



<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study,
without prior permission or charge

This work cannot be reproduced or quoted extensively from without first
obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any
format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author,
title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk

CONTROL OF TESTOSTERONE 5 α -REDUCTASE
ACTIVITY IN
THE HUMAN HYPERPLASTIC PROSTATE.

by

HELEN B. McINTYRE

THESIS

Submitted for the Degree of
Doctor of Philosophy

in the Department of Biochemistry,
University of Glasgow, Scotland.

November, 1978.

ProQuest Number: 10646334

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10646334

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS.

| | <u>Page</u> |
|--|-------------|
| <u>ACKNOWLEDGMENTS</u> | (xv) |
| <u>LIST OF ABBREVIATIONS</u> | (xvii) |
| <u>STEROID NOMENCLATURE</u> | (xviii) |
| <u>SUMMARY</u> | (xx) |
| <u>CHAPTER I.</u> | |
| <u>GENERAL INTRODUCTION.</u> | |
| 1. Structure and Function of the Prostate Gland. | 1 |
| A. Structure. | 1 |
| B. Function. | 5 |
| 2. The Prostate as an Androgen Dependent Organ. | 8 |
| 3. Testosterone 5 α -Reduction within the Prostate Gland. | 16 |
| 4. Further Androgen Metabolism within the Prostate. | 23 |
| 5. Factors Controlling Androgen Metabolism within the Prostate Gland. | 29 |
| 6. The Mode of Action of Androgens. | 33 |
| 7. Benign Prostatic Hyperplasia and Prostatic Carcinoma. | 39 |
| A. Benign Prostatic Hyperplasia. | 39 |
| B. Prostatic Carcinoma. | 42 |
| 8. Outline of Research. | 44 |
| <u>CHAPTER II.</u> | |
| <u>MATERIALS AND METHODS.</u> | |
| 1. Materials. | 46 |

| <u>CHAPTER II.</u> (continued) | <u>Page</u> |
|--|-------------|
| A. Radiochemicals | ... 46 |
| B. Chemicals | ... 46 |
| C. Steroids | ... 48 |
| D. Solvents | ... 48 |
| E. Scintillation Materials | ... 48 |
| F. Miscellaneous | ... 49 |
| 2. <u>Methods.</u> | ... 50 |
| A. Chromatography Methods. | ... 50 |
| (i) Paper Chromatography. | ... 50 |
| (ii) Thin Layer Chromatography. | ... 51 |
| (iii) Gas Liquid Chromatography. | ... 52 |
| B. Measurement of Radioactivity. | ... 53 |
| (i) Paper and Thin Layer Chromatogram Scanners. | ... 53 |
| (ii) Liquid Scintillation Counting. | ... 54 |
| C. Processing of Scintillation Counter Output. | ... 55 |
| D. Purification of Steroids. | ... 56 |
| (i) Non-radioactive Steroids | ... 56 |
| (ii) Radioactive Steroids | ... 56 |
| E. Chemical Methods. | ... 57 |
| (i) Acetylation of Steroids | ... 57 |
| (ii) Chromic Acid Oxidation of Steroids.. | 57 |
| F. Preparation of Buffers. | ... 58 |
| (i) Tris Buffers | ... 58 |
| (ii) Krebs-Ringer Bicarbonate Buffer | ... 59 |
| (iii) Phosphate Buffers | ... 60 |
| G. Preparation of Prostate Tissue | ... 60 |
| H. Assay of Testosterone 5 α -Reductase | ... 61 |
| (i) Enzyme Preparation | ... 61 |
| (ii) Incubation | ... 62 |
| (iii) Calculation of Results | ... 64 |

| <u>CHAPTER II.</u> (continued) | <u>Page</u> |
|---|-------------|
| I. Assay of 17 β -Hydroxysteroid Dehydrogenase | ... 65 |
| (i) Enzyme Preparation | ... 65 |
| (ii) Incubation | ... 65 |
| J. Superfusion Techniques | ... 66 |
| (i) Tissue Preparation | ... 66 |
| (ii) Superfusion Apparatus | ... 67 |
| (iii) Superfusion -General Details | ... 67 |
| (iv) Analysis of Fractions Following Superfusion with T/DHT | ... 68 |
| K. Miscellaneous Methods | ... 69 |
| (i) Colourimetric Estimation of Heparin | ... 69 |
| (ii) Protein Determination | ... 71 |

CHAPTER III.

HEPARIN AND RELATED COMPOUNDS IN THE CONTROL OF PROSTATIC TESTOSTERONE 5 α -REDUCTASE.

| | |
|---|--------|
| 1. Introduction | ... 72 |
| 2. Results | ... 77 |
| A. Initial Experiments | ... 77 |
| B. The Effect of Heparin on the <u>In Vitro</u> 5 α -Reduction of Testosterone | ... 79 |
| C. The Effect of Selected Monosaccharides, Polysaccharides and Polyionic Substances on Prostatic Testosterone 5 α -Reductase | ... 84 |
| D. Prostatic Testosterone 5 α -Reductase Following the <u>In Vivo</u> Administration of Heparin to Rats | ... 86 |
| E. Preliminary Investigation of the Endogenous Heparin-like Content of Benign Hyperplastic Human Prostate | ... 88 |
| 3. Discussion | ... 95 |

CHAPTER IV.PageTHE INTERACTION OF SPIRONOLACTONE
METABOLITES WITH PROSTATIC TESTOSTERONE
5 α -REDUCTASE.

| | |
|---|---------|
| 1. Introduction | ... 105 |
| 2. Experimental and Results | ... 108 |
| A. Interaction of Aldadiene and Potassium Canrenoate with Testosterone 5 α -Reductase | ... 108 |
| B. The Purity of Aldadiene | ... 108 |
| C. Kinetics of Aldadiene Inhibition of Testosterone 5 α -Reductase Using Homogenate Preparations | ... 109 |
| D. Preparation of Microsomes and Validation of the Method. | ... 110 |
| E. Kinetics of Aldadiene Inhibition of Testosterone 5 α -Reductase Using Microsome Preparations | ... 111 |
| F. Metabolism of [1,2- ³ H]-Aldadiene | ... 112 |
| 3. Discussion | ... 113 |

CHAPTER V.THE INVESTIGATION OF AN ISOTOPE EFFECT
IN THE CHEMICAL AND BIOLOGICAL METABOLISM
OF 17 α -TRITIATED ANDROGENS.

| | |
|--|---------|
| 1. Introduction | ... 115 |
| 2. Results | ... 119 |
| A. Initial Observations | ... 119 |
| B. Systematic Study of the Oxidation of Testosterone | ... 120 |
| C. The Purity of [17 α - ³ H]-Testosterone | ... 123 |
| (i) Assessment of Purity by Paper Chromatography | ... 123 |
| (ii) Assessment of Purity by Thin Layer Chromatography | ... 123 |

| <u>CHAPTER V.</u> (continued) | <u>Page</u> |
|--|-------------|
| (iii) Assessment of Purity by Recrystallisation | ... 124 |
| (iv) Assessment of Purity by Derivative Formation | ... 124 |
| D. Assessment of the Stereochemical Purity of $[17\alpha\text{-}^3\text{H}]$ -Testosterone | ... 125 |
| E. Time Course of the Chromic Acid Oxidation of Isotopically Labelled Species of Testosterone | ... 126 |
| F. The Enzymatic Metabolism of $[17\alpha\text{-}^3\text{H}]$ - Testosterone | ... 128 |
| (i) Metabolism of $[17\alpha\text{-}^3\text{H}]$ -Testosterone by the 17β -Hydroxysteroid Dehydrogenase of <u>Pseudomonas</u> <u>Testosteroni</u> | ... 128 |
| (ii) Metabolism of $[17\alpha\text{-}^3\text{H}]$ -Testosterone by Human Prostatic 5α -Reductase | .. 130 |
| 3. Discussion | ... 131 |

CHAPTER VI.

THE INTERACTION OF PROGESTERONE AND RELATED
COMPOUNDS WITH PROSTATIC TESTOSTERONE
 5α -REDUCTASE.

| | |
|---|---------|
| 1. Introduction | ... 135 |
| 2. Results | ... 141 |
| A. The Inhibition of Testosterone 5α -Reductase by Progesterone and Other C21 Steroids | ... 141 |
| B. The Inhibition of Testosterone 5α -Reductase by Naturally Occurring Androgens | ... 142 |
| C. The Kinetics of Progesterone Inhibition of Testosterone 5α -Reductase | ... 143 |
| D. The Effect of Progesterone on Testosterone Metabolism in Superfused Human Prostatic Tissue | ... 144 |
| (i) A Time Course for the Uptake of Testosterone, DHT and Progesterone. | 145 |

| <u>CHAPTER VI.</u> (continued) | <u>Page</u> |
|--|-------------|
| (ii) The Parameters Calculated from the Superfusion of Testosterone and DHT and the Errors in their Measurement . . . | 147 |
| (iii) The Effect of Added Progesterone on the Parameters of Testosterone and DHT Metabolism . . . | 152 |
| E. Uptake and Retention of Progesterone, Testosterone and Cortisol within the Prostate . . . | 154 |
| (i) Uptake and Retention of Testosterone and Cortisol . . . | 157 |
| (ii) Uptake and Retention of Testosterone and Progesterone . . . | 158 |
| F. The Metabolism of Progesterone within Human Hyperplastic Prostate Cells . . | 160 |
| (i) Preliminary Investigations . . . | 161 |
| (ii) Time Course of Progesterone Uptake and Metabolism . . . | 162 |
| 3. Discussion . . . | 165 |
| <u>CHAPTER VII.</u> | |
| <u>CONCLUSIONS AND SCOPE FOR FUTURE INVESTIGATION.</u> . . . | 172 |
| <u>LIST OF REFERENCES</u> . . . | 180 |

LIST OF FIGURES.

| <u>Figure I - 1</u> | <u>Following Page</u> |
|--|-----------------------|
| Anatomical Location of the Human Prostate ... | 1 |
| <u>Figure I - 2</u> | |
| (a) Anatomical Divisions of the Prostate | |
| (b) Histological Divisions of the Prostate ... | 2 |
| <u>Figure I - 3</u> | |
| Histology of Normal, Benign Hyperplastic and Carcinomatous Human Prostate ... | 4 |
| <u>Figure I - 4</u> | |
| Major Pathway for Reduction of Testosterone within the Prostate ... | 17 |
| <u>Figure I - 5</u> | |
| Metabolism of Testosterone within the Prostate ... | 28 |
| <u>Figure I - 6</u> | |
| Scheme for the Mode of Action of Testosterone within the Prostate ... | 35 |
| <u>Figure II - 1</u> | |
| Superfusion Apparatus ... | 67 |
| <u>Figure II - 2</u> | |
| Flow diagram for Superfusion Work Up ... | 69 |
| <u>Figure III - 1</u> | |
| Suggested forms for the Basic Structure of Heparin ... | 73 |
| <u>Figure III - 2</u> | |
| (a) The Effect of NADPH Concentration on Testosterone 5 α -Reductase Activity | |
| (b) The Effect of Ionic Strength on Testosterone 5 α -Reductase Activity ... | 78 |

| <u>Figure III - 3</u> | <u>Following Page</u> |
|---|-----------------------|
| The Inhibitory effect of Heparin on Rat and Human Prostatic Testosterone 5 α -Reductase | ... 79 |
| <u>Figure III - 4</u> | |
| The Effect of Heparin on Glucose-6-Phosphate Dehydrogenase Activity | ... 80 |
| <u>Figure III - 5</u> | |
| Heparin Inhibition of Human Prostatic Testosterone 5 α -Reductase from Different Glands | ... 81 |
| <u>Figure III - 6</u> | |
| Lineweaver Burke Plot showing Heparin Inhibition of Human Prostatic Testosterone 5 α -Reductase | ... 82 |
| <u>Figure III - 7</u> | |
| The Effect of Various Compounds on Prostatic Testosterone 5 α -Reductase | ... 83 |
| <u>Figure III - 8</u> | |
| The Effect of Selected Saccharides and Polyanions on Human Prostatic Testosterone 5 α -Reductase | ... 85 |
| <u>Figure III - 9</u> | |
| Polysaccharide Inhibition of Human Prostatic 5 α -Reductase | ... 86 |
| <u>Figure IV - 1</u> | |
| Steroid Structures | ... 105 |
| <u>Figure IV - 2</u> | |
| Inhibition of Testosterone 5 α -Reductase by Aldadiene and Canrenoate | ... 108 |
| <u>Figure IV - 3</u> | |
| Time course of Testosterone 5 α -Reductase using a Microsome Preparation | ... 111 |

| <u>Figure IV - 4</u> | <u>Following Page</u> |
|---|-----------------------|
| Double Reciprocal Plots of 5 α -Reductase Inhibition by Aldadiene | ... 112 |
| <u>Figure V - 1</u> | |
| The Chromic Acid Oxidation of [1,2- ³ H]-DHT and [17 α - ³ H]-DHT | ... 119 |
| <u>Figure V - 2</u> | |
| Panax Radiochromatogram Scans of the Chromic Acid Oxidation Products of Testosterone | ... 121 |
| <u>Figure V - 3</u> | |
| Panax Radiochromatogram Scans of the Products of Chromic Acid of a Mixture of [17 α - ³ H]- and [4- ¹⁴ C]-Testosterone | ... 122 |
| <u>Figure V - 4</u> | |
| Time Course of Chromic Acid Oxidation of Testosterone Isotopes | ... 127 |
| <u>Figure V - 5</u> | |
| Time Course of Oxidation of Testosterone Isotopes by the 17 β -Hydroxysteroid Dehydrogenase from <u>P. Testosteroni</u> | ... 129 |
| <u>Figure VI - 1</u> | |
| Time Courses for the Reaction A \rightarrow B \rightarrow C when Studied by Static Incubation and Tissue Superfusion | ... 138 |
| <u>Figure VI - 2</u> | |
| Parameters Measured by Superfusion | ... 139 |
| <u>Figure VI - 3</u> | |
| Progestins Assessed for Their Ability to Inhibit the Testosterone 5 α -Reductase Activity of Human Prostate | ... 141 |

| <u>Figure VI - 4</u> | <u>Following Page</u> |
|--|-----------------------|
| The Inhibition of Prostatic Testosterone 5 α -Reductase by Naturally Occurring Progestins | ... 141 |
| <u>Figure VI - 5</u> | |
| Androgens Examined as Possible Inhibitors of the Testosterone 5 α -Reductase Activity of Human Prostate | ... 142 |
| <u>Figure VI - 6</u> | |
| The Inhibition of the 5 α -Reduction of [1,2- ³ H]-Testosterone by Naturally Occurring Androgens | ... 142 |
| <u>Figure VI - 7</u> | |
| The Inhibition, by Progesterone, of Testosterone 5 α -Reductase from a Prostatic Microsome Preparation | ... 144 |
| <u>Figure VI - 8</u> | |
| Time Course for the Uptake of Testosterone and DHT | ... 146 |
| <u>Figure VI - 9</u> | |
| Time Course for the Uptake of Progesterone | ... 146 |
| <u>Figure VI - 10</u> | |
| The Parameters Measured in Human Prostatic Tissue after Superfusion with Testosterone and DHT | ... 147 |
| <u>Figure VI - 11</u> | |
| Uptake and Retention of Testosterone and Cortisol by Human Hyperplastic Prostate | ... 158 |
| <u>Figure VI - 12</u> | |
| Uptake and Retention of Testosterone and Progesterone by Human Hyperplastic Prostate | ... 159 |
| <u>Figure VI - 13</u> | |
| The Metabolism of Progesterone by Superfused Human Prostatic Tissue | ... 163 |

LIST OF TABLES.

| <u>Table</u> | <u>Following Page</u> |
|--|-----------------------|
| <u>Table II - 1</u> | |
| Solvent systems used for the separation of steroids, and the Rf values obtained. | ... 52 |
| <u>Table II - 2</u> | |
| Counting efficiencies, in each channel of scintillation fluids. | ... 54 |
| <u>Table III - 1</u> | |
| The effect of enzyme dilution on the conversion of testosterone to 5 α -dihydrotestosterone using fresh human prostatic homogenate. | ... 77 |
| <u>Table III - 2</u> | |
| Composition of polymeric compounds tested for inhibition of testosterone 5 α -reductase. | ... 85 |
| <u>Table III - 3</u> | |
| Rat prostatic testosterone 5 α -reductase activity following the <u>in vivo</u> administration of heparin. | ... 87 |
| <u>Table III - 4</u> | |
| Composition of final heparin extracts of human hyperplastic prostate tissue. | ... 91 |
| <u>Table IV - 1</u> | |
| Distribution of testosterone 5 α -Reductase activity in different preparative fractions. | ... 110 |
| <u>Table V - 1</u> | |
| ³ H/ ¹⁴ C Ratios following the chromic acid oxidation of a mixture of [17 α - ³ H]-testosterone and [4- ¹⁴ C]-testosterone. | ... 122 |
| <u>Table V - 2</u> | |
| Metabolism of testosterone by different enzyme concentrations of the 17 β -hydroxysteroid dehydrogenase of <u>P. Testosteroni</u> . | ... 129 |

Table V - 3Following Page

Time course of testosterone metabolism
by the 5 α -reductase of human hyperplastic
prostate tissue. ... 130

Table VI - 1

The Errors involved in the measurement of
the superfusion parameters. ... 151

Table VI - 2

Comparison of parameters from six different
Prostates. ... 152

Table VI - 3

The effect of progesterone at different
concentrations on the parameters obtained
by simultaneous superfusion with testosterone
and DHT. ... 152

Table VI - 4

Radioactive products obtained from prostatic
tissue after superfusion with [1,2,6,7-³H]-
progesterone. ... 161

ACKNOWLEDGMENTS.

