nine weeks old. The results of this study will be given later.

Baille et al. (1966) and Pearce (1972) have written of the problems of demonstrating HSD's histochemically. Some of the difficulties will be mentioned here. Once mixed the constituents of the reaction fluid are inclined to interact, even in the absence of enzyme and result in non-specific deposition of coloured formazan. The formazan is otherwise the final product of the enzyme initiated reaction and therefore this non-enzymic deposit has to be kept to a minimum. The substrate itself is not involved in this non-enzymic reaction. A duplicate slide subjected to the histochemical technique but with the omission of the steroid substrate in its reaction mixture will show the weak non-enzymic deposition and this reaction in the slide is called the 'nothing dehydrogenase'. This level of reaction has to be borne in mind and mentally subtracted from any result given in the test slide. The interference from 'nothing dehydrogenase' is increased if a pH above 7.0 is used for the reaction mixture. The 'non enzymic' formazan is produced throughout the fluid covering the slide. However,
if it is kept in suspension by increasing the viscosity of the fluid with polyvinylpyrrolidone and/or arranging the slide so that the formazan gravitates away from the section, then the 'nothing dehydrogenase' reaction can be kept fairly weak.

The last stage of the histochemical reaction that is initiated by a HSD is dependant on another enzyme called nicotinamide adenine dinucleotide (reduced) (abbreviated to NADH) diaphorase. This enzyme is widely distributed throughout tissues, and the final formazan will not be formed in its absence, (apart that is from nothing dehydrogenase levels). If another section is studied for diaphorase content any area that is positive in the test and yet negative for diaphorase cannot be due to a HSD. On the other hand if all areas positive for diaphorase are positive for a HSD this is also suspect. This second condition most probably indicates a soluble HSD which is forming NADH in the body of the reaction fluid and this NADH is then reacting with all the sites of diaphorase activity.

These last two paragraphs may be summarised in practical terms as follows:–

(1) Each HSD studied was controlled by a 'nothing dehydrogenase' slide which was subjected to a reaction mixture in which only the steroid was omitted. Taking 3pHSD as an example sufficient reaction mixture was prepared to use two steroid substrates separately (the
fifth and sixth in figure 2.01) and to do a single 'nothing dehydrogenase' which would control both substrates.

(2) Two slides were used to demonstrate NADH diaphorase. These slides were a control for all the other slides taken from this block of tissue at the one cutting session. The slides taken for diaphorase study were the first and last to be cut.

2.12 Aminopeptidase

Human and animal tissues contain a variety of peptidases with a wide range of catalytic abilities. In biochemistry many authors have used peptidases which although synthesised artificially could be expected to be found naturally in the tissues, whereas in histochemical studies to date only non biological synthetic substrates have been utilised. This is probably the main reason why histochemical peptidase cannot be related to any E.C. numbered peptidase.

The substrate used in this study was L-leucyl-\(\beta\) naphthylamide. Most of the published work refers to the enzyme(s) reacting with this substrate as aminopeptidase and sometimes leucine aminopeptidase. In a series of papers Sylven and his co-workers (Sylven and Bois 1962, 1964; Sylven and Snellman 1964, 1968; Sylven and Bois-Svenson 1964) have shown that hydrolysis of L-leucyl-\(\beta\) naphthylamide (LNA) could be due to any number of the following: (1) A metal dependant group of naphthylamidases. (2) A leucinamide-splitting manganese activated peptidase.
(3) Chymotrypsin. (4) Prolinase. (5) Carboxypeptidase and (6) Cathepsin B. It is probably best if reactions using LNA are spoken of as being due to hydrolysis by LNAase whilst bearing in mind the reaction can be due to a number of widely differing peptidases. It is worth mentioning here that Burstone and Folk (1956) maintained that in freeze-dried sections magnesium and manganese were contained in quantities able to activate LNAase. Cryostat sections as prepared for this study would retain magnesium and manganese in the tissue in the same way as freeze drying.

It was a paper by Muntzing and Nilsson (1972) that drew attention to the level of peptidase in the prostate and its increase (ten times) in prostatic hyperplasia. LNAase in the hyperplastic prostate was studied to see if the histochemical level could be related to another simple factor.

2.13 Acid Phosphatase

Acid phosphatase is widely distributed in human and animal tissues; prostate, spleen and liver being three of the richest sources. A number of different enzymes exist and although these are more easily distinguished from each other than those having peptidase activity, histochemically they are generally treated as if they were one enzyme. Prior to 1960 the histochemical methods were poor, but now a number of methods and modifications are available giving clear localisation of enzyme reaction.
They all use synthetic substrates. The feature usually regarded as typifying human prostatic acid phosphatase is its inhibition by DL-tartrate, although this inhibition is influenced by the substrate used (Smith and Whitby 1967). In fact the acid phosphatase of the adrenal cortex is equally sensitive to DL-tartrate and the liver enzyme also shows marked sensitivity (Goodlad and Mills 1957).

2.14 **Cholesterol**

Cholesterol is present in varying amounts in all animal tissues. Under certain pathological conditions cholesterol is freely deposited in the tissues in crystalline structure and it can then be easily demonstrated histochemically by Lieberman Burchard-type reactions. Where cholesterol is not so concentrated its demonstration is far more difficult and the results are variable. In humans, apart from well defined pathological conditions, a generalised mild deposition of cholesterol can occur with increasing age or be found following haemorrhage.

In hyperplasia cholesterol is occasionally found as crystals in the lumen of prostatic acini (Ogilvie 1967). The cholesterol content of prostatic fluid can be over 600 mg/100 ml (Documenta Geigy 5th ed.) and this may indicate a high cholesterol level in the tissue. Free cholesterol was demonstrated in tissue sections.
2.20 Methods

2.21 Preparation of Tissue

Fresh human hyperplastic tissue was obtained from retro-pubic prostatectomy operations. Hydroxysteroid dehydrogenases, acid phosphatase, amino peptidase and cholesterol in the tissue were all examined histochemically.

For the enzyme studies two pieces of tissue about 2x2x0.3 cm were taken from different areas of each specimen. These were frozen separately with liquid CO₂ from a gas cylinder and sectioned at 8 microns in a cryostat. The sections were allowed to visibly dry at room temperature and were then stored in a dessicator at 5 torr over silica gel at 4°C. All the enzyme studies were completed within 24 h with the hydroxysteroid dehydrogenases (HSD) being examined first. Rat tissue was examined as part of the HSD study and this was frozen, sectioned and stored in the same way.

Two separate pieces of hyperplastic prostate tissue were taken from each specimen for cholesterol examination and the processing of these pieces is given in the method.

Tissue from four normal prostates was studied for hydroxysteroid dehydrogenases, acid phosphatase, L-leucyl-β-naphthylamidase and cholesterol content. This tissue was the remains of the specimens used in chapter 3, and had been taken from men under 35 years of age a few hours after
death. The tissue had been stored at minus 40°C. It contained the three types of acinar glands found embedded in the prostate whereas benign hyperplastic prostate contains only peri-urethral (or mucosal) and submucosal glands. One piece of tissue from each normal prostate was sectioned in a cryostat and the slides stored as for the hyperplastic tissue. The piece taken was of necessity smaller than those taken from hyperplastic glands. After the section had been cut the remains of the piece of tissue was thawed and processed for cholesterol demonstration.

2.22 Technique for demonstrating NADH Diaphorase

Preparation of stock 0.2 M Buffer (pH 7.4).

To 20.7 ml of 0.2 M Tris (hydroxymethyl) aminomethane (24.2 g/litre) add 79.3 ml of 0.2 M-HCl.

Preparation of stock incubating solutions.

- Nitro-blue tetrazoleum (4mg/ml) 2.5 ml.
- Tris buffer (pH 7.4) 2.5 ml.
- MgCl₂ (5mM) 1.0 ml.
- Distilled water 3.0 ml.

Check the pH of these solutions and adjust to between pH 7.0 and 7.2 using stock 0.2 M-Tris or 0.2 M-HCl. These solutions, stored at -20°C are stable for several months.
Preparation of incubating media (Volume 1 ml.)

NADH  2 mg.
Stock solution 0.9 ml.
Distilled water 0.1 ml.

Check pH and adjust, if necessary, to pH 7.0 to 7.1 before incubating tissues.

Method

(1) Mount cryostat sections on coverslips.
(2) Deliver on to each section sufficient incubating medium to cover the tissue completely (approximately 0.2 ml.). Incubate (aerobically) for 10-60 minutes at 37° C.
(3) Pour off incubating medium and immerse sections in 15 per cent formol saline for 15 minutes.
(4) Wash in running tap water for 2 minutes.
(5) Rinse well in distilled water.
(6) Dehydrate in alcohols, clear in xylene and mount in synthetic resin.

Result Purple formazan deposits indicate enzyme activity.
2.23 Method for 3 Beta-Hydroxysteroid Dehydrogenase
(after Wattenberg, 1958 modified)

Incubating Medium (Volume 1.0 ml.)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD*</td>
<td>6.6 mg/ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>4.8 mg/ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Nitro-BT</td>
<td>2 mg/ml in DMF*</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Steroid</td>
<td>5 mg/ml in DMF</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Tris-HCl*</td>
<td>(pH 7.5, 0.2M)</td>
<td>0.6 ml</td>
</tr>
</tbody>
</table>

All the above solutions except MgCl₂ and Tris-HCl should be freshly prepared.

* NAD - niotinamide-adenine dinucleotide
* DMF - dimethylformamide
* Tris - Tris(hydroxymethyl)methylamine

Method

1. Remove lipids from cryostat sections by treatment with cold acetone (-20°C) for 20 minutes.
2. Dry sections in air.
3. Incubate at 37°C for 20-30 minutes.
4. Rinse briefly in distilled water.
5. Fix in neutral formol saline for 30 minutes.
6. Rinse in distilled water and treat for 2 minutes with 20 per cent ethanol.
7. Rinse in distilled water and counterstain nuclei (if required) in 2 per cent methyl green.
8. Wash and mount in glycerine jelly.
Result Purple formazan deposits indicate localization of the enzymes.

2.24 Method for 3 Alpha-Hydroxysteroid Dehydrogenase
(after Balogh, 1966)

Preparation of incubating medium.

Nitro-BT 5 mg, NAD 5 mg, EDTA 10 mg, add in order and all dissolved in 2 ml of 0.2M Tris-HCl pH 7.0. Then mix with 2 ml of 50% (w/v) polyvinylpyrrolidone in the same buffer. And finally add 5 mg of steroid in 1.0 ml of DMF.

Method

(1) Remove lipids from cryostat sections by treatment with cold acetone (-20°C) for 20 minutes.
(2) Dry sections in air.
(3) Incubate at 37°C for 20-30 minutes.
(4) Rinse briefly in distilled water.
(5) Fix in neutral formol saline for 30 minutes.
(6) Rinse in distilled water and treat for 2 minutes with 20 per cent ethanol.
(7) Rinse in distilled water and counterstain nuclei (if required) in 2 per cent methyl green.
(8) Wash and mount in glycerine jelly.

Result Purple formazan deposits indicate localization of the enzymes.
2.25 **LNA Method for Leucine Naphthylamidase**

(Nachlas, Crawford and Seligman, 1957 modified)

**Incubating Medium**

Dissolve 8 mg. per ml. of L-leucyl-β-naphthylamide in distilled water. This solution can be stored at 0-4° for several months.

- Stock substrate solution 1 ml.
- Acetate buffer (0.1 M, pH 6.5) 10 ml.
- Sodium chloride (0.85% w/v) 8 ml.
- Potassium cyanide (20mM) 1 ml.
- Fast blue B salt or Fast Garnet GBC salt or Fast Blue BB salt 10 mg.

**Method**

1. Incubate sections for 15 minutes to 2 hours at 37°.
2. Rinse in saline for a few seconds.
3. Immerse in 0.1 M cupric sulphate for 2 minutes.
4. Rinse again in saline.
5. Dehydrate in increasing strengths of alcohol to absolute, clear in xylene and mount in Canada balsam.

**Result** Using Fast Blue B salt the LNAase reaction sites are purplish. If step 3 is omitted the results are red. The results are red if Fast Blue BB salt is used but the colour can be changed towards blue by prolonging step 3. When Fast Garnet GBC salt is used the results are golden brown. All three dyes were tried and gave essentially
similar results. The precise details are covered in section 2.32. Fast blue BB salt was used for the majority of the study.


Preparation of Solutions

(1) Substrate solution
Napthol AS-B1 phosphate 50 mg
DMF 5 ml

(2) Buffer solution
Sodium acetate 1.17 g
Sodium barbitone 2.94 g
Distilled water to 100 ml

(3) Sodium Nitrite
Sodium nitrite 400 mg
Distilled water 10 ml

(4) Pararosanilin-HCl stock
Pararosanilin hydrochloride 2 g
2 N-Hydrochloric acid 50 ml

Heat gently, cool to room temperature and filter.

(5) Distilled water

Preparation of Incubation Solution

Solution 1 0.5 ml
Solution 2 2.5 ml
It is necessary for the success of the technique that equal parts of solutions 3 and 4 are mixed together and allowed to stand for two minutes before being added to the rest of the incubating medium.

The final pH should be between 4.7 - 5.0. It is adjusted if necessary with 0.1 N NaOH.

Incubation Method
(1) Incubate sections at 37°C for 15-60 min.
(2) Wash in distilled water.
(3) Counterstain in 2% methyl green (chloroform extracted).
(4) Wash in running water.
(5) Either (a) mount in glycerin jelly
    (b) dehydrate rapidly through fresh alcohols to xylene and mount in D.P.X.

Results Acid phosphatase activity: red
Nuclei: green.

2.27 Bismuth Trichloride Method for Cholesterol
(Grundland, Bulliard and Maillet, 1949 modified)
Preparation of the Reagent
Bismuth trichloride 0.2 g
<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl chloride</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Anhydrous nitrobenzene</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

**Method**

1. Fix small pieces of tissue for 24-48 hours in 70% (w/v) alcohol saturated with digitonin.
2. Place tissue in distilled water for 12 to 24 hours and change the water at least once during this time.
3. Cut cryostat sections 10μ-thick.
4. Dry in a dessicator with silica-gel overnight at 4°C.
5. Expose to the bismuth trichloride reagent for 15-45 minutes.
6. Rinse rapidly in 10% (w/v) acetyl chloride in nitrobenzene.
7. Wash in benzene.
8. Mount in liquid paraffin.

**Result** Cholesterol appears brown, its esters are not revealed. (The preparations are stable for a long period.)

Further treatment was employed to accentuate the colour of the reaction. For this purpose proceed thus after stage 6:

1. Wash in absolute alcohol/conc. nitric acid (1:3) to remove excess bismuth.
2. Wash rapidly in absolute alcohol.
(3) Immerse in a dilute solution of yellow ammonium sulphide.
(4) Wash in absolute alcohol.
(5) Clear in xylene and mount in Canada balsam.

This accentuation stage was always found to be necessary.

2.30 Results and Discussion

2.31 Hydroxysteroid dehydrogenase (HSD)

The methods and substrate were first tested against rat tissue as mentioned in section 2.11. Where a steroid proved to be a satisfactory substrate it did so for both the testes and the adrenals. The results are shown in table 2.01.

Figures 2.02, 2.03 and 2.04 show the appearance of the reactions obtained. Lording and Kretser (1972) suggested that two generations of interstitial cells exist in the young rat. The varying reaction shown with dehydroepiandrosterone in Table 2.01 indicates that there are changes in at least the level of a 3αHSD between one and five weeks of life. The photographs (figures 2.02 - 2.04) show the changing appearances of HSD activity in sections of rat testis with age. Just after birth the reaction is intense and confined to single or small groups of cells easily distinguished from the rest of the interstitial tissue. Within a few weeks the previously non reactive tissue starts to exhibit HSD reactivity
<table>
<thead>
<tr>
<th>Order No. in figure</th>
<th>Name</th>
<th>Reaction of Testis</th>
<th>Reaction of Adrenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Pregnenolone</td>
<td>++++ throughout the age range tested</td>
<td>++++ throughout the age range tested</td>
</tr>
<tr>
<td>4</td>
<td>4-pregnen-3,20βdiol</td>
<td>++++ throughout the age range tested</td>
<td>++++ throughout the age range tested</td>
</tr>
<tr>
<td>5</td>
<td>Androsterone</td>
<td>++ throughout the age range tested</td>
<td>++++ throughout the age range tested</td>
</tr>
<tr>
<td>7</td>
<td>Dehydroepiandrosterone</td>
<td>++++ to week 4, +++ at week 5 and ++ thereafter</td>
<td>++++ throughout the age range tested</td>
</tr>
<tr>
<td>8</td>
<td>Epiandrosterone</td>
<td>++++ throughout the age range tested</td>
<td>++++ throughout the age range tested</td>
</tr>
</tbody>
</table>

Table 2.01 The reactions given by rat testis and adrenals (taken from animals between one and seven weeks old) in hydroxysteroid dehydrogenase studies.
**Fig. 2.02** Hydroxysteroid dehydrogenase reaction using epiandrostosterone as substrate in two week old rat testis x200 magnification

**Fig. 2.03** Hydroxysteroid dehydrogenase reaction using epiandrostosterone as substrate in four week old rat testis x200 magnification
Fig. 2.04 Hydroxysteroid dehydrogenase reaction using epianandrosterone as substrate in seven week old rat testis x200 magnification
(figure 2.03) and usually by seven weeks the compact areas of reaction seen in the younger tissue can no longer be distinguished. The same changes were shown by the fluorescently labelled and peroxidase labelled steroid antisera used in chapter four. On two occasions epiandrosterone gave a weak (+) reaction in some sertoli cells of the tubules. It did not do so when tested against testicular tissue from the twelve week old rats. A $\Delta 5,3^\beta$ HSD can be shown to exist separately from $3\beta$HSD (Baillie et al. 1966) and this is probably being demonstrated by dehydroepiandrosterone.

As previously stated a steroid found to demonstrate a HSD in one tissue will not necessarily work as well or at all in another, therefore all the eleven steroids listed in figure 2.01 were used on sections of hyperplastic prostate.

Twenty operation specimens were examined for HSD activity. The diaphorase studies showed the presence of enzyme throughout the tissue although the epithelial cells were the most active. The reactions obtained for prostatic HSDs have to be classed as inconclusive. There is so much scientific evidence for steroid metabolism in the prostate that there can be no doubt that the tissue contains HSDs. With the $3\beta$hydroxy substrates the formazan deposits were stronger than those present in the 'nothing dehydrogenase' slide; but with the experience gained from the study of the testicular and adrenal reactions in rats, to have even called the results 'weak' would have been overstating the case. Such reactions as were seen were obtained following
prolonged incubation and were only present in the epithelial tissue. Pearce (1972) does not recommend the use of phenazine mephosulphate and its inclusion in the reaction mixture did not seem to help. The four normal prostates gave negative results for HSD activity. This normal tissue had been stored for several weeks at minus 40°C and the storage may have affected the activity. In the study with rat tissues the freshest sections did seem to work best, however Baillie et al. (1966) claimed results from museum specimens which were presumably months if not years old.

2.32 \textit{L-leucyl-\beta\-Naphthylamidase (LNAase)}

The LNAase results obtained were not assessed easily. Using a microscope with a low power lens the activity could be seen to change from field to field, although the reaction was always predominately in the epithelial tissue. Furthermore the precise location of the enzyme reaction differed between Fast Blue BB salt and Fast Garnet GBC salt. These different points can best be considered by studying the photographs of figures 2.05 to 2.07.

Figure 2.05 is a medium magnification of mainly epithelial tissue where Fast Garnet GBC salt has been used and the nuclei are counterstained with haematoxylin. The dye gives rust-brown areas of activity. Not all the cells have reacted and further round the same acinus the results were in fact completely negative. Figure 2.06 shows
Fig. 2.05 L-leucyl-β-naphthylamidase reaction in human hyperplastic prostate using Fast Garnet GBC salt. Nuclei are stained with haematoxylin. x820 magnification

Fig. 2.06 L-leucyl-β-naphthylamidase reaction in human hyperplastic prostate using Fast Garnet GBC salt. x820 magnification
Fig. 2.07  L-leucyl-\(\beta\)-naphthylamidase reaction in human hyperplastic prostate using Fast Blue BB salt. 
\(\times 820\) magnification
another mainly epithelial area with the same dye, but without the haematoxylin staining of the nuclei. The nuclei can be distinguished in this photograph as pale homogenous agranular areas. Figure 2.07 in contrast shows the results obtained using Fast Blue BB salt. Here some of the nuclei (those of the basal layer of acinar cells) are strongly positive, while other epithelial nuclei show no reaction. The cytoplasmic reactions involve all the cytoplasm of every cell in this part of the tissue. This is in contrast with figures 2.05 and 2.06 where the reaction involved only part of the cytoplasm of some cells and where some cells gave no apparent reaction at all. Overall Fast Blue BB salt gave as many positive areas as Fast Garnet GBC salt and in duplicate slides the same areas reacted.

A possible explanation is that some dyes can penetrate membrane barriers more easily than others. This enables them to be involved in the reaction at the site of the enzyme whereas they might otherwise have to wait for the products of the enzymic reaction to diffuse through the membrane in the other direction before being involved. If from the photographs one concludes that the Fast Garnet GBC salt reaction has occurred within a membrane (which is the most likely from appearances) the nuclear reaction of the Fast Blue BB salt remains unexplained. Characteristically the basal cells of the prostatic acini disappear in areas of carcinomatous change of the prostate.
Twenty operation specimens were examined for LNAase activity using Fast Garnet GBC salt. As already described, two pieces of tissue from each specimen were sectioned. None of the slides gave a negative result and the weakest reaction was graded +. The strongest reaction was graded ++++ and on this scale all the results were assessed. Table 2.02 shows the results obtained and other details relative to the patients. It will be seen that it was quite common for one slide of a pair to give a low reaction while the other gave a strong reaction.

Table 2.02 shows how the patient's details were grouped because it was hoped that by this means a pattern would be more obvious. Apart from visual study, punched cards were used in conjunction with a statistician's needle utilising the grouped figures, but no pattern or correlation was found.

2.33 Acid Phosphatase

Fig. 2.08 shows a typical result of acid phosphatase histochemistry by the method of Barka (1960). Two other methods (Gomori 1950 and Grogg and Pearse 1952) were tried but the results were not so sharp. The enzyme reaction was extremely strong. After only two to three minutes it had to be stopped to prevent diffusion from the active acinar cells into those of the stroma. It can be seen from the methods quoted that the author found that with other tissues the reaction can take up to sixty minutes.
<table>
<thead>
<tr>
<th>Age in years</th>
<th>Weight of prostatic tissue removed in grams</th>
<th>Patients weight in kg</th>
<th>LNAase activity (scale 1 to 4) 2 slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>31</td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>59</td>
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<td>86</td>
<td>63</td>
<td>66</td>
<td>1</td>
</tr>
</tbody>
</table>

(1) 30 grams and below  
(2) 31 - 50  
(3) 51 - 70  
(4) 71 - 90  
(5) 91 grams and over

(1) 60 - 69 kilograms  
(2) 70 - 79  
(3) 80 - 89  
(4) 90 - 99

Table 2.02 A comparison table in which some details of patients undergoing retropubic prostatectomy are compared with the L-leucyl-β-naphthylamidase reaction of the tissue removed. No correlation was found.
Fig. 2.08  A demonstration of acid phosphatase activity in the human hyperplastic prostate by the method of Barka (1960)  x200 magnification
The strong level of activity was found in every prostate examined. Altogether twenty-four glands were examined. Four were from young men under the age of thirty-five and were therefore considered to be normal prostatic tissue.

The enzyme level of these specimens was indistinguishable histochemically. The normal tissue seemed to have the same level of activity as the hyperplastic tissue. A few pieces of hyperplastic tissue were re-sectioned after being stored for two months at minus 40°C and their acid phosphatase activity was unimpaired. It was shown however that freezing and thawing the tissue six times reduced the acid phosphatase activity (besides damaging histological appearances of the tissue).

2.34 Cholesterol

The histological demonstration of cholesterol is comparatively easy where deposition has occurred. Where cholesterol is dispersed through the tissues without areas of concentration in what can be considered 'normal levels', the demonstration is far more difficult. Fig. 2.09 shows one of the best results. The fibro-muscular stroma has given a weak positive reaction but the glandular tissue is negative. All the slides studied were of this type, namely positive stroma and negative glands. The demonstrations were inclined to be patchy and although this may well have reflected a variation in cholesterol concentration from area to area it was felt that the variation was due to the method being capricious.
Fig. 2.09  Cholesterol in the human hyperplastic prostate by the modified method of Grundland et al. (1949).

x370 magnification
Consecutive cryostat sections were cut prior to fixation and 'stained' by a modified method and these were also patchy, but the results varied from section to section. This does not prove the patchiness is an artefact but it does make it quite likely. The reaction was graded using a microscope with a low power objective. A strong result was judged as one where a good proportion of the stroma was positive with a good intensity of reaction. The best results seen were given (++++) and the weakest positives seen a (+). No specimen was completely negative. The two pieces of tissue taken for cholesterol demonstration from each specimen were processed together and usually gave the same reaction grade in the final sections. The results did not relate to any of the other stated parameters applied to the tissue. Another disappointment was a failure to relate the findings with the level of free cholesterol extracted from the tissue (see chapter 3) the results of which were available for some of the specimens.
CHAPTER 3.

IMMUNO-HISTOLOGICAL STUDY OF ENDOGENOUS STEROIDS IN HYPERPLASTIC PROSTATIC TISSUE.