Over the years of my studying, many people have given me advice and encouragement, and I would like to thank here all those who have helped me throughout this time, and especially those who have helped in the preparation of this thesis.

My Supervisor, Dr. G.H. Beastall, I would like to thank especially. His support and encouragement throughout the work and suggested improvements during the writing of this thesis were invaluable, and his spelling is much better than mine.

The Department of Steroid Biochemistry proved a stimulating place to be based, and the many discussions with Drs. J.K. Grant, B. Cock, R.A. Cowan and other members of the laboratory provided inspiration and rationalisation on many occasions.

I would like to thank the Urological Surgeons and Theatre staff of the Royal Infirmary, Western Infirmary and Stobhill General Hospital, for providing the human prostatic tissue, and the staff of the Pathology Department at the Royal Infirmary who also performed the histological examinations upon it. I am indebted also, to the technicians of the Department of Surgery Animal House who maintained and sacrificed the rats used in this study.

I would like to thank Hoffmann-La Roche Inc.

for providing me with the synthetic polysaccharides used in Chapter III; and Searle Pharmaceuticals for the aldadiene, canrenoate and [^3H]-Aldadiene, used in Chapter IV.

I am very grateful to the Scottish Hospitals Endowment Research Trust for funding this work.

Finally, I would like to thank my parents and my husband, not only for giving me support and encouragement, but also for providing an environment which made my studying very pleasant and comfortable. My mother also deserves a great deal of thanks for her patience in typing this thesis.

LIST OF ABBREVIATIONS.

The following list of abbreviations has been used throughout this Thesis where, for either lack of space or unnecessary repetition of lengthy terms, they have been found more appropriate.

| <u>Abbreviation</u> | <u>Full Term</u> |
|---------------------|-------------------------------------|
| ACTH | - Adreno-corticotrophic hormone |
| BPH | - Benign prostatic hyperplasia |
| BSA | - Bovine serum albumin |
| CBG | - Cortisol-binding globulin |
| CPC | - Cetyl pyridinium chloride |
| DHAS | - Dehydroepiandrosterone sulphate |
| DHT | - 5 α -Dihydrotestosterone |
| FSH | - Follicle stimulating hormone |
| GLC | - Gas liquid chromatography |
| LH | - Luteinising hormone |
| S.A. | - Specific enzyme activity |
| SBG. | - Sex steroid binding globulin |
| Sp.Act. | - Specific activity (radioactivity) |
| T | - Testosterone |
| TCA | - Trichloroacetic acid |
| TLC | - Thin layer chromatography |

STEROID NOMENCLATURE.

The following list gives the trivial names of steroids mentioned in this Thesis, along with their corresponding correct names. The nomenclature is in agreement with that proposed by the IUPAC - IUB Commission on Biochemical Nomenclature as published in the Biochemical Journal (1969) 113, 5 -28.

| <u>Trivial Name</u> | <u>Correct Name</u> |
|---|--|
| aldadiene | - 17-hydroxy-3-oxo-17 α -4,6-pregnadiene-21-carboxylic acid- γ -lactone |
| aldosterone | - 18,11-hemiacetal of 11 β ,21-dihydroxy-3,20-dioxo-4-pregnen-18-al |
| androstenedione | - 4-androstene-3,17-dione |
| canrenoate | - 17-hydroxy-3-oxo-17 α -4,6-pregnadiene-21-carboxylic acid |
| cortisol | - 11 β ,17 α ,21-trihydroxy-4-pregnene-3,20-dione |
| cortisone | - 17 α ,21-dihydroxy-4-pregnene-3,11,20-trione |
| cyproterone acetate | - 6-chloro-17 α -hydroxy-1 α ,2 α -methylene-4,6-pregnadiene-3,20-dione-17-yl acetate |
| 5 α -dihydrotestosterone | - 17 β -hydroxy-5 α -androstane-3-one |
| dehydroepiandrosterone sulphate | - 3 β -hydroxy-5-androsten-17-one sulphate |
| deoxycortisol | - 17 α ,21-dihydroxy-4-pregnene-3,20-dione |
| epitestosterone | - 17 α -hydroxy-4-androsten-3-one |
| 17 α -hydroxyprogesterone caproate | - 17 α -hydroxy-4-pregnene-3,20-dione caproate |

| <u>Trivial Name</u> | <u>Correct Name</u> |
|---------------------|--|
| medrogestone | - 6,17-dimethyl-4,6-pregnadiene-3,20-dione |
| megestrol acetate | - 17 α -hydroxy-6-methyl-4,6-pregnadiene-3,20-dione acetate |
| progesterone | - 4-pregnene-3,20-dione |
| spironolactone | - 17 α -hydroxy-7-mercapto-3-oxo-17 α -4-pregnene-21-carboxylic acid- γ -lactone,7acetate |

SUMMARY.

The prostate is a male secondary sex organ dependent on a supply of androgens from the blood for its growth, maintenance and function. Within the gland, testosterone is reduced to 5α -dihydrotestosterone (DHT) by the enzyme testosterone 5α -reductase, and it is the DHT which appears to be the active hormone within this organ. Benign prostatic hyperplasia is a common condition in elderly men, and a raised tissue concentration of DHT has been implicated as a possible cause.

In this thesis, several compounds which decrease DHT production, by inhibition of the testosterone 5α -reductase, were investigated for their possible usefulness in the treatment of benign prostatic hyperplasia.

The polysaccharide, heparin, showed competitive kinetics with respect to both substrate and cofactor of the prostatic enzyme. The inhibition was not reversed by zinc ions. Several other natural and synthetic monosaccharides and polysaccharides were tested with the enzyme system, but no pattern, of either monosaccharide unit, degree of sulphation, molecular weight or glycoside linkage, could be discerned as the prime cause of the observed inhibition. Administration of heparin to

rats caused no significant effect on the testosterone 5 α -reductase activity of the gland. An uronic acid containing substance was extracted from human prostatic tissue which was able to inhibit the testosterone 5 α -reductase.

Metabolites of spironolactone, the aldosterone antagonist, were also tested with the testosterone 5 α -reductase. Canrenoate showed no significant effect, and aldadiene proved to be a poor inhibitor. The kinetics of this inhibition could not be resolved even with a more homogeneous microsome preparation. The implications of the observations are discussed.

An isotope effect was shown to occur during both chemical and biological oxidation of [17 α -³H]-testosterone. No isotope effect was observed in the 5 α -reduction of this compound.

Progesterone and other hydroxylated progestins were incubated with testosterone 5 α -reductase. The inhibition observed with each compound depended on the number and position of hydroxyl groups present. Progesterone exerts competitive inhibition. The addition of progesterone to an in vitro superfusion system showed that the progesterone was capable of inhibiting the conversion of testosterone to DHT but only at much higher concentrations than expected from homogenate incubations. The lack of potency

of progesterone could not be accounted for by lack of entry of this steroid into the tissue.

Progesterone was shown to be extensively metabolised by prostatic tissue to 5α -reduced metabolites. The implications of these observations are discussed in terms of the value of testosterone 5α -reductase inhibitors in the control of benign prostatic hyperplasia.

CHAPTER I.

CHAPTER I.

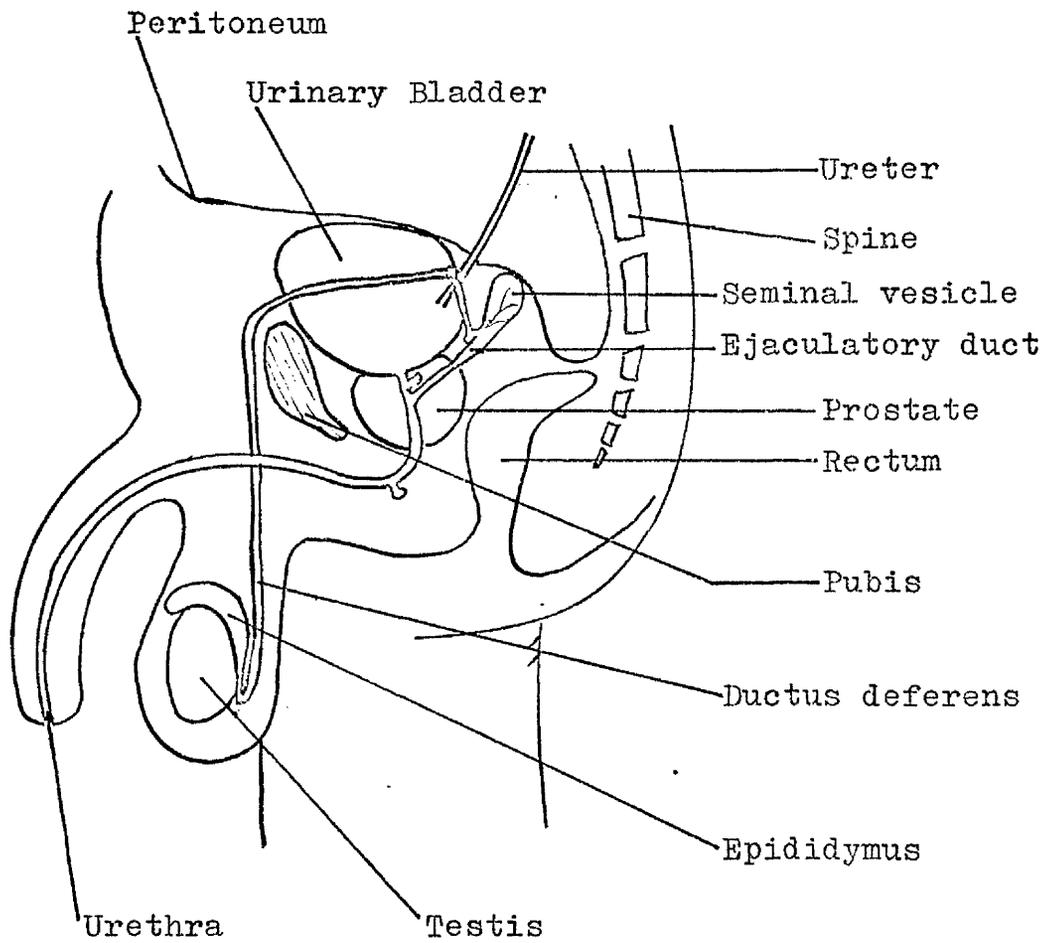
GENERAL INTRODUCTION.

1. Structure and Function of the Prostate Gland.

The prostate gland is an integral part of the male reproductive tract, being an accessory sex organ whose normal function is to manufacture and secrete many of the components of semen. In man the prostate gland is also the site of two common pathological conditions and as such has attracted considerable clinical attention for many years. Benign prostatic hyperplasia (BPH) is a condition which often causes urinary retention, whilst prostatic carcinoma is second only to lung cancer as the most common malignancy found in males. The discovery that the prostate gland is an androgen dependent organ has prompted widespread research in an attempt to understand the aetiology of these two conditions and it is the purpose of this introduction to summarise the present state of knowledge.

A. Structure.

In man the prostate gland is situated at the neck of the bladder surrounding the urethra (fig. I-1). It weighs only a few grams at birth and consists of a

Fig.I - 1Anatomical Location of the Human Prostate.

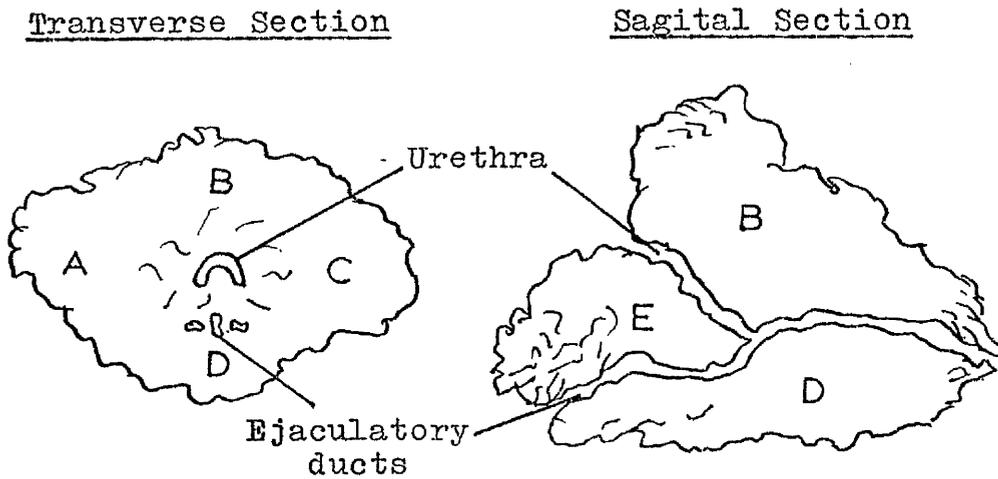
duct system embedded in a stroma of muscle and connective tissue. At puberty the gland undergoes an androgen induced growth, the increase in size being due to the modification of ducts and development of the end buds of the follicles. A reduction in the volume of the stroma in relation to the epithelium results. These changes in the fine structure occur in six to twelve months, after which growth continues slowly until, by about twenty years of age, the gland has reached fifteen to twenty grams (Warwick & Williams, 1973). During the third decade of life the foldings of the epithelium become more complex, although the size of the prostate remains stable until fifty - sixty years of age, when it may either progressively atrophy or undergo a second growth spurt (Sitteri & Wilson, 1970).

The prostate has five lobes which can be traced from the openings of the ducts into the urethra (Fig.I - 2a). The posterior lobe lies behind the urethra and the ejaculatory ducts; the median lobe lies between the ejaculatory ducts; the anterior lobe (which may be absent or atrophic) lies in front of the urethra; and two lateral lobes which lie on either side of the urethra (Price, 1963). However, on superficial examination the whole structure appears uniform and no functional or histological differences are seen between the divisions which are based purely

Fig.I - 2.

(a) Anatomical divisions of the prostate.