3.10 Introduction

Before studying the biochemical function of any substance a specific and sensitive assay method for it (and sometimes for its metabolites) is necessary. In the case of testosterone (and its metabolites) the existing techniques of radioimmunoassay and gas chromatography are not sufficiently specific and sensitive for widespread use. For this reason, radioactive labels are frequently used in metabolic studies of these steroids in animals. When radioactively labelled testosterone is injected into a living animal it will mix with and be metabolised as endogenous testosterone. Thus the label can be used to mark and measure the testosterone and its metabolites in the animal. Such an injection will however temporarily increase the circulating testosterone level and one can never be certain that under these conditions totally normal transport, binding, and metabolism are being maintained.

I felt that the immuno-histological demonstration of endogenous steroids in prostatic tissue sections could reveal valuable new information and support (or cast doubt) on existing published work where injections of radioactively
labelled testosterone have been used.

3.11 Brief Background History of the Techniques to be Utilised.

A number of dyes combine with antibodies and a few will do so quite strongly to give a stable complex. The free dye can be separated from the dye-protein complex using a Sephadex column and the specificity and avidity of the antibody is often little altered by the whole process. Unfortunately there is a limit to the number of dye molecules that can be attached to an antibody and in turn a limit to the number that can be combined with an antigen.

There is therefore a ceiling to the amount of pigment that can be united with a tissue element by this means and consequently when examined under the ordinary light microscope only the strongest reactions are detectable. Coons et al., (1941) showed that very small quantities of fluorescent dyes could be readily seen under ultra violet light and in 1950 Coons and Kaplan published a paper which opened up the whole field of fluorescent labelling of antibodies in a very practical way. Nevertheless the search for other sensitive methods of immunohistochemical staining continued with an emphasis on producing permanent preparations. The common feature of these methods was the use of a marker substance bound to the antibody, which would by itself, or after a
further reaction be detectable using the electron or light microscope or both. Radioactive substances (Berenbaum 1959), ferritin (Singer 1959), heavy metals (Pepe 1961) and enzymes (Nakane and Pierce 1966) were all used.

During the late 1950's and early 60's considerable progress was made in the assay of hormones and vitamins. The production of specific antibodies to the hormone or vitamin played an invaluable part in this work and a much quoted example of the first use of this technique is the paper by Yalow and Berson (1960) which describes the measurement of insulin using an insulin antibody. However even earlier Erlanger et al., (1957) had started to solve the problems of producing an antibody to non-antigenic hormones by combining them with bovine serum albumin (BSA) and then using this complex to stimulate the production of antibodies cross reacting with the hapten and BSA.

Specific binding sites are thought to play an important part in the effects steroids have on their target organs. I felt that if the binding of testosterone and dihydrotestosterone in the prostate were strong and yet left sufficient of the steroid free to be recognised by specific antibody, it would be possible to demonstrate the steroid by locating the antibody attached to it. Details of the most satisfactory way of doing this are given in the technical section of this chapter.
3.20 Methods.

3.21 Preparation of Steroid-Protein Conjugates.

3.211 Preparation of Testosterone-17-Chlorocarbonate (Midgley et al., 1969)

One gram of testosterone was dissolved in 100 ml of diethyl ether. Phosgene gas from a small gas cylinder was bubbled through the solution for one hour at room temperature. Most of the waste gas after bubbling was absorbed by passing over porous pot soaked with 5M-NaOH solution. The testosterone solution was allowed to react to form testosterone-17-chlorocarbonate for twenty-four hours. Then the ether was evaporated by immersing the solution in a water bath at 37°C. To speed the rate of evaporation a venturi pump was used to reduce the pressure, and to draw off the vapour and phosgene.

A chlorocarbonate group will react with water, and it was thought that attempts to purify the testosterone-17-chlorocarbonate by crystallisation from acetone could reduce the amount of chlorocarbonate present and add more contamination than they removed. The steroid was dissolved in dioxane to form a 5% (w/v) solution and this was used to produce the steroid protein complex.

Figure 3.01 shows the reaction sequence for this and the next section.
Fig. 3.01 - Reaction sequence for the production of testosterone - 17 - bovine serum albumin conjugate.
2.5 ml of water was added to 100 mg of crystalline bovine serum albumin (BSA). The solution was cooled to 4°C and the pH adjusted to between 9.0 and 10.0 with 1M-NaOH. The solution was mixed gently whilst 2 ml of the testosterone-17-chlorocarbonate solution was added very slowly. Sufficient 1M-NaOH was added at frequent intervals in order to maintain the pH at between 9.0 and 10.0. When the addition of the testosterone-17-chlorocarbonate was complete the low temperature was maintained and gentle mixing continued for a further two hours. The solution was dialysed for three days against daily changes of 500 ml of 60% (v/v) dioxane in water and for a further ten days against frequent changes of distilled water. Finally the solution was concentrated by dialysing for three days against 6% (w/v) dextran solution. The resultant amber fluid was centrifuged to remove a slight turbidity and the supernatant lyophilised.

3.213 Ultraviolet Spectra - Characterisation of Testosterone-17-Bovine Serum Albumin (T17BSA).

The absorption characteristics of T17BSA and BSA in 0.05 M tris buffer at pH 8.4 had maxima at 222 nm (fig. 3.02). Their absorption curves were widest apart at 243 nm. At this second wavelength and a concentration of 15 mg per 100 ml of tris buffer, T17BSA had an extinction of 1.040.
Fig. 3.02 Ultraviolet absorption Spectra of T17BSA and BSA.
Under the same conditions BSA alone gave a reading of 0.120. The difference in extinction therefore was 0.920. Using the molar extinction coefficient, found by Erlanger et al., (1957), to be 15650 litre mole$^{-1}$cm$^{-1}$, the concentration of steroid residues was $15650 \div 0.920$ litres per mole (or $5.88 \times 10^{-5}$M). The molecular weight of the steroid residue was 315 so its concentration was 18.67 mg per litre. Therefore 13.13 mg of BSA were present in the 15 mg of complex or 131.3 mg per litre of tris buffer. If the molecular weight of BSA is taken as 70,000 the number of moles of BSA per litre was $\frac{131.3 \times 10^{-3}}{70,000}$ or $18.76 \times 10^{-7}$. The number of moles of testosterone per mole of BSA was calculated using the above figures as $\frac{5.88 \times 10^{-5}}{18.76 \times 10^{-7}} = 31.3$. Effectively this means that by this analytical method an average of 31.3 testosterone molecules were attached to every molecule of BSA. The ε-amino groups of the lysine residues represent the most abundantly available sites in BSA for coupling and there are 59 such groups in each molecule (Erlanger et al. 1957). From the analysis probably about half of these were in combination with testosterone.

3.2.14 Preparation of Dihydrotestosterone-17-chlorocarbonate and its combination with BSA.

As for testosterone, dihydrotestosterone-17-chlorocarbonate was formed and combined with BSA by the Schotten-Baumann reaction, except that dihydrotestosterone was
3.215 Preparation of Testosterone 3 (O-Carboxymethyl) oxime Erlanger et al. (1957).

A solution containing 200 mg of testosterone and 218 mg of O-carboxymethyl hydroxylamine in 40 ml of ethanol was made alkaline by adding 4 ml of 5% w/v NaOH, and the mixture refluxed for 90 minutes. The volume was reduced to about 5 ml by evaporation and extracted once with 20 ml of ether. The lower aqueous layer was taken off and adjusted to a pH of about 1.0 by the addition of concentrated hydrochloric acid. The precipitate that formed was dissolved by shaking with another 20 ml of ether. The ethereal solution was removed, washed with water, dried with anhydrous sodium sulphate and finally evaporated to dryness. The residue was recrystallised three times from benzene-ligroin. When dissolved in 0.05M-tris buffer at pH 8.5 it had a maximum absorption peak at 252 nm as expected. Fig.3.03 covers this and the following section.

3.216 Preparation of Testosterone-3-BSA conjugate.

109 mg of testosterone-3-(O-carboxymethyl) oxime were dissolved in 3.5 ml of dioxane and 0.5 ml of dioxane containing 0.143 ml of tri-n-butylamine were added. The mixture was cooled to 4°C and 0.5 ml of a solution containing 6.4% (v/v) isobutyl chlorocarbonate in dioxane added. After standing for 30 min at this temperature a
Fig. 3.03 - Reaction sequence for the production of testosterone - 3 - bovine serum albumin conjugate.
pre-cooled solution of 350 mg of bovine serum albumin in 9.2 ml water, 6.2 ml dioxane and 0.35 ml molar sodium hydroxide was added rapidly. The low temperature was maintained and gentle stirring continued for a further 4 hours. The solution was dialysed against running water overnight and then adjusted to pH 4.6 with M-HCl. The resultant precipitate was removed by centrifugation. It was resuspended in 10 ml of water and solution occurred when the pH was adjusted to 7.0 with M-NaOH. 15 ml of acetone was added and reprecipitation occurred on adjusting the pH to 4.5 with M-HCl. The precipitate was collected by centrifugation and the complete solution and precipitation from acetone repeated twice. After suspending the precipitate in 15 ml of water, a solution was obtained by adjusting the pH to 7.8 with M-NaOH. The fluid was then dialysed against running water for 5 hours, concentrated by dialysing against 6% (w/v) dextran, and lyophilised.

3.2.17 Preparation of Dihydrotestosterone-3-BSA

Dihydrotestosterone-3(0-carboxymethyl) oxime was prepared and bound to bovine serum albumin by the same method used for testosterone except that dihydrotestosterone was used as the starting material.
3.22 PRODUCTION OF STEROID ANTIBODIES

3.221 In Rabbits.

The following four steroid-BSA conjugates were used to prepare antisera.

Testosterone-3-BSA  Dihydrotestosterone-3-BSA
Testosterone-17-BSA  Dihydrotestosterone-17-BSA

Each conjugate was injected into two rabbits.

For each rabbit 12 mg of the appropriate steroid-BSA conjugate were added to 2.1 ml of pyrogen free distilled water and the solution divided into seven parts.

To one part was added an equal volume of complete Freund's adjuvant and the mixture emulsified by squirting forcefully through a fine hyperdermic needle using a syringe. The emulsion was injected intramuscularly. On the same occasion another part was mixed with two drops of washed aluminium hydroxide gel and given intravenously. The remaining five parts were deep frozen at -40°C until needed.

Five weeks later two parts were emulsified with incomplete Freund's adjuvant and given intramuscularly. The last three parts were given intramuscularly after a further ten weeks.
The rabbits given testosterone-17-BSA were tested for antibody production ten days after the last injection, but the steroid binding levels were only a little above the control serum. (Faced with the possibility that the rabbits would not produce satisfactory antibodies a separate programme of injecting guineapigs with the four conjugates was started at this time. See 3.222).

The immunisation schedule for the rabbits was extended. At monthly intervals for three months, 3 mg of conjugate were given intramuscularly in saline. Then after a further ten days the rabbits receiving testosterone-17-BSA were again tested for antibody production. The results were as before.

The rabbits were rested for ten weeks and then given an intramuscular injection of 0.2 mg of conjugate in 0.5 ml of saline every three weeks for nine weeks. Two weeks after the fourth and last injection all the rabbits were tested but the testosterone-3-BSA and 17-BSA antisera were still too weak to use and the dihydrotestosterone-3-BSA and 17-BSA antisera were no better, so the immunisation was stopped.

3.222. In Guineapigs.

The four steroid-BSA compounds listed above were injected into guineapigs. The 17-BSA compounds were each given to two guineapigs and the 3-BSA compounds were each given to three guineapigs. Each guineapig
was started with 0.5 mg of steroid-BSA conjugate in 0.2 ml of saline and emulsified with an equal volume of Freund's complete adjuvant. This emulsion was injected half into muscle and half into the peritoneum. At monthly intervals for the next four months 0.5 mg of steroid-BSA conjugate was mixed with incomplete adjuvant and injected intramuscularly half into each rear leg.

Some of the animals were tested for antibody production during the immunisation programme and all the conjugates proved to be antigenic in guineapigs. Three weeks after the last injection the animals were anaesthetised and bled out by cardiac puncture.

3.230 CHARACTERISATION OF THE GUINEAPIG ANTISERA TO TESTOSTERONE AND DIHYDROTESTOSTERONE.

All the guineapig antisera were tested in a competitive binding radioimmunoassay technique to establish the titre and the weakest T-3-BSA and the weakest DHT-3-BSA antisera were discarded. The remaining eight were assessed for avidity, titre and cross-reactivity with androgens other than the hapten used. There were two antisera for each of the four conjugates listed below.

testosterone-17-bovine serum albumin (T17BSA)
testosterone-3-bovine serum albumin (T3BSA)
dihydrotestosterone-17-bovine serum albumin (DHT17BSA)
dihydrotestosterone-3-bovine serum albumin (DHT3BSA)
Establishing the 50% Binding Titre.

The following buffer of pH 7.0 was used to prepare all dilutions of serum:

5.35 g NaH$_2$PO$_4$.2H$_2$O
16.4 g Na$_2$HPO$_4$.7H$_2$O
1.0 g NaN$_3$.
9.0 g NaCl
1.0 g gelatin
distilled water to one litre.

Incubation mixture for the serum titrations was as follows:

0.3 ml buffer
0.1 ml diluted serum
0.1 ml of (3H)steroid (51 pg of testosterone and 22,000 d.p.m., or 61 pg of dihydrotestosterone and 22,000 d.p.m.)

Polystyrene tubes were used to avoid possible absorption of testosterone onto glass.

Non specific blanks were prepared by adding 0.3 ml of buffer containing 500 ng of the appropriate steroid.

The following control tubes were also prepared.
(1) Diluted serum replaced by buffer.
(2) 0.2 ml of buffer instead of dextran charcoal (see later).
(3) Diluted serum replaced with 0.1 ml of buffer containing 500 ng of the appropriate steroid.

Mixtures were incubated for two hours at 4°C.

Dextran charcoal was prepared by mixing equal parts of a 1% (w/v) suspension of charcoal (Norit A) in water and 0.1% (w/v) Dextran, (BDH Grade B) also in water and allowed to stand overnight.

(A constant temperature of 4°C and the timing were found to be critical for this next section)

Not more than twenty tubes were treated at a time. The dextran/charcoal was mixed on a magnetic stirrer and 0.2 ml volumes quickly added to each tube at zero time. As soon as this had been done each tube was mixed for about a second using a vortex stirrer. This stirring was repeated once every minute. At five minutes the tubes were centrifuged without delay at 4°C and 2000 revs (900 g) for ten minutes. Then 0.4 ml of the supernatant fluid was removed and placed in a counting vial.

When all the incubation tubes had been so treated 10 ml of scintillation fluid was added to each vial and the contents mixed. The composition of the scintillation fluid was:-
The radioactive disintegrations were counted on a liquid scintillation counter and where necessary the counting time was extended so that at least 1000 disintegrations were counted. Counts were expressed as disintegrations per minute.

Antibody dilution tests were done in triplicate, but cross reactions were only duplicated.

Typical titration curves are given in Fig. 3.04. An antibody dilution is that dilution existing in the incubation tube and a titration figure for an antiserum is taken as that dilution which will bind 50% of the steroid. In the method given the charcoal combines with any steroid not already bound to protein. The dextran coats the charcoal and helps to prevent the unwanted binding with protein. It is the protein-bound fraction of the (3H)steroid that is counted, so that the weaker the antiserum dilution the lower the count.


Taking the titration results obtained from section 3.231 (e.g. establishing the 50% binding titre) the
Titration curves of antisera to DHT-BSA.

Two antisera to dihydrotestosterone 3BSA diluted to find the titre at which they would bind 50% of the 3H labelled dihydrotestosterone.
antisera to T3 & T17BSA and DHT3 & DHT17BSA were diluted so that in the incubation mixture used in that method they would have a 50% binding capacity.

Tubes were prepared to contain the following:

0.1 ml serum diluted to give 50% binding
0.1 ml of (3H)steroid in buffer (51 pg of testosterone and 22000 dpm, or 61 pg of dihydrotestosterone 22000 dpm)
0.3 ml buffer containing non-radioactive testosterone or dihydrotestosterone to cover the range 1 pg to 100 μg using 'ten times' dilutions, (e.g. 1 pg, 10 pg, 100 pg ... 100 μg)

Therefore using an antiserum to T3BSA as an example nine tubes were prepared each with a different level of non-radioactive testosterone over the range 1 pg to 100 μg. Each tube received the antiserum to T3BSA diluted to give a 50% binding and 51 pg of 3H testosterone (22000 dpm). The non-radioactive testosterone competed with the (3H) labelled testosterone for binding sites on the antibody molecules. The more non-radioactive steroid there was present the less radioactive steroid was able to bind to the protein. Incubation times and temperatures and charcoal extraction procedures were followed as in section 3.231 (establishing the 50% binding titre). As before only the (3H) steroid bound to protein was eventually subjected to scintillation counting and lower
counts were obtained where higher levels of non-radioactive steroid had existed in the incubation tubes. Non-specific blanks and the three controls listed in section 3.231 were also prepared.

In addition a standard tube was prepared where non-radioactive testosterone was omitted. This was the 'non-competitive' situation and competitive results were expressed as a percentage of the count obtained from this tube. Typical results are shown in Fig. 3.05 and 3.06.

The procedure was actually duplicated for each antiserum. The other antisera were tested in the same way except that 61 pg of (3H)dihydrotestosterone (22000 dpm) and non-radioactive dihydrotestosterone were used with the DHT3BSA and DHT17BSA antisera.


The method given in the preceding section was repeated with modifications. In the case of T3BSA and T17BSA antisera; dihydrotestosterone, androstene 3,17-dione, androsterone, epiandrosterone, 5α androstane 3,17-dione, 3α androstanediol and 3α androstanediol were all used one at a time in place of non-radioactive testosterone. This gave results which showed their ability in competition with (3H)testosterone to bind with antibody. The more specific the antibody the less able were these androgens in competition. The same steroids were tested against the antisera to dihydrotestosterone except that testosterone
Cross reactions of antisera to DHT3BSA.

Standard curve and cross reaction curves showing how the
3H dihydrotestosterone is displaced from its binding with
the two dihydrotestosterone 3BSA antisera by other steroid
Cross reactions of antisera to DHT\textsubscript{3BSA}

Standard curve and cross reaction curves showing how the
\textsuperscript{3}H dihydrotestosterone is displaced from its binding with
the two dihydrotestosterone-3BSA antisera by other steroid
was substituted for dihydrotestosterone. Figs. 3.05 and 3.06 give the results of the cross reactions for the two DHT3BSA antisera and the other graphs are contained in the Appendix.

3.24 DEMONSTRATING STEROIDS IN TISSUE

SECTIONS USING T3BSA, T17BSA, DHT3BSA AND DHT17BSA ANTISERA

3.241 Establishing the Optimum conditions for an Indirect Fluorescent Antibody Method.

A tissue known to be involved with androgen production was needed to test the method and testis was used. The tissue was obtained from rats. Preliminary studies were done on 6 μm cryostat sections cut from fresh tissue and dried for one hour in a blast of air at 37°C. The slides used were teflon coated except for a 15 mm circular area and the sections were picked up on this untreated spot. Since teflon has water repellent properties the reagents were confined to the islands of clear glass containing the sections and minimal volumes were needed.

The principle was that the steroid antisera raised in guineapigs could combine with androgen bound to the tissue. Subsequently this guineapig globulin would itself be located by a rabbit antiguineapig globulin serum which
had a fluorescent label (Wellcome Reagents Ltd.).

At the start the optimum titres for the guineapig antisera and the rabbit antiserum were unknown so they were cross reacted by a method which will be referred to as the 'cross-titration' technique. This technique is depicted below and it can be seen that five dilutions of the guineapig antiserum were each matched with five dilutions of the rabbit antiserum.

Fluorescently labelled rabbit guineapig-globulin antiserum.

<table>
<thead>
<tr>
<th>Guineapig Anti-steroid serum</th>
<th>1/5</th>
<th>1/10</th>
<th>1/20</th>
<th>1/40</th>
<th>1/80</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/5</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1/10</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1/20</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1/40</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1/80</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Twenty seven sections were cut to cover the cross titration pattern plus two negative controls; one omitting the guineapig anti-steroid serum and another omitting the rabbit anti-guineapig. Sections were treated for 10 min with the appropriate dilution of anti-steroid serum and then washed for 30 min with continual gentle agitation in a dish of phosphate
buffered saline at pH 7.4 (Coons 1958). The appropriate dilution of anti-guineapig globulin rabbit serum with a fluorescent label (Wellcome Reagents Ltd.) was allowed to react with the section for 30 min, followed by a further 30 min wash in buffered saline with agitation. The sections were then mounted with nine parts glycerol and one part of buffered saline and examined by dark-ground ultra-violet microscopy.

3.242 Establishing the Optimum conditions for an Indirect Peroxidase Labelled Antibody Method.

The technique used was in principle that used for the indirect fluorescent antibody method given above. A cross-titration pattern was used but instead of the fluorescently labelled rabbit anti guineapig globulin serum, an antiserum labelled with peroxidase was prepared after Avrameas (1969).

To 1.0 ml of 0.1 M phosphate buffer pH 6.8, containing 5 mg of rabbit anti guineapig globulin (Wellcome Reagents Ltd.), were added 12 mg of peroxidase. The mixture was stirred gently until solution was complete, and the stirring continued while adding 0.05 ml of 1% (w/v) aqueous glutaraldehyde. The liquid was left for two hours at room temperature and was then dialysed against 100 ml of buffered physiological saline with several changes for 24 hours. The precipitate which formed was removed by centrifugation at 4°C and discarded,
and the final reagent stored at the same temperature.

This reagent having been applied to the tissue sections in the place of the fluorescent labelled antibody the peroxidase label was demonstrated by the method of Graham and Karnovsky (1966). 25 mg of 3,3'-diaminobenzidine tetrahydrochloride (Sigma 97.9%) were dissolved in 50 ml of 0.05 M tris/HCl buffer of pH 7.6. This solution was prepared freshly and 0.15 ml of 3% (w/v) hydrogen peroxide was added immediately before use. Sections were stained for 10 minutes in the above reagent at room temperature. They were then washed briefly, first in the tris/HCl buffer and then in distilled water. Finally they were dehydrated in absolute alcohol, cleared in xylene and mounted in DePeX.

3.30 RESULTS AND DISCUSSION

The steroid-bovine serum albumin conjugates were made so that steroid antibodies could be established in rabbits and later in guineapigs. The object was to produce a conjugate that was very antigenic and produced highly avid and specific antisera to the steroids used. During the conjugation process the structure of the bovine serum albumin (BSA) would almost certainly be altered and a variety of different forms result. Furthermore the numbers and positions of the steroid attachments would vary. The only aspect that could be loosely controlled was the average ratio between steroid molecules and BSA molecules in the conjugate. Without
careful testing the best ratio to use was a matter of speculation. In the case of testosterone-bovine serum albumin it was decided to follow the opinion of Lieberman et al. (1959) and try and exceed a 50% combination with the lysine residues (the most likely sites for conjugation to occur). Three preparations were made before this objective was achieved and I suspect the failures were due to denaturation while crystallising from acetone, a step which was omitted on the third attempt. This step was also omitted in the preparation of the DHT17 BSA conjugate.

Rabbits proved to be unsuccessful animals for the production of the steroid antisera. They were chosen initially because they were large enough for 10 ml of blood to be taken without harming the animal and yet small enough that they could be housed conveniently. Furthermore a number of workers had used them successfully in the production of steroid antisera. Once it was found that the testosterone-17-BSA and the dihydrotestosterone-17-BSA preparations were slow to work in rabbits a programme of immunising guineapigs was started. The fact that the guineapigs responded where the rabbits failed could have been due to the former being more sensitive to tuberculosis and consequently more sensitive to an antigen mixed with complete Freunds adjuvant.

The volumes of antisera needed for the tests
described were not large, but more than could be obtained from the guineapigs without killing them. The animals were bled out under anaesthesia by cardiac puncture. Two of the guineapigs died rather from haemorrhax and the blood sample at first obtained from them was small. The situation was recovered by waiting a few hours for the natural haemolysins to have effect and then opening the thorax and aspirating the blood.

The antisera titres and cross reaction studies were necessary to show that the immunisation had been successful and that some degree of specificity existed. The structure of the steroids used is shown in Figure 3.07. If the active groups on carbon 17 are ignored it will be seen that testosterone has the same structure as 4 androstene 3,17-dione and dihydrotestosterone as 5α-androstanedione. If instead carbon 3 is ignored testosterone is still different from all the others, but dihydrotestosterone is the same as 3α and 3β androstanediol. In fact in use the antisera did not demonstrate all these differences and similarities. The antisera to the 17-BSA conjugates (those recognising the carbon 3 end of the steroid molecule) had the more predictable reactions, although they cross reacted weakly when there was a keto group on carbon 17 of the steroid being tested. The DHT 17BSA antisera did not react well with testosterone and 4 androstenedione, probably because of the double bond at carbon 4, whereas the T17BSA antisera probably
Fig. 3.07
The structure of androgens used in testing the steroid antisera.

H

testosterone

dihydrotestosterone

4 androstene 3, 17-dione

androsterone

3β androstane-17-diol

3α androstane-17-diol

5α androstane 3, 17-dione

epiandrosterone
did not recognise the difference and reacted well.