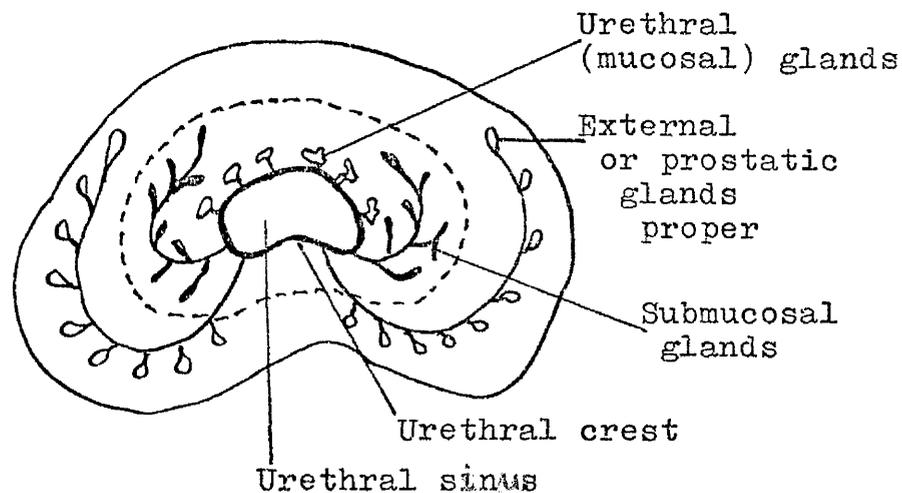
(Kerr et al., (1960)



- A - Right lateral lobe
- B - Anterior lobe
- C - Left lateral lobe
- D - Posterior lobe
- E - Median lobe

(b) Histological divisions of the prostate.

(Franks, 1954)



on foetal anatomy (Mann, 1964).

The prostate may more easily be divided into inner and outer zones (fig. I-2b). The inner zone consists of mucosal and submucosal glands which have short duct systems, and is more susceptible to the estrogen induced metaplasia which is often seen in new born infants as a result of circulating maternal estrogens. It is in this inner region that benign prostatic hyperplasia most often arises in elderly men (Blandy, 1976). The outer region or prostate gland proper has a longer and more branched duct system and is the usual site of origin of prostatic carcinoma (Franks, 1954; Scott, 1963). A true capsule separates the outer zone from the loose pelvic fascia and is adherent to the underlying gland.

Examination of the fine structure of the human prostate gland shows it to have a tubulo-alveolar structure, with a secretory epithelium lining the alveoli. The epithelial cells have a basement membrane resting on a stroma of connective tissue containing smooth muscle which surrounds the ducts. There is a rich blood supply, and nerve endings are found in both the smooth muscle and epithelium. At ejaculation the smooth muscle fibres contract under stimulation from the sympathetic nervous system and the alveoli are partially emptied of their secretions.

A typical section of normal human prostate is shown in fig. I-3a. Benign prostatic hyperplasia results in an increase in stromal/epithelial ratio of the tissue as is shown in fig. I-3b and in prostatic carcinoma there is a loss of structural differentiation (fig. I-3c) (Price, 1963).

Apart from man the two animals most commonly used in studies of the prostate are the dog and the rat. The dog, like man, has a single compact prostate which completely surrounds the urethra at the neck of the bladder. The dog is the only other animal commonly reported to have naturally occurring hyperplasia and for this reason has been intensively studied (King & Mainwaring, 1974). However, the hyperplasia arises in the outer region of the canine gland and has a cystic rather than nodular type of development (Price, 1963) and involves the acini rather than stroma (Blandy, 1976). This significant difference means that caution must be exercised in the extrapolation of experimental data obtained in dogs to the situation in man. It is only in the ageing rhesus monkey that anything is seen which can be compared to benign nodular hyperplasia in man (Blandy, 1976).

In the rat the prostate is composed of paired ventral lobes at the neck of the bladder, and paired dorsal and paired lateral lobes which surround the

Fig.I - 3Histology of Normal, Benign Hyperplastic and
Carcinomatous Human Prostate.(a) Normal Prostate.

The gland has a tubulo-alveolar structure, with the secretory columnar epithelial cells lining the alveoli: the epithelial cells have a basement membrane resting on a stroma of connective tissue containing smooth muscle cells which surround the alveoli.

(b) Benign Hyperplastic Prostate.

There is an increase in the stromal/epithelial ratio, although there is still structural differentiation.

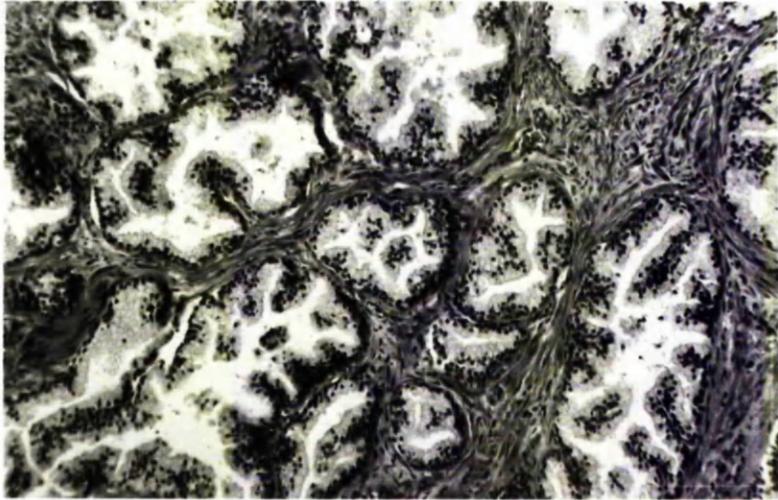
(c) Carcinomatous Prostate.

There is loss of structural differentiation, although both epithelial and stromal elements are present.

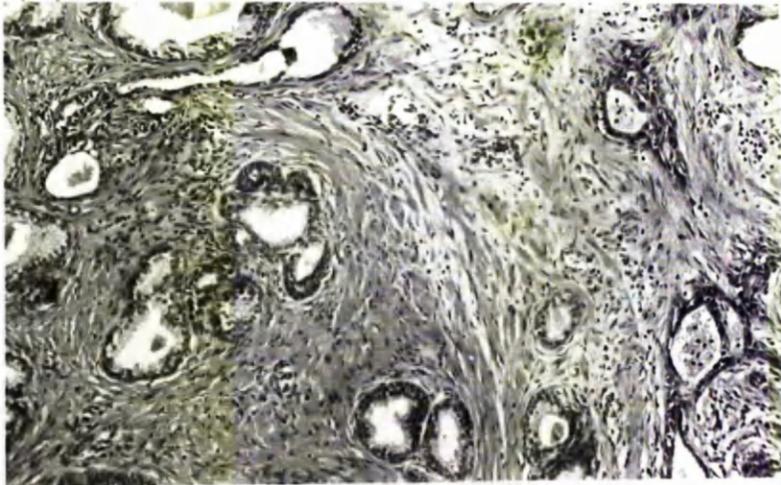
Magnification: x 300.

Fig. I - 3

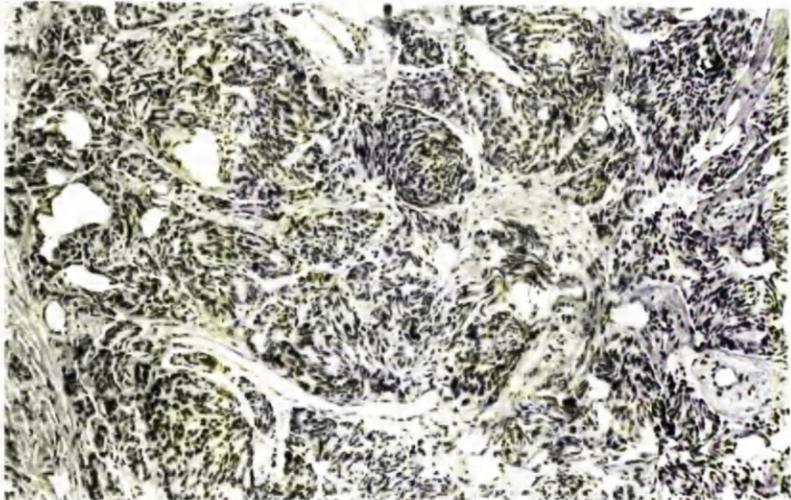
(a)



(b)



(c)



urethra (Price, 1963). It is the rat ventral prostate which is used in most experimental work as it is easily dissected free of other tissues and responds rapidly to hormone manipulations. The histology of the rat ventral prostate differs from that of man and dog, being a less compact tissue and having a higher epithelial to stromal cell ratio. Thus whilst this gland is useful in many ways for the study of androgen action it should not be seen as a model for human prostate.

B. Function.

The ejaculated semen is a suspension of spermatozoa in a fluid medium. This fluid is made up of the secretions of the male accessory organs of reproduction and the mixture is known as the seminal plasma.

The composition of the seminal plasma and the relative contribution of the different accessory sex glands to it varies from one species to another, and is discussed extensively by Mann (1964). The semen volume and sperm density is also subject to wide species differences. In man 2-6ml is the average volume range for ejaculates and sperm density averages 10^5 cells/ml, so that only 10% of the ejaculate is sperm and the rest is seminal plasma (Mann, 1964).

Human seminal plasma is made up of secretions from the prostate, epididymi, vas deferentia,

ampullae, seminal vesicles, bulbo-urethral and urethral glands. Between 13 and 33% of seminal plasma is due to the prostatic secretion (Lundquist, 1949) which is a colourless fluid, usually slightly acid, pH6.5 (Huggins, 1947), has high proteolytic enzyme activity and is noted for an almost complete absence of reducing sugars. The prostatic secretion is the main source of the citric acid and acid phosphatase of the human semen, and analysis of these two components provides a convenient chemical indicator test for the functional state of the gland. Citric acid formation in the prostate appears to be androgen dependent (Humphrey & Mann, 1948, 1949), disappearing after castration but reappearing after testosterone administration. The exact function of citric acid is not certain. It does not appear to influence markedly the aerobic or anaerobic metabolism of spermatozoa (Humphrey & Mann, 1949), but may have a beneficial effect on sperm motility (Lardy & Phillips, 1945), function as a calcium-binding substance (Huggins, 1945) or have a role in maintaining the osmotic equilibrium in semen (Mann, 1954).

Acid phosphatase production, like that of citric acid, is androgen dependent. Levels of the enzyme are low in childhood but increase rapidly at puberty (Gutman & Gutman, 1938,b). Under physiological

conditions acid phosphatase does not pass into the blood stream, although handling of the prostate during clinical procedures may cause a transient rise in blood level of the enzyme (Marberger et al., 1957). Malignant growth of the prostate and metastases of prostatic cancer in the bones also result in an increased blood level, and the determination of this prostatic acid phosphatase activity in the blood has proved to be a useful tool in the diagnosis and treatment of prostatic carcinoma (Gutman & Gutman, 1938,a; Huggins et al., 1941; Watkinson et al., 1944).

Other notable constituents of the human prostatic secretion are the nitrogenous bases, spermine and spermidine. The physiological role of these substances is not yet clear but there are observations which suggest that spermine has a stabilising influence on whole cells, mitochondria, ribosomes, certain bacteriophages and certain enzymes, including prostatic acid phosphatase (Mann, 1964).

The prostate and prostatic secretion have extremely high levels of zinc. The mean zinc content of human prostate is 69.2mgZn/100g dry tissue (Mawson & Fisher, 1952), which is considerably higher than that found in other tissues such as liver, muscle, brain, testis or blood. Only a small percentage (10%) of this zinc can be accounted for

by metalloproteins such as carbonic anhydrase or acid phosphatase, the remainder being either free ionic zinc or zinc non-covalently bound to proteins or small molecules. The zinc content of the prostate like so many of the components of the gland is controlled by the circulating androgens and indicates the general androgen dependence of the gland as a whole.

2. The Prostate as an Androgen Dependent Organ.

In the male, the testes not only produce spermatozoa, but also have an endocrine role in the production of the steroid hormone testosterone. Within the testis steroid production is localised to the Leydig (interstitial) cells which lie between the seminiferous tubules and adjacent to blood capillaries and the lymphatic system. Like the ovaries in the female, the testes are under the control of the pituitary gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH), which are in turn, controlled by a releasing hormone from the hypothalamus. The role of LH is to control growth and to stimulate testosterone secretion by the Leydig cells. The testosterone, in conjunction with FSH, promotes spermatogenesis and the growth of the seminiferous tubules (Turner & Bagnara, 1971).

Apart from its role in spermatogenesis

testosterone, and its principal metabolite 5α -dihydrotestosterone (DHT), have important anabolic and androgenic effects on muscle and hair growth, maintain the prostate, seminal vesicles and other accessory organs and play an important role in mating behaviour of the male.

The dependence of secondary sex organs on the testis was shown in the classical study of Hunter (1786) when he observed the post castration involution of the prostate and seminal vesicles. Later Pézard (1911) showed that extracts from the testis contained some biologically active substance which was capable of preventing the atrophy of the accessory sex glands caused by castration. This active substance was eventually isolated (David et al., 1935) and shown to be testosterone. The androgen dependent nature of the prostate gland in particular led Huggins (Huggins & Hodges, 1941) to employ castration and estrogen therapy in the treatment of carcinoma of the prostate.

According to classical endocrinology the testosterone secreted by the Leydig cells passes into the adjacent blood capillaries and thence into the general circulation via the spermatic vein. For many years it was assumed that this was the only route for the transport of androgens to the prostate gland, but more recently Pierrepoint and co-workers

(1973, 1975) have shown that in the rat and dog there may be passage of testosterone along the vas deferens to the prostate, and that the deferential vein may also act as a local transport system for androgens. Support for this latter route was given by Lewis & Moffat (1975) who described a mechanism for blood flow from the deferential vein into the prostatic venous plexus when conditions of raised central venous pressure prevailed.

In blood, testosterone is transported mainly bound to proteins. In man this binding is principally to one specific β -globulin, sex steroid binding globulin (SBG), which has a high affinity but low capacity not only for testosterone but also for estradiol and other sex steroids (Murphy, 1968). Only in certain clinical syndromes, where there is an abnormally high level of steroid secretion, is there significant binding of androgen to high capacity but low affinity proteins such as cortisol binding globulin (CBG) or albumin (King & Mainwaring, 1974). Not all higher animals have high affinity binding proteins related to SBG. For example in the rat and dog the high affinity binding of testosterone is to CBG (Murphy, 1968).

The effect of androgen binding within the plasma is to reduce the amount of hormone available to target tissues, including the prostate gland, and

it has been suggested that only the relatively small fraction of unbound steroid is readily available to the tissue, and hence biologically active (Vermeulen & Verdonck, 1972). Data obtained from organ culture (Lasnitzki & Franklin, 1974; Mercier-Bodard et al., 1976), and in vitro superfusion (Giorgi & Moses, 1975) would seem to support this, as in both cases, addition of plasma to the medium decreased the uptake of radioactive testosterone and DHT by prostate tissue.

The availability of testosterone to the prostate gland is thus determined not only by the total plasma concentration of the steroid but also by the concentration and extent of binding to SBG.

It would appear that the entry of testosterone into the cell is achieved by passive diffusion, as the amount of steroid found in the cytoplasm has been shown to be proportional to the amount injected into castrated rats (Rennie & Bruchovsky, 1973). However in vitro work with superfused human prostate tissue slices has demonstrated that not all steroids enter the tissue at the same rate (Grant & Giorgi, 1972) and it is possible that some element of selectivity towards testosterone may be involved. Within the cell the transport of androgens is controlled by specific binding proteins or receptors, which will be discussed more fully in a later section.

It is now generally accepted that for the

maintenance of normal prostatic function testosterone is only a prehormone which requires reduction within the gland to the active hormone 5 α -dihydrotestosterone (DHT). Evidence for this comes from many workers and from several different approaches. (For reviews see Wilson, 1972; King & Mainwaring, 1974.)

In 1961 Pearlman & Pearlman first showed that the prostate and other male accessory sex glands were capable of reducing the double bond in the A ring of testosterone. Such a metabolic step is not unique, for testosterone reduction also occurs in the liver (Tomkins, 1957), but a fundamental difference exists between the metabolism in this gland and in the accessory sex organs, in that the liver produces both 5 α and 5 β reduced androgens whilst the sex organs produce almost exclusively 5 α reduced steroids (King & Mainwaring, 1974). This difference may be explained in terms of the relative functions of the organs for whilst in the liver steroid reduction is a method of diminishing the biological potency of a variety of hormones, in the sex organs the reduction of testosterone is the key metabolic step in the normal control of those glands.

That DHT is a more potent androgen than testosterone in the prostate can be demonstrated by the relative ability of these steroids to maintain epithelial cell heights and secretory activity

(Lasnitzki, 1970; Robel et al., 1971). Furthermore, administration of DHT to immature castrated rats produces a greater proliferative effect on the ventral prostate than does testosterone (Lesser & Bruchovsky, 1973; Schmidt et al., 1972) and after prolonged castration of male rats there is a very slow regeneration of the prostate during the first week of testosterone administration compared to the immediate response to DHT administration. Lesser & Bruchovsky (1973) suggest that the delay observed with testosterone replacement may represent the time necessary for the induction of the 5α -reductase enzyme which facilitates the conversion of testosterone to DHT.

When ^3H -testosterone is injected into castrated rats there is rapid and selective uptake of the steroid into the prostate. The radioactivity can be detected in the prostatic nuclei as ^3H -DHT bound to an acid nuclear protein as early as 15min. after administration (Anderson & Liao, 1968; Bruchovsky & Wilson, 1968,b; Hansson et al., 1971). There is selective retention of the DHT in the prostate for at least six hours although ^3H -testosterone disappears from the blood long before this time (Fang et al., 1969).

Whilst there is much evidence to show that DHT is the most potent androgen in the prostate and certain other androgen dependent tissues of man

this is probably not a universal finding for all androgen dependent or responsive tissues. For example in rabbit and rat embryos DHT formation is rapid only in the urogenital sinus (Wilson & Lasnitzki, 1971), and may be an inherent property of the tissue rather than a result of androgen action. In contrast the mullerian and wolfian ducts acquire the ability to form DHT only after the initial stages of differentiation implying that the acquisition of testosterone 5 α -reductase activity in these particular tissues may be the result of testosterone itself (Wilson & Lasnitzki, 1971).

The human embryo exhibits a similar pattern of events (Siiteri & Wilson, 1974), DHT formation in the urogenital tract being greatest at the onset of male differentiation, whereas in the wolfian ducts DHT formation is not demonstrated until late in male differentiation. This suggests that testosterone itself may have a role to play in this important stage of development, a suggestion which is supported by Gupta & Bloch (1976), who showed a testosterone binding protein which increases in concentration with progressive wolfian duct differentiation. From measurements of testosterone and DHT in plasma and various tissues Robel et al., (1973) propose that in the adult animal testosterone is the active hormone in muscle, kidney, hypothalamus and pituitary, whereas

it is a prehormone, requiring reduction to DHT, in the prostate, seminal vesicles and other accessory glands.

This complex situation concerning the relative physiological roles of testosterone and DHT in androgen action at target tissues has in part been clarified by Imperato-McGinley et al., (1974), who have located a group of male pseudohermaphrodites in a village in the Dominican Republic. These affected males (46XY) are born with ambiguity of the external genitalia and are mostly raised as females. At puberty there is marked virilisation and the subjects assume their true male phenotype. A geneological table indicates that the syndrome exhibits autosomal recessive inheritance. Biochemical evaluation reveals a decreased plasma DHT concentration secondary to a decrease in testosterone 5 α -reductase activity. By studying the clinical details linked to this deficiency it has been possible to distinguish between organs which are sensitive to testosterone and others which are sensitive to DHT. Testicular-testosterone acts directly on wolfian ducts to cause differentiation to vas deferens, epididymi and seminal vesicles, but in the urogenital sinus and urogenital tubercle testosterone functions as a prehormone where its conversion to DHT results in the differentiation of the external genitalia and

prostate. Again at puberty both testosterone and DHT are necessary for the androgen induced changes. In particular increase in muscle mass, growth of phallus and scrotum and deepening of the voice appear to be mediated by testosterone and occur in the affected males. Prostate growth, facial hair, temporal regression of the hairline and acne do not occur and appear to be mediated by DHT. Sexual orientation after puberty is male even although the subjects have been raised as females, thus male sex drive appears to be testosterone rather than DHT related.

3. Testosterone 5 α -Reduction within the Prostate Gland.

Bruchovsky (1971) compared the fate of seven natural androgens administered to castrated, functionally hepatectomised rats and found that for all these steroids the major metabolite in the prostatic nuclei was DHT, and he suggested that all natural androgens possessing a significant degree of potency are metabolised in varying amounts to DHT. The initial 5 α -reduction of testosterone thus plays a central role in the mechanism of androgen action and since this fact has been recognised considerable research has been directed towards a study of the various steroid metabolising enzymes in the prostate and other androgen dependent tissues, and especially

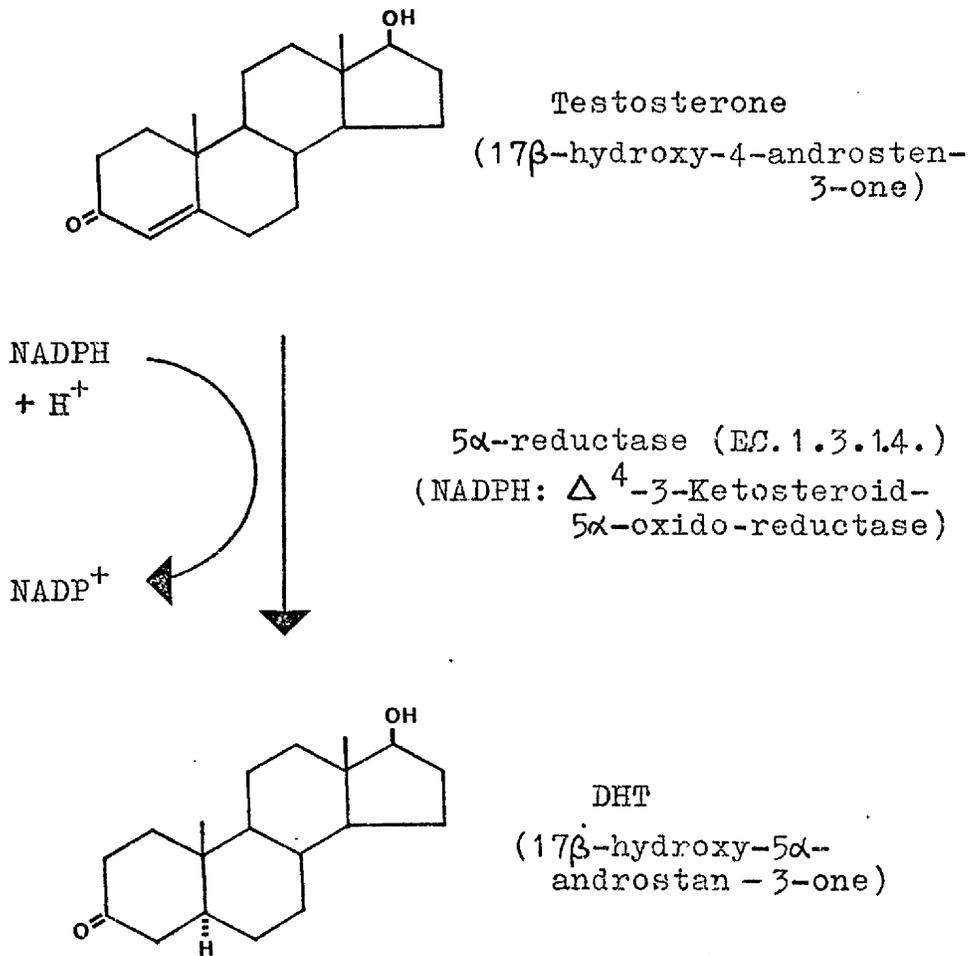
to the enzyme which facilitates the conversion of testosterone into DHT, the NADPH: Δ^4 -3-ketosteroid-5 α -oxidoreductase (E.C.1.3.1.4.) or as it is more conveniently called, testosterone 5 α -reductase.

This work has been reviewed by King & Mainwaring, (1974) and Wilson & Gloyna, (1970). A scheme for this key reaction is shown in fig. I-4.

Testosterone 5 α -reductase is located predominantly in the nuclear and microsomal fractions of the cell (Nozu & Tamaoki, 1973), the nuclear enzyme being located on the outer nuclear membrane (Moore & Wilson, 1972). This is in contrast to testosterone 5 α -reductase in liver which is located in the cytoplasmic fraction (Hajj, 1972).

Testosterone 5 α -reductase has an absolute specificity for NADPH as its cofactor (Chamberlain et al., 1966) and for both the microsomal and nuclear enzymes of rat ventral prostate the stereospecific transfer of the proton is from the 4-pro-S-proton of NADPH (Nozu & Tamaoki, 1974).

The kinetic properties of the nuclear and cytoplasmic (microsomal) 5 α -reductases of the rat prostate have been studied in great detail and on the grounds of pH optima and Km values for testosterone it was suggested that the two forms are identical (Frederiksen & Wilson, 1971). This is not surprising since the outer nuclear membrane in which

Fig.I - 4.Major Pathway for Reduction of Testosterone
within the Prostate.

the 5 α -reductase is located is merely an extension of the endoplasmic reticulum which results in the microsomal fraction.

It is not yet known whether the location of the 5 α -reductase on the outer nuclear membrane serves any physiological function. Nozu & Tamaoki (1973) observed that cytosol caused the inhibition of ³H-testosterone reduction in microsomal incubations but not in nuclear incubations. This appears to be the only major difference between the two enzymes and they suggest that in vivo the nuclear membrane may be the more important site of DHT production.

Moore & Wilson (1974) have partially purified the rat prostatic 5 α -reductase using digitonin and potassium chloride. They report a 90 fold purification of the NADPH stabilised enzyme which sediments at 13.5 - 15s and has a molecular weight of 250,000 - 350,000. The pH optima of 6.9 and Km of 0.7×10^{-6} M (nuclear) and 0.8×10^{-6} M (microsomal) are similar to those found in intact nuclei and microsomes. Digestion of the nuclear membrane by phospholipase A was the method used by Shimazaki et al., (1973) to obtain a 20 fold purification of the enzyme. The pH optima here was reported to be around 7.2 and again there is a requirement for NADPH as the obligatory hydrogen donor.

The liver also contains a high testosterone

5 α -reductase activity. However, the intracellular distribution is different in the liver with only about 1% of the activity being associated with the nuclei, the vast bulk being recovered from the cytoplasmic membranes (Roy, 1971). It also appears that hepatic 5 α -reductase is not a single species, and at least five 5 α -reductases have been detected, each having different substrate specificities, and regulated to different extents by protein and steroid hormones (McGuire & Tomkins, 1960; McGuire et al., 1960).

From a comparison of kinetic parameters it would appear that the liver and prostatic nuclear testosterone 5 α -reductases are not only functionally but probably structurally different, and regulatory control of the enzymes also appears distinctly different (Gustafsson & Pousette, 1974) since the prostate enzyme, unlike that of the liver, cannot reduce steroids with oxygen substitutions at C₁₁ (e.g. cortisol and corticosterone) (McGuire & Tomkins, 1960), but can reduce 17 α -hydroxy-4-pregnene-3,20-dione and 20 α -hydroxy-4-pregnene-3one at a greater rate than the liver enzyme (Frederiksen & Wilson, 1971). The liver enzyme is also stimulated by administration of medroxyprogesterone, (Gordon et al., 1971) whereas the prostate enzyme is not affected (Frederiksen & Wilson, 1971).

Recently Moore & Wilson (1976) have reported that

in fibroblasts from genital skin there are two distinct 5α -reductase enzymes. The major activity is located in the microsomal fraction and has a narrow pH optimum around pH 5.5. The second activity which is not limited to genital skin and found largely in the 100,000g supernatant has maximum activity over the range pH 7-9. The physiological significance of this observation is not certain.

The testosterone 5α -reductase of rat prostate is itself androgen dependent since castration causes a decreased concentration of the enzyme which can be reversed by androgen replacement therapy (Gustafsson & Pousette, 1974; Moore & Wilson, 1973).

A study of DHT formation in the prostates of several species before and after puberty reveals a fall in enzyme activity in the cat and bull, and an increase in the dog, whilst in man and rat the levels remain high (Gloyna & Wilson, 1969). These authors suggest that the high levels of DHT after maturation in the prostate glands of man and dog may have implications in the development of benign hyperplasia, although DHT levels are also high in the rat prostate which is not normally subject to this condition. In the same study a wide range of rat tissues were assayed for 5α -reductase activity and were:-

prostate > epididymus > seminal vesicle >
penis > scrotum > kidney > skin > liver > others.

A similar study by Verhoeven et al., (1974) showed the order of activity to be:-

lung > prostate > kidney > submaxillary gland >
midbrain.

It is probable that androgens exert their effects by different mechanisms in androgen dependent tissues, such as prostate, and in androgen responsive tissue such as liver (Tomkins, 1957) and kidney (Verhoeven & De Moore, 1971) and this may account for the different constraints imposed on the 5 α -reductases from the various tissues studied.

Moreover, the in vitro demonstration of the testosterone 5 α -reductase activity within a specific tissue does not necessarily imply that the enzyme has a key role to play in the intact cell since discrepancies between in vitro and in vivo DHT formation have been observed in liver and kidney (King & Mainwaring, 1974).

An important feature of the testosterone 5 α -reductase enzyme is that the reaction it catalyses appears to be irreversible both in vitro (McGuire et al., 1960; Roy, 1971) and in vivo (Robel et al., 1971). The reaction is usually assumed to be a direct transfer of hydrogen from NADPH to testosterone. However Roy (1971) has suggested that

this in fact may not be the case, especially since most enzymes involved in the reduction or formation of a carbon-carbon double bond require a flavin, e.g. acyl CoA dehydrogenases, succinic dehydrogenase, and the 5β -steroid dehydrogenase from P.testosteroni (Davidson & Talalay, 1966). Also, most reactions involving NADPH directly are freely reversible. There is however no evidence for the involvement of a flavin, since atebirin is not an inhibitor of the reaction (McGuire et al., 1960; Roy, 1971). It has also been suggested by Roy (1971) that 5α -reductase activity may not be due to a single enzyme but ^{may} be a function of the cytochrome P₄₅₀ system and although he showed that carbon monoxide does not inhibit the testosterone 5α -reductase of rat prostate microsomes, the involvement of some cytochrome cannot be totally excluded.

The distribution of testosterone 5α -reductase activity between the different cellular components of the prostate has been investigated, but agreement on the matter has not yet been reached. Becher and co-workers (1972) reported that in human benign prostatic tissue the amount of testosterone 5α -reductase activity depended on the proportion of epithelial elements in the tissue, but Jenkins & McCaffery (1974) reported no such correlation. Two groups of workers have separated stromal and

epithelial elements of human prostatic tissue and whilst Harper et al., (1974) report higher activity in the epithelial fraction, Cowan et al., (1977) found enrichment of testosterone 5α -reductase activity in the stromal fraction.

4. Further Androgen Metabolism within the Prostate.

Although testosterone 5α -reductase may be regarded as being of central importance in androgen action in the prostate, it is not the only steroid metabolising enzyme present, and DHT is far from being the only androgen isolated from the tissue. Numerous reports have been made of the capacity of prostatic tissue to metabolise steroids (King & Mainwaring, 1974) and a detailed description is beyond the scope of this section. However, an attempt will be made to summarise the concensus view of androgen metabolism in the human gland, and differences between species will be illustrated.