Although one or two of the antisera could have been used for radioimmunoassay they would all be regarded as inferior for this purpose. However, there could be no assurance that the antisera found to be best for radioimmunoassay would give the strongest and best reaction on tissue sections. Accordingly, all the antisera were tried against sections of rat testis. Four antisera to testosterone given by Dr Jeffcoate of St Thomas' Hospital were also tested. Rats twelve weeks old were used for the experiment, but once the best method was established, testes from younger rats were also studied. There was a chance that the reaction between tissue bound steroid and the antisera would be very weak, possibly to the point where the fluorescent method would fail to work. For this reason, a method was set up in which peroxidase was utilised instead of the fluorescent label, and for a while this method was used in parallel with the fluorescent technique. Figures 3.08 to 3.15 show the type of reaction obtained. The tubular structure of the tissue can just be seen in the photographs and the testosterone producing Leydig cells that are found in the connective tissue between the tubules are seen to react strongly. This peroxidase method produced a permanent preparation but unfortunately the results were inconsistent. The cross titration results varied and non-specific background staining often occurred. Against this, the fluorescent technique proved
Fig. 3.08 and 3.09. Testis from two week old rat showing reaction between peroxidase labelled rabbit antiguineapig serum, guineapig anti-T3BSA serum and interstitial tissue. Two 'generations' of Leydig cells ('condensed circular' and 'streaky' areas) are demonstrated and these are discussed in chapter two (section 2.31). Two magnifications of one area, x40 and x200.
Figs. 3.10, 3.11, 3.12.

Testis from a one week old rat showing the reaction between guineapig anti-\(^3\)HSA serum, peroxidase labelled rabbit anti-guineapig serum and interstitial tissue. Condensed areas of first 'generation' Leydig cells are demonstrated.

Three magnifications of one area, x40, x200 and x400.
Figs. 3.13, 3.14, 3.15.  

Testis from 12-week-old rat showing reactions between guineapig anti-T3BSA serum, peroxidase labelled rabbit antiguisneapig serum and interstitial tissue. The reaction is seen to be granular when highly magnified and a background activity was often present in these preparations. Three magnifications of one area, x40, x200 and x400.
more reliable. If the steroid antisera were used too strongly a weak positive reaction was found throughout the tissue. Once this reaction was diluted out, further dilution simply decreased the time taken for the fluorescence to 'burn-out' under the ultra violet light, but the intensity of the fluorescence was not greatly affected.

In the mature rat testis all the interstitial tissue reacted (Figs. 3.16 to 3.20). The fluorescence also weakly involved the germinal cells of the seminiferous tubules. The weak reaction could be prevented by further dilution of the steroid antisera, but in view of the studies with other tissues (described later) this pale area was almost certainly correct. When the testis was prepared for sectioning an adrenal gland from the animal was implanted in the edge of the tissue so that adrenal was included in the study. This organ exhibited considerable bright blue autofluorescence which seemed to be an artifact in that occasionally it was quite slight though never absent. When the autofluorescence was not masking the tissue, some reaction could be seen. The zona glomerulosa gave a weak diffuse reaction which by itself might well have been ignored. The zona fasciculata gave a similar weak reaction except that a few cells stood out as being obviously weakly fluorescent. The zona reticularis gave a moderate fluorescent reaction throughout with a thin strongly reacting halo around the nucleus. The medulla never reacted. The vessel walls of the cortex reacted whereas those in the medulla did not.
Fig. 3.16  x40  Fig. 3.17  x200
Sections of testis stained with haematoxylin and eosin.

Fig. 3.18  Testis from a five week old rat showing reaction with anti-T3BS and fluorescently labelled rabbit anti-guineapig serum.  x425
Fig. 3.19 Testis from twelve week old rat showing reaction with anti-T3BSA and fluorescently labelled rabbit anti-guineapig sera. x950

Fig. 3.20 Testis from one week old rat showing reaction with anti-T3BSA and fluorescently labelled rabbit anti-guineapig sera. x950
Certain tests were made to try and confirm the specificity of the fluorescent reaction. A trial had shown that cryostat sections after six weeks storage at 4°C and less than 50 torr over calcium chloride still gave a +++ fluorescence. Using this information sections of testis were gathered and stored, from man, guineapig and mouse. These were compared with rat sections under the optimum conditions. The results were equally strong with all four tissues. Fears that the guineapig tissue would react in a non specific way with the rabbit antiguineapig serum were not realised.

To provide negative controls for the method soft tissues from the rat were also tested. The tissues used were heart, lung, liver, spleen, kidney and small bowel. They were tested by the full cross-titration method and although they gave an overall fluorescence at the strongest dilutions this became negative before the optimum dilutions for testis were reached.

The last control experiment involved adding testosterone or dihydrotestosterone (10mg % w/v) as appropriate to the buffered saline used for diluting the steroid antisera in the cross-titration method. This experiment was repeated with each steroid antiserum using a different sample of testis. The results were consistent and showed a reduction in titre, but not complete neutralisation of the antiserum. A deposit of fluorescent staining material was found on the sections and it may be that this was precipitated antibody/steroid complex.
All the steroid antisera reacted with rat testis by the fluorescent method but some were better than others. (See below.) Some of the dihydrotestosterone antisera

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Label</th>
<th>Fluorescent reaction strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti T17BSA</td>
<td>a</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>+</td>
</tr>
<tr>
<td>Anti T3BSA</td>
<td>a</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>+++</td>
</tr>
<tr>
<td>Anti DHT17BSA</td>
<td>a</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>++</td>
</tr>
<tr>
<td>Anti DHT3BSA</td>
<td>a</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>++</td>
</tr>
<tr>
<td>T3BSA antiserum from St Thomas' Hospital</td>
<td>a</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>+++</td>
</tr>
</tbody>
</table>

Range-negative, +, ++, +++.

**Table 3.1 Performance of Steroid Antisera in the Indirect Fluorescent Method**

could equal the antisera formed against testosterone but overall they were not so good when used with the rat testis. Nearly all the androgen in rat testis would exist as testosterone so this result was quite to be expected. There was some comparison between the serum titration results obtained by radioimmunoassay and their suitability for the fluorescent technique. The high titre antisera were more likely to react strongly with the tissue sections.

For the prostatic study all four anti-dihydrotestosterone sera were used and also the four best anti-testosterone sera (i.e. anti T3BSA a & b, and St. Thomas' T3BSA a & d).
The sera were used at their optimum titre and these titres were checked in use. Sections were prepared from seventeen human hyperplastic prostates obtained freshly from the operating theatre. These were normally sectioned and used immediately in the fluorescent method, but sometimes the sections were stored overnight as previously described and used the following day.

There was a reproducible fluorescent result in all the specimens tested. Unfortunately, compared with the results obtained using rat testis these were very weak and 'burnt out' quite quickly under the action of the ultra violet light, having a half life of about ten seconds. The fastest colour film available required a two minute exposure and of course the fluorescence could no longer be seen after half a minute. This burning out was a problem with all the fluorescent photographs but only in the case of the prostate (see Fig. 3.21) has the photography really failed to represent the original appearance.

Apart from some stroma close to the acini only the epithelial cells were positive, so the acini of the tissue were picked out against a dark background. The brightest area was a nuclear halo. This had quite a sharp definition and was probably the nuclear membrane. This appearance is in agreement with the findings of Moore and Wilson (1973) who have shown that an appreciable amount of the enzyme $\Delta^4$-3-$\beta$-ketosteroid-5-reductase, which is responsible for the conversion of testosterone to
Fig. 3.21  Human hyperplastic prostate showing weak reaction with anti-T3BSA and fluorescently labelled rabbit anti-guineapig sera. Cytoplasm of epithelial cells shows a weak fluorescence with a slightly stronger nuclear 'halo'.  x425

Fig. 3.22  Human hyperplastic prostate showing the reaction with guineapig anti-T3BSA serum and peroxidase labelled rabbit anti-guineapig serum.  x40
Dihydrotestosterone, is located in the prostatic epithelial nuclei. By comparison the cytoplasm was paler but evenly stained, while the rest of the nucleus was the least fluorescent part of the cell.

Despite the unreliability experienced with the peroxidase method it was used so that a record of the reaction between steroid antisera and human prostatic tissue could be made. Figure 3.22 shows the best result obtained. At high magnification definition was lost in granularity. The nuclear 'halo' reaction seen with the fluorescent method could not be distinguished separately from the overall peroxidase staining of the epithelial tissue.

These results find agreement with other studies done on the prostate, where injected rather than endogenous androgen has been studied. They differ slightly from those of Tveter and Attramadal (1969) who found the greatest concentration of dihydrotestosterone in the epithelial nuclei. These authors were using tritium labelled testosterone injected into castrated rats and showed radioactive androgen by autoradiography. Bruckovsky and Wilson (1968) injected tritium labelled testosterone into rats and separated a nuclear and a cytoplasmic fraction from the prostates. The radioactivity recovered was mainly from the cytoplasm and the fluorescent study was in accord with this.

Androgens have been shown to have quick and variable
effect on prostatic epithelium (Robel et al., 1971). Because the organ in man and animals is sensitive to steroids and anti-androgens, it has been an attractive area for study. The work of Franks et al. (1970) highlights the weakness of considering the epithelium on its own and not in conjunction with its stroma.
CHAPTER 4

4.00 THE COMPARISON OF CHOLESTEROL, PEPTIDASES
AND ACID PHOSPHATASE FROM HYPERPLASTIC
PROSTATE WITH NORMAL GLAND.

4.10 Introduction

About 200 years ago it was discovered that human
gallstones consist largely of a white crystalline
substance. This substance was at first named cholesterine,
but after the presence of an alcohol group was established
the more descriptive name of cholesterol became accepted
in most countries. Practically all mammalian tissues are
capable of synthesising cholesterol. The main site of
formation is in the liver and this is the only tissue shown
to monitor cholesterol levels and to balance production
against dietary intake. Cholesterol is a precursor of all
the steroids found in man and the liver, adrenal and gonads
are important sites of its production. The prostate is not
recorded as being very active in forming cholesterol. The
cholesterol level of prostatic secretion varies between 86
and 618 mg/100 ml (Huggins 1945). This is a wider range
than is found in blood or serum where the normal is 130-
350 mg/100 ml. The level of dihydrotestosterone in the
prostate is increased when hyperplasia is present (Sitteri
and Wilson 1970). It was felt that there might be a
similar association between cholesterol and prostatic
hyperplasia. The cholesterol was extracted from a number
of hyperplastic and a few normal prostates and then estimated as free and ester cholesterol.

Peptidases are found in all tissues but because they do not seem to have any medical significance they are rarely examined.

A tissue leucine aminopeptidase was recorded by Berger and Johnson in 1939. Smith and Bergman (1944) extracted this enzyme from pig intestine and noted the splitting of leucinamide, leucyl glycine and leucylglycyl glycine at almost equal rates. When they further purified the extract however they observed different activities towards the three compounds. The work of Lewis and Harris (1967) and Harris (1969) on red cells, and Rapley et al. (1971) on tissue has clearly defined a number of peptidases designated A, B, C, D, E, F and S. This identification was done after separation by starch gel electrophoresis. The demonstration of aminopeptidases in tissue sections is far less satisfactory. This is largely due to the action of other non-specific enzymes and because the amide substrates that are used only react with E and S peptidases (Rapley et al. 1971). Muntzing and Nilsson (1972) in a paper on histochemical enzyme activity in the prostate found a wider range and larger amounts of aminopeptidase activity in hyperplasia than in cancerous tissue.

I have compared the level of aminopeptidase activity in hyperplasia with that in normal tissue.
The term phosphoesterase covers a wide range of enzymes which catalyse the hydrolysis of phosphorus ester links. Some of them only hydrolyse monoesters and are called phosphomonoesterases. Human and animal tissues contain some phosphomonoesterases with optimum activity at about pH 9.5 and these are commonly called alkaline phosphatases. Similarly another group of phosphomonoesterases are active at about pH 5.0 and are called acid phosphatases. Human acid phosphatases are mainly produced in the liver and erythrocytes and additionally in the prostate gland in men. The acid phosphatases found in these tissues are differently affected by inhibitors (Everson Pearce 1968) and two or more acid phosphatases can be found in the same tissue (Goodland and Mills 1957, Moore and Angeletti 1961). Human prostatic acid phosphatase from normal and from hyperplastic glands was studied and the results compared.

Due to the acid phosphatase from the prostate gland, serum acid phosphatase is higher in men than in women. Under normal conditions only a small proportion of the enzyme is released from the gland and despite a possible twenty times increase in size during hyperplastic growth the serum level changes very little. In contrast, carcinoma of the prostate causes an increase in the release level despite a generally reduced production of acid phosphatase per cell. A number of scientific papers covering the histochemical demonstration of acid phosphatase can be found, but only a few of these are concerned with
4.20 Methods

4.21 Selection and Homogenisation of Normal and Hyperplastic Prostatic Tissue.

Hyperplastic prostatic tissue was obtained from routine surgical cases for prostatectomy and a few normal prostates were taken during post mortem examination of males under thirty years of age. Because in routine prostatectomy cases the capsule is left inside the patient and only glandular tissue removed, the capsule tissue was carefully avoided when taking samples from the post mortem material. Furthermore because prostatic hyperplasia is centered on the periurethral glands, this was the area principally selected in the normal organ.

Most of the surgical tissue was received as one piece that had been enucleated from the capsule without cutting. A transverse slice about 3 to 5 mm thick was cut from each specimen. The slice was cut into small pieces using scissors and placed into a universal container together with two to three volumes of distilled water. This container was cooled by standing it in crushed ice and the cooling maintained while the tissue and water were blended to a smooth paste using a Silverson blender. Where a surgical specimen was received in more than one piece, each piece was sampled. The size of the sample under these circumstances was judged to proportionally represent...
the piece from which it was taken.

This method of sampling and blending was used for the extraction of acid phosphatase and aminopeptidase as well as for cholesterol.

4.22 Extractable Total and Ester Cholesterol From Prostatic Tissue

4.221 Extraction Method

Tissue was selected and blended as in section 4.21.

A stoppered conical centrifuge tube (of about 12ml volume) was weighed, and about 1.0ml of prostatic tissue paste added. Together with 8.0ml of 25% (v/v) diethyl ether in ethanol. The contents of the tubes were shaken to thoroughly disperse the tissue particles prior to incubation at 45°C (with gentle mixing at about five minute intervals) for a total time of 30 minutes. The tube was then centrifuged to pack the cellular material, (2,000 revs for ten minutes in a bench centrifuge being sufficient,) and the supernatant fluid poured into a 25ml stoppered measuring cylinder. The tissue plug was loosened with a thin glass rod and the extraction procedure repeated with a further 8ml of ether/ethanol mixture. After centrifugation the second supernatant fluid was added to the first in the stoppered measuring cylinder.
The plug of tissue left after the second extraction was dried in the tube at 60°C to a constant weight. From the two weighings the dried weight of tissue was calculated and this figure was used to express the cholesterol results as cholesterol/100 grams of dried tissue.

The pooled extractions in the measuring cylinder were mixed and divided equally between two 50ml beakers using a graduated pipette. In order to wash out any remaining cholesterol containing solvent, 2ml of fresh ether/ethanol mixture were added to the cylinder and after shaking and draining, this fluid was also divided between the beakers. To one beaker was added, with mixing, 1.0ml of a freshly prepared 0.5ml (w/v) solution of digitonin in ethanol. The beakers were covered and left for 10 minutes at room temperature for cholesterol in the digitonin beaker to precipitate as digitonide.

The contents of the beakers were evaporated to dryness in a water bath at 80°C. During the evaporation, air that had been dried by passing over calcium chloride was blown over the surface of the extract. This prevented steam entering the beaker and kept the duration of heating to a minimum by encouraging the evaporation of the solvent mixture.
4.222 Cholesterol Estimation

The following reagents were prepared.

(I) Colour reagents: 0.05g FeCl₃ 6H₂O in 100 ml of acetic acid.

(II) 1.0ml of a 0.1g/100ml solution of cholesterol (BDH) in A.R. acetic acid, added to 24ml of reagent (I).

(III) A.R. sulphuric acid.

About 3.0ml of petroleum ether (boiling range 90% 40° to 60°C) were added to the 'digitonin' beaker to dissolve the ester cholesterol present. The beaker was warmed gently and the fluid carefully decanted into a clean test tube. The extraction was repeated twice with the extraction fluids being added to the test tube.

The pooled petroleum ether extract in the test tube was evaporated to dryness as had been the ether/ethanol extract, by using an 80°C water bath and a current of dried air. The residue contained only cholesterol esters. The tube was allowed to cool at room temperature and 5ml of colour reagent was added to this ester tube. Also 10ml of colour reagent was added to the other beaker. This beaker was covered with parafilm and both test tube and beaker left at room temperature for 10 minutes, with occasional mixing, for the extracts to dissolve in the colour reagent. Then 5ml of fluid was transferred from the beaker to a clean test tube and this was used for a total 'cholesterol estimation'.
Three standard tubes were prepared. The first contained 1 ml of solution (11) and 4 ml of solution (1). The second contained 3 ml of solution (11) and 2 ml of solution (1). The third contained 5 ml of solution (11) only. A blank was prepared containing 5 ml of reagent (1).

To the six tubes (total, ester, three standards and blank) were added 3 ml of reagent (111), the contents of each tube being mixed vigorously with a glass rod immediately after the addition. The tubes were placed in a water bath at 80°C for 5 minutes and then left for 15 minutes at room temperature. The absorbance was then read at 560 nm in an EEL Spectra. Where a number of tubes were to be read, a one minute spacing was introduced at the sulphuric acid stage so that the 5 and 15 minute incubation times could be closely adhered to.

4.223 Preparation of a Standard Curve.

The reagents were those used for estimating the cholesterol content of prostatic tissue except that reagent (11) was prepared by adding 3 ml of the cholesterol solution in acetic acid to 27 ml of reagent (1). 5 ml of the above working standard which contains 0.5 mg of cholesterol was placed in a test tube. By taking 4.5 ml of working standard and adding 0.5 ml of reagent (1), a standard tube was prepared containing 0.45 mg of cholesterol. This method of dilution was
continued to a tenth tube containing 0.05 mg of cholesterol. Tube number 11 was a blank and contained 5 ml of reagent (1).

3 ml of sulphuric acid (111) was added to each tube (with mixing as described before), allowing exactly one minute between each tube. After heating for 5 minutes in a water bath at 80°C and leaving for 15 minutes at room temperature the absorbance of each tube was read at 560 nm using an EEL Spectra.

4.224 Comparison of Absorption Curves of Final Colours Produced by Cholesterol and the Tissue Extract.

The absorption curves of the final coloured product from cholesterol and three extracts were traced using a Unicam SP 800. The curves covered more than 200 nm (see Fig. 4.01).

4.225 The Value of Another (Third) Extraction.

The technique under "cholesterol estimation" (4.222) was followed except that a third extraction of the prostatic tissue was made. The supernatant of this third extract was placed in a clean beaker and then treated as a test sample.

4.226 Recovery of Cholesterol by the Previous Method.

1.0 ml of acetic acid containing 2.0 mg of cholesterol
Fig. 4.01 Absorbance spectra of the final coloured solution (A) and a cholesterol solution (B) given in the method for total cholesterol given in section 4.
(BDH) per ml was measured into a centrifuge tube and evaporated to dryness. Then 1.0 ml of distilled water was added and thereafter the sample was treated as if it were tissue extract.

4.227 Cholesterol Distribution in the Hyperplastic Prostate

A hyperplastic prostate from a man of 76 years was obtained post mortem and was sliced as shown in Fig. 4.02. Tissue was taken from either side as also shown in the figure. The lowest slice was from an area not showing hyperplasia and was sampled centrally.

The pieces were homogenised separately and cholesterol and cholesterol esters estimated as previously described.

4.230 Electrophoretic Separation of Amino Peptidase From Normal and Hyperplastic Human Prostates.

4.231 Extraction of Enzymes.

Tissue was taken from the mucosal and submucosal gland area and homogenised as described under section 4.21. Where necessary the tissue samples were stored at minus 70°C before homogenisation. The peptidases did not appear to be affected by up to two months of storage at this temperature. To obtain the peptidases the homogenate was centrifuged for ten minutes at about 2,000 revs in a bench centrifuge and the supernatant
Fig. 4.02 Drawing showing how a hyperplastic prostate gland was sampled to study cholesterol distribution.
fluid containing the enzymes removed.

4.232 Preparation of Starch Gel.

Starch gel was used for electrophoresis. A tris-maleate buffer at pH 7.1 was prepared by adding 0.2 M maleic anhydride to 0.2 M tris with continual mixing until the pH dropped to 7.1. This stock buffer was diluted to 0.01 M for use in the starch gel preparation. The gel was prepared by gently heating a suspension of 44 g of hydrolysed starch (Connaught Laboratories) in 375 ml of buffer until it boiled. Constant mixing was necessary during the heating process. The heating was done in an Erlenmeyer flask and when the contents had boiled the top was plugged with a rubber bung and the side arm connected to a venturi pump which was used to reduce the pressure in the flask and induce degassing. The contents of the flask were inclined to recommence boiling so the pressure was reduced carefully. The hot liquid was then poured into a previously prepared mould, the surface covered with a sheet of Melinex (I.C.I.) and the gel allowed to set at room temperature.

The mould was prepared from 6.25 mm plate glass. The base consisted of a 304x178 mm sheet and the sides of two 304x12.75 mm strips and the ends of two 152x12.75 mm strips. The strips were 'glued' in place on the edges of the base with silicone stopcock grease (Edwards High Vacuum). The strips were carefully abutted again using
stopcock grease, so as to contain the gel. Using cold glass there was rarely any leakage.

4.233 Electrophoresis

When the gel had set and cooled the samples of tissue extract were inserted on filter paper wicks. Eight wicks (Whatman 3M) of size 7.5x6.25mm could be inserted with suitable gaps between them in a line across the width of the plate. The paper wicks were dipped into the tissue extract, lightly blotted, and pushed into vertical slots made with a short length of razor blade. The inserts were made in a line, evenly spaced, 76mm from one end of the gel and parallel to the shorter side. Samples had to be duplicated so four cases could be studied at a time and were inserted in the order 1, 2, 3, 4, 1, 2, 3, 4 across the gel. Fig. 4.03 shows how the gel on the glass plate was fitted for electrophoresis with the end nearest the wicks being made the cathode. The cooling was essential for the electrophoretic separation of the peptidases. The water pumped through the cooling plate was taken from a refrigerated bath and was at 7°C. The perspex tanks were filled with tris/maleate buffer 0.1M forming a discontinuous system and the filter paper wicks made from two layers of Whatman 3M. A potential was applied to the gel at 8V DC per cm width from a constant voltage source for 16 hours. Under these conditions the operating current was about 2mA per cm².
Fig. 4.03 The water cooled electrophoresis apparatus used for peptidase and acid phosphatase starch gel electrophoresis.

Fig. 4.04 Drawing showing how the starch gel was cut following peptidase electrophoresis prior to enzyme demonstration.
At the end of the run the assembly was dismantled and the gel in its glass mould removed. The peptidases were contained in the gel between the sample wicks and the anode so the 76mm of gel on the cathode side of the wicks was cut off at this time. Similarly the 38mm of gel at the anodal edge were waste and were removed. The next step was to cut the gel in half through its thickness so as to produce two approximately 178x152mm pieces of gel but only 3mm thick, (see Fig.4.04). First the wicks were carefully removed and the glass side and end pieces taken off the base. The longer glass side pieces were replaced with strips of perspex 3mm thick and these were used as a guide so that a thin polished knife could be used to cut through the thickness of the plate. A sheet of saran wrap was placed on top of the gel to give the top slice support and this slice was then smoothly and quickly peeled off the bottom half. After some practice the gel separated without tearing or cracking. Both layers were then cut in the middle lengthways between samples 4 and 1 to give four pieces of gel of equal size, shape and thickness. The cut surfaces were used for the detection of enzyme activity.

The method for the detection of peptidase activity is based on the series of reactions shown below in Fig. 4.05.
peptidase + peptide $\rightarrow$ L-amino-acids (1)

\[
\text{L-amino-acid oxidase}
\]

L-amino-acid + O$_2$ $\rightarrow$ keto-acid-NH$_3$ + H$_2$O$_2$ (2)

\[
\text{peroxidase}
\]

H$_2$O$_2$ + O-dianisidine $\rightarrow$ oxidized dianisidine (3)

**Figure 4.05** The reaction sequence of the method used for the detection of peptidase activity after starch gel electrophoresis.

The L-aminoacids released in reaction 1 by the peptidase activity are oxidised by the L-aminoacid oxidase in reaction 2. The hydrogen peroxide which is one of the products of reaction 2 is reduced by the peroxidase with the accompanying oxidation of o-dianisidine. This results in a brown area of oxidised dianisidine at the site of activity. A crude preparation of the venom from the rattlesnake Crotalus adamanteus was used as a source of the enzyme L-aminoacid oxidase.

The different peptidases were distinguished first by their electrophoretic mobilities but also by their differing abilities to 'split' certain di- and tri-peptides. In selecting these di- and tri-peptides for the locating reagents it was also necessary to remember that the snake venom oxidised some L-amino acids more effectively than others. (This will be dealt with further under section 4.62.) In practice four separate locating reagents were
prepared to the formula given below. The peptide used in the first was L-leucyl L-alanine (Sigma), in the second L-leucyl L-proline (Cyclo), in the third L-lysyl L-leucine 2HBr (Miles-Yeda) and in the fourth the tripeptide L-leucyl L-leucyl L-leucine HCl (Cyclo). Each formula was applied to a different piece of gel and by this means five different peptidases were demonstrated, (some by more than one locating reagent).