Quantitatively the 5α -androstanediols are major constituents of human prostatic tissue and although 5α -androstan- $3\alpha,17\beta$ -diol is the most abundant of these, the isomeric diol 5α -androstan- $3\beta,17\beta$ -diol is also formed in small quantities (Bruchovsky & Wilson, 1968(b)) indicating clearly that both 3α -hydroxysteroid dehydrogenase and 3β -hydroxysteroid dehydrogenase are present in the tissue.

Whilst Taurog and co-workers (1975) could detect no 5α -androstane- $3\beta,17\beta$ -diol formation from DHT in the rat prostate, Levy and associates (1974) on the other hand did show reduction of DHT to this androstenediol in the cytosol fraction, but activity of the 3β -hydroxysteroid dehydrogenase was much lower than that of the 3α -hydroxysteroid dehydrogenase and would indicate that the pattern of 5α -androstenediols is very similar to that of the human gland. In the canine prostate there is a notable difference, where in addition to the two specified 5α -androstenediols, both 5α -androstane- $3\alpha,17\alpha$ -diol and 5α -androstane- $3\beta,17\alpha$ -diol have also been isolated as products of testosterone metabolism (Griffiths ^{et al.}, 1970; Harper et al., 1971). The possession of a 17α -hydroxyl group suggests that DHT is not involved as an intermediary in the formation of these steroids. It has been claimed that very low levels of the 17α -hydroxy- 5α -androstenediols can also be found in cultures of rat prostatic tissue (Griffiths, 1970), but superfusion of human hyperplastic tissue with labelled precursor failed to demonstrate significant levels and at best the 17α -hydroxy- 5α -androstenediols are minor metabolites of testosterone in the human gland (Beastall, 1975).

The conversion of DHT into 5α -androstane- $3\alpha-17\beta$ -diol is a reversible reaction in vivo since the administered diol is rapidly taken up by the prostate

and converted readily to DHT. In a similar experiment the 3β -isomer remained almost unchanged (Horst et al., 1975). These findings confirm the earlier in vitro observations of Lasnitzki (1970). More recently Malathi & Gurpide (1977,a) have shown significant interconversion of DHT and 5α -androstane- $3\alpha,17\beta$ -diol in superfused human prostate, with the equilibrium favouring DHT formation. However, a partially purified preparation of 3α -hydroxysteroid dehydrogenase from rat prostate favoured the reduction of DHT (Inano et al., 1977).

The intracellular location of 3α - and 3β -hydroxysteroid dehydrogenases has not been finalised for whilst Bruchovsky & Wilson (1968b) report that both the enzymes of rat prostate are located in the soluble fraction, Levy and associates (1974) produce evidence that the 3α -hydroxysteroid dehydrogenase of the same tissue is found in all subcellular fractions but is concentrated in the microsomes. In the canine prostate both 3α - and 3β -hydroxysteroid dehydrogenase have been found in microsomal and soluble fractions (Jacobi & Wilson, 1976). The cofactor requirements of the two enzymes are also a little uncertain for whilst both NADH and NADPH are effective cofactors for both reactions in canine prostate (Jacobi & Wilson, 1976), the former is preferred for the 3β -hydroxysteroid dehydrogenase of rat prostate (Levy et al., 1974) and

the latter preferred for the 3α -hydroxysteroid dehydrogenase of the same tissue (Inano et al., 1977).

The physiological function of the 5α -androstane-diols can at present only be speculative. By analogy to the liver it may be tempting to suppose that reduction of the ketone group on carbon atom 3 is merely an effective way of inactivating DHT prior to excretion, but the accumulation of these diols in the tissue contraindicates such a supposition, as does the reversible properties of the 3α -hydroxysteroid dehydrogenase itself.

Whilst possessing some androgenic activity, 5α -androstane- $3\alpha,17\beta$ -diol is much less potent than either testosterone or DHT in maintaining the structure and function of the prostate. In castrated rats the androgenicity may be explained by its conversion to DHT (Schmidt et al., 1973). Baulieu et al., (1968) have suggested that whilst DHT and 5α -androstane- $3\alpha,17\beta$ -diol stimulate cell division and perhaps hyperplasia, 5α -androstane- $3\beta,17\beta$ -diol maintains epithelial cell height and stimulates secretory activity. Such a conclusion must, however, be treated with caution since the data on which it was based was obtained by the addition of non-physiological amounts of steroid to tissue culture preparations. The role of the hydroxysteroid dehydrogenases in the aetiology of human benign

prostatic hyperplasia is thus uncertain although Geller et al., (1976) suggest that the accumulation of DHT in BPH may in part be related to a relative reduction in the activity of the 3α -hydroxysteroid dehydrogenase.

In canine prostate it seems more likely that a role may exist for 5α -androstane- $3\alpha,17\alpha$ -diol since Evans and Pierrepont (1975) have demonstrated a specific cytosol receptor for this diol which does not bind either testosterone or DHT, and the same steroid was the only one of the four isomeric 5α -androstane diols which maintained the epithelial height and secretory activity of explants of tissue cultured for up to 5 days (Sinowatz & Pierrepont, 1977).

Although DHT and the androstane diols are the major products of testosterone metabolism in the prostate, in vitro incubations of radioactive testosterone have produced a multiplicity of products. For example Chamberlain et al., (1966) identified ten metabolites during long term incubations of human prostatic homogenates. The incubation conditions of high substrate concentration and long incubation times (2h) permitted the full metabolic potential of the tissue to be expressed, and thus it was shown to contain 3α , 3β and 17β -hydroxysteroid dehydrogenases, and 5α - and 5β -reductases and some unidentified

hydroxylases.

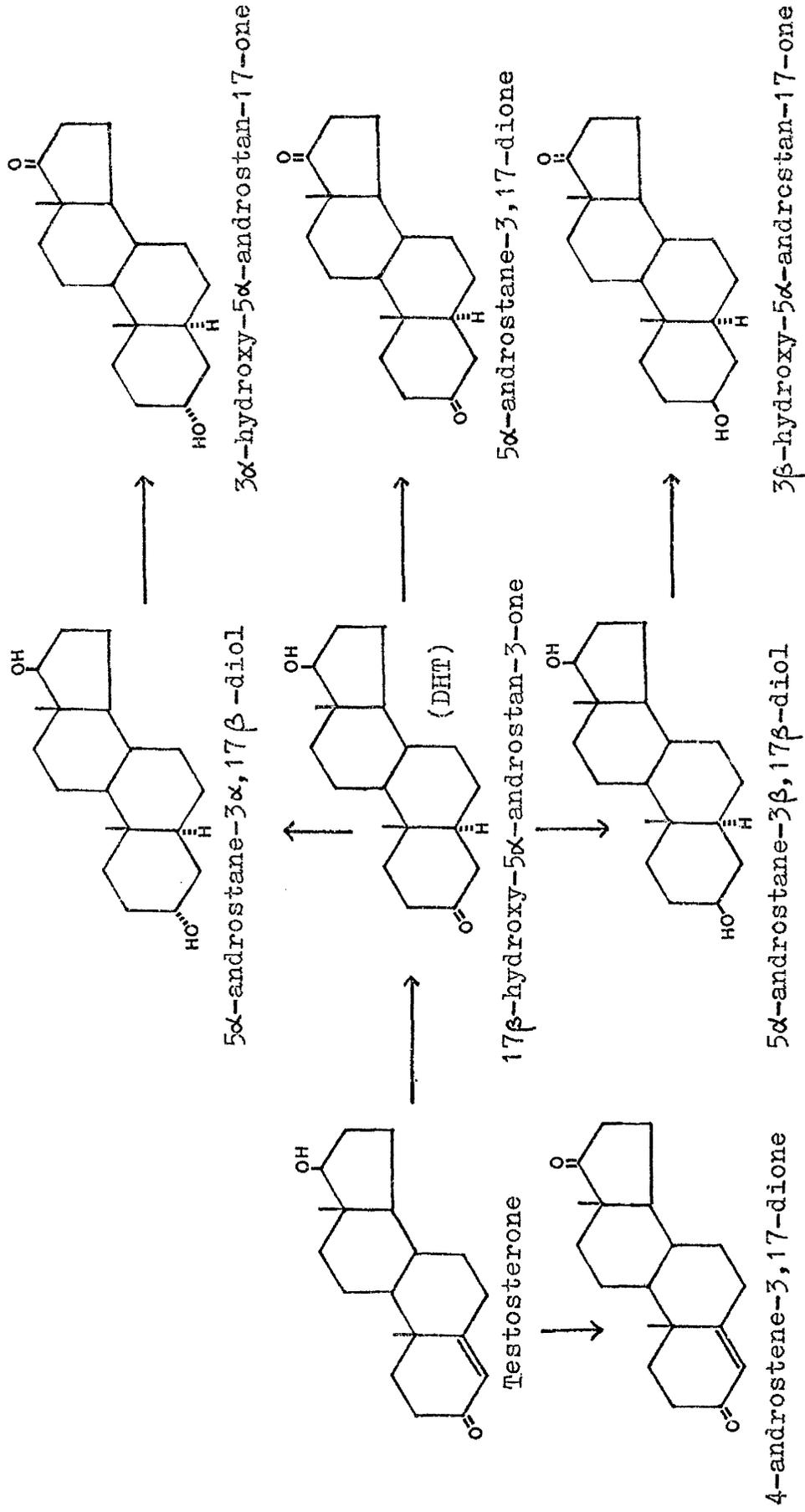
In vivo perfusion studies of human hyperplastic tissue have confirmed the existence of the three hydroxysteroid dehydrogenases but not the 5β -reductase (Morfin et al., 1970). In vitro perfusion of hyperplastic human prostatic tissue also failed to confirm 5β -reduction and Beastall (1975) suggests the pattern of metabolism shown in fig. I-5 as the most appropriate for the human gland. From this scheme all the 5α -reduced steroids containing at least one hydroxyl group appear to be retained in the tissue more avidly than testosterone whilst the two diketones are apparently liberated from the tissue more readily than testosterone.

To this point the discussion of the steroid metabolising capacity of the prostate gland has been centred around its ability to convert testosterone as substrate. The human prostate gland contains a very active dehydroepiandrosterone sulphate sulphatase and as such is capable of hydrolysing some of the large number of conjugated steroids transported in plasma (Farnsworth, 1973). However the ability of the tissue to metabolise steroids unsaturated between carbon atoms 5 and 6 is very limited (Beastall, personal communication) although small amounts of dehydroepiandrosterone have been reported to be converted into DHT (Harper et al., 1974). Human

Fig. I - 5.

Metabolism of Testosterone in the Prostate.

(from Beastall, 1975)



prostate is unable to convert C₂₁ steroids such as pregnenolone and progesterone into the C₁₉ androgens (Struthers, 1975) and although the C₂₁ steroids may well have a key role in the control of androgen metabolism this results from their ability to inhibit the enzymes of androgen metabolism rather than from conversion into the androgens themselves.

5. Factors Controlling Androgen Metabolism within the Prostate Gland.

Testosterone metabolism within the prostate is of central importance for the maintenance of structure and function of the gland. Control of this androgen metabolism is essential in maintaining the proper concentration of each metabolite and integrating the function of the gland with that of the whole animal. Several compounds, both synthetic and natural, have been shown to influence this metabolism, and a brief discussion of these is appropriate at this point.

It was stated earlier that the prostate has a high zinc content, and that the concentration and metabolism of this metal is under androgenic control (Mann, 1964). At least three specific zinc binding proteins from the prostate have been reported (Habib & Stitch, 1975; Reed & Stitch, 1973), and one of these has been purified and amino acid analysis shows it to have a high molar proportion of histidine, which

probably accounts for its zinc binding capacity (Heathcote & Washington, 1973). Although the zinc content of the gland is under androgen control, the zinc itself appears to influence testosterone metabolism. In human hyperplastic prostate there is an inverse relationship between the total zinc content and testosterone 5 α -reductase activity (Habib et al., 1975). Other divalent cations such as H_g⁺⁺, Cu⁺⁺, Cd⁺⁺ and Mg⁺⁺ have also been shown to affect the activity of this enzyme in both the human (Wallace, 1975) and rat prostate (Frederiksen & Wilson, 1971). These cations exhibit competitive kinetics with respect to the NADPH cofactor, and the explanation for this may be due to the inactivation of an -SH group at or near the cofactor binding site as reasoned by similar results being obtained with specific thiol group blocking agents (Wallace, 1975).

Circulating hormones may also influence prostatic androgen metabolism. The responsiveness of rat accessory sex organs to androgens is diminished by hypophysectomy and augmented by several pituitary hormones, particularly prolactin (Baker et al., 1977). These workers suggest that their results of increased ³H-DHT uptake into the nuclei may be due to an alteration in the androgen receptor mechanism. Negro-Vilar et al., (1977) also obtained evidence for a role of prolactin in the growth of rat prostate

and seminal vesicles by the use of anterior pituitary grafts. However, at physiological concentrations the conversion of testosterone to DHT in cultured rat ventral prostate cells was not affected by prolactin (Johansson, 1976).

It has been suggested that the adrenal glands also may influence prostatic growth (Fingerhut & Veenema, 1967; Tisell, 1970; Tullner, 1963). Tullner (1963) showed that ACTH affected the ventral prostate of immature castrated rats, but not if they were also adrenalectomised.

Estrogen therapy has been widely used in the treatment of prostatic cancer to suppress growth. Pharmacological doses of the steroid results in a lowering of testosterone secretion from the testis through suppression of pituitary gonadotrophins. However, there is also evidence of a direct influence of estrogens on the androgen metabolism within the prostate. Changes in metabolic clearance rate and transport have been reported (Bird, et al., 1971) and it is suggested that the beneficial effects of estrogen therapy in patients with prostatic carcinoma may be related to this increase in testosterone metabolism. Estrogens, both natural (estradiol) and synthetic (stilboestrol and ethinylestradiol) also inhibit the testosterone 5α -reductase in vitro, but at pharmacological concentrations (Bonne &

Raynaud, 1973; Jenkins & McCaffery, 1974; Lee et al., 1974).

Progestins as a group also have distinct antiandrogenic properties, and, like estrogens, part of this activity almost certainly includes suppression of pituitary LH (Chisholm, 1970). Cyproterone acetate acts by selectively blocking the binding of DHT to the androgen receptor in the rat prostate but has no effect on the testosterone 5 α -reductase activity (Fang & Liao, 1969; Orestano et al., 1975). Progesterone itself may bind to the androgen receptor but in vitro has been shown to be a potent inhibitor of the testosterone 5 α -reductase (Jenkins & McCaffery, 1974; Tan et al., 1974). The effect of these and other progestins will be discussed more fully in Chapter VI.

Some aldosterone antagonists have progestin like structures and in this context have aroused interest as possible antiandrogens. Basinger & Gittes (1974) reported a 40% atrophy of the prostate and seminal vesicles of mature rats injected with spironolactone for ten days. Apart from a direct antiandrogenic effect on the prostate by binding to the androgen receptor (Bonne & Raynaud, 1974; Corval et al., 1975), spironolactone also decreases the activity of testicular 17 α -hydroxylase (Menard et al., 1974) resulting in increased plasma

progesterone and 17α -hydroxyprogesterone (Stripp et al., 1975). These compounds also will be discussed more fully in the appropriate chapter (Chapter IV).

Heparin is a naturally occurring polyanionic mucopolysaccharide, and is a drug in common use in anticoagulation therapy. It has been reported to inhibit the steroid 5α -reductases in several organs, including the testis (Kizanowski & Troop, 1968), adrenals (Lakatos et al., 1970), liver (Steinberger & Fisher, 1971) and more relevant to the considerations here, in rat prostate (Blaquier & Calandra, 1973). Most of these observations have been made in vitro but in vivo small effects on liver 5α -reductase have been reported (Carter et al., 1968) and heparin was also observed by these authors to have an antiinflammatory effect in intact, but not in adrenalectomised rats. Like progesterone and spironolactone, heparin will be discussed more fully in the appropriate following chapter (Chapter III).

6. The Mode of Action of Androgens.

Steroid hormones have been known for many years to exert dramatic effects on their target cells, but it is only in recent years that their mode of action has been elucidated.

Early work by Gorski & Morgan (1967) and

Jensen et al., (1968) followed the progress of 17β -estradiol within its target organ, the rat uterus, and showed that it bound to two distinct types of protein. The proteins which bound the steroid with low capacity but high affinity were called specific receptors, and were responsible for the transportation of the bound steroid from the cytoplasm to the nucleus. The other proteins, with high capacity and low affinity were called storage receptors (Wurtman & Jensen, 1968) and were probably plasma binding globulins. Considerable interest was stimulated by these findings and it was not long before a general picture for the action of all steroid hormones emerged. (See King & Mainwaring, 1974; O'Malley & Schrader, 1976, for general reviews.)

The steroid must first pass through the plasma membrane before binding to the specific receptor in the cytoplasm. A transformation of the hormone receptor complex takes place followed by transfer to the nucleus, where the steroid can interact with the chromatin, ultimately causing the synthesis of new proteins. In the case of 17β -estradiol the plasma steroid is bound directly to the cytoplasmic receptor. On the other hand steroids such as testosterone in the prostate, require modification by cytoplasmic enzymes before binding to the specific receptor takes place. A scheme for the

mode of action of testosterone within the prostate is shown in fig. I-6.

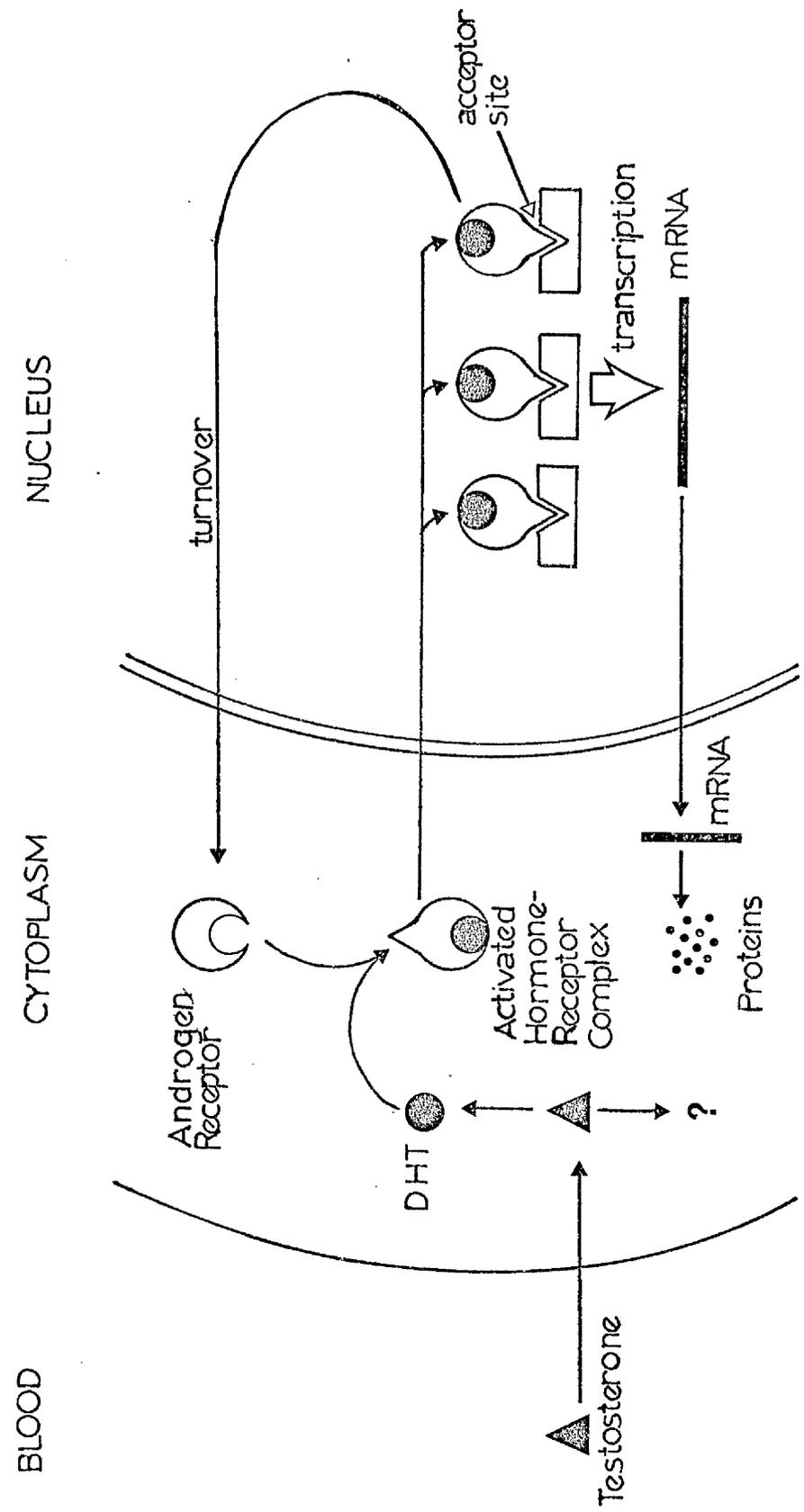
It is apparent from the above brief account that the mechanism of action of the steroid hormone is fundamentally different from that of the protein hormones whose receptors are contained in the plasma membrane, and it is therefore perhaps not surprising that cyclic AMP cannot mimic the androgen effect (Craven et al., 1974; Lesser & Bruchovsky, 1973; Sanborn et al., 1975).

When the prostatic cytoplasmic receptor was isolated two to five minutes after the administration of [3 H]-testosterone to rats, binding was found predominantly to a protein with a sedimentation coefficient of 8S, whereas at longer times of labelling a 3.5S receptor was predominant (Mainwaring & Peterkin, 1971), indicating that some transformation takes place after the binding. This conversion from 8S to 3.5S form of receptor has been shown to be temperature dependent, and to precede the transfer of the DHT-receptor complex into the nucleus.

The specificity of the prostatic androgen receptor has been studied and the apparent binding constants for a number of androgens, antiandrogens and estrogens showed that all the common prostatic metabolites of testosterone had a measurable affinity for the rat cytoplasmic receptor and demonstrated

Fig.I - 6

Scheme for the Mode of Action of Testosterone within the Prostate.
(Adapted from Leake, 1976)



that the presence of a 3-keto or 17 β -hydroxy function on the steroid nucleus is a strong determinant of the binding efficiency (Shain & Boesel, 1975). Skinner et al., (1975) showed that DHT binding was inhibited by several steroid analogues, the most effective had generally planar geometry and were known to possess potent androgenic activity.

The receptors in various organs other than the prostate have also been studied. Krieg & Voigt (1976) showed that whilst the androgen receptor has the same physiochemical properties in the prostate, levator ani and skeletal muscles, the dissociation constants for testosterone, DHT and 19-nortestosterone were different in the different tissues. Conversely a comparison of the binding properties of the androgen receptor for testosterone and DHT in the rat testis, epididymis and prostate (Wilson & French, 1976) suggested that in these three tissues the receptors are identical. These differing results may perhaps be explained by the fact that in the first study a mixture of androgen dependent and androgen responsive tissues were studied whereas in the second study only androgen dependent tissues were investigated.

The presence of the androgen receptor in androgen responsive cells is of prime importance, and it might be expected that at least a small

receptor concentration would be present at all times, regardless of the androgen status of the animal. Whilst this may be partially true there is evidence that external factors also exert an influence on the receptor level. Ageing in the rat has a marked effect on the prostatic androgen receptor concentration. A decrease in receptor content is seen to occur abruptly at about 300 days of age, falling to about 57% of the level found in the younger animals. The histology of the prostates from the older group revealed an increase in the number of alveoli containing low epithelial cells, and a decrease in secretion, indicating a regression in the gland as a whole (Shain et al., 1975). Castration has also been shown to have an effect with an initial fall in the rat androgen receptor concentration to virtually undetectable levels being seen after four days (Sullivan & Strott, 1973) or seven days (Bruchovsky & Craven, 1975) post surgery. The difference in the rate of decrease is probably due to the different experimental methods employed. Sullivan & Strott (1973) reported that whilst the receptor level in the prostate as a whole remained low after the fourth day, the levels per cell returned to near normal by the eighth day, and were maintained for six weeks at least. This can only occur if there is a net synthesis of protein, or

a loss of DNA without loss of receptor, the latter being unlikely. As these observations were also made with animals which had been either adrenalectomised or hypophysectomised at the time of castration it would indicate that the observed restoration of receptor is independent of androgens. Bruchovsky & Craven (1975) indicate by tissue mixing experiments that day 7 prostates contain factors, presumably proteolytic enzymes from disrupted lysosomes, which can eradicate the binding of DHT to the receptor and may in part explain the initial fall in receptor level.

To date, the majority of information about the androgen receptor has been obtained from the rat prostate. This tissue is easily obtainable, and homogenises well, and the hormone status of the animal can be changed by castration, adrenalectomy, hypophysectomy or drug administration. The human prostate on the other hand is more difficult to obtain, especially normal tissue, and because of the large proportion of stromal and connective tissue the human gland is much more difficult to work with. Good liaison with surgeons and improved techniques have resulted in the use of benign hyperplastic and carcinomatous human tissue by several groups of workers, and one hopes that this will lead to a rapid increase in our knowledge about

androgen receptors in the human gland.

7. Benign Prostatic Hyperplasia and Prostatic Carcinoma.

A. Benign prostatic hyperplasia.

It has been estimated that up to 80% of men over 70 years of age have enlarged prostates resulting from benign prostatic hyperplasia (BPH) (Franks, 1954). This is a nodular hyperplasia which arises in the inner region of the prostate near the urethra, and is distinct from prostatic carcinoma which arises in the outer region (Scott, 1963). The majority of hyperplasias seen are stromal in origin, but there are also a substantial number of cases of adenomatous (epithelial) hyperplasias. The factors responsible for the initiation of BPH have not been fully elucidated. After middle age the production of androgen falls and the relative ratio of estrogens increases and it may be that prostatic enlargement results from this change. However, if the enlargement were due simply to reduction in androgen secretion, then it would be expected that hyperplasia would follow castration, but this never occurs. Thus it appears that some element of testicular secretion may be required (Newsam & Thomson, 1974).

There is evidence that 5 α -dihydrotestosterone may be implicated, as the concentration of this steroid

in hyperplastic tissue (0.60 g/100 tissue) was found to be higher than in normal tissue (0.13 g/100g tissue) and that in prostates with early hyperplasia the DHT content in the periurethral (inner) area where hyperplasia usually commences, was two and three times greater than in the outer region of the gland (Siiteri & Wilson, 1970). However whether this increased DHT content is a cause or an effect of the benign hyperplasia remains to be decided by further experimentation.

The development of a hyperplastic prostate in itself poses no great danger to the patient as this is a benign condition. However, enlargement of the gland may interfere with the proper functioning of the bladder thus causing discomfort and greatly increasing the risk of urinary tract infection and kidney disorders.

Growth of the median lobe into the bladder can cause an increase in residual urine behind the projection, or may cause obstruction of the bladder opening into the urethra. Growth of the lateral lobes in the area surrounding the urethra gradually compresses the outer region of the prostate into what is known as the false capsule and continued growth will cause pressure on the urethra making urination more difficult. These symptoms may be temporarily removed by catheterisation, but in the long term the

only satisfactory method of treatment is the surgical removal of either the whole or the offending part of the prostate.

Prostatectomy for benign enlargement consists of removal of the enlarged inner glands and leaves intact the compressed outer group of glands (false capsule) and the true capsule. A plane of cleavage allows easy digital enucleation by several approaches. After removal, the raw prostatic cavity becomes lined with epithelium in seven to ten days (Newsam & Thomson, 1974).

Hormonal therapy has also been advocated as it has been repeatedly shown that growth of the prostate depends on its hormone environment. This would be especially attractive as many patients with benign prostatic hyperplasia fall into the poor surgical risk class.

Pituitary hormones exert a direct effect on the prostate, as was shown by Grayhack (1963) using hypophysectomised and castrated rats. The hypophysectomy causing greater prostatic regression than castration, Lawrence & Landau (1965) showed that prostatic uptake of ^3H -testosterone was much less in hypophysectomised animals, and Farnsworth (1970) suggested that prolactin may also be involved since slices of BPH tissue take up more ^3H -testosterone when prolactin is present in the culture medium,

than do controls.

In the past, treatments using estrogens or androgens (Heckel, 1944) or combinations of both, have been tried but conflicting results were obtained. Synthetic estrogens, such as ethinyl estradiol have proved valuable in halting or reversing the growth of prostatic carcinoma, but have little effect on benign hyperplasia (Briggs & Briggs, 1973) and unpleasant side effects including breast enlargement are known to occur.

Progestins as a group have a similar effect to estrogens. Their mode of action almost certainly includes the suppression of pituitary hormones (Chisholm, 1970) and thus testicular testosterone, as well as any direct effect. Results of drug trials give conflicting results as to the usefulness of many of the compounds, but some encouraging results have been observed. Further discussion of progesterone treatment will be found in Chapter VI.

B. Prostatic Carcinoma.

Prostatic carcinoma is the second most common cancer of the male, and causes approximately 7% - 8% of all deaths due to tumours in men over fifty years of age in the United Kingdom (Franks, 1974). The cause of prostatic cancer, like that of most other cancers remains obscure, but the incidence appears to be subject to some racial and

geographical differences (Franks, 1974).

The outer region of the gland is the most common site of origin of the carcinoma (Scott, 1963) and many of these growths are androgen dependent. The androgen dependent nature of the disease led to the widespread use of estrogens for the clinical control of the disease (Huggins & Hodges, 1941). Pharmacological quantities of estrogens lower testicular secretion of testosterone, probably through suppression of pituitary gonadotrophin release. Although estrogen therapy causes shrinkage of the primary lesion in the prostate it is not a cure, but only delays the fatal consequences of the disease.

The prostatic tumour commonly invades local tissues such as the bladder, seminal vesicles and less commonly the rectum; and transport by both blood and lymphatics may cause involvement of more distant lymph nodes. Bone metastases are also an important feature of prostatic carcinoma, the head of the femur, the pelvis, the lumber spine and the ribs being the most common sites involved (Newsam & Thomson, 1974).

Diagnosis of the condition may be aided by rectal examination and biopsy. High acid phosphatase levels are also an indication of the condition, although actual levels do not have a

prognostic value. Bone scanning also has a useful place in detecting metastases (Shuttelworth & Blandy, 1976).

Treatment of the disease depends to a great extent on the advancement of the tumour and to the condition of the patient. Radical prostatectomy for a localised growth may be successful, and estrogen therapy or removal of the testis may give rapid and prolonged relief of symptoms. Radiotherapy has also become more popular in recent years. Varying success rates have been reported for each of these treatments, and with continuing research one hopes that the prognosis for these patients will improve dramatically in future years.

8. Outline of Research.

The initial aims of this project were to investigate the effects of heparin and related mucopolysaccharides on the prostatic testosterone 5 α -reductase enzyme in vitro and in vivo with a view to assessing these compounds as potential non-steroidal antiandrogens. Measurement of prostatic tissue content of heparinoids was also planned.

The work was expanded to investigate the effects of the aldosterone antagonists, aldadiene and canrenoate, on the 5 α -reductase enzyme, since these compounds had previously been shown to have

antiandrogenic side effects.

Many antiandrogenic compounds have a steroid nucleus based on that of progesterone and hence have progestational actions. Experiments were designed to determine the effect of progesterone itself on the 5α -reductase enzyme both on homogenised tissue preparations and using in vitro superfusion techniques on tissue slices.