15ml phosphate/HCl buffer 0.2M pH 7.5
(0.2M Na$_2$HPO$_4$ adjusted to pH 7.5 with 1M HCl)
5mg L-aminoacid oxidase (crude Crotalus adamanteus venom)
2.5mg peroxidase (POD 11 Boehringer)
2.5mg O-dianisidine hydrochloride
10mg peptide (one of the three di-peptides or the tri-peptide)
When dissolved, 15ml of 2% agar at 60°C was stirred in, and the mixture used immediately.

The agar containing reagent was poured quickly over the surface of the gel slab to give an even depth. To make this possible the slabs were boxed-in using the long glass strips on edge along the sides, and for the ends, suitable pieces of gel cut from the waste three inches that had been removed from the starch gel block after electrophoresis. Once poured the agar set quickly and the surface was covered with saran wrap to reduce evaporation. All four slabs were incubated for up to 2 hours at 37°C. As each agar
overlay reached its optimum for colour and sharpness of bands the reaction was stopped by flooding the surface with 10 to 20 ml of the following reagent.

\[
\begin{align*}
11 \text{ parts } & \text{66 O.P. spirit} \\
8 \text{ parts } & \text{distilled water} \\
1 \text{ part } & \text{acetic acid}
\end{align*}
\]

A sheet of Whatman 3M filter paper was then placed on top of the agar and peeled back removing the agar layer from the starch gel. The paper plus agar was allowed to dry at room temperature and became a semi-permanent record of the results.

4.240 Electrophoretic Separation of Prostatic Tissue Acid Phosphatase From Normal and Hyperplastic Human Prostates.

4.241 Extraction of Enzymes

Hyperplastic prostatic tissue was compared with normal prostatic tissue sampled from the mucosal and submucosal gland area. As described under section 4.21, tissues were stored at \(-70^\circ\)C, (study having shown that acid phosphatase was not adversely affected by storage at this temperature.)

The procedure used for electrophoretic separation was based on that used for aminopeptidases (4.23) and only the differences will be covered here in any detail.
A weighed sample of tissue was homogenised with three times its weight of distilled water. The homogenate was centrifuged and the supernatant used for electrophoresis.

4.242 Preparation of Starch Gel.

The gel used for electrophoresis was prepared from hydrolysed starch using 0.01M tris/maleate buffer at pH 7.1 and poured into a mould made from plate glass. The pH of the buffer was later changed as detailed at the end of this section. Samples were applied to the set gel using wicks made of Whatman 3M filter paper. Unlike the method for aminopeptidases, up to eight samples were applied singly across the gel.

As the method became established the buffer was prepared at a lower pH so as to reduce the competition with the pH of the locating reagent buffer which was at 4.9. A pH of 5.4 was found to be best for electrophoretic separation (see Fig. 4.6, 4.7, 4.8) and this was prepared by adding 0.2M maleic anhydride to 0.2M tris with mixing until the correct pH was reached. This buffer was diluted to 0.01M for preparing the starch gel. The change in buffer pH affected the acid phosphatase separation and instead of being all anodal the slower bands became cathodal. For this reason the wicks were inserted 12.7cm from the cathode end and not at 7.6cm as with the aminopeptidases.
Acid phosphatase activity demonstrated following electrophoretic separation in starch gel using tris/maleate buffer at three different pH levels.
4.243 Electrophoresis

The plate was fitted to the electrophoretic/cooling apparatus and run for 16 hours with a D.C. potential of 8V per cm width.

4.244 Cutting Of Gel Prior To Enzyme Demonstration

At the end of the run the glass sides and ends of the mould were removed and the sample wicks replaced with 3mm thick perspex. Using these as a thickness guide the gel was sliced in two horizontally. A sheet of saran wrap was laid on the top layer of the gel to give it support and the layers separated by peeling back the top slice. The cut surface was used for the detection of enzyme activity.

4.245 Detection of Acid Phosphatase Activity in the Starch Gel.

A locating reagent was prepared by mixing equal parts of the following:

1. A 0.1% w/v solution of sodium α-naphthyl phosphate (Sigma) in 0.5M acetate buffer at pH 4.9.
2. A 0.1% w/v solution of Fast Garnet GBC (Lamb) in 0.5M acetate buffer at pH 4.9.

The surface of the gel was flooded with 40ml of this
mixture and because the fluid drained off, it was collected with a pasteur pipette and re-applied every five to ten minutes. Within a few minutes of treatment the bands started to appear. The results could only be recorded by photography. Adjacent bands of high activity started to run together before the weaker bands could be seen. For this reason, the photographs do not show all the bands at their best resolution. The photographs were generally taken about two hours after the start of treatment with the locating reagents. Replacing the locating reagent in use with fresh fluid did not improve the speed of development or resolution of the bands.

4.246 Some Aspects of the Character of Prostatic Acid Phosphatase

A small number of experiments were carried out to see if the many bands of acid phosphatase were due to conditions of electrophoresis, the storage of the enzyme, or the presence of more than one basic enzyme.

4.2461 Electrophoresis of Prostatic Acid Phosphatase

When establishing the method for electrophoretic separation of prostatic tissue acid phosphatase (4.240) it had been found that changing the pH of the discontinuous buffer only affected the mobility of the enzyme. The banding of the enzyme in the starch gel was not affected. Other buffer systems were tried whilst retaining the pH of
5.4 and the molar strength of 0.01M for the gel. As before the buffer constituents were dissolved to give 0.2M solution in distilled water and the 'acidic' solution added to the 'basic' until a pH of 5.4 was reached. Three different buffers were prepared in this way. They were tris/phosphate (0.2M tris and 0.2M phosphoric acid), tris/acetate (0.2M tris and 0.2M acetic acid) and sodium acetate/acetic (0.2M sodium acetate and 0.2M acetic acid). These buffers were used separately, the same buffer being used for tank and gel. The dilutions established for tris/maleate buffer were used namely 0.01M for the gel and 0.1M for the tanks.

4.2462 Electrophoresis of the same Prostatic Tissue Extract at Different Dilutions

A single tissue extract was diluted by five doubling dilutions using distilled water to give the following; 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32. These, together with an undiluted sample, were subjected to the standard starch gel electrophoresis technique (4.50) at pH 5.4 and the enzyme demonstrated.

4.2463 Electrophoresis of a Fresh Tissue Extract Compared With One That Had Been Stored

An homogenate was prepared from a surgical specimen of prostate and the extract subjected to starch gel electrophoresis within two hours of the operation. For comparison another specimen was separated on the same
plate. This was an older tissue extract that had been stored overnight at 40°C after first being prepared and had then been frozen for four weeks at -20°C, being thawed and refrozen twice during this time.

4.2464 Double Electrophoresis of a Prostatic Tissue Extract

When electrophoresis is done using a tris/acetate buffer pH 5.4 for sixteen hours, the separation is approximately half anodal and half cathodal. A starch gel was prepared using this buffer system and a prostatic tissue extract was applied three times. The extract was subjected to electrophoresis but for only one hour. The apparatus was then dismantled and the tissue wicks removed. Knowing the distance between the most anodal and the most cathodal bands after sixteen hours, it was expected that the enzyme would be contained in the 1cm of gel on either side of the wicks. To be sure of not missing any enzyme a 2cm wide block was taken on the anode side of each wick and a 2cm wide block taken from the cathode side. The pieces of anode gel were bottled together and those from the cathode were combined in a second bottle. The gel was gently broken down and 6ml of physiological saline was added. The contents were mixed and the bottle placed in a refrigerator at 4°C for an hour with the contents being mixed gently from time to time. The pieces of gel were allowed to settle and the supernatant extract removed. This extraction was repeated twice with the extracts of the
anodal bottle and cathodal bottle being pooled separately. The extracts were then dialysed for two hours against 2 litres of physiological saline at 4°C to reduce the amount of tris/acetate buffer present and then concentrated with the use of polyethylene glycol 400 also at 4°C. When the volumes were down to about 2ml they were carefully transferred to narrower cellophane tubing and the concentration continued.

When about 0.25ml remained in each tube a standard pH 5.4 tris/maleate buffer starch gel electrophoresis system was set up. A sample wick was soaked in each concentrate and subjected to 16 hours electrophoresis in the standard way described under 4.50. Enzymic activity was then demonstrated using sodium α-naphthyl phosphate, and Brentamine Fast Garnet as usual.

4.2470 Minor Experiments Concerning Prostatic Acid Phosphatase

4.2471 A Study of the Electrophoretic Pattern of Serum Acid Phosphatase (from a patient with carcinoma of the prostate)

The standard starch gel electrophoresis method previously given (4.240 to 4.245) was used for the separation. The serum was obtained from a patient with carcinoma of the prostate, and it had a level of over 20KA units of acid phosphatase per 100ml. The serum was frozen and allowed
to thaw slowly. Previous experiments had shown that the first few drops to separate from the ice would contain all the soluble material, so this concentrate was used for electrophoresis.

Despite being concentrated in this way, the locating reagents failed to show enzyme activity in the gel (Fig. 4.15). As explained in 4.244 before demonstrating the enzyme activity the gel is cut in half producing two full size pieces of gel, but of only half thickness. The cut surface of one half having been used unsuccessfully to demonstrate the enzyme an alternative approach was tried on the other half. That part of the gel which contained the serum was cut into 7.5mm strips, (the strips being parallel to the sample wick). Each strip was separately bottled and frozen slowly. The acid phosphatase content of each was measured as follows by modifying the method of Abdul-Fadl and King (1948). The strips were thawed and broken up in buffered substrate, and incubated for \(3\frac{1}{2}\) hours at \(37^\circ\)C. After adding Folin and Ciocalteau's reagent and centrifuging, a colour was produced with sodium carbonate. The tubes were read and a block graph made from the results.

4.2472 The Level of Acid Phosphatase Activity in a Hyperplastic Prostate

A piece of prostatic tissue from a fresh operation specimen was weighed and four times its weight of distilled water was added. Tissue and water were then homogenised as explained under 4.20 with a Silverson blender. The
material was centrifuged and the supernatant removed for analysis.

The supernatant was diluted 1 in 2000 and the acid phosphatase activity measured by the method of Abul-Fadl and King (1948). In this method the enzyme was used to liberate phenol from disodium phenyl phosphate (M/200) at pH 5.0. Folin and Ciocalteau's reagent was added after 15 minutes which both precipitates any protein (thus stopping the reaction) and combines with the liberated phenol. After the precipitate had been removed by centrifugation and the supernatant made alkaline with sodium carbonate a blue colour developed which was proportional to the phenol first liberated. This was read at 680 nm.

4.2473 Electrophoresis of prostatic tissue extracts by the standard technique for acid phosphatase with L(+)-tartrate included in the locating mixture

Prostatic tissue extracts were separated in starch gel by the standard technique. 1M sodium hydroxide solution was added to 8% (w/v) L(+)-tartaric acid until a pH of 4.9 was reached. The tartrate solution was added to the Fast Garnet GBC/sodium phenolphthalein phosphate reagent to give a 0.04M solution. This reagent was used to demonstrate the areas of acid phosphatase activity.

4.30 Results and Discussion

In this chapter results obtained from hyperplastic
prostates were compared with those obtained from a limited number of normal prostates from young men of under 33 years. Cholesterol and the peptidases were known to be reasonably stable and the fact that the normal tissue had been in a cadaver for a few hours before receipt was not expected to affect the results. Acid phosphatase on the other hand is a rather labile enzyme so its study was qualitative rather than quantitative.

Twenty-one surgical cases were compared with six normal prostates taken post mortem. The same normal prostates were used for the cholesterol and enzyme studies but only twelve of the surgical cases were common to all the studies. Six unenlarged prostates from older men (over 70 years) were used in the cholesterol study.

4.31 Cholesterol

The results of total cholesterol and ester cholesterol estimations are shown in tables 4.01 and 4.02. The young and old unenlarged prostates were rather few in number and insufficient to define a normal range but their results are quite closely grouped. By comparison the hyperplastic tissue encompasses the range covered by these 'normals' for both cholesterol and cholesterol ester, but occasionally exceeds the latter quite strikingly.
<table>
<thead>
<tr>
<th>Hyper-gram of Grams of</th>
<th>Grams of</th>
<th>Ratio of</th>
</tr>
</thead>
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<td>extractable</td>
<td>cholesterol</td>
</tr>
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<td>specimen number</td>
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<td>per 100gm (dry weight) of prostatic tissue</td>
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<td>3.11</td>
<td>0.44</td>
</tr>
<tr>
<td>2</td>
<td>1.83</td>
<td>0.15</td>
</tr>
<tr>
<td>3</td>
<td>3.04</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>3.15</td>
<td>0.50</td>
</tr>
<tr>
<td>5</td>
<td>2.37</td>
<td>0.37</td>
</tr>
<tr>
<td>6</td>
<td>3.18</td>
<td>0.32</td>
</tr>
<tr>
<td>7</td>
<td>3.01</td>
<td>0.30</td>
</tr>
<tr>
<td>8</td>
<td>5.24</td>
<td>0.91</td>
</tr>
<tr>
<td>9</td>
<td>2.62</td>
<td>0.50</td>
</tr>
<tr>
<td>10</td>
<td>4.80</td>
<td>1.60</td>
</tr>
<tr>
<td>11</td>
<td>2.41</td>
<td>0.28</td>
</tr>
<tr>
<td>12</td>
<td>2.82</td>
<td>0.30</td>
</tr>
<tr>
<td>13</td>
<td>3.43</td>
<td>0.41</td>
</tr>
<tr>
<td>14</td>
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<td>0.22</td>
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<tr>
<td>15</td>
<td>3.04</td>
<td>0.34</td>
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<td>16</td>
<td>2.76</td>
<td>0.41</td>
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<td>17</td>
<td>2.58</td>
<td>0.28</td>
</tr>
<tr>
<td>18</td>
<td>4.09</td>
<td>0.60</td>
</tr>
<tr>
<td>19</td>
<td>4.65</td>
<td>0.36</td>
</tr>
<tr>
<td>20</td>
<td>2.80</td>
<td>0.43</td>
</tr>
<tr>
<td>Mean</td>
<td>3.179</td>
<td>0.452</td>
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<tr>
<td>Standard Deviation</td>
<td>0.873</td>
<td>0.314</td>
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Table 4.01 Levels of total cholesterol and cholesterol ester found in a series of hyperplastic prostates.
<table>
<thead>
<tr>
<th>Young normal prostate specimen number</th>
<th>Grams of extractable cholesterol per 100gm (dry weight) of prostatic tissue</th>
<th>Grams of extractable cholesterol ester per 100gm (dry weight) of prostatic tissue</th>
<th>Ratio of cholesterol to ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.68</td>
<td>0.39</td>
<td>6.87</td>
</tr>
<tr>
<td>2</td>
<td>1.88</td>
<td>0.38</td>
<td>4.95</td>
</tr>
<tr>
<td>3</td>
<td>2.50</td>
<td>0.39</td>
<td>6.44</td>
</tr>
<tr>
<td>4</td>
<td>2.45</td>
<td>0.51</td>
<td>4.80</td>
</tr>
<tr>
<td>5</td>
<td>2.20</td>
<td>0.44</td>
<td>5.00</td>
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<tr>
<td>6</td>
<td>2.59</td>
<td>0.37</td>
<td>7.00</td>
</tr>
<tr>
<td>Mean</td>
<td>2.383</td>
<td>0.413</td>
<td>5.843</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.295</td>
<td>0.053</td>
<td>1.034</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old normal prostate specimen numbers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.52</td>
<td>0.65</td>
<td>5.42</td>
</tr>
<tr>
<td>2</td>
<td>3.94</td>
<td>0.88</td>
<td>4.48</td>
</tr>
<tr>
<td>3</td>
<td>2.82</td>
<td>1.30</td>
<td>2.17</td>
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<td>4</td>
<td>2.65</td>
<td>0.40</td>
<td>6.63</td>
</tr>
<tr>
<td>5</td>
<td>2.57</td>
<td>0.54</td>
<td>4.76</td>
</tr>
<tr>
<td>6</td>
<td>2.66</td>
<td>0.29</td>
<td>9.17</td>
</tr>
<tr>
<td>Mean</td>
<td>3.027</td>
<td>0.677</td>
<td>5.438</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.567</td>
<td>0.368</td>
<td>2.341</td>
</tr>
</tbody>
</table>

Table 4.02 Levels of total cholesterol and cholesterol ester in a series of normal prostates from young men (under 35 years of age) and old men (over 70 years of age).
Although the number of specimens tested was small the results of Table 4.03 when studied with Tables 4.01 and 4.02 indicate that the total cholesterol level increases with age both in the small and the hyperplastic prostate. In the old but small prostate the ester fraction increases at the same rate as the total and the ratios found in young and old are essentially the same. However in the hyperplastic prostate the ester fraction is not increased in proportion (if at all) so that the ratio of total cholesterol to ester is raised.

The cholesterol extraction, ester separation, and colour formation methods were developed from data given by Henry (1966). The standard curve produces a straight line but not quite passing through zero. The standard curve was repeated twice and once more over a year later with completely different reagents, but the failure to pass through zero persisted. There was no apparent reason for this and as the error was not great (see Fig.4.09) it was not investigated. Absorbance figures were converted to milligrams of cholesterol using a graph. The colours produced by the cholesterol reaction have a wide absorption as shown in Fig.4.01. The tissue extracts differ from the standard but they are comparable between 550 and 570 nm and so permit estimation of cholesterol at the 560 nm point. Henry (1966) states that the presence of more than 4% (v/v) of water in the extraction mixture adversely affects the removal of cholesterol. Two extractions theoretically reduced the water concentration to an acceptable level, but a third extraction was measured separately to test this.
<table>
<thead>
<tr>
<th>Data from Tables 4.01 and 4.02</th>
<th>'T' test for Total</th>
<th>'T' test for Ester</th>
<th>'T' test for Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old small prostates compared with young prostates</td>
<td>0.035</td>
<td>0.11</td>
<td>0.70</td>
</tr>
<tr>
<td>Young prostates compared with hyperplastic prostates</td>
<td>0.002</td>
<td>0.60</td>
<td>0.003</td>
</tr>
<tr>
<td>Old small prostates compared with hyperplastic prostates</td>
<td>0.61</td>
<td>0.18</td>
<td>0.03</td>
</tr>
</tbody>
</table>

'T' test results became increasingly significant below 0.05.

Table 4.03 The results of a Students 'T' Test study on the figures contained in Tables 4.01 and 4.02.
Fig. 4.09  Standard graph for cholesterol obtained by the method detailed in section 4.223.
The absorbance of this extraction after colour production was 0.005. Because the standard graph does not pass through zero, readings this low cannot be interpreted with accuracy, but it indicates something in the order of 0.02g per 100g prostatic tissue dry weight; so low in fact that two extractions were counted sufficient.

The test for recovery also supported the method. 2mg of cholesterol were put in and 2.01mg were calculated from the final colour. According to the suppliers any ester present in the cholesterol standard would have been in trace amounts and 0.039mg were calculated; again, difficult to measure with any accuracy at this level. This was equivalent to less than 2%.

The range of results obtained for cholesterol in hyperplastic prostate could have reflected regular differences at different points in the gland or considerable random variation. For this reason a number of samples were taken from one gland as shown in Fig. 4.02. The results are given in Table 4.04. Judging from this specimen, there is a slight increase in total cholesterol in the middle of the gland as compared with the poles and an increase in cholesterol ester at the base. These differences are not great enough to invalidate the results given in Table 4.01 and 4.02 as samples were taken from the middle of the gland and never from the base. The differences do show that care would be needed if a highly critical assessment of cholesterol and its esters were planned.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Grams of extractable cholesterol per 100gm (dry weight) of prostatic tissue</th>
<th>Grams of extractable cholesterol ester per 100gm (dry weight) of prostatic tissue</th>
<th>Ratio of cholesterol to ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7</td>
<td>0.17</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>1.7</td>
<td>0.16</td>
<td>10.6</td>
</tr>
<tr>
<td>3</td>
<td>2.1</td>
<td>0.16</td>
<td>13.1</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>0.17</td>
<td>11.8</td>
</tr>
<tr>
<td>5</td>
<td>1.9</td>
<td>0.21</td>
<td>9.1</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>0.25</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Mean 1.817

Standard Deviation 0.222

Table 4.04  The variation in total cholesterol and ester level found in different samples from a hyperplastic prostate (see also Fig.4.02)
4.32 Peptidases

The peptidase study was often carried out on fresh specimens but where storage was necessary this was done at -70°C. When specimens are stored unfrozen the peptidases slowly combine with glutathione from red cells and the electrophoretic pattern changes. These changes can be reversed by the addition of mercaptoethanol to the stored sample to give a 0.02M concentration (Lewis 1969). The combination with glutathione causes an increase in the number of sub-bands of the peptidases. These sub-bands are present in the freshest samples but are much weaker than the main enzyme and give 'shadow' reactions by comparison. In Fig. 4.10 these are best seen with regard to peptidases S and C. Mercaptoethanol was added to some samples and even to half a specimen so that a comparative electrophoretic separation could be made. Its regular inclusion was not necessary. There is also evidence (Lewis 1969) that certain ions namely manganese, cobalt or zinc ions can activate or inhibit peptidases. However when included at 0.01M in the agar based locating mixture, no effect was demonstrated. Fig. 4.05 shows that the coloured reaction at the site of the peptidases depends on L amino acid oxidase being added. Lewis (1969) has shown that in red cells each peptidase shows a greater ability to hydrolyse some di- and tripeptides rather than others. In turn the rattle-snake venom (source of L amino acid oxidase) was more able to deaminate some amino acids than others. Both these separate abilities had to be brought together to get
Fig. 4.10 Peptidase activity demonstrated in agar gel overlays following electrophoretic separation of four prostatic tissue extracts.
the strongest final colour. Furthermore by choosing the right di- or tripeptide two or more separate peptidases were demonstrated. With one exception, namely F, all the peptidases present in red cells were found in the prostate. Similarly with one exception, namely S, all the peptidases present in the prostate can be found in red cells. In a limited study the enzymes from both hyperplastic and normal prostate had the same pattern of reactions with the different substrates and the same electrophoretic mobilities.

Comparing the normal and the hyperplastic prostate, no differences were found in electrophoretic mobility of extracted acid phosphatase, or in the reaction given by the locating reagent. Similarly although the study was qualitative there was no suspicion of quantitative differences between the groups or even of a single specimen for that matter. Much of the scientific information on peptidases in the prostate is based on histological study. The most successful and widely used substrates have been combinations of leucine and naphthylamine (Burstone and Folk 1956, Nachlas et al. 1957, Nachlas et al. 1960, Gomori 1950). These substrates only demonstrate peptidase E and S (Rapley et al., 1971). Close attention was given to the comparative reaction strengths of E and S bands from normal and hyperplastic tissue but no obvious differences were seen. Despite the limited number of specimens two probable genetic variants were found. Both were in normal prostates so by the time they were discovered they could
not be confirmed with a blood sample.

4.33 Acid Phosphatase

The electrophoresis of acid phosphatase was a natural follow-on of the work with peptidases. Initially the experimental conditions used were identical except that the samples were only applied once and not twice to the gel. Encouraging results were obtained from the start and the first changes to be studied were pH and buffer salts. The objective in reducing the pH of the electrophoretic gel was to try and close the gap between it and the pH of the locating mixture. The tris/maleate buffer was retained, but the pH was reduced. Satisfactory electrophoretic separation was achieved at pH 5.4 which was very near the lower end of the range of this buffer (Figs. 4.06 to 4.08). Alternative buffer systems were tried at pH 5.4 to see if even better separations was possible (Figs. 4.11 to 4.13). Of the three alternative buffers tried tris/acetate was the best and sodium acetate/acetic acid gave imperfect separation. When the enzyme bands from the tris/acetate gel were located it seemed that the anodal bands were not as well separated as with the tris/maleate buffer. Therefore tris/maleate was maintained and a pH of 5.4 used. The locating reagent was prepared at pH 4.9 but was fifty times stronger than that used in the electrophoretic gel. The formula for the locating reagent was based on the method of Grogg and Pearce 1952 for demonstrating acid phosphatase in tissue sections. An
Three figures showing the electrophoretic separation of acid phosphatase from prostatic tissue using different buffer systems.
adaptation of the method of Barka and Anderson (1962) using Napthol AS phosphate was tried once on the other half of a gel for comparison. The demonstration was not as good but the bands demonstrated coincided with the half stained by the standard sodium o-anapthyl phosphate/Fast Garnet GBC reagent.

There was a possibility of an electrophoretically 'fast' or 'slow' acid phosphatase existing which was either cut off with the waste gel or not identified by the reagents used. For this reason a sample of the waste gel on the anode and cathode ends of the gel plate plus gel from the ends of the spare half plate beyond the known enzymes area were incubated in a manner similar to the method given under 4.2471. No enzyme activity was demonstrated.

Three experiments were performed to see if the multiple banding was a phenomenon due to the preparation and electrophoresis of the specimen. In the first a tissue extract was diluted 1 in 2, 1 in 4, 1 in 6, 1 in 8, 1 in 16, and 1 in 32 prior to electrophoresis. Diluting the tissue extract did not affect the separation or the number of bands found (Fig.4.14). However the strength of reaction with the colour reagent did seem to be disproportionately affected by the dilution. In the second experiment a stored extract was compared with a very fresh extract. The two reactions 'fresh' and 'stored' are quite comparable (Fig.4.15). In the third experiment an extract was run for only one hour to split it, half to the anode and half to the cathode. Each half was then eluted from
Fig. 4.14 The effect of diluting the tissue extract prior to electrophoresis and demonstrating acid phosphatase activity. (Doubling dilutions from 1 in 2 to 1 in 32.)