At the conclusion of this project it was hoped that a greater understanding would be gained of the control of testosterone 5α -reduction within the human prostate, which in turn might contribute to the elucidation of the aetiology of benign prostatic hyperplasia.

CHAPTER II.

CHAPTER II.

MATERIALS AND METHODS.

1. MATERIALS.

A. Radiochemicals.

Radiochemicals were obtained from the Radiochemical Centre, Amersham, as follows:-

| | |
|--|--|
| [1,2- ³ H] -Aldadiene (Gift from Searle) | .. 53 Ci/mmole |
| [4- ¹⁴ C] -Androstenedione | .. 60mCi/mmole |
| [1,2- ³ H] -5 α -Dihydrotestosterone | .. 47 Ci/mmole |
| [4- ¹⁴ C] -5 α -Dihydrotestosterone | .. 50mCi/mmole |
| [1,2,6,7- ³ H] -Cortisol | .. 85 Ci/mmole |
| [³⁵ S] -Heparin | .. 36.7mCi/g on 9/9/1974 ($t_{1/2}$ = 87.1 days) |
| [1,2,6,7- ³ H] -Progesterone | .. 80 Ci/mmole |
| [1,2- ³ H] -Testosterone | .. 49 Ci/mmole |
| [4- ¹⁴ C] -Testosterone | .. 59mCi/mmole |
| [17 α - ³ H] -Testosterone | .. 5.5 Ci/mmole |

The purity of all radiochemicals was tested by chromatography and purification was carried out where necessary, before use.

B. Chemicals.

General chemicals were obtained from BDH Chemicals

Ltd., Poole, England, or from Sigma, London, and were of the highest quality available. In addition, the following specific chemicals were used:-

Chondroitin Sulphate (Sigma) Grade III
from whale and shark cartilage.

D-Glucose-6-Phosphate (Sigma)
monosodium salt.

D-Glucose-6-sulphate (Sigma)
potassium salt.

D-Glucuronic Acid (Sigma) Grade II
sodium salt.

D-Glucuronolactone (BDH)

Heparin (Sigma) Grade I 158 J-A units/mg.
from porcine intestinal mucosa.

N-Acetyl Glucosamine (Sigma)

NAD (Boehringer Mannheim GmbH) Grade I
Free Acid.

NADP (Sigma)
monosodium salt.

NADPH (Sigma)
tetra sodium salt.

Protamine Sulphate (Sigma) Grade I
from Salmon - essentially histone free.

Poly-L-Glutamic Acid (Sigma) Type 1
sodium salt. Degree of polymerisation - 90.

Ro1 - 8307 (Hoffmann - Roche, gift)
N-formyl-chitosan polysulphuric acid.

Ro2 - 3053 (Hoffmann - Roche, gift)
mepesulphate.

Ro2 - 7509 (Hoffmann - Roche, gift)
sulphated cyclohepta amylase.

Ro2 - 7529 (Hoffmann - Roche, gift)
sulphated cyclohexa amylase.

C. Steroids.

Non-radioactive steroids were obtained from Steraloids Ltd., Croydon. These were of the purest commercially available quality and were tested by recrystallisation followed by observation of the melting point of the crystals obtained.

Aldadiene and Potassium Canrenoate were gifts from Searle.

D. Solvents.

Ethanol and methanol, A.R. grade, were obtained from Burroughs Ltd. All other solvents were purchased from BDH Chemicals Ltd., and were used without further purification.

All aqueous solutions were prepared from glass distilled water.

E. Scintillation Materials.

Toluene and Triton X-100 were obtained from BDH Chemicals Ltd., Poole, England.

2,5-Diphenyloxazole (P.P.O.) activator and 1,4Di[2-(5-phenyl-oxazolyl)]-benzene (P.O.P.O.P.) were obtained from Koch-Light Laboratories Ltd., England.

F. Miscellaneous.

Alumina

Aluminoxide 60, PF 254+366 (Type E)
Merck, Darmstadt.

Silica Gel

Kieselgel HF 254+366 nach Stahl (Type 60)
Merck, Darmstadt.

Chromatography paper

Whatman No.1, 5cm wide rolls.

Nybolt, Nylon Bolting Cloth

25T, 45 μ pores, obtained from
John Shaniar & Co., Manchester

The scintillation vials used throughout the study, unless otherwise stated, were of the plastic disposable type and were in general, obtained from Intertechnique Ltd., Middlesex.

Several commercially available enzymes were used in this study. Glucose-6-phosphate dehydrogenase (360 units/mg protein) and 6-phosphogluconate dehydrogenase (20 units/mg protein) were obtained from Sigma; Pronase (45,000 PUK Units/mg) and Trypsin (reagent grade) were obtained from BDH Chemicals Ltd., Poole, England.

2. METHODS.

A. Chromatographic Methods.

(i) Paper Chromatography.

Paper chromatography was used for the purification of steroid substrates before use and for the separation of metabolic products after experimentation.

The fraction to be chromatographed was evaporated under nitrogen, dissolved in a few drops of acetone and transferred to a 5cm x 50cm strip of Whatman No.1 chromatography paper before being placed in a chromatography tank to equilibrate for at least two hours. The tank walls were lined with heavy chromatography paper saturated with the mobile phase, and another strip of chromatography paper was suspended into a trough of stationary phase. After equilibration, the paper was developed for 2h by descending chromatography, taken from the tank and dried in air.

For all the paper chromatography in this study a Bush B-3 solvent system was employed (Bush, 1952). This has a stationary phase of Methanol/Water:4/1 and a mobile phase of Benzene/Pet.Ether (Bpt. 80-100°C): 2/1. Both phases were equilibrated with each other before use.

The strips were scanned with a Packard Radioautogram scanner to locate the steroids. The

appropriate regions were cut into 1cm squares and eluted overnight with 5-10ml of methanol/H₂O:29/1. The eluate was decanted and the paper rinsed with a further 5ml of the solvent. The pooled methanol extracts were evaporated under a stream of nitrogen.

(ii) Thin Layer Chromatography (TLC).

Glass thin layer plates (20cm x 20cm) were coated with a slurry of either Alumina PF 254+366 or Silica Gel HF 254+366 using a Shandon plate spreader adjusted to give layers 0.3mm thick. The powdered adsorbents were mixed with distilled water in the ratio Alumina 50g/water 55ml or Silica 25g/water 55ml, to give sufficient slurry for 5 plates. The coated plates were activated by drying at 115°C for 1½hrs. and stored in airtight cabinets over silica gel crystals prior to use.

Samples to be chromatographed were dissolved in a few drops of an appropriate solvent and applied to the plates in 2cm streaks with a Pasteur pipette. A maximum of five lanes per plate were employed to ensure there was no overlapping of samples when the plates were developed. The plates were placed in a glass tank with a saturated atmosphere and containing 1-2cm of solvent and developed by ascending chromatography until the solvent front was within 4-5cm of the top of the plates. The plates were removed from the tank and allowed to dry in the air

before the spots were visualised under UV light. Steroids containing conjugated double bond systems were visualised under a 254nm lamp whilst other substances were visualised under a 350nm wavelength light.

Where appropriate, steroids were removed from the plate by scraping the adsorbent into an inverted Pasteur pipette plugged with cotton wool and attached to a water suction pump. The steroids were then eluted either into a scintillation vial or a test tube with 2x2ml diethyl ether.

The solvent systems used for separating the various steroids are listed in Table II - 1 along with their Rf values.

(iii) Gas Liquid Chromatography (GLC).

A Pye 104, type 1 chromatograph fitted with an autosolid injection device was used for monitoring the recovery of certain steroids.

For the quantitation of 5 α -androstanediols and their diacetates, a 7ft. x $\frac{1}{4}$ inch column filled with 1% SE30 on Gas chromQ was used at 240 $^{\circ}$ C, whilst for progesterone, 5 α -pregnane-3,20-dione, and 3 β -hydroxy-5 α -pregnane-20-one the column was 6ft. x $\frac{1}{4}$ inch filled with 3% SE30 on Gas chromQ and maintained at 210 $^{\circ}$ C. In both cases the carrier gas was Nitrogen used at a flow rate of 60ml/min.

A standard flame ionisation detector was employed,

Table II - 1

Solvent systems used for the separation of steroids,
and the Rf values obtained.

| Solvent System | Type of TLC Plate | Steroid | Rf |
|--|-------------------|--|------|
| 1. Cyclohexane/ Ethylacetate 3/1 | Alumina | 5 α -pregnane- 3-ol,-20-one | 0.22 |
| | | progesterone | 0.29 |
| | | testosterone acetate | 0.33 |
| | | 5 α -pregnane- 3,20-dione | 0.41 |
| | | DHT acetate | 0.45 |
| | | 5 α -androstane-3 α , 17 β -diol diacetate | 0.64 |
| 2. Chloroform/ Acetone 7/1 | Silica Gel | testosterone | 0.31 |
| | | DHT | 0.42 |
| | | androstene-3,17- dione | 0.54 |
| | | 5 α -androstane- 3,17-dione | 0.62 |
| 3. Chloroform/ methanol 99/1 | Silica Gel | 5 α -pregnane- 3 β -ol-20-one | 0.25 |
| | | 5 α -pregnane- 3 α -ol-20-one | 0.31 |
| | | Progesterone | 0.54 |
| | | 5 α -pregnane- 3,20-dione | 0.57 |

the flame being supported by a mixture of hydrogen from a generator, and air. Peak areas were measured by an Autolab 6300 digital integrator, and steroid recovery determined by reference to the peak areas of relevant standards.

B. Measurement of Radioactivity.

(i) Paper and thin layer chromatogram scanners.

(a) Paper Scanner.

Location of radioactivity on paper strips after chromatography was facilitated by a Packard Radio-chromatogram Scanner. The high voltage was set at 1200 V, the gas flow rate at 200ml/min, and a time constant of 1-3 was used throughout. A scanning speed of 2cm/min was used for routine purposes, or 1cm/min when overnight scanning was necessary. The sensitivity setting depended on the concentration of steroid on the paper strip.

(b) Thin Layer Chromatogram Scanner.

A Thin Layer Chromatogram Scanner (Panax Equipment Ltd., Surrey) was used for the location of radioactivity on thin layer plates after chromatography.

The E.H.T. was set at 1052 volts, the disc-bias at 100mV and the time constant at 0.1 seconds. The gas flow rate was adjusted to 60ml/min. Scanning conditions were constant throughout the data presented and the range (c.p.s.) adjusted to give peaks of

suitable height for measurement.

(ii) Liquid Scintillation Counting.

A Tri-Carb liquid scintillation spectrophotometer (Model 3380 Packard Instrument Co.) was used throughout all experiments for the counting of radioactive samples. Unless otherwise stated, plastic vials were used for counting. Two types of scintillation fluid were used. Samples which had previously been dried were counted after the addition of "non-aqueous" scintillator (6ml). The scintillation fluid had a toluene base and contained 2,5 diphenyloxazole (P.P.O.) activator (4g/l) and 1,4Di[2-(5-phenyl-oxazolyl)]-benzene (P.O.P.O.P) spectrum shifter (0.2g/l). This scintillator was chosen because the compounds used with it were completely soluble in toluene, the efficiency was high, and minimal quenching occurred. Samples which were water soluble or had a high water content were counted in "aqueous" scintillator, which was made by the addition of Triton-X100 emulsifier in a proportion of 2 parts 'non-aqueous' scintillator to 1 part Triton-X100. Aqueous samples were counted in this fluid by adding 6ml scintillator to 0.5ml sample, or 10ml scintillator to 1ml sample followed by thorough mixing. The efficiency of these scintillation fluids are shown in Table II-2.

Double isotope counting was performed in two

Table II - 2

Counting efficiencies, in each channel, of
scintillation fluids.

| Scintillation Fluid | Isotope | Efficiency % | | |
|---------------------|-----------------|--------------|-------|-------|
| | | Red | Green | Blue |
| Non-aqueous | ^3H | 27-30 | - | 35-40 |
| | ^{14}C | 7-8 | 50-56 | 24-28 |
| Aqueous | ^3H | 20-25 | - | 23-28 |
| | ^{14}C | 12-14 | 30-33 | 38-42 |

Non-aqueous scintillator was used with dried, toluene soluble compounds.

Aqueous scintillator was used with water soluble compounds in a ratio of either 6ml scintillator to 0.5ml sample, or 10ml scintillator to 1ml sample.

channels (red and green) of the scintillation spectrophotometer. The width of the green channel was adjusted to count carbon-14 alone, whilst in the red channel both tritium and carbon-14 were counted. The exact degree of spillover of carbon-14 into the red channel was determined under experimental counting conditions by the inclusion of a carbon-14 standard in each set of samples. The spillover value was subtracted from the total counts in the red channel to give the true value for tritium. In cases where tritium alone was to be counted the blue channel was used as the window setting allowed the total tritium spectrum to be counted thus maximising the efficiency.

The channel selection controls were set as follows:-

| | <u>Channel</u> | <u>Window Setting</u> | <u>Amplification</u> |
|-------|----------------|-----------------------|----------------------|
| Red | A - B | A50 - B195 | 50% |
| Green | C - D | C210 - D1000 | 77% |
| Blue | E - F | E50 - F1000 | 50% |

Counts were accumulated in each channel to give an S.E.M. of less than one per cent, which in most samples required counting for 10min.

C. Processing of Scintillation Counter Output.

The requirement of correction for procedural losses, and the involved but repetitive nature of many of the calculations meant that the use of a

programmable calculator was important in reducing the time required for processing data obtained from the scintillation counter.

Output from the scintillation counter was simultaneously typed in numerical form and on punch tape, and calculations performed on a Wang 600 series programmable calculator with a high-speed Optical Paper Tape Editor. Programmes were written and stored on magnetic tape for standard calculations such as $^3\text{H}/^{14}\text{C}$ ratios, correction of procedural losses, calculation of specific enzyme activity and of superfusion parameters.

D. Purification of Steroids.

(i) Non-Radioactive steroids.

The steroids used were of the purest quality commercially available, and the purity was tested before use by recrystallisation followed by observation of the melting point of the crystals obtained.

(ii) Radioactive steroids.

Radioactive steroids to be used as substrates were required to be of the highest possible purity. Purity was tested by recrystallisation from hexane/acetone after the addition of approximately 10mg of pure unlabelled steroid and pure samples of the appropriate [^3H]-steroid for [^{14}C]-samples or [^{14}C]-steroid for [^3H]-samples. The samples were

deemed pure if the isotope ratio of the original solution, the crystals and the mother liquor were within 5% of each other. If the steroids were outwith this limit appropriate chromatography was performed followed by another test recrystallisation until the required purity was attained.

E. Chemical Methods.

(i) Acetylation of Steroids. Bush (1961,a)

Steroids to be acetylated were evaporated to dryness under air before the addition of 100 μ l of dry pyridine and 100 μ l acetic anhydride. The samples were incubated for either 1hr at 60 $^{\circ}$ C or 18hr at room temperature. The acetylation reaction was stopped by adding 2ml water, the steroids extracted with 8ml chloroform, the aqueous phase removed and the solvent evaporated to dryness.

In later experiments it was found that the addition of 2ml of ethanol assisted the evaporation of the pyridine and acetic anhydride mixture and thus permitted the omission of the extraction step.

(ii) Chromic Acid Oxidation of Steroids. Bush (1961,b)

Steroids requiring oxidation were first evaporated to dryness. Glacial acetic acid (1ml) and chromium trioxide (0.5ml, 2% in water) were added and the solution incubated at room temperature for 30min. It was important that this time was not

exceeded since destruction of the steroid nucleus could take place. The steroids were extracted following the addition of 2ml water and 8ml ethyl acetate. The aqueous phase was removed and the ethyl acetate layer washed repeatedly with 2ml fractions of distilled water, potassium carbonate (2ml of 2% in water) and finally distilled water. Evaporation to dryness was carried out under a stream of nitrogen.