Fig. 4.15 The effect of storage on a tissue extract for acid phosphatase and the failure to demonstrate serum acid phosphatase of approximately 20KU units/100ml by this method.
the buffer, re-concentrated, and subjected to normal electrophoresis. The anodal portion has remained anodal and the cathodal portion has remained cathodal, showing that the many bands are in a stable state under the conditions of electrophoresis.

Fig. 4.15 also shows (by blank areas) the results of trying to compare the serum from a patient with a high acid phosphatase (over 20KA units) and a normal serum with tissue extracts. The 'high serum' experiment was recovered by segmenting the unused half of the electrophoresis gel in the area of the high serum run and subjecting these segments to a more traditional test tube analysis. From the results a block graph was drawn and in length and intensity of reaction it compared with the tissue extract (see Fig. 4.16). Banding could not be confirmed by this means. The initial failure to demonstrate a high serum acid phosphatase by electrophoresis raised the question of 'What is the approximate tissue level of acid phosphatase in prostatic hyperplasia?' As shown by Muntzing and Nilsson (1972) this will vary, but one hyperplastic tissue extract gave a figure of 2285KA units of acid phosphatase per gram wet weight of tissue. L(+)-tartrate is added to serum samples in biochemical analysis and this inhibits prostatic acid phosphatase. By this means a high serum level can be shown to have come mainly from the prostate and support a diagnosis of carcinoma of the prostate gland. L(+)-tartrate was included in a quantity of locating reagent and the spare half of gel from an electrophoretic separation
Photograph showing typical acid phosphatase activity following electrophoresis of a prostatic tissue extract.

Fig. 4.16 Graphic presentation of serum acid phosphatase activity obtained after starch gel electrophoresis and subsequent extraction of the enzyme (see section 4.2471)
of acid phosphatase was treated with it. Fig. 4.17 shows the result. The gel was in fact the other half of that shown in Fig. 4.11 with which it may be compared. Inhibition resulted in a delayed reaction. Smith and Whitby 1967 recorded the substrate used (sodium α-naphthyl phosphate) as being the one least inhibited by L(+)-tartrate. As can be seen from Fig. 4.14 (diluted tissue extract) the enzyme is present to excess and the strength of reactions is not completely related to its dilution, therefore the fact that in this experiment the inhibition was judged at about 30% is not in conflict with the 70% obtained by assay by these authors.
Fig. 4.17  The effect of adding L(+)−tartrate to the locating reagent used on prostatic acid phosphatase following starch gel electrophoresis. Six samples are shown.
CHAPTER FIVE

5.00 TESTOSTERONE METABOLISM IN ORGAN CULTURE OF THE HYPERPLASTIC PROSTATE

5.10 Introduction

More than 80 years ago it was realised that testes influenced the growth of the prostate. As stated in Chapter 1, Ramm in 1894 and White in 1895 reported the results of orchidectomy on the treatment of prostatic hypertrophy. Since that time the hormonal control of the prostate has received growing attention particularly during the last ten to twenty years. Testosterone is the main vehicle of interaction between the two tissues. It is carried free and bound to testosterone-binding globulin in the blood and possibly also travels to the prostate via the vasa deferentia. However, once in the prostate there seems little doubt that the effects of testosterone are through dihydrotestosterone (DHT) and other androgenic metabolites. A significant discovery was found by Siiteri and Wilson (1970) concerning the concentration of DHT in the human prostate. They found that whereas the testosterone and androstenedione content of normal and hyperplastic prostates was the same, the DHT level was higher in the latter. They also found that the DHT content was 2-3 fold greater in the periurethral area of the prostate where hyperplasia starts than in the rest of the prostate. There was no
correlation between age and testosterone level in the prostate but that after the age of 60 years there was a striking increase in DHT. Though much more is now understood on the mechanism of this increased binding of DHT its full significance in relation to prostatic hyperplasia remains obscure.

In 1970 Baulieu drew attention to 5α-androstane 3β,17β diol (3β-androstanediol). He said that in organ culture DHT favours hyperplasia of epithelial cells and that 3β-androstanediol favours hypertrophy. He was of the opinion that pathological disorders could reflect disturbance in the subtle balance of these two steroids.

In the same year Mauvais-Jarvis et al. (1970) found no 3β-androstanediol normally present in male urine and found that when the steroid was injected into men it was substantially converted to 3α forms (mainly androsterone glucuronide).

A search of the literature on organ culture of the prostate and steroid metabolism showed that (I) much of the work involved animal and not human tissue, (II) often only two or three metabolites of testosterone were examined, and (III) the reports on 3α and 3β androstanediol were very few. This chapter covers the organ culture of human hyperplastic prostate with a study of eight metabolites of testosterone including the two androstanediols.

Tissue culture could be considered to have started in 1898 when Ljienggren demonstrated by reimplantation
that human skin could survive in vitro for many days if stored in ascitic fluid. The cultivation of tissues as distinct from cells was initiated in 1914. Once antibiotics became readily available the methods which had for so long demanded the tedium of strict aseptic technique were in part superceded by more tolerant methods. Due to the painstaking work that has been done mainly on media it is now possible to perform short-term organ culture successfully by purchasing commercially available culture fluids and using basic laboratory equipment. Fig.5.01 shows a flow chart of how an organ culture incorporating ($^3$H)testosterone provides information on testosterone metabolites. Following organ culture the fluid was always examined but where a tissue study was also to be included the tissue was homogenised prior to extraction of the steroids. The early extraction work was done with chloroform but later dichloroethane was found to be almost as efficient and less likely to form emulsions. The thin layer chromatography (TLC) technique was only developed after considerable trial and error.

The literature on steroid metabolism in the prostate concentrates on a small number of androgens. The structures of these were studied and in combination with further information from text books and experimental work on interconversions, a possible metabolic pathway was constructed. The metabolism of all the nine steroids shown (Fig.5.02) was investigated using organ culture.
Fig. 5.01  Flow diagram of the methods used in studying the metabolism of ($^3$H)testosterone by the prostate.

Fig. 5.02  Proposed steroid interconversions in the human prostate.
In order to make the pathway complete, a few other steroids had to be included in addition to the androgens first selected from the literature.

5.20 Methods

5.21 Organ Culture

Only very fresh tissue (with the exception of the 18 hour prostate in section 5.31) was used for organ culture. The specimens came from retro-pubic prostatectomy operations and were collected from the theatre as soon as they were removed. As previously explained the hyperplastic tissue usually consisted of one large piece amounting to perhaps 90% of the specimen and a number of smaller lumps. Aseptic technique was used as far as possible.

5.211 Preparation of Tissue

The hyperplastic prostate received from the operating theatre was cut in half and the thinnest possible slices were cut free with a scalpel from the cut surface. The very edge of the prostate (capsule) was not sliced but otherwise adenoma and connective/myomatous tissue alike was taken. The greatest emphasis was placed on the 'thinness' of the sections and as satisfactory pieces were difficult to cut they were rarely larger than 3cm$^2$ even
with a large prostate. Two cultures were prepared from each specimen (see section 5.22) so the slices were placed as cut, into two piles. The object was to place slices from the same area into alternate piles so that the two cultures might be as similar as possible. 2.5g of tissue per pile was more than sufficient for the culture.

5.212 Stainless-steel Supports

The tissue slices were placed on woven stainless-steel wire mesh supports in petri dishes. The supports were rectangles 4.4 x 3.5cm. The longer sides were bent downwards to an angle of about 45°, about 0.4cm from the edge. This bending gave two short side legs and so the mesh formed a shallow bridge about 0.3cm high with a flat surface approximately 3.6 x 3.5cm. The mesh when new was well cleaned with organic solvents, scrubbed with detergent, rinsed with industrial spirit, autoclaved in distilled water, and finally allowed to dry. Just before use it was boiled in distilled water for ten minutes to provide some degree of sterilisation. After use the mesh was washed well in distilled water, stored dry, and boiled as before, just prior to being used again. Two supports were used per petri dish.

5.213 Culture Media

Two different culture media were used with each
In 1950 Morgan, Morton and Parker produced a culture medium called 199. The authors have slightly modified the formula a number of times since then and the constituents are shown in Table 5.01. The fluid was kept in the dark at $4^\circ$C and used within two months. The medium is widely used for maintaining tissue for virus production. In the absence of serum, cells will not survive in this medium for more than two days, but with serum added some cell strains can be maintained indefinitely (Paul 1965).

The second medium used was Trowell's T8 (1955). This is simple in composition (Table 5.02) comparison with other media for cell and tissue culture and the inclusion of serum is not essential. It will maintain many adult tissues from several days to weeks in a healthy condition. It is intended for use where mitotic activity is not great and consequently where very little increase in mass is expected. It contains insulin.

Just before use 1000 i.u. of penicillin and 1000 i.u. of streptomycin together with 1ml of horse serum were added to 19ml of both medium. Also added at this time were 0.15 mols of $^3$H labelled testosterone (0.2 Ci) in 10$\mu$l of ethanol. The media were mixed well and about 18ml was poured carefully into separate petri dishes containing two supports per dish. Sufficient was added to flood under the stainless-steel wire bridges even when
### Medium 199 (Modified)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>mg/litre</th>
<th>Ingredient</th>
<th>mg/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine*</td>
<td>25.000</td>
<td>ATP, Na$_2$</td>
<td>10.000</td>
</tr>
<tr>
<td>L-arginine . HCl</td>
<td>70.000</td>
<td>5-adenylic acid</td>
<td>0.200</td>
</tr>
<tr>
<td>L-asparic acid*</td>
<td>30.000</td>
<td>L-ascorbic acid</td>
<td>0.050</td>
</tr>
<tr>
<td>L-cysteine . HCl . H$_2$O</td>
<td>0.110</td>
<td>D-biotin</td>
<td>0.010</td>
</tr>
<tr>
<td>L-cystine</td>
<td>20.000</td>
<td>Calciferol</td>
<td>0.100</td>
</tr>
<tr>
<td>L-glutamic acid*</td>
<td>66.820</td>
<td>D-Ca pantothenate</td>
<td>0.010</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>100.000</td>
<td>Choline Chloride</td>
<td>0.500</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.050</td>
<td>Folic Acid</td>
<td>0.010</td>
</tr>
<tr>
<td>Glycine</td>
<td>50.000</td>
<td>L-inositol</td>
<td>0.050</td>
</tr>
<tr>
<td>L-histidine . HCl . H$_2$O</td>
<td>21.881</td>
<td>Menadione</td>
<td>0.010</td>
</tr>
<tr>
<td>Hydroxy-L-proline</td>
<td>10.000</td>
<td>Niacin</td>
<td>0.025</td>
</tr>
<tr>
<td>L-isoleucine*</td>
<td>20.000</td>
<td>Nicotinamide</td>
<td>0.025</td>
</tr>
<tr>
<td>L-leucine*</td>
<td>60.000</td>
<td>p-aminobenzoic acid</td>
<td>0.050</td>
</tr>
<tr>
<td>L-lysine . HCl</td>
<td>70.000</td>
<td>Pyridoxal . HCl</td>
<td>0.025</td>
</tr>
<tr>
<td>L-methionine*</td>
<td>15.000</td>
<td>Pyridoxine . HCl</td>
<td>0.025</td>
</tr>
<tr>
<td>L-phenylalanine*</td>
<td>25.000</td>
<td>Riboflavin</td>
<td>0.010</td>
</tr>
<tr>
<td>L-proline</td>
<td>40.000</td>
<td>Thiamin . HCl</td>
<td>0.010</td>
</tr>
<tr>
<td>L-serine*</td>
<td>25.000</td>
<td>DL-X-tocopherol phosphate</td>
<td>0.010</td>
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<tr>
<td>L-threonine*</td>
<td>30.000</td>
<td>Vitamin A acetate</td>
<td>0.1147</td>
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<td>L-tryptophan*</td>
<td>10.000</td>
<td>Cholesterol</td>
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</tr>
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<td>L-tyrosine</td>
<td>40.000</td>
<td>2-Deoxy-D-Ribose</td>
<td>0.500</td>
</tr>
<tr>
<td>L-valine*</td>
<td>25.000</td>
<td>D-ribose</td>
<td>0.500</td>
</tr>
<tr>
<td>Adenine Sulphate</td>
<td>10.000</td>
<td>Na Acetate . 3H$_2$O</td>
<td>60.000$^a$</td>
</tr>
<tr>
<td>Guanine . HCl</td>
<td>0.300</td>
<td>Tween 80</td>
<td>5.000$^b$</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.300</td>
<td>Fe(NO$_3$)$_3$ . 9H$_2$O</td>
<td>0.100$^b$</td>
</tr>
<tr>
<td>Thymine</td>
<td>0.300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>0.300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>200.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose D-Glucose</td>
<td>1000.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>98.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>6800.00</td>
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<td></td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ . 3H$_2$O</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>400.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol Red Na</td>
<td>17.00</td>
<td></td>
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</tr>
</tbody>
</table>

*Table 5.01 The constituents of culture medium 199*
Trowell’s T8 Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>mg/litre</th>
<th>Ingredient</th>
<th>mg/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine HCl</td>
<td>21.00</td>
<td>L-Valine</td>
<td>23.00</td>
</tr>
<tr>
<td>L-Cysteine HCl</td>
<td>47.00</td>
<td>Thiamine HCl</td>
<td>17.00</td>
</tr>
<tr>
<td>L-Histidine HCl</td>
<td>10.00</td>
<td>p-aminobenzoic acid</td>
<td>35.00</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>26.00</td>
<td>Insulin</td>
<td>50.00</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>26.00</td>
<td>NaCl</td>
<td>6100.00</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>36.00</td>
<td>KCl</td>
<td>450.00</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>7.50</td>
<td>CaCl₂</td>
<td>220.00</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>16.50</td>
<td>MgSO₄.7H₂O</td>
<td>250.00</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>24.00</td>
<td>NaH₂PO₄.2H₂O</td>
<td>450.00</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>4.00</td>
<td>Glucose</td>
<td>4000.00</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>18.00</td>
<td>Phenol Red</td>
<td>10.00</td>
</tr>
</tbody>
</table>

Table 5.02 The constituents of culture medium T8
the dish was very slightly tilted, and yet insufficient for the level to be above the bridges. The slight differences in the volume required reflected the slight disparity in bridge height.

At this point then, there were two piles of thinly sliced tissue and two petri dishes containing different basic media but with the same additives. Each petri dish had two wire supports. One pile of tissue was used for each dish. The tissue slices were laid out on the supports to cover them as completely as possible without stretching, bunching or overlapping the tissue. There was always tissue left over from the 2.5g.

5.214 Incubation

A small piece of polystyrene about 1 x 20mm was cut from the lip of both petri dish bases with a hot scalpel. This was to permit easy gaseous exchange between the dish and outside. The dishes were then lowered with their lids on into the glass bottom of a McIntoch and Fildes jar.

A different lid had been designed for the jar. This lid consisted of a circle of 5mm perspex big enough to cap the jar. Two holes 9mm in diameter were drilled through the circle on opposite sides and about 8cm apart. A short length of copper tube was pushed through each hole and fixed with 'Araldite'. Both tubes had short lengths
of rubber tubing on the outside of the lid and the one used for 'gas in' had rubber tubing on the inside of such a length that the gas was delivered to the bottom of the jar.

The new lid of the McIntoch and Fildes jar was placed on the jar and gas sealed with heavy duty silicone stopcock grease. The 'gas in' tube was connected to a gas cylinder containing 6% carbon dioxide in oxygen and freely gassed for five minutes. At the end of this time the cylinder was turned off. The rubber tubing on 'gas in' and 'out' was pinch clipped (screw type), the cylinder disconnected and the jar incubated at 37°C. The usual incubation time was 6 hours but other times were used as required.

After incubation the culture fluid was poured into a bottle. The tissue was also placed in a bottle and both fluid and tissue were stored at -40°C. When histological examination was required the piece or pieces selected were not frozen but fixed for at least 18 hours in 4% (w/v) formaldehyde in physiological saline at room temperature.

5.22 Extraction of (3H)Androgens from Culture Fluid and Tissue

Benzene, toluene, chloroform, and dichloroethane were tested for their ability to extract (3H)dihydrotestosterone from aqueous solution. Five universal
containers were prepared each containing 0.2μCi of dihydrotestosterone in 15 ml of water. 5ml of each solvent was added per bottle, the fifth bottle acting as a standard. All the bottles were put on a Kahn shaker for 15 minutes, centrifuged and 0.5ml of the aqueous layer added to 9.5ml of the scintillation fluid used in section 5.252.

Tissue that had been used for tissue culture was rinsed briefly in distilled water and homogenised with a Silverson blender in about two volumes of distilled water. The homogenate was distributed amongst five universals and one third homogenate volumes of each solvent added, shaken, separated, and the aqueous layer counted as before.

The results of these tests are given in section 5.31 and as a consequence dichloroethane was routinely used to extract culture fluids and tissue homogenates, three extractions being given to each specimen. The three extracts were pooled and evaporated to dryness. The residue was dissolved in ethanol prior to being 'spotted' onto a chromatography plate. When tissue was extracted it was found that the extract contained too much lipid to permit chromatography. The lipid was removed by dissolving the residue of the dichloroethane extraction in 0.05ml of chloroform. To this was quickly added 5ml of ethanol. After 10 minutes the cloudy mixture was centrifuged and the clear supernatant solvent decanted and evaporated to dryness. The residue was dissolved in
a minimal volume of ethanol and 'spotted' onto a chromatography plate.

5.230 Chromatography of the Steroids.

5.231 The Steroids Involved

As explained in section 5.10 eight androgenic steroids are commonly recognised as metabolites of testosterone in the prostate gland. The names and abbreviations of the nine steroids to be studied are given below.

<table>
<thead>
<tr>
<th>Trivial name</th>
<th>Abbreviation</th>
<th>Systematic Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Testosterone</td>
<td>testost.</td>
<td>17β-hydroxy-androst-4-en-3-one</td>
</tr>
<tr>
<td>2. Dihydrotestosterone</td>
<td>DHT</td>
<td>17β-hydroxy-5-androstan-3-one</td>
</tr>
<tr>
<td>3. 4-Androstenedione</td>
<td>4A.enedione</td>
<td>Androst-4-ene-3,17-dione</td>
</tr>
<tr>
<td>4. 3α-androstane-diol</td>
<td>3αA.diol</td>
<td>5α-androstane-3α.17β-diol</td>
</tr>
<tr>
<td>5. 5α-androstane-dione</td>
<td>5αA.dione</td>
<td>5α-androstane-3,17-dione</td>
</tr>
<tr>
<td>6. Androsterone</td>
<td>A.erone</td>
<td>3α-hydroxy-5α-androstan-17-one</td>
</tr>
<tr>
<td>7. Dehydroepiandrosterone</td>
<td>DHA</td>
<td>3β-hydroxy-androst-5-en-17-one</td>
</tr>
<tr>
<td>8. Epiandrosterone</td>
<td>ΕΑ.erone</td>
<td>3β-hydroxy-5α-androstan-17-one</td>
</tr>
<tr>
<td>9. 3β-androstane-diol</td>
<td>3βA.diol</td>
<td>5α-androstane-3β.17β-diol</td>
</tr>
</tbody>
</table>

0.5mg of each steroid was dissolved in 1.0ml of
ethanol. 10μl of this solution was used for a chromatogram spot.

5.232 Paper Chromatography

To the best of my knowledge no details of a single two way paper or TLC method have ever been published to separate these nine steroids. I did not even find a method to separate six of them. Lisboa (1969) has reviewed TLC of steroids and provides a mine of correlated information on the subject. From the $R_F$ values he quotes it seemed unlikely that TLC would separate all of them. Cathro et al. (1964) have quoted $R_F$ values for five of the steroids using paper and four Bush solvent systems. The details of the systems and the results obtained are given below.

Bush L/85: Light petroleum (b.p. 100-120)-methanol-water (100:85:15, by vol.). Temperature 23°C Equilibration 15h.


Bush T/75: Toluene-methanol-water (100:75:25, by vol.). Temperature 28°C Equilibration 5h.

In all cases, Whatman No. 42 paper was used.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>L/85</th>
<th>LB21/80</th>
<th>T75</th>
<th>LB21/A85</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Testosterone</td>
<td>0.10</td>
<td>0.42</td>
<td>0.84</td>
<td>0.33</td>
</tr>
<tr>
<td>2. Dihydrotestosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 4 Androstenedione</td>
<td>0.31</td>
<td>0.78</td>
<td>0.90</td>
<td>0.42</td>
</tr>
<tr>
<td>4. 3α-androstanediol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 5α-androstanedione</td>
<td>0.50</td>
<td>0.83</td>
<td>0.93</td>
<td>0.62</td>
</tr>
<tr>
<td>6. Androsterone</td>
<td>0.34</td>
<td>0.69</td>
<td>0.89</td>
<td>0.58</td>
</tr>
<tr>
<td>7. Dehydroepiandrosterone</td>
<td>0.19</td>
<td>0.57</td>
<td>0.88</td>
<td>0.47</td>
</tr>
<tr>
<td>8. Epiandrosterone</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. 3β-androstanediol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rfs obtained by D.M. Cathro et al. (1964) using paper chromatography and Bush systems.

All four systems were tried. Squares of Whatman No 42 paper for two dimensional chromatography were spotted, one steroid to each, and a tenth sheet containing all the nine steroids in one spot. The ten sheets were mounted in an aluminium frame and run in a 'cubic' chromatography tank. The first system was run at room temperature but the other three were run in an incubator set at 28°C.

The results of the paper chromatography are dealt with in section 5.32. It was concluded that a thin layer system was needed.

After many trials during which different solvent
systems, tanks, thin layer media, and some conjugations were tried the following method was established.

5.233 Final Chromatographic Technique

Merck 20 x 20cm silica gel on glass plates (non fluorescent) were soaked in ethanol for three changes of 2 hours each. The plates were then dried and stored until required for use.

The plates were marked carefully with a soft pencil for use in two dimensional chromatography and activated for 1 hour at 120°C. The extraction of the residual testosterone and its metabolites from the culture fluid has been described in section 5.22 but the level of some of the metabolites was so low that identification after chromatography would have been impossible. A composite solution of the eight metabolites was prepared using the steroids purchased from Koch Light and Sigma, to contain 0.01mg of each steroid in 0.2ml of ethanol. This solution was used to dissolve the culture fluid extract and was then carefully spotted onto the plate. Evaporation of the solvent was enhanced with warm air from a hair dryer. The plates were finally dried for 15 minutes at 80°C and once they had cooled to room temperature they were loaded into a previously prepared Gelman-Hawksley chromatography tank (see Fig.5.03).

The first solvent system was 10% (v/v) ethanol in
benzene. The tank is so shaped that it is possible to have a different vapour phase to the solvent phase but for this first system benzene/ethanol was used for both. A large pad of filter paper which like the plates had received 1 hour at 130°C just prior to use, was placed in the back of the tank and charged with a separate reservoir of the same solvent system as was used for the run. The glass door was heavily sealed with Edwards silicone stopcock grease. Without either the seal or the vapour pad the solvent front stopped before reaching the top of the plate and the separation was poor. The tank was allowed to equilibriate for a short time even though it had to be opened to introduce the plates.

Nearly all the experiments involved two cultures and thus produced at least two extracts for chromatography. The tank was designed for one plate but with the help of two paper clips and a bent glass rod an extra plate could be run at the same time. Fig. 5.03 shows the tank and how this was done. The plates were allowed to run until the solvent front reached the top. The plates were then removed and dried thoroughly in an oven at 80°C. The solvent levels for both vapour phase and chromatography were topped up and the plates run again in the same direction as before. Although it would probably have made no difference the plate previously put in first was this time second. The run was completed as before and the plates thoroughly dried.
Fig. 5.03 The photograph shows a Gelman Chromatography tank. When loading it with two plates the first is put in facing the back. The bent paper-clips seen at the top hold the plate off the tank. The inverted 'V' of glass rod separates the second plate (which is put in to face the front) from the first. A plate glass door seals the tank.
The second solvent system to run at right angles was 40% (v/v) cyclohexanone in cyclohexene. A freshly dried filter paper pad was used for the vapour phase but only soaked with cyclohexene. If the complete solvent system was used it was found that the top of the plate 'refluxed' towards the end of a run and the plate became spoilt. Again the plate was run twice in this direction with a thorough drying at 80°C between runs.

All chromatography and the drying-off of the solvents was done in a fume cupboard as the solvents were toxic, but the smell of the cyclohexanone was so unpleasant it would have been necessary for this reason alone.
Metabolites of (3H)testosterone were located, eluted and estimated as described under section 5.240.

5.240 Experiments Concerned With Locating And Eluting the (3H)Testosterone And its Metabolites

5.241 Iodine

While the final chromatography technique was being developed iodine and phosphomolybdic locating reagents were used consecutively to demonstrate the nine androgenic steroids. Iodine heated to about 80°C gives off a purple vapour. At first, plate and iodine were heated together but it was later found that carefully lowering a cold plate into iodine vapour was much more effective. All the steroids except 3α androstaneediol and testosterone took up the iodine and so became brown on a yellow background.
Once removed from the vapour the iodine staining started to fade and spots had to be ringed quickly using a soft pencil. When heated in an oven at 80°C the plate very quickly lost visible traces of iodine but the treatment was continued for 15 minutes.

5.242 Phosphomolybdic Acid

The reagent used was a saturated solution of phosphomolybdic acid in ethanol. Plates were sprayed with this reagent and heated at 120°C for five minutes or more. When the first development was pale it was possible to spray again and re-heat. Seven of the steroids developed as blue green spots and fortunately these included 3α-androstanediol and testosterone (see 5.241). Later the effects of this locating reagent on scintillation counting were studied and the results are given in section 5.248.

5.243 Phosphoric Acid

50% (v/v) orthophosphoric acid in ethanol was sprayed on the plates and a reaction developed with the steroids at 120°C for 10 minutes. All nine steroids were demonstrated and the speed of development and the colours given could be used to assist in the identification of the spots. The colours seen were purple, green, and orange. Stored reagent produced poor results and excess reagent made the plate glutinous. The effects of this locating
reagent on scintillation counting were studied and the results are given in section 5.33.

5.244 Anisaldehyde-Sulphuric Acid (Miescher 1946)

1ml anisaldehyde
2ml sulphuric acid
98ml acetic acid

Plates were sprayed and heated at 95-100°C for 15 minutes.

5.245 Sulphuric Acid

50% (v/v) sulphuric acid in water was sprayed onto the plates and the plates heated at 100°C for up to ten minutes.

5.246 'Beta-Graph' (Panax Nucleonics)

The 199 and T8 fluids from a five day culture were extracted and TLC chromatograms prepared. The chromatography solvent phase used was ethyl acetate 5% (v/v) in diethyl ether. This was used once in two dimensions and this ensured that the steroids would be found on a line starting from the 'spotting' corner and drawn diagonally across the plate.
Two plates (a 199 and a T8 from the same tissue) were taken to Panax of Mitcham. Polaroid photographs showing the radioactive areas were made using a spark chamber on a 'beta-graph'. The photographs were projected on to the plate and the areas ringed with a soft pencil.

A strip 3cms wide was marked out across the diagonal of the plate and the waste corners of the plate cut off to make it easier to handle. The strip was marked off cross-wise in 5mm steps and then these were scraped off into vials. Scintillation fluid was added and the contents counted.

5.247 Thin Layer Radiochromatogram Scanner

The equipment used would have needed considerable alteration (and this is mentioned in section 5.33) to be used for this work. In view of the problems and that satisfactory alternatives existed it was never used.

5.248 Recovery of \(^{3}\text{H}\)Steroid after Chromatography and Location

Recovery of \(^{3}\text{H}\)steroid was investigated by six experiments which required the repeated use of a sample of \(^{3}\text{H}\)testosterone solution in ethanol containing 10\(\mu\text{Ci/ml}\).
10µl of an ethanolic solution of (³H)testosterone was spotted on a narrow 20cm strip of TLC plate. An equal volume of the same solution was placed in a counting vial and allowed to dry out. The TLC was run once using the benzene/ethanol solvent. Using an RF value for testosterone which had been established with this solvent system, a thin pencil line was marked across the strip where the steroid could be expected to be found. A band of silica gel was then removed centering on this line and counted as described under section 5.252.

One 10µl volume of the (³H)testosterone solution was concentrated with drying on TLC in the usual way and another was added all at once and allowed to spread and dry without heat. These spots were then scraped off and counted as described under section 5.252.

Six 10µl spots were made with the (³H)testosterone solution on separate pieces of silica gel TLC plate and dried. Ethanol/orthophosphoric acid 50:50 was sprayed on each, but in increasing quantity. The colour was developed at 120°C and the spots were removed and counted as described under section 5.252.

The first experiment described in this section was repeated except that 0.02mg of cold testosterone was added to the (³H)testosterone. Phosphoric acid reagent was sprayed on and the plate heated at 120°C to produce the colour. The spot was then transferred to a vial and
counted in the usual way.

Six 10μl spots were made with the (3H)testosterone solution on separate pieces of silica gel TLC plate and dried. Phosphomolybdic acid reagent as described in section 5.242 was sprayed on each but in increasing quantity. The colour was developed at 120°C. The spots were then scraped off and counted as described under section 5.252.

Two 10μl volumes of the (3H)testosterone solution were spotted onto a piece of silica gel TLC plate. The spots were dried and sprayed with 50% (w/v) orthophosphoric acid in ethanol and heated at 120°C to develop the colour. Both spots were then scraped off into separate scintillation vials. One was thoroughly powdered and 10ml of scintillation fluid (section 5.252) added. The other was similarly powdered but 0.2ml of distilled water was added, mixed, and left for five minutes. 10ml of scintillation fluid was then added with a further mix and the vials subjected to scintillation counting.

5.249 Specific Activities

A six hour organ culture was prepared containing 15μM/litre of testosterone, but with a higher (3H)testosterone level (150μCi/litre) than was routinely used. The steroids were extracted using dichloroethane and separated by thin layer silica gel chromatography using benzene-ethanol and cyclohexene-cyclohexanone as previously
The steroids were identified using iodine vapour. Once the iodine sensitive spots were marked, the position of the non-reactive spots could be predicted. The steroids were eluted from the plate and 30 mg of the authentic commercial steroid added. Crystals were allowed to form from an acetone/ligroin solution and specific activities (dpm/mg) were calculated. The results are given later.

5.250 Location, Elution, and Estimation of the (³H)Testosterone and its Metabolites Following Organ Culture and Chromatography

5.251 Location

The steroids were located following chromatography by using the 50% (v/v) phosphoric acid solution described in section 5.249. This reagent had some quenching effect on the scintillation count and a standard technique was developed when spraying so that minimal quantities were used and an even coating of the plate was achieved.

The colours were developed by heating at 110 to 120°C for up to 20 minutes. At the lower temperature the difference in the colours seemed more obvious but some were very pale. At the higher temperature the spots were darker but were inclined to start to fade as heating was continued. The first colouration would start at about 3 minutes at the low temperature with maximum possibly
achieved between 15 and 20 minutes. The spots were marked with a pencil as a precaution against subsequent fading.

5.252 Elution

The spots were scraped off with a scalpel blade. The powder from a spot was tipped first onto a piece of highly glazed paper and then funelled into a counting vial. The lumps of powder were then gently pulverised with a glass rod and then 0.2ml of distilled water added. The silica 'dust' was soaked in the water for at least 5 minutes before 10ml of scintillation fluid was added. The scintillation fluid was the one used in section 3. The formula is repeated here.

Triton x 100 333ml
Toluene 666ml
Butyl PBD 6g

The vial was well mixed and left for at least 30 minutes before being placed in the counter.

5.253 Interpretation of the Scintillation Counts

The counts were corrected as recommended by the manufacturer of the machine and the external standard ratio facility regularly used.
There were two simple methods of expressing the results which are readily interconvertible.

Method A. Counts expressed as a % of the combined results of all nine steroids.

Method B. Counts expressed as a % of the combined results of the eight metabolites, and the testosterone count related to this combined figure. The results in this chapter will be given in 'B' form only, unless otherwise stated.

5.30 Results and Discussion

5.31 Organ Culture

The objective during organ culture is to maintain the metabolism of the tissue as if it were still in vivo. Tissues can be maintained in culture for years and then by reimplantation can be proved to be still viable. Nevertheless, it seems improbable, that once divorced from the hormonal control of the body and without the influence of other tissues and a circulating blood supply, that metabolism will go on as normal. It was hoped that by control of technique and careful study of the results any harmful influences would be detected and eliminated. The various experiments undertaken with this end in mind will be detailed next.

The first organ culture experiments maintained the
tissue in media with twice daily changes of the atmosphere for five days. A histological comparison of the tissues before and after five days of culture can be seen in Figs. 5.04, 5.05, 5.08 and 5.09. The stromal tissue is surviving well (as might be expected) but the glandular epithelium has undergone obvious change. The epithelium has been shed into the lumen of the acini. The nuclei of the epithelial cells seem a little more homogenous after culture than before and yet no worse in this respect than the Figs. 5.06 and 5.07 which are after 24 hours of culture. It has been explained elsewhere that the ratio of glandular to stromal tissue and the appearance of acinar structure will vary even from field to field down a microscope. Therefore no significance should be placed on such differences in the photographs being studied.

After 24 hours culture epithelial height seems to be unaffected but the division between one cell and the next seems less pronounced (Fig. 5.06 and 5.07). The majority of the experimental work was based on a six hour culture and after such a short period of time no cellular differences could be seen.

Tissue thickness is important in organ culture. The prostate is a very tolerant organ yet ideally the slices needed to be less than 0.1 cm thick. Direct measurement of slices seemed difficult but a simple yet reliable method of assessing the average thickness was found. The stainless-steel grids were examined after culture and the area of grid covered by tissue estimated. Altogether
Photographs of prostatic hyperplasia stained with haematoxylin and eosin.
Photographs of prostatic hyperplasia. Shows slight changes due to organ culture for 24 hours. Stained with haematoxylin and eosin.
Photographs of prostatic hyperplasia. Shows changes due to organ culture for 5 days. Stained with haematoxylin and eosin.
eight cultures were examined and in the worst case there was at least 90% coverage. The tissues were weighed after each culture and the mean weight used was 2.381gms. Using the area of the grid covered and an approximate specific gravity figure of 1.0 for the tissue, the tissue was calculated at being less than 0.104cm thick. The coverage of the stainless-steel was judged after the culture because there was a slight tendency for the tissue to contract during the six hours. A separate investigation has shown that the tissue weight does not change significantly during culture.

The prostatectomy specimens were collected from the theatre as soon as they were available. When everything went smoothly the culture could be set up within 20 minutes. For many and varied reasons it could take up to 2 hours. When any delay was foreseen the specimens were stored at 4°C. It was not known if these delays would significantly affect the results of the culture, so therefore a specimen that arrived rather later than expected was deliberately left at 4°C overnight before culturing. It was by then about 18 hours old. The results of the testosterone metabolism following this delay are shown in Table 5.03 and can be compared with the results shown in Fig.5.14 and Tables 5.07 and 5.08.

The most obvious difference can be seen with the level of androstenedione in both fluid and tissue. It is not possible for one androgen to be altered in the system without having an effect on all the others. This may
<table>
<thead>
<tr>
<th>Steroid</th>
<th>Fluid</th>
<th>T8</th>
<th>Tissue</th>
<th>T8</th>
</tr>
</thead>
<tbody>
<tr>
<td>testost.</td>
<td>385.9</td>
<td>354.3</td>
<td>48.5</td>
<td>29.3</td>
</tr>
<tr>
<td>DHT</td>
<td>17.1</td>
<td>24.0</td>
<td>15.6</td>
<td>10.1</td>
</tr>
<tr>
<td>4A.enedione</td>
<td>14.4</td>
<td>12.0</td>
<td>5.0</td>
<td>3.4</td>
</tr>
<tr>
<td>3 A.diol</td>
<td>29.5</td>
<td>30.1</td>
<td>31.5</td>
<td>26.5</td>
</tr>
<tr>
<td>5 A.dione</td>
<td>4.0</td>
<td>5.0</td>
<td>3.6</td>
<td>3.4</td>
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<td>6.1</td>
<td>5.9</td>
<td>9.1</td>
<td>10.9</td>
</tr>
<tr>
<td>DHA</td>
<td>1.7</td>
<td>3.0</td>
<td>4.3</td>
<td>6.0</td>
</tr>
<tr>
<td>EA.erone</td>
<td>9.9</td>
<td>6.4</td>
<td>7.4</td>
<td>13.2</td>
</tr>
<tr>
<td>3 A.diol</td>
<td>16.4</td>
<td>13.5</td>
<td>23.5</td>
<td>26.4</td>
</tr>
</tbody>
</table>

Table 5.03: The results of a 6 hour organ culture in two different culture media where the prostate gland had been stored for 18 hours at 4°C before the culture was set up.
be a real change due to the interconversions that exist in the pathway (Fig.5.02), or an apparent one due to the results being expressed as a percentage. However, as these minor changes represent an eighteen hour delay in culturing, it seems unlikely that significant differences would result from the short delays that sometimes occurred.

It is perhaps best stated now that this last investigation showed that the freshest post mortem normal prostate from young men could be used in a comparative study with hyperplastic prostate. Indeed much of this work was undertaken in the belief and expectation that when kidneys for transplanting were removed from cases of sudden death the prostate would be taken at the same time. Although I was reassured on more than one occasion, the months went by but no prostates were ever received. Normal prostates were used as detailed in chapter 4 but they were from routine post mortem examinations at a distant hospital. They were some hours old on removal and yet this disadvantage was in part offset for the investigations of chapter 4, because the tissue could be frozen immediately to -40°C and transported at leisure. Tissue from this distant hospital would have been unsuitable for organ culture due to the unavoidable delays.

One prostatectomy specimen that was received was used for a short term experiment. Cultures were prepared
for 1 and 2 hour incubations and the results of these cultures are shown in Fig. 5.15.

Although they have deeper implications which will be discussed later the results show that when combined with figures for 6 hour culture the overall metabolism of testosterone is progressive.

Lastly two culture media were used to try and prove in part, that the design of the organ culture technique was satisfactory. The literature shows that a number of different media have been used for prostate culture in studies of steroid metabolism. Different findings could have been the result of using different media. Two dissimilar though popular media were used and despite this the results were substantially the same. The horse serum used in the culture media was measured very carefully to keep the proportions correct (Lasnitzki and Franklin 1972).

The importance of completely replacing the natural atmosphere with the gas mixture is stressed by most workers. The McIntoch and Fildes jar was altered because the standard lid has very small gas ports which create turbulence. With a newly designed lid the jar could be freely gassed with delivery to the bottom of the jar and venting air from the top without turbulence continually mixing the 'incoming' with the 'existing' atmosphere.

The results of the solvent extraction of (3H) DHT
dissolved in water are given here.

<table>
<thead>
<tr>
<th></th>
<th>Percentage Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>78%</td>
</tr>
<tr>
<td>Toluene</td>
<td>76%</td>
</tr>
<tr>
<td>Chloroform</td>
<td>85%</td>
</tr>
<tr>
<td>Dichloroethane</td>
<td>84%</td>
</tr>
</tbody>
</table>

The figures show that chloroform and dichloroethane are the best solvents tested. In use the culture fluids were inclined to form emulsions with chloroform and so the later work was done with dichloroethane.

5.32 **Chromatography**

The paper chromatography using Bush systems was unsatisfactory. The explanation was inadequate technique as the method is known to require a carefully controlled environment and to be very exacting. Table 5.04 shows some results compiled by Lisboa (1969) for seven of the steroids under study using TLC. Benzene with ethanol had a good range of $R_P$ values in three different proportions and after trial a ratio of 9:1 was used in one dimension. Further work showed that although the spots were slightly bigger after a second run, the separation was enhanced. The main group of steroids had $R_P$ values between 0.3 and 0.4 which were low enough to justify a second run. Table 5.05 shows some of the solvent systems examined in the search for one to use in the second dimension. Finally cyclohexene/cyclohexanone was used in the ratio of 6:4 and was also run twice.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf Value (ethyl acetate, cyclohexane, ethanol)</th>
<th>Rf Value (ethyl acetate, cyclohexane)</th>
<th>Rf Value (chloroform, ethanol)</th>
<th>Rf Value (benzene, ethanol)</th>
<th>Rf Value (ethyl acetate, n-hexane, glacial acetic acid)</th>
<th>Rf Value (benzene, ethanol)</th>
<th>Rf Value (chloroform, ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>0.46</td>
<td>0.46</td>
<td>0.23</td>
<td>0.58</td>
<td>0.46</td>
<td>0.58</td>
<td>0.23</td>
</tr>
<tr>
<td>1. Testosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Dihydrotestosterone</td>
<td>0.52</td>
<td>0.52</td>
<td>0.58</td>
<td>0.58</td>
<td>0.52</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>3. Androsterone</td>
<td>0.55</td>
<td>0.55</td>
<td>0.50</td>
<td>0.50</td>
<td>0.55</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>4. Androstenedione</td>
<td>0.50</td>
<td>0.50</td>
<td>0.48</td>
<td>0.48</td>
<td>0.50</td>
<td>0.48</td>
<td>0.48</td>
</tr>
<tr>
<td>5. Androstanediol</td>
<td>0.55</td>
<td>0.55</td>
<td>0.57</td>
<td>0.57</td>
<td>0.55</td>
<td>0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>6. Androstenedione</td>
<td>0.57</td>
<td>0.57</td>
<td>0.57</td>
<td>0.57</td>
<td>0.57</td>
<td>0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>7. Epiandrosterone</td>
<td>0.63</td>
<td>0.63</td>
<td>0.61</td>
<td>0.61</td>
<td>0.63</td>
<td>0.61</td>
<td>0.61</td>
</tr>
<tr>
<td>8. Epiandrosterone</td>
<td>0.66</td>
<td>0.66</td>
<td>0.67</td>
<td>0.67</td>
<td>0.66</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>9. Androstanediol</td>
<td>0.63</td>
<td>0.63</td>
<td>0.64</td>
<td>0.64</td>
<td>0.63</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>10. Androstanediol</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Table 3.04 Rfs obtained by B.P. Lisboa 1964 with Merck silica gel thin layer chromatography plates.
<table>
<thead>
<tr>
<th>Substance</th>
<th>R&lt;sub&gt;p&lt;/sub&gt;s</th>
<th>Ethyl Acetate, Hexane, 3:2</th>
<th>Ethyl Acetate, 2:2:4</th>
<th>Ethanol, Pyridine</th>
<th>Acetic Anhydride, Chloroform, Ethanol, 9:1</th>
<th>Benzene, Ethanol, Pyridine, Chloroform, Dioxane, 8:2</th>
<th>Benzene, Acetone, 7:1</th>
<th>1,1,1-Trichloroethane, Acetone, 1:1</th>
<th>1,1,1-Trichloroethane, Dioxane, 4:1</th>
<th>1,1,1-Trichloroethane, Ethyl Acetate, 1:1</th>
<th>1,1,1-Trichloroethane, Ethanol, 9:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>0.30, 0.27</td>
<td>0.40, 0.42</td>
<td>0.61</td>
<td>0.50</td>
<td>0.12, 0.40</td>
<td>0.11, 0.25, 0.40</td>
<td>0.065</td>
<td>0.23, 0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>0.42, 0.37</td>
<td>0.44, 0.61</td>
<td>0.49</td>
<td>0.27</td>
<td>0.54, 0.19</td>
<td>0.17, 0.19, 0.36</td>
<td>0.027</td>
<td>0.33, 0.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Androstenedione</td>
<td>0.40, 0.35</td>
<td>0.48, 0.72</td>
<td>0.76</td>
<td>0.27</td>
<td>0.54, 0.25</td>
<td>0.26, 0.17, 0.34</td>
<td>0.027</td>
<td>0.33, 0.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Androstaneidol</td>
<td>0.31, 0.27</td>
<td>0.29, 0.38</td>
<td>0.48</td>
<td>0.34</td>
<td>0.51, 0.065</td>
<td>0.085, 0.07, 0.34</td>
<td>0.026</td>
<td>0.22, 0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Androstaneidone</td>
<td>0.53, 0.46</td>
<td>0.73, 0.54</td>
<td>0.76</td>
<td>0.38</td>
<td>0.65, 0.37</td>
<td>0.34, 0.23, 0.065</td>
<td>0.026</td>
<td>0.34, 0.41</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Androsterone</td>
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<td>0.50, 0.60</td>
<td>0.61</td>
<td>0.43</td>
<td>0.37, 0.51</td>
<td>0.17, 0.17, 0.32</td>
<td>0.026</td>
<td>0.33, 0.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0.38, 0.31</td>
<td>0.47, 0.52</td>
<td>0.56</td>
<td>0.17</td>
<td>0.49, 0.16</td>
<td>0.17, 0.41, 0.02</td>
<td>0.026</td>
<td>0.38, 0.39</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>0.34, 0.30</td>
<td>0.45, 0.59</td>
<td>0.53</td>
<td>0.15</td>
<td>0.42, 0.15</td>
<td>0.15, 0.38, 0.21</td>
<td>0.026</td>
<td>0.31, 0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Androstaneidol</td>
<td>0.31, 0.27</td>
<td>0.33, 0.50</td>
<td>0.59</td>
<td>0.09</td>
<td>0.33, 0.07</td>
<td>0.021, 0.28, 0.28</td>
<td>0.026</td>
<td>0.21, 0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.05  R<sub>p</sub>s obtained using Merck silica gel thin layer chromatography plates in a Gelman Tank at 4°C
When the choice of solvents had been decided another difficulty became apparent. Whichever locating method was used, a dark band was obtained occupying one third of the plate and nearest to the side that was the top when the cyclohexene/cyclohexanone was being run. Fig. 5.10 shows a plate after the steroids have been located and ringed, and in fact the band nearly obscures the 5α-androstanedione spot. It can also be seen that there is a concentration in the top-right corner. This indicated that the benzene/ethanol had also contributed to the effect. When first discovered the band ruined the plates. The TLC plates did not contain additives such as fluorescent material. If the plates were soaked in ethanol as described in 5.2.3 the interference was reduced to the tolerable level seen in Fig. 5.10.

The steroid spots did not seem to separate quite as expected. The Rp figures that applied when run separately did not hold when they were separated from a common origin. This gave some doubt at first as to which spot was which. The different colours given with the locating reagents, the fact that testosterone was the largest spot and that 4-Androstenedione and 5α-androstanedione were well away from the others all helped in the identification. However where doubt existed a chromatogram was run using a pool of eight steroids and omitting the one under examination. By this means the two androstanediol spots were distinguished and the main block of four steroids (Dihydrotestosterone, Androsterone, Dehydroepiandrosterone
Fig. 5.10 The photograph shows a TLC silica gel plate after it has been used to separate the nine steroids and they have been located with phosphoric acid reagent. The spots have been ringed with a soft pencil because they start to fade quite quickly. The dark band is discussed in the text (5.32).
and Epiandrosterone) were identified. Table 5.06 shows the specific activities obtained from a culture following chromatographic separation. In this culture the proportion of radio-active testosterone was increased but the total amount of testosterone was unchanged. After silica gel thin layer chromatography the steroids were located with iodine vapour as detailed in section 5.241. Further to confirming that the chromatographic spots were due to single steroids and not a mixture, the specific activities also confirm the success of the identification procedure undertaken in section 5.32.

5.33 **Locating Reagents and Other Methods.**

Three of the locating reagents worked better than the other two. Of those that worked the iodine was not the easiest to use as the iodine was only effective on cold plates and the spots disappeared in seconds once removed from the vapour. Only seven of the steroids reacted with iodine, but from these, the position of the other two could be accurately predicted. The phosphomolybdic acid was easier to use but quenched the tritium (as will be explained later). The ethanol/orthophosphoric acid reagent had disadvantages but it did show all the steroids and was the reagent regularly used. The anisaldehyde reagent was discovered some months after the complete culture and chromatography method was functioning. Although it looked the most promising reagent it made the whole plate a deep beetroot red and no further time was spent with it. The
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**Table 5.06**  Recrystallization to constant specific activity (dpm/mg) of testosterone and its metabolites from organ culture fluid.
50% sulphuric acid was also unsuccessful. The spray was unpleasant to use, the plate went nearly black on heating and the silica gel became soft.

Figs. 5.11 and 5.12 show copies of the photographs made by a Panax Nucleonics (of Mitcham) Beta-Graph. The firm generously agreed to run two of the thin layer chromatography plates through their machine. The two heavy round spots are for location purposes and originate from the machine and not from the plates. One of the plates was put in the machine upside down relative to the other. The results were not equal to those of the chemical sprays which on similar plates showed a more precise localisation of steroid. The silica gel across the diagonal of the plate was then removed and subjected to scintillation counting as described in section 5.246. Fig. 5.13 shows a block graph prepared from the counts obtained and its comparison with the diagonal portion of the plate showing Beta-Graph plots and the marking into 5mm bands.

The culture used for the Beta-Graph had been up for five days. This was done so that much of the radioactively labelled testosterone would be converted into metabolites. The block graph shows that the Beta-Graph has not detected the lower levels of radioactivity. If the usual six hour culture period had been used the Beta-Graph would have been even less effective. This could in part be off-set by increasing the proportion of ($^3$H)testosterone to non radioactive testosterone in the culture. There would
Fig. 5.11  Panax Beta-graph photograph of T8 culture TLC plate.

Fig. 5.12  Panax Beta-graph photograph of 199 culture TLC plate.
Fig. 5.13 The comparison of a Beta-Scan and the scintillation counts obtained from sectional scrapings of a silica gel thin layer plate.

Block graph of counts obtained from systematic scrapings of 199 chromatography plate

Beta-scan of 199 chromatography plate
remain however a discrepancy in the equipment when the polaroid photograph is projected onto the plate so that areas of radioactivity might be ringed. This discrepancy only amounted to a few millimetres random difference each time the projection was set up, but looking again at the chromatographic pattern shown in Fig.5.04 this would have been unacceptable even if the Beta-Graph could have demonstrated all the metabolites.

The high radioactivity shown in Fig.5.13 near the origin is due to conjugated forms of steroid that are not produced in any quantity during the first six hours of culture.

Thin layer radiochromatogram scanners are less sensitive than the Beta-Graph for \( \beta \) radioactivity. In order to get such a machine to discriminate between steroids so closely positioned, a sampling slit much narrower than the manufacturers supply would need to be made. The level of radioactivity would have to be considerably increased to overcome the insensitivity and increased again because of the narrower slit. The scanner would have to run at its slowest and therefore the diagonal would need to be cut from the plate so that the machine would not waste many hours on blank areas. Because the locating reagents were so effective at identifying the steroids these modifications were never made.

A number of tests were done to determine the effect of the chromatographic and spraying techniques on the
final $^3$H count. One test involved spotting ($^3$H)testosterone onto TLC plate and then scraping off the gel for counting. The concentrated spot gave 4% loss but the spot allowed to spread to about the size reached after chromatography gave a 16% loss. This could mean that not all the steroid was eluted from the gel by the scintillation fluid, or that some soluble material from the gel was quenching the counts. Either cause could be difficult to overcome. The culture and chromatography were undertaken that different prostates might be compared. Therefore, small effects could be tolerated providing they were consistent.

Another test involved a chromatographic run of ($^3$H)testosterone but without any locating reagents. This showed a 19.8% loss of radioactivity. Such a result could mean that not all the $^3$H was present as ($^3$H)testosterone and the deficit was elsewhere on the plate.

The ethanol/orthophosphoric acid locating reagent was found to have a binding or quenching effect. Spots of ($^3$H)testosterone of equal size were placed on the TLC plate and without any chromatography were sprayed with phosphoric acid reagent in varying quantity. After heating and scraping, the spot given the lightest treatment with phosphoric acid showed a 14% loss and that with the heaviest treatment a 54% loss. Bearing in mind the ability of the gel to reduce the count by 16% (as recently stated) this represents a range of -2 to 38% due to the phosphoric acid alone. All the plates from
culture fluids when treated with phosphoric acid locating reagent were sprayed evenly in a draught free atmosphere.

The phosphomolybdic acid spray had a very heavy quenching effect on the scintillation counts. At first it was thought that the steroid might have been bound to the silica gel by the reagent and not free to be counted, but a vial that contained a little silica gel treated with phosphomolybdic acid quenched an external standard of 10000 disintegrations to 3.

In the introduction it was explained how the eight metabolites of testosterone were chosen. There was a possibility that other metabolites were being formed in substantial quantity. Therefore after the nine spots were removed from a TLC plate a broad diagonal strip about 6cm wide was marked across the plate. The back of the plate was scored along the lines and the strip broken free. Strips 0.5cm wide were marked gently across the strip with a pencil and then one by one scraped into counting vials. The silica gel was then pulverised, wetted, mixed with scintillation fluid and counted as previously described. The counts obtained were very low and were rarely more than back-ground figures. The results were therefore rather inaccurate but in total constituted another 5%.

A purity test was run on the (\(^{3}H\))testosterone by subjecting a sample to chromatography with added metabolites as markers as in the usual technique following organ culture.
When this check was done only 2.4% of the tritium was recovered from the metabolites and the distribution was fairly even amongst them. However, it was later found that ($^3$H)4-androstenedione was slowly formed in the ($^3$H)testosterone on storage. This was only found when the results of organ culture were being affected by its formation and a number of experimental results had to be rejected on suspicion of being wrong. Thereafter the stock ($^3$H)testosterone was checked and replaced regularly.

It has already been stated that two different culture media were used for organ culture. Once the methods of organ culture and metabolite separation and estimation were satisfactory, six cultures were set up using both fluids. The figures were analysed statistically and the block graphs of Fig. 5.14 show some of these results.

Two graphs are shown, one relating to the fluid and the other to the tissue. The blocks in most cases can be seen to have stepped tops. The right side is the mean result for medium 199 and the left the mean result for medium T8. The rods show ± standard error of the mean (SEM).

The two culture media gave very similar results. The greatest difference was found with epiandrosterone both in fluid and in tissue. It can be seen that the SEM's have little if any overlap for this steroid. When comparing 199 and T8 the use of different culture media may affect steroid metabolism in vitro, but in this
Fig. 5.14  Block graph showing means and ± SEM calculated from six organ cultures using T8 medium (left side of columns) and 199 medium (right side of columns).
instance (with the exception of epiandrosterone) the differences were not significant. In subsequent experiments only medium 199 was used.

Table 5.07 shows the results of the next sixteen organ culture fluids and Table 5.08 the statistical data relating to them. There is wide variation in the testosterone level remaining after six hours. It is mainly in the epithelial cells that the testosterone is metabolised, but Franks et al. (1970) have shown that the stroma is necessary for epithelial activity. However, with the stroma intact the quantity of testosterone converted to metabolites is dependant on the quantity and activity of the epithelial tissue. This is an uncontrollable variable between the organ cultures and it is for this reason that method B presentation of results is generally preferred to method A (section 5.253).

The last figure (5.15) is constructed from the results of the one and two hour cultures mentioned in section 5.31 and the results depicted in Fig.5.14. The results are in the A form (section 5.253) and show the rate at which the level of each metabolite is changing. The graphs are drawn from only three points. The points are the mean of the results obtained. The changing level in the fluid suggests a continual build-up of some metabolites over the six hours while others have possibly reached a plateau after only one hour. This shows the importance of keeping conditions well controlled within a series of experiments.
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Table 5.07  Results obtained from sixteen 6 hour culture fluids using medium 199. The results are calculated from the scintillation counts by method B (see text of section 5.253).
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**Table 5.08** Statistical presentation of Data given in Table 5.07.
Fig. 5.15.
Distribution and build-up of labelled metabolites of testosterone during six hours of organ culture. The results are calculated from the scintillation counts by method A (see text of section 5.235).
and the difficulty of trying to compare the results of
groups of workers who used differing times and ratios
of tissue to culture fluid.

The distribution of metabolites in tissue shows a
steady build-up of all 8 testosterone metabolites in the
slices of hyperplastic prostate. The ratio of tissue to
fluid graphs are drawn from corrected scintillation counts.
The mass of tissue was just over one seventh that of the
fluid. Therefore the scintillation counts of fluid and
tissue were mathematically calculated as concentrations
and then their ratio worked out. This ratio was plotted
on the graphs. A level graph means that any change in
fluid level is being matched by a similar change in tissue
level.

The object of the experimental work in this chapter
was to provide a system in which the steroid metabolism
of different samples of prostatic tissue could be compared
and to use it for this purpose. The first objective has
been achieved but the full potential of the second has
not, because normal prostate from young and old men was
not available and only hyperplastic prostate has been
tested. Had the tissue been available the differences
found between the groups would have been an excellent
starting point for assessing the factors governing the
metabolism of testosterone within the prostate. As it
is one is tempted to try such an assessment using the
graphs in Fig. 5.15 when the information is inadequate.
However the following can be said within the culture system:-

(1) At six hours testosterone is still being strongly metabolised and the quantity of the other androgens is still increasing. Six hours is therefore a satisfactory period for the culture to run.

(2) In Fig. 5.02 the metabolic pathway shown ends with the androstanediols. In accord with this the build-up of these androgens is most marked despite the reactions being reversible.

(3) The metabolites of testosterone are retained within the tissue against an increasing gradient. This is most likely to be due to inability to cross cell membranes or to binding within the cell.
6.00 THE EFFECT OF ANTI-ANDROGENS ON THE METABOLISM OF TESTOSTERONE IN ORGAN CULTURE OF HYPERPLASTIC PROSTATE

6.10 Introduction

The point that the prostate is androgen dependant has already been made. This fact has stimulated pharmaceutical laboratories to try and produce drugs that would produce a 'pharmacological orchidectomy' when administered. A few have given encouraging results initially and Cyproterone is such a substance. At first it was thought that this synthetic steroid, which is structurally rather similar to progesterone, acted as a competitive inhibitor of the enzymes involved in testosterone metabolism. However later work has indicated that in the prostate cyproterone combines with and blocks receptor sites in the cytoplasm and nucleus of the epithelial cells, without affecting the enzymes (Hansson and Treter 1971, Neumann et al. 1975). Schering (Berlin) the manufacturers are not proposing to market the drug because when it is used there is an enhanced negative feed-back to the hypothalamus/pituitary which calls for the release of more gonadotrophins (Sturde 1971). It is possible that other hormones such as growth hormone and thyroid stimulating hormone are also increased. Huggins and Russell (1946)
using dogs showed that hypophysectomy resulted in a more marked prostatic atrophy than castration and therefore the level of testosterone is not the only factor. It is possible that another drug could be found to block the feed-back and then it and cyproterone could be used together.

However Schering have found that adding an acetate at carbon 17 (see fig. 6.01 (a) and (b)) has made the drug more versatile. Cyproterone acetate is simultaneously a potent gestagen, inhibiting the gonadotrophic secretions at the hypothalamic/pituitary level and an androgen antagonist (Neumann et al. 1975). Cyproterone acetate at 30mg per day causes aspermia at between 6 and 10 weeks with a histological picture of arrested maturation. The testis shows no apparent changes following cyproterone acetate at 200mg/day (Petry et al. 1970). About 30% of cyproterone acetate administered by mouth is absorbed in the gastro-intestinal tract (Kolb and Koepke 1968). Doses of up to 150mg of the acetate have been given per day. It is available for the treatment of hypersexuality under the name of Androcur.

Anti-androgen therapy using diethylstilboestrol (hereafter simply called stilboestrol) has been the conventional procedure for the management of patients with carcinoma of the prostate for many years. The structure of the oestrogenic drug is shown in fig. 6.0 (c). It is assumed that its activity is due to the two unsaturated carbon rings containing a single hydroxyl group each,
Fig. 6.01 (a) (b) and (c) show the structures of the three antiandrogens incorporated into the hyperplastic prostate organ cultures.
because this is the only similarity that it has with the natural oestrogens found in the body. When used for carcinoma of the prostate at least 100mg is given daily. Side effects such as loss of libido, breast development and cardiovascular disease are not uncommon.

It was decided to add these three anti-androgens separately to organ cultures of hyperplastic prostate tissue and study their effect on the metabolism of testosterone.

6.20 Methods

6.21 Tissue Used for Organ Culture

The tissue used in the organ culture experiments was hyperplastic prostate and was cultured quickly, following removal by retro-pubic prostatectomy. The objective was for the culturing procedure to be started within fifteen minutes of the tissues removal and to be completed by one hour.

6.22 Organ Culture

The organ culture was almost identical to that described under section 5.21 of the previous chapter. The differences were as follows. Two cultures were set up and the same basic culture fluid (namely 199) was used for both. To one of the culture fluids was added cypionate acetate (or cypionate, or stilboestrol).
The tissue slices were then cultured and any difference demonstrated in the metabolism of \(^3\text{H}\)testosterone between the two cultures (and not due to experimental variations) was attributed to the drug.

Cyproterone and its acetate were used at two concentrations namely 75 and 150\(\mu\text{M}/1\) and stilboestrol only at the former concentration.

6.23 **Extraction, Chromatography, Location, Elution and Counting of Tritiated Steroids**

These stages were all followed as detailed in chapter five, sections 5.22, 5.233, 5.251, 5.252 and 5.253.

6.24 **Statistical Analysis**

This analysis was designed and calculated by Mr C.G. Hawes, who was until recently Head of the School of Mathematics at Kingston Polytechnic. The details and explanation of this work are contained in the appendix to this chapter.

6.30 **Results and Discussion**

6.31 **Organ Culture**

The results of the organ cultures following the use of cyproterone, cyproterone acetate and stilboestrol are shown in tables 6.01 to 6.05. The levels of these drugs used in the culture fluid are high but not excessive.
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<td>4.99</td>
<td>5.58</td>
<td>2.83</td>
<td>8.06</td>
<td>18.60</td>
</tr>
</tbody>
</table>

### Culture No. 2

<table>
<thead>
<tr>
<th></th>
<th>DHT</th>
<th>A.ene</th>
<th>3αA.</th>
<th>5αA.3.17</th>
<th>androst</th>
<th>DHA</th>
<th>Epi</th>
<th>3βA.diol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control of Fluid 199 with testost</td>
<td>37.17</td>
<td>7.28</td>
<td>18.11</td>
<td>6.69</td>
<td>5.80</td>
<td>3.38</td>
<td>6.99</td>
<td>14.59</td>
</tr>
<tr>
<td>+ 75 M/l of cyproterone acetate</td>
<td>34.87</td>
<td>6.41</td>
<td>21.73</td>
<td>6.00</td>
<td>5.92</td>
<td>2.84</td>
<td>6.57</td>
<td>15.66</td>
</tr>
</tbody>
</table>

Table 6.01 Results of two organ cultures showing the effect of incorporating 75μM/l of cyproterone acetate. The table shows the percentage of each metabolite of testosterone present in the culture fluid after six hours.
<table>
<thead>
<tr>
<th>Culture No.1.</th>
<th>DHT</th>
<th>A.ene dione</th>
<th>3αA. diol</th>
<th>5αA.3.17 dione</th>
<th>androst</th>
<th>DHA</th>
<th>Epi androst</th>
<th>3βA.diol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control of Fluid 199 with testost</td>
<td>26.01</td>
<td>5.96</td>
<td>22.44</td>
<td>7.23</td>
<td>8.72</td>
<td>4.07</td>
<td>11.57</td>
<td>14.00</td>
</tr>
<tr>
<td>+ 150 μM/l of cyproterone acetate</td>
<td>16.84</td>
<td>4.65</td>
<td>28.71</td>
<td>5.69</td>
<td>9.27</td>
<td>3.72</td>
<td>12.99</td>
<td>18.13</td>
</tr>
<tr>
<td>Culture No.2.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control of Fluid 199 with testost</td>
<td>27.00</td>
<td>7.67</td>
<td>23.74</td>
<td>3.86</td>
<td>9.67</td>
<td>3.27</td>
<td>8.15</td>
<td>16.64</td>
</tr>
<tr>
<td>+ 150 μM/l of cyproterone acetate</td>
<td>32.26</td>
<td>5.03</td>
<td>25.91</td>
<td>2.98</td>
<td>9.98</td>
<td>2.41</td>
<td>6.22</td>
<td>15.21</td>
</tr>
<tr>
<td>Culture No.3.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control of Fluid 199 with testost</td>
<td>35.99</td>
<td>6.68</td>
<td>21.14</td>
<td>4.83</td>
<td>7.28</td>
<td>3.73</td>
<td>6.43</td>
<td>13.92</td>
</tr>
<tr>
<td>+ 150 μM/l of cyproterone acetate</td>
<td>32.99</td>
<td>3.95</td>
<td>24.60</td>
<td>3.91</td>
<td>7.89</td>
<td>3.04</td>
<td>5.27</td>
<td>18.35</td>
</tr>
</tbody>
</table>

Table 6.02 Results of three organ cultures showing the effects of adding 150 μM/l of cyproterone acetate. The table shows the percentage of each metabolite of testosterone present in the culture fluid after six hours.
**Table 6.03** Results of two organ cultures showing the effects of adding 75μM/l of cyproterone. The table shows the percentage of each metabolite of testosterone present in the culture fluid after six hours.

<table>
<thead>
<tr>
<th>Culture No.1.</th>
<th>DHT</th>
<th>A.ene' dione</th>
<th>3αA. diol</th>
<th>5αA.3.17 dione</th>
<th>androst</th>
<th>DHA</th>
<th>Epi androst</th>
<th>3βA. diol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control of Fluid 199 with testost</td>
<td>25.24</td>
<td>5.49</td>
<td>25.12</td>
<td>5.81</td>
<td>6.75</td>
<td>3.39</td>
<td>13.28</td>
<td>14.92</td>
</tr>
<tr>
<td>+ 75 M/l of cyproterone</td>
<td>26.87</td>
<td>4.54</td>
<td>30.79</td>
<td>5.29</td>
<td>4.73</td>
<td>3.58</td>
<td>8.63</td>
<td>15.66</td>
</tr>
<tr>
<td>Culture No.2.</td>
<td>DHT</td>
<td>A.ene' dione</td>
<td>3αA. diol</td>
<td>5αA.3.17 dione</td>
<td>androst</td>
<td>DHA</td>
<td>Epi androst</td>
<td>3βA. diol</td>
</tr>
<tr>
<td>Control of Fluid 199 with testost</td>
<td>30.71</td>
<td>7.77</td>
<td>22.63</td>
<td>4.75</td>
<td>7.95</td>
<td>3.01</td>
<td>9.78</td>
<td>13.40</td>
</tr>
<tr>
<td>+ 75 M/l of cyproterone</td>
<td>31.90</td>
<td>6.53</td>
<td>27.18</td>
<td>4.37</td>
<td>5.69</td>
<td>3.35</td>
<td>9.09</td>
<td>11.89</td>
</tr>
<tr>
<td>Culture No.1.</td>
<td>DHT</td>
<td>A.ene dine</td>
<td>3αA. diol</td>
<td>5αA.3.17 dione</td>
<td>androst</td>
<td>DHA</td>
<td>Epi androst</td>
<td>3βA.diol</td>
</tr>
<tr>
<td>---------------</td>
<td>-----</td>
<td>------------</td>
<td>-----------</td>
<td>----------------</td>
<td>----------</td>
<td>-----</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>Control of Fluid 199 with testost</td>
<td>36.57</td>
<td>7.10</td>
<td>19.90</td>
<td>4.62</td>
<td>6.03</td>
<td>4.24</td>
<td>9.31</td>
<td>12.23</td>
</tr>
<tr>
<td>+ 150 M/l of cyproterone</td>
<td>38.07</td>
<td>2.64</td>
<td>25.04</td>
<td>4.17</td>
<td>4.38</td>
<td>3.80</td>
<td>8.41</td>
<td>13.49</td>
</tr>
<tr>
<td>Control No.2.</td>
<td>Control of Fluid 199 with testost</td>
<td>25.78</td>
<td>7.24</td>
<td>22.47</td>
<td>5.79</td>
<td>7.72</td>
<td>2.87</td>
<td>14.40</td>
</tr>
<tr>
<td>+ 150 M/l of cyproterone</td>
<td>28.24</td>
<td>5.93</td>
<td>24.49</td>
<td>5.30</td>
<td>5.39</td>
<td>3.06</td>
<td>12.11</td>
<td>15.48</td>
</tr>
</tbody>
</table>

Table 6.04  Results of two organ cultures showing the effects of adding 150μM/l of cyproterone. The table shows the percentage of each metabolite of testosterone present in the culture fluid after six hours.
<table>
<thead>
<tr>
<th></th>
<th>DHT</th>
<th>A.ene dione</th>
<th>3αA. dionol</th>
<th>5αA.3.17 dione</th>
<th>androst</th>
<th>DHA</th>
<th>Epi androst</th>
<th>3βA. diol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control fluid 199</td>
<td>20.7</td>
<td>5.9</td>
<td>26.2</td>
<td>6.5</td>
<td>10.4</td>
<td>4.2</td>
<td>10.7</td>
<td>19.3</td>
</tr>
<tr>
<td>+ stilboestrol</td>
<td>22.0</td>
<td>9.4</td>
<td>20.3</td>
<td>6.5</td>
<td>9.1</td>
<td>4.7</td>
<td>14.1</td>
<td>13.8</td>
</tr>
<tr>
<td>Control fluid 199</td>
<td>22.4</td>
<td>13.9</td>
<td>16.7</td>
<td>9.4</td>
<td>9.2</td>
<td>6.3</td>
<td>12.3</td>
<td>9.8</td>
</tr>
<tr>
<td>+ stilboestrol</td>
<td>18.3</td>
<td>18.2</td>
<td>12.2</td>
<td>9.4</td>
<td>8.0</td>
<td>6.8</td>
<td>15.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Control fluid 199</td>
<td>22.7</td>
<td>6.3</td>
<td>23.2</td>
<td>7.3</td>
<td>7.7</td>
<td>3.9</td>
<td>13.2</td>
<td>15.6</td>
</tr>
<tr>
<td>+ stilboestrol</td>
<td>21.9</td>
<td>9.8</td>
<td>20.6</td>
<td>6.6</td>
<td>6.3</td>
<td>5.1</td>
<td>15.6</td>
<td>14.1</td>
</tr>
<tr>
<td>Control fluid 199</td>
<td>19.8</td>
<td>9.0</td>
<td>24.8</td>
<td>7.2</td>
<td>9.1</td>
<td>4.1</td>
<td>9.6</td>
<td>16.4</td>
</tr>
<tr>
<td>+ stilboestrol</td>
<td>17.6</td>
<td>9.3</td>
<td>20.2</td>
<td>8.4</td>
<td>9.2</td>
<td>5.5</td>
<td>11.6</td>
<td>18.2</td>
</tr>
<tr>
<td>Control fluid 199</td>
<td>23.5</td>
<td>8.1</td>
<td>22.0</td>
<td>7.2</td>
<td>9.0</td>
<td>4.4</td>
<td>13.0</td>
<td>12.8</td>
</tr>
<tr>
<td>+ stilboestrol</td>
<td>24.7</td>
<td>10.6</td>
<td>17.0</td>
<td>8.9</td>
<td>7.7</td>
<td>5.7</td>
<td>13.7</td>
<td>11.6</td>
</tr>
</tbody>
</table>

Table 6.05  Results of five organ cultures showing the effects of adding 75μM/l of stilboestrol. The table shows the percentage of each metabolite of testosterone present in the culture fluid after six hours.
It is not unusual to find a competitive substance being included at a ratio of 200 to 1 in 'in vitro' experimental work when such levels would be intolerable in vivo. The ratio of antiandrogen to testosterone in the cultures is 5 to 1 where \(75 \mu M/l\) was used and 10 to 1 where \(150 \mu M/l\) was used. Kolb and Roepke (1968) found a 30% absorption of oral cyproterone acetate and on this basis the ratios represent doses of about 150 and 300mg/day in an adult male. Stilboestrol was only used at the lower concentration as early analysis of the figures suggested that significant results were being obtained.

6.32 Statistical Analysis

The results of the statistical analysis are contained in Appendix 6.0.

6.33 Interpretation of Results

To make reference to the tables easier some of the statistical work is reproduced here. The table numbering that is used in the appendix remains unchanged.

The purpose of this statistical study was to show whether antiandrogens affect the metabolism of testosterone in the prostatic organ culture. If fresh non hyperplastic tissue was available from young and old men, the incorporation of antiandrogens into the culture system would give a much wider picture on the changing nature of
the tissue with both maturity and hyperplasia. The work of this chapter is therefore best described as a 'feasibility study'. No firm conclusions can be drawn from the small samples and the interpretation must remain tentative even in the face of statistical probabilities. Furthermore the effects of the antiandrogens have only been studied in the culture fluid. In a full study the tissue would have to be included because it is reasonable to expect an antiandrogen to affect the binding. Such an investigation would be a large project.

Below is the second table given within the text of section Appendix 6.2. A highly significant difference between cultures from different specimens is shown by the variance ratio for 'cultures'. The main reason for this is because the results used are in the 'B' form (see section 5.253)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>Variance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>136.0</td>
<td>295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroids</td>
<td>122.0</td>
<td>7</td>
<td>17.4</td>
<td>483***</td>
</tr>
<tr>
<td>Cultures</td>
<td>4.8</td>
<td>36</td>
<td>0.133</td>
<td>3.69***</td>
</tr>
<tr>
<td>Error</td>
<td>9.2</td>
<td>252</td>
<td>0.036</td>
<td></td>
</tr>
</tbody>
</table>

and this heavily reflects the amount of testosterone metabolism rather than the pattern of testosterone metabolism. Therefore the statistics of chapter five show that the pattern of testosterone metabolism is fairly consistent in basic 199 cultures and the statistics
of chapter six that the amount of testosterone metabolism is very inconsistent.

The first table (Table of Variance) contained in the text of section Appendix 6.3 combines experiments that a biologist would probably never group together. It is included because there may be some virtue in justifying an assumption that could have been taken for granted. It shows that there is a strong probability that the anti-androgens differ in the effect they have on testosterone metabolism.

Table Appendix 6.1 shows where the formation of a metabolite has been inhibited (minus figure) or occasionally enhanced (positive figure). From it the following statements can be made.

75µM/l cyproterone acetate shows no results significantly different from those of 199 with testosterone only.

150µM/l cyproterone acetate causes steroids dihydrotestosterone, androstenedione, 5α-androstane 3,17dione, dehydroepiandrosterone and epiandrosterone to be produced more slowly in the culture fluid than when testosterone is used alone.

75µM/l cyproterone causes steroids andosterone and possibly epiandrosterone to be produced more slowly in the culture fluid than when testosterone is used alone.
150\(\mu M/l\) cyproterone causes steroids androstenedione and androsterone to be produced more slowly in the culture fluid than when testosterone is used alone.

With stilboestrol present steroids dihydrotestosterone, 3\(\alpha\)androstanediol, 5\(\alpha\)androstane 3,17dione, androsterone and 3\(\beta\)androstanediol are formed more slowly.

Epiandrosterone is formed more readily in T8 than in 199 medium

On the whole 150\(\mu M/l\) cyproterone acetate, 75\(\mu M/l\) cyproterone, 150\(\mu M/l\) cyproterone and stilboestrol have significant inhibiting effects and T8 a more stimulating effect.

The fact that the significant increase in epiandrosterone shown with 75\(\mu M/l\) of cyproterone is not maintained at 150\(\mu M/l\) is not easily explained. It may be that the significant increase would not have been found had the sample been larger.

When the 'probabilities' given above are studied in relation to the metabolic pathway of figure 5.02 (reproduced here), the following suppositions may be made.

Cyproterone acetate may inhibit the conversion of C17 hydroxy to C17 keto in the C3 ketosteroids studied but not in C3 hydroxysteroids. It may also inhibit the reduction of unsaturated A and B rings and 4-5 isomerase.
Cyproterone may inhibit the conversion of the C17 hydroxysteroids studied to C17 keto. If this is so the failure to affect 5 androstane 3,17 dione may be due to the formation of this steroid from androstenedione and androsterone without the need for such conversion.

The obvious structural features of stilboestrol are the two unsaturated rings with hydroxyl groups. As the production of androstane diols are reduced when it is used, a feed-back mechanism is indicated either affecting the production of a specific enzyme or the enzyme is regulatory.

In all the above conclusions, it has been assumed
that tissue binding of the steroids has been unaffected by the inclusion of the antiandrogens.

In the case of T8 versus 199, changing the constituents of a culture medium might reasonably lead to enhanced or reduced steroid metabolism, but for only one or possibly two steroids to be affected is unexpected and rather baffling. It may be that this is due to some non-competitive inhibitions being seen in the 199 medium.
CHAPTER 7

FINAL DISCUSSION

With the exception of the first one, each of the preceding chapters covers a separate aspect of the research. Although these chapters were written as a part of the whole, they are generally complete in themselves and have a discussion section at the end of each. The aim of this chapter is to draw them together and also to go beyond the experimental work in theoretical consideration.

In the second chapter the main histological study was described. It seemed improbable that this approach had not previously been used at depth and yet the scientific papers using it were few and far between. Apart from the size of the gland, the histological appearance shows the most distinctive differences between benign hyperplasia and the normal prostate gland. Certain enzymes and cholesterol had become associated with the prostate in the literature and these were therefore studied histologically. Hydroxysteroid dehydrogenases, amino peptidases, acid phosphatase and cholesterol were all studied in the hyperplastic prostate and when possible compared with young normal prostate. It was soon apparent that obvious differences were not going to be found.

In order to get the most from the study the results were categorised according to reaction strength; and
because hyperplasia is a progressive condition of later life, patient age and weight, and the weight of prostatic tissue removed were included. It can now be said that although a very much longer study might reveal a tendency, there is no definite relationship between any of the factors studied and the commonest type of benign prostatic hyperplasia found; furthermore of the cellular substances studied, an increased variation in level from cell to cell was seen to accompany hyperplasia.

These results although disappointing did not cause surprise. In a well differentiated carcinoma it may be difficult if not impossible to find secretory differences in the cells and yet the criteria of malignancy may be clearly seen. Generally the hyperplasia in malignancy is far more active than that found in benign prostatic hyperplasia. There can be no doubt that there are biochemical differences between normal and hyperplastic prostatic cells but it is probable that they are so fine that they will be very difficult to demonstrate.

Chapter three is a further histological study but aimed at demonstrating the location of androgens. Androgens are undeniably connected with prostatic hyperplasia but the more the relationship is studied the more they appear secondary to other factors. At the time that this part of the research was in hand, papers on cellular binding sites in the prostate were frequent and either relied on autoradiography or the recovery of radioactively labelled androgens from cell fractions.
Also at this time fluorescently labelled antisera to gamma globulins were available, but these were mostly being used to demonstrate auto-immune diseases. The use of antisera to polypeptides for histological demonstration was well documented, but those made to non-protein molecules were only used for test-tube assays. The binding of androgens to prostatic tissue is very strong as the fluorescent antibody method proved. The opportunity for the steroids to be eluted during the staining method are so great that success seemed unlikely. The explanation does not lie in the cell membranes providing a retentive barrier, for if the small steroid molecule could not get out the much larger protein antibody would never get in and be able to combine with it. Furthermore the trauma within the cell from microtomy must be so severe that no reliance could be placed on the physical integrity of the barriers active in life. In practice there was little difference in the reaction strength of the cells in the epithelial tissue and it was concluded that cells cut through the middle were reacting in proportion with those untouched by the knife. The photographs in chapter three show that the nuclear membrane of the epithelial cells is strongly fluorescent and that no reaction occurs within it. If this were solely a matter of permeability then nuclei cut in half might have been expected to react differently from those that were still complete. There now exist a number of papers demonstrating nuclear binding of androgens in the prostate. The possibility remains that such a differential reaction may occur during staining but that the intranuclear binding of
the steroid is so weak that the complex is lost at the washing stage. The control methods used almost certainly eliminate the possibility that the results are demonstrating anything other than androgens. This means that the binding of one or more of the androgens to testis and prostate is strong enough to resist being washed out by the staining procedure. In the case of the prostate this is partly supported by the evidence of organ culture. Only a few authors have compared the steroid content of prostate and culture fluid, following organ cultures with added steroid, and most of these used animal tissue for their study. It seems reasonable to suppose that the tissue binding sites can be saturated. In a culture with a physiologically very high steroid content this is most likely to occur. When the tissue volume is small compared to the incubation fluid volume the androgen level in fluid is barely affected by tissue uptake, particularly where a physiologically high concentration of androgen exists. In this case the binding ratio will be low. With low steroid concentration and a high tissue to fluid ratio the binding ratio is much higher. This latter situation has been the case with the organ cultures employed in this thesis and explains in part the high ratios obtained when the results are compared with those of other workers. I believe that under more favourable conditions even higher binding ratios could be demonstrated because the present published figures reflect saturation and not the ability to bind against a gradient. In the case of the testis the high levels of androgen may reflect storage but as the steroid molecule is very small
and therefore presumably difficult for the cell to store within vacuoles, active binding could be expected.

Since this part of the research was completed a reliable anti-guinea pig globulin complete with peroxidase marker has become commercially available. This would not alter the results obtained but could provide a permanent record which the fluorescent method was unable to do.

No general study of the prostate gland would be complete without a section on acid phosphatase. Its level in human serum is quite low and until recently, difficult to measure accurately. In the male at least half the serum acid phosphatase is of prostatic origin and this portion is strongly inhibited by tartrate. When a prostatic carcinoma exists the increase in enzyme containing cells and the fact they probably release the enzyme more easily causes an increase in the total acid phosphatase level of the serum and significantly an increase in the tartrate labile fraction. Consequently, in hospital work acid phosphatase is as strongly associated with the prostate as bilirubin is with the liver. Recently radioimmuno assay has been applied to acid phosphatase estimation with a much increased sensitivity and it is claimed that an even earlier diagnosis of carcinoma prostate is now possible. It is well known that serum acid phosphatase is not increased in benign prostatic hyperplasia and the study of acid phosphatase activity in tissue sections had shown no differences in siting or activity between normal and hyperplastic tissue.
A characterisation study was therefore undertaken to provide a more complete picture. Electrophoresis was used to see if there was a discernable difference between the enzyme in hyperplastic tissue and normal tissue. The multiple banding that was demonstrated enabled a more satisfactory visual comparison between samples than could have been made if only a few bands had been demonstrated. The study showed no qualitative or gross quantitative differences between enzyme from normal and from hyperplastic prostate. No conclusion was drawn as to why so many bands were present but the arrangement proved stable during separation, recombination, concentration and repeated separation. The enzyme in serum is very labile and more than 50% of the activity is lost during 24 hours storage at room temperature. The electrical energy required to separate the enzyme generates heat in the starch gel equivalent to the same area of domestic electric blanket. Cooling is therefore essential in order to preserve enzyme activity and any method which ignores this only separates altered enzyme. As it is, serum acid phosphatase deteriorates when stored at 4°C, so unfortunately the banding may only indicate a slightly stable and active intermediate stage in the breakdown of the enzyme. Further testing of stored enzyme samples may have shed more light in this area but were considered to be beyond the scope of this research.

By comparison with acid phosphatase, the peptidases are very stable and cooling is only required as an aid to
separation. The tissue sections that were studied were limited by the substrate only demonstrating amino peptidases E and S. Such demonstrations showed no differences between hyperplastic and normal prostate. Electrophoretic separation following tissue extraction separated more fractions but without cellular localisation. Without complex measurement, electrophoresis is limited when used to compare the amount of enzyme in different samples. The acid phosphatase study showed that the depth of final colour was unaffected until the tissue extract was very substantially diluted. With the enzyme obviously present to excess in most of the bands perhaps the extracts should have been routinely used in a more diluted form, possibly at the cost of the few weaker bands, but increasing the ability to demonstrate differences of strength in the others. Dilution experiments were not tried on the peptidases because the reaction colour given by some of them was pale from the outset. However it may have been that this was due to factors other than enzyme concentration. As stated in the relevant chapter no differences were demonstrated between normal and hyperplastic prostates. If the study of either amino peptidase or acid phosphatase had been prolonged and exceptions found, they would have had their root in some other condition and have not been of any significance in establishing the mechanism of human benign prostatic hyperplasia.

It has been suggested that the increased level of
dihydrotestosterone found in hyperplastic prostate is due to a reduced metabolism of this steroid and the other metabolites of testosterone. If this is true, it could be caused by a generalised loss of efficiency or partial failure at a specific point such as conjugation and a feed-back affecting metabolism. The metabolism of 3H testosterone in six hour organ culture was quite rapid and yet negligible quantities of 3H conjugated steroids were found. Had young normal human prostate been available for culture it is possible that more 3H testosterone would have been metabolised and a significant proportion recovered as conjugated steroid. The study of cholesterol extracted from human prostates also fits this pattern, in that the total level of cholesterol in hyperplastic prostates is higher than found in the prostates of normal men in the same age group and conversely the esterified (conjugated) fraction is lower. The enzymes that esterify sterols are not well defined and some show a wide range of activity. B-glucuronidases affecting sterols are probably more active in hydrolysing glucuronides than in forming them and certainly glucuronides of steroids are still formed when B-glucuronidases are being inhibited.

The possibility exists that the increased dihydro-testosterone and cholesterol found in prostatic hyperplasia is due to reduced activity of one enzyme within the esterase system. Whatever the cause of the increases the question remains 'Where does the chain of events start; in the prostate or outside?'
A small extension of the cholesterol study would be to separate epithelium from stroma and to measure cholesterol and esters in both fractions.

The widespread use of organ culture encourages the experimenter to the complacent belief that his or her results are valid for the *in vivo* situation but there is little justification for this attitude. Apart from the fact that the tissue is subject to compression and stretching during preparation and that a large proportion of the cells are cut or ruptured with consequent escape of constituents, there is no active circulation through the tissue, the degree of anoxia varies through the tissue and the substance under study is almost invariably at a concentration many times higher than that found in life. If a normal metabolism continued under such circumstances the experimenter would indeed be fortunate. Therefore organ culture is most wisely considered only as an experimental system within which the effect of changing one or more of the variables can be considered. The object was to see what range of results could be counted 'normal' for human hyperplastic prostatic tissue in this particular organ culture system and then to see if non hyperplastic tissue produced results within or outside the range. Any differences found might only be present due to the artificial stresses such as the abnormally high steroid concentration and not actually exist *in vivo*. On the other hand it would at least show a difference when most of the other studies undertaken showed nothing. The
organ culture study undertaken seemed most likely to show-up any differences that might exist in view of:— (1) the number of testosterone metabolites under study, (2) the fact that both incubation fluid and tissue concentrations could be examined, (3) that a comparative assessment of testosterone binding could be made, and (4) that formation rate studies were possible.

In the organ culture study the main factor influencing how much testosterone was converted to other androgens was the number of epithelial cells. There was no simple or accurate way to compare this factor in the organ cultures and assuming all other aspects of tissues to be identical, one culture could have twice the number of epithelial cells of another and convert twice as much testosterone. Without a cell count the one would appear hyper-active in comparison to the other. However it was expected that hyperplasia would cause changes in the metabolic pattern of the androgens rather than to affect only the amount of testosterone converted. For this reason the amount of say androstenedione relative to the amount of the other testosterone metabolites was more important than the absolute quantity. If it represented 3% of the metabolites it is most probable that if the culture had been accidently left for a further half an hour the proportion would be unchanged despite the increase in the total mass of metabolites formed. By the same yard-stick a highly glandular specimen would convert more testosterone in the standard time of six hours but with the same proportional changes.
Proportional figures were used for the study but the results allow absolute figures to be calculated.

Without normal tissue with which to compare the results the main thrust of the organ culture is lost. The chapter on organ culture with anti-androgens added demonstrated their activity. The level of anti-androgens used in the culture was selected after considering the physiological level of these substances when used clinically and the level of testosterone in the culture. The number of cultures used for this work was small but the final object was to justify its suitability for this study rather than to comment on the mode of action of the anti-androgens as it stands.

Part of the objective of this research was to identify the cause of human benign prostatic hyperplasia and this has not been achieved. This objective must be nearer than it was, but does not seem to be close at hand. The changes that occur in the prostate take many years and could result from an imbalance so slight that everyone has looked and yet not seen it. The two areas that I feel should have had more attention from workers in this field are the part the stroma has to play, (particularly as the hyperplasia could start there,) and the increased number of PAS positive cells in the pituitaries of men with benign prostatic hyperplasia (Haugen 1973).
APPENDIX TO CHAPTER 3

This appendix contains graphs establishing the 50% binding titre and cross reactions of six steroid antisera described in chapter four.
Two antisera to Testosterone 3BSA diluted to find the titre at which they would bind 50% of the 3H labelled testosterone.

Fig A.5.1

Titration curves of antisera to 3BSA.
Fig. A3.2
Titration curves of antisera to T17BSA.
Two antisera to Testosterone 17BSA diluted to find the titre at which they would bind 50% of the 3H labelled testosterone.
Two antisera to Dihydrotestosterone 17BSA diluted to find the titre at which they would bind 50% of the 3H labelled dihydrotestosterone.
Fig. A.3.4

Cross reactions of antisera to T3BSA.

Standard curve and cross reaction curves showing how the 3H testosterone is displaced from its binding with the two testosterone 3BSA antisera by other steroid.
Cross reactions of antisera to T3BSA
Standard curve and cross reaction curves showing how the 3H testosterone is displaced from its binding with the two testosterone 3BSA antisera by other steroid.
Cross reactions of antisera to T17BSA.

Standard curve and cross reaction curves showing how the 3H testosterone is displaced from its binding with the two testosterone 17BSA antisera by other steroid.
Fig. A3.7
Cross reactions of antisera to DHT17BSA

Standard curve and cross reaction curves showing how the
3H dihydrotestosterone is displaced from its binding with
the two dihydrotestosterone 17BSA antisera by other steroid
Fig. A3.8
Cross reactions of antiserum to DHT17BSA
Standard curve and cross-reaction curves showing how the 3H dihydrotestosterone is displaced from its binding with the two dihydrotestosterone 17BSA antisera by other steroid.
A.6.1 The Statistical Treatment of the Data

The data includes controlled factors eg. culture fluid, the steroid measurement in the experiment, and the time over which the experiment lasted. In addition, the data contains uncontrolled factors of a variable kind, responsible for "error variance" in the results, even when all controlled factors are kept the same. Differences between mean results associated with particular controlled factors are "significant", i.e. significantly great, if it is improbable that such differences would arise through error variation alone. A standard threshold of probability for this purpose is taken as 5%; and a result "significant" at 5% means that error variation alone could produce such a difference only in one experiment out of 20 average. The Techniques of analysis of Variance are described by Armitage (1) 1971. In particular, the normality, independence, and equality of error variance in various groups had to be investigated.

The technique is valid for non-normal data, provided this is not too severe, and the data was satisfactory in this respect. The figures given as percentages among metabolites (numbers 2 to 9) were not independent as each culture would necessarily have a total response of 100, so they were expressed as percentages of all nine, so including variations due to different rates of
metabolism. We indicate these percentages as \( x_2, x_3, \ldots, x_9 \) for each culture (see note 1).

A.6.2 The Results Concerned with Basic Fluid "199" Only

From the 16 results of Table 5.07 and those involving "199" only in the paired experiments, 37 results were available (see note 2).

The following Table shows that the variance among cultures for the same steroid increases with the mean (\( \bar{x} \)) for this steroid. Using \( y = \log x \) (or \( \ln x \)) as the variable, the stability of the variance is greatly, (though not completely,) restored.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{x} )</td>
<td>6.1</td>
<td>1.7</td>
<td>5.2</td>
<td>1.3</td>
<td>1.8</td>
<td>.8</td>
<td>2.4</td>
<td>3.3</td>
</tr>
<tr>
<td>( S_x^2 ) (for Steroid)</td>
<td>40.0</td>
<td>3.0</td>
<td>29.0</td>
<td>2.0</td>
<td>3.5</td>
<td>1.0</td>
<td>6.0</td>
<td>11.0</td>
</tr>
<tr>
<td>( \bar{y} )</td>
<td>1.8</td>
<td>.5</td>
<td>1.6</td>
<td>.2</td>
<td>.6</td>
<td>-0.2</td>
<td>.8</td>
<td>1.2</td>
</tr>
<tr>
<td>( S_y^2 ) (for Steroid)</td>
<td>3.3</td>
<td>.3</td>
<td>2.8</td>
<td>.1</td>
<td>.4</td>
<td>.1</td>
<td>.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

For this reason the variable \( y \) is used in the analysis rather than \( x \) (Armitage (2) 1971).

The basic fluid ("199") results were then analysed as a two factor experiment, the factors being steroids versus cultures. The results from the 37 cultures (8 \( y \)-values for each) were:
The results indicate not only highly significant differences between steroids (as expected) but also between cultures; and an error variance of 0.036 (see Note 3).

A.6.3 The Results Concerned With Other Culture Fluids Compared With "199". (i.e. paired cultures)

These results were all in the form of pairs, being tissue from one specimen used in two cultures, so differences between the \( y \) result for each member of the pair were taken. Calling this \( z \) we have \( z = y_c - y_{199} \) for each steroid (\( y_c = y \) reading for the steroid in the new culture fluid) (See Note 4).

In this case several results were available from each culture and a two factor analysis with replication was possible enabling an estimate of interaction between steroid and culture fluid responses to be made.

The variance table was as follows:-

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>Variance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>136.0</td>
<td>295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroids</td>
<td>122.0</td>
<td>7</td>
<td>17.4</td>
<td>483***</td>
</tr>
<tr>
<td>Cultures</td>
<td>4.8</td>
<td>36</td>
<td>0.133</td>
<td>3.69***</td>
</tr>
<tr>
<td>Error</td>
<td>9.2</td>
<td>252</td>
<td>0.036</td>
<td></td>
</tr>
</tbody>
</table>

*...P 0.05     **...P 0.01     ***...P 0.001
<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Variance Estimate</th>
<th>Variance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>10.46</td>
<td>167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultures</td>
<td>3.07</td>
<td>5</td>
<td>0.614</td>
<td>19.7***</td>
</tr>
<tr>
<td>Steroids</td>
<td>0.48</td>
<td>7</td>
<td>0.068</td>
<td>2.18*</td>
</tr>
<tr>
<td>Interaction</td>
<td>3.17</td>
<td>35</td>
<td>0.091</td>
<td>2.9***</td>
</tr>
<tr>
<td>Error</td>
<td>3.74</td>
<td>120</td>
<td>0.031</td>
<td></td>
</tr>
</tbody>
</table>

The highly significant interaction indicates that in addition to the culture fluids having different inhibitory effects, these differences are not the same for all steroids.

The main conclusions from Table A6.1 are

(a) Cyproterone acetate 75μM/l and cyproterone 150μM/l show little or no evidence that they inhibited metabolism more than "199".

(b) Cyproterone acetate 150μM/l, stilboestrol 75μM/l and T8 do appear to have this inhibitory effect. Of these stilboestrol is significantly more effective than cyproterone acetate 150μM/l, which is significantly more effective than T8.
Table of Means With Significant Difference From Zero

<table>
<thead>
<tr>
<th>Fluids</th>
<th>DHT</th>
<th>A.ene</th>
<th>3αA.</th>
<th>5αA.3.</th>
<th>androst.</th>
<th>DHA</th>
<th>Epi.</th>
<th>3βA.</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 M/l.cyprot.ac. (2)</td>
<td>-.06</td>
<td>-.11</td>
<td>.11</td>
<td>.095</td>
<td>0</td>
<td>-.175</td>
<td>-.05</td>
<td>.14</td>
<td>-.03</td>
</tr>
<tr>
<td>150 M/l.cyprot.ac. (3)</td>
<td>-.21*</td>
<td>-.5**</td>
<td>.06</td>
<td>-.33**</td>
<td>-.04</td>
<td>-.30**</td>
<td>-.22**</td>
<td>.05</td>
<td>-.19**</td>
</tr>
<tr>
<td>75 M/l.cypionate (2)</td>
<td>.035</td>
<td>-.195</td>
<td>.175</td>
<td>-.10</td>
<td>-.36**</td>
<td>.065</td>
<td>-.27*</td>
<td>-.09</td>
<td></td>
</tr>
<tr>
<td>150 M/l.cypionate (2)</td>
<td>-.04</td>
<td>-.70**</td>
<td>.06</td>
<td>-.20</td>
<td>-.44**</td>
<td>-.115</td>
<td>-.235</td>
<td>.005</td>
<td>-.21**</td>
</tr>
<tr>
<td>75 M/l.stilboestrol (5)</td>
<td>-.32**</td>
<td>.02</td>
<td>-.51**</td>
<td>-.23**</td>
<td>-.41</td>
<td>-.09</td>
<td>.12</td>
<td>-.26**</td>
<td>-.24**</td>
</tr>
<tr>
<td>TB medium (7)</td>
<td>-.07</td>
<td>-.01</td>
<td>.14*</td>
<td>.07</td>
<td>.07</td>
<td>.11</td>
<td>.23**</td>
<td>.01</td>
<td>.07**</td>
</tr>
</tbody>
</table>

* ...P 0.05,        ** ...P 0.01.

Table A6.1
The statistical design and work of this appendix has been contributed by Mr C.G. Hawes who was, until recently, Head of the School of Mathematics at Kingston Polytechnic. It has only been slightly edited in two places and therefore the following notes are included in explanation of certain points in the text.

(1) In the material given to Mr Hawes for analysis, the eight metabolites of testosterone were numbered rather than named. In the rest of the thesis an order is maintained in listing the metabolites and this order was unchanged even though numbers were used.

Therefore:-  Number 2 was Dihydrotestosterone  
Number 3 was Androstenedione  
Number 4 was 3α-Androstanediol  
Number 5 was 5α-Androstan-3,17-dione  
Number 6 was Androsterone  
Number 7 was Dehydroepiandrosterone  
Number 8 was Epiandrosterone  
Number 9 was 3β-Androstanediol

(2) Mr Hawes has extracted from the information provided, all the results of cultures using 199 medium (+ the standard amount of $^3$Htestosterone) on its own and examined them as a group.

(3) 0.036 is a very small figure for error variance apparently, (as is 0.031 in section A6.3). The reader
is referred back to the first two sentences of section A6.1 therefore, to make the conclusion that the experiments were scarcely subject to uncontrolled factors.

(4) The paired experiments were based on a comparison of (1) a basic 199 culture (containing \(^{2}\)Htestosterone) and (2) a second culture having some difference such as the inclusion of stilboestrol. Mr Hawes in his text has referred to these second cultures as 'new' cultures.
REFERENCES

Abul-Fadl, M. A. M. & King, E. J. (1948) J. Clin. Path. 1, 80
Anderson, W. A. D. (1961) Pathology, 4th edn., p. 643,
C.V. Mosby Co., St Louis
Armitage, P. (1971) Statistical Methods in Medical
350-352, Blackwell, Oxford
Avrameas, S. (1969) Immunochemistry, 6, 43-52
Developments in Steroid Histochemistry, pp. 11-13,
Perspect Biol. Med. 11, 384-421
Berenbaum, M. D. (1959) Immunology 2, 71-83
Berger, J. & Johnson, I. V. (1939) J. Biol. Chem. 130, 641
Normal and Abnormal Growth of the Prostate, (Goland, M.
Cathro, D. M., Cameron, J. & Birchall, K. (1965)
J. Chromatogr. 17, 362-372
(1943) J. Urol. Baltimore, 50, 680
Clarke, R. (1937) Brit. J. Urol. 9, 254
Coons, A.H. (1958) General Cytochemical Methods,


Documenta Geigy Scientific Tables (1970) 7th.edn.,
p.685, Geigy, Basle, Switzerland

Erlanger, B.F., Borek, F., Beiser, S.M. & Liebermann, S.
(1957) J.Biol.Chem. 228, 713-727

Farnsworth, W.E. (1975) Normal and Abnormal Growth of the
Prostate, (Goland, M. ed.) pp. 160-169, Thomas,
Springfield, Illinois, U.S.A.


Franks, L.M., Riddle, P.N., Carbonell, A.W. & Gey, G.O.
(1970) J.Path. 100, 113-119

Cytochem. 14, 291-302

Gomori, G. (1950) Stain Tech. 25, 81

346-354

Honor of William Wallace Scott, pp. 39-49


Biol., Paris, 143, 771

411-424

Hansson, V. & Tveter, K.J. Acta Endocrinol. (Kbh) (1971)
68, 69-78

Henry, R.J. (1966) Clinical Chemistry Principles and

Huggins, C. (1945) Physiol.Rev. 25, 281

Loeschke (1920) Muenchen Med.Wschr. 67, 302
Churchill Livingstone, Edinburgh and London
Arzneimittelforschung 20, 545-547
Ramm, F. (1894) Kastrationens Betydning i
prostatahypertrofien Behandling. H. Aschehoug & Co.,
Kristiania.
34, 307-320
Endocrinol Clin. 12, 220-222
1737-1745
151, 607-618
Sturde, H.C. (1971) Arch Dermatol Forsch 241, 86-95
Sylven, B. & Bois, I. (1964) Histochem. 3, 341
Sylven, B. & Snellman, O. (1964) Histochemie 3, 484
Sylven, B. & Snellman, O. (1968) Histochemie 12, 240
Theodorides, P., Bourke, J.B. & Griffen, J.P. (1972)
Proc. Royal Soc. Med. 65, 12
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