F. Preparation of Buffers.

In all cases glass distilled water was used in the preparation of the following buffers:-

(i) Tris buffers.

(a) Tris/HCl.

This basic buffer contained 50mM Tris and 30mM Nicotinamide and was brought to pH 7.4 before making to its final volume.

(b) Tris/HCl + Sucrose.

For the preparation of crude tissue homogenates Tris/HCl buffer was supplemented with 0.25M Sucrose to produce an isotonic solution.

(c) TKM.

The preparation of a microsome fraction requires the presence of divalent cations to prevent clumping. Thus, for work involving microsomes Tris/HCl was supplemented with 25mM KCl and 5mM MgCl₂.

(d) TKM + Sucrose

TKM + 0.25M Sucrose

Storage of Tris Buffers.

Owing to the unpredictable arrival of human prostate tissue for experimentation, it was found prudent to have stocks of prepared buffers at hand. Tris/HCl and TKM buffers were made at X10 concentration, stored in suitable aliquots and kept frozen at -20°C . These were made up to the correct concentration, supplemented with sucrose as required and kept at 4°C for only limited periods of time.

(ii) Krebs-Ringer Bicarbonate buffer.
(Cohen, 1957)

This buffer was prepared freshly before use from six stock solutions which were stored at 4°C and replaced at intervals of less than six months. Prior to use the buffer was bubbled with a gas mixture of 95% O_2 and 5% CO_2 for 30min. and the pH adjusted to 7.4 by adding 0.1M NaOH dropwise.

| Solution | Vol. used (ml) | Concentration of stock (g/100ml) | Final Concentration (mM) |
|---|-------------------|--|--------------------------------|
| NaCl | 10 | 6.780 | 116.00 |
| KCl | 10 | 0.347 | 4.65 |
| NaHCO_3 | 10 | 2.050 | 24.40 |
| Glucose | 10 | 1.000 | 0.055 |
| KH_2PO_4 | 1 | 1.573 | 1.16 |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 1 | 2.860 | 1.16 |
| $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ | 1 | 5.300 | 2.42 |
| H_2O | 57 | | |

(iii) Phosphate buffers.

(a) Standard phosphate buffer.

Solutions of 50mM KH_2PO_4 and 50mM K_2HPO_4 were mixed in the appropriate ratio to give a pH of 7.0, the resultant buffer was supplemented with 3mM EDTA, 0.05% β -mercaptoethanol and 20% glycerol, and stored at 4°C.

(b) Pyrophosphate buffer.

Solutions of 20mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ and 20mM $\text{Na}_3\text{H P}_2\text{O}_7$ were mixed to give a solution of pH 8.9. The buffer was stored at 4°C awaiting use.

G. Preparation of Prostate Tissue.

Human or rat ventral prostate was dissected free of fat and membranes, chopped into small (5mm) cubes, washed in saline (0.9% NaCl) and either homogenised immediately or frozen in liquid nitrogen and stored at -70°C until use. The latter method results in little if any loss of testosterone 5 α -reductase activity during freezing, a loss of 30% activity during the first few days of storage at -70°C followed by a much more gradual decline to 60% of the original activity at day 30, after which the activity remains constant for at least another month (Wallace, 1975). Freezing and storing the tissue in this way helps to overcome some of the problems of an irregular supply of human tissue

although it does mean that in preparing enzyme solutions the age of this tissue must be taken into account.

H. Assay of Testosterone 5 α -Reductase. E.C. 1.3.1.4.

(i) Enzyme Preparation.

Two different enzyme preparations were used in the assay of testosterone 5 α -reductase:

(a) Homogenate enzyme (b) Microsomal enzyme

(a) Homogenate enzyme.

For preliminary experiments a crude homogenate preparation was found to be adequate. This was prepared by suspending finely chopped tissue in ice cold Tris/HCl+Sucrose buffer in a ratio of 1g rat prostate/20ml or 1g fresh human prostate/100ml, and homogenised by two ten second bursts of an Ultra-turax homogeniser, with one minute for cooling between bursts. The homogenate was then filtered through fine mesh gauze (Nybolt No.25T, 45 μ pores) to remove any large clumps of cells and fibrous material. Throughout the process the tissue and solutions were kept on ice. On microscopic examination the filtrate was seen to contain cellular fragments, nuclei, whole cells and some small clumps of cells (probably epithelial sheets).

(b) Microsomal enzyme.

For kinetic experiments a more purified enzyme

preparation was employed. Testosterone 5 α -reductase is a membrane bound enzyme, located in the outer nuclear membrane and in the endoplasmic reticulum. A microsome fraction, consisting largely of endoplasmic reticulum fragments, was found to give a suitably enriched enzyme preparation.

Fresh tissue was suspended in TKM + sucrose buffer in a ratio of 1g tissue/10ml buffer and homogenised and filtered as above. The suspension was then centrifuged at 15,000g for 10min. to precipitate whole cells, nuclei and mitochondria. The low spin supernatant was then recentrifuged for a further 60min. at 105,000g which caused most of the membrane fragments to sediment. The supernatant was decanted and the pellet resuspended in TKM+Sucrose buffer. The most suitable volume for resuspension was found to be 5n ml where n is the number of grams of tissue homogenised.

The suspensions were made as homogeneous as possible with 5 strokes of a Thomas homogeniser. Again all solutions were kept on ice and refrigerated centrifuges were employed.

(ii) Incubation.

The basic incubation medium contained 0.25mM NADPH and 4.3nM [1,2-³H]-testosterone (S.A. 12.5Ci/mole) in Tris/HCl or TKM buffer (depending on the enzyme preparation used). All solutions were kept on ice

and the reaction started by adding 0.5ml enzyme solution, and plunging the tubes into a shaking water bath at 37°C. Unless otherwise stated, final incubation volumes were 1.6ml.

The reaction was stopped by the addition of 2ml ethanol, followed by rapid mixing. For monitoring recoveries, carrier testosterone (100µg), DHT (100µg) and 5α-androstane-3α,17β-diol (100µg) were added together with 2000cpm each of [4-¹⁴C]-testosterone and [4-¹⁴C]-DHT. Lipids were extracted with chloroform (8ml), evaporated to dryness under air, and acetylated. Separation of the acetylated steroids was carried out on alumina TLC plates using solvent system 1. Three spots were visualised, corresponding to testosterone acetate, DHT acetate and 5α-androstane-3α,17β-diol diacetate. These were scraped from the plates, eluted in diethyl ether, evaporated to dryness and radioassayed in a liquid scintillation spectrophotometer using a non-aqueous scintillator. Half of the 5α-androstane-3α,17β-diol diacetate was retained for monitoring recovery by gas liquid chromatography.

The metabolism to 5α-androstane-3α,17β-diol was always checked in control incubations, but fractions were only fully worked up for the rest of any particular experiment if the incorporation of radioactivity was more than 5% of the total product.

(iii) Calculation and expression of results.

Correction for procedural losses was necessary for each individual incubation. This was done for testosterone and DHT by the addition of a constant amount of [^{14}C]-steroid after the incubation, and counting the separated steroid acetates in the liquid scintillation spectrophotometer with window settings such that the two isotopes could be distinguished. Standard ^{14}C was also counted to allow correction for spillover between channels. The equation used for the calculation was corrected cpm in sample:

$$\left[\frac{\text{cpm Rch A}}{\text{cpm Gch A}} - \frac{\text{cpm Rch Std}}{\text{cpm Gch Std}} \right] \times \text{cpm Gch Std}$$

where Rch = channel measuring ^3H
 Gch = " " ^{14}C
 A = sample
 Std = ^{14}C standard

Background radiation was subtracted from each value used in the calculation.

The 5α -androstane- $3\alpha,17\beta$ -diol diacetate recoveries were measured by gas liquid chromatography.

After correction for recoveries the corrected cpm of the products were summed and the results expressed in terms of constant time and protein concentration i.e. pmoles substrate reduced/h/ng protein. This allowed comparison of activity between different enzyme preparations.

I. Assay of 17 β -Hydroxysteroid Dehydrogenase.
(E.C. 1.1.1.51.)

(i) Enzyme preparation.

Lyophilised powder of Pseudomonas Testosteroni (0.25g) was mixed with 3.5ml of standard phosphate buffer, placed in an ice bath, and sonicated at 20 kHz for 15 mins. using an MSE sonicator. The resulting preparation was centrifuged at 20,000g for 60min. at 4 $^{\circ}$ C. The supernatant was mixed with glycerol to a final concentration of 50% and stored at -20 $^{\circ}$ C. This preparation is stable for several weeks and was diluted in pyrophosphate buffer immediately before use. Aliquots (0.5ml) of a 1/4,000 dilution were found to be most suitable.

(ii) Incubation.

The incubation (final volume 2ml) was carried out in pyrophosphate buffer containing 50ng testosterone, 0.5mM NAD and 0.5ml enzyme solution.

The testosterone contained either [17 α - 3 H]-testosterone or [1,2- 3 H]-testosterone and [4- 14 C]-testosterone in a 3 H / 14 C ratio of approximately 10/1, and non-radioactive testosterone to make up the required concentration.

The assay tubes were preincubated at 37 $^{\circ}$ C and the reaction started by adding the enzyme solution. The reaction was stopped after the appropriate time by the addition of 5ml ethyl acetate followed by 15sec. vortexing. The aqueous phase was removed and

after the addition of carrier steroid the remaining liquid was evaporated to dryness under air. The steroids were separated on silica TLC plates in solvent system-2. Bands corresponding to testosterone and 5 α -androstane-3,17-dione were eluted with diethyl ether, evaporated to dryness and radioassayed in a liquid scintillation spectrophotometer using non-aqueous scintillator.

J. Superfusion Techniques.

(i) Tissue preparation.

Fresh prostatic tissue was cut into 2x1x1 cm blocks and sliced by hand with a Stadie-Riggs microtome. The mean thickness of the slices was calculated to be 0.54mm, by measurement of area and weight of slices taken at random. The slices were transferred from the microtome to ice-cold petrie dishes lined with saline moistened filter paper. Blocks of tissue to be sliced were chosen at random, but slices were transferred to the petrie dishes in a non-random fashion so that each superfusion channel could contain tissue of as similar composition as possible.

The tissue slices were weighed and divided into 0.5g - 1g aliquots depending on the type of experiment being performed. In each separate experiment all the chambers contained equal amounts of tissue.

Tissue aliquots were washed three times in 2-3ml saline to remove small debris and excess prostatic secretion, prior to transfer to the superfusion chamber.

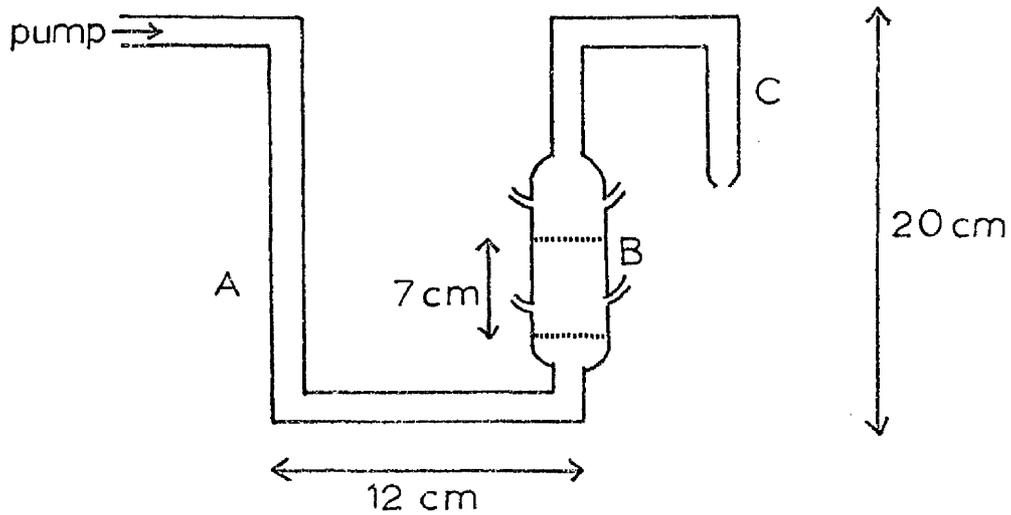
(ii) Superfusion apparatus.

The apparatus employed is shown in Fig.II-1. An all glass system was used, the various pieces having ground glass joints which are held together by sprung metal clips. The tissue chamber is formed by the space between two sintered glass filters. In experiments where tissue had to be removed during the perfusion an open chamber was used. The chambers were submerged in a water bath at 37°C, and connected via a 100ml glass syringe to a pump (Multisyringe Attachment, F135, C.F. Palmer Ltd., London) which was set to deliver the medium through the chamber at a constant rate of 25ml/h.

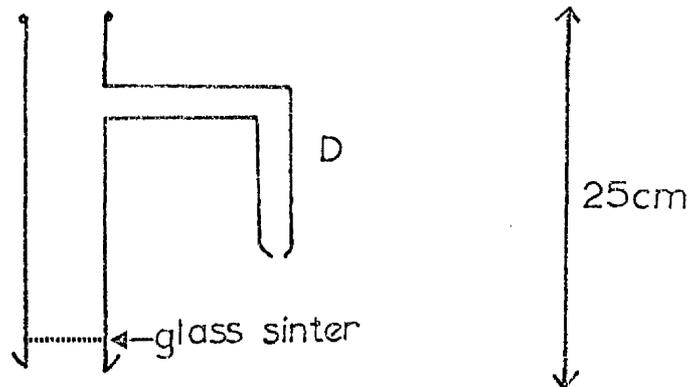
(iii) Superfusion - general details.

The perfusion medium was Krebs-Ringer bicarbonate buffer, prepared freshly before use. Purified radioactive and non-radioactive steroids were made up in ethanol and added to the buffer such that the final concentration of alcohol was less than 1%.

In order to prevent further metabolism of steroid by any enzymes which may have been released from the tissue slices, perfusates were collected in tubes standing in crushed ice.

Fig.II - 1.The Superfusion Apparatus.

An all glass superfusion apparatus is employed. The three sections A, B and C are held together with spring clips. Tissue slices are placed between the glass sinters.



Section D replaces Sections B and C when tissue is to be removed during the superfusion.

Medium from the syringes was also kept for analysis.

The tissue was removed from the apparatus either at intervals during, or at the end of the perfusion, rinsed in ice-cold Krebs-Ringer bicarbonate buffer (2 x 3ml) and for metabolic experiments suspended in acetone.

All samples were kept at -20°C until processed.

(iv) Analysis of fractions following superfusion with T/DHT.

The perfusates and medium were thawed at room temperature and shaken well to disperse any precipitated salts, before a measured aliquot (5ml) was removed and extracted with ethyl acetate (10ml) which contained [^{14}C]-testosterone and [^{14}C]-DHT to allow for the correction of procedural losses.

Tissue slices were removed from the acetone, minced, suspended in glass distilled water and homogenised with a fine bladed Ultra-turax homogeniser. The homogenate and acetone were combined and extracted together as described for the perfusates.

The aqueous phase of all the samples was removed, the organic phase washed twice with distilled water (5ml) and evaporated under N_2 at less than 60°C . The residue was dissolved in $250\mu\text{l}$ acetone and spotted onto a paper chromatogram,

which was developed and eluted as described previously. Cold carrier steroids were added at this stage (100 μ g testosterone and 250 μ g DHT) to assist visualisation during subsequent stages.

The DHT fraction was divided into two parts and all tubes evaporated to dryness under air, before acetylation of the testosterone and one DHT fraction, and oxidation of the second DHT fraction. The acetylation products were chromatographed on Alumina TLC plates in solvent system 1, the testosterone acetate, or DHT acetate bands visualised, eluted and counted in non-aqueous scintillator until the SEM was 0.5%. The oxidation products were chromatographed on Silica TLC plates in solvent system 2.

The spots corresponding to 5 α -androstane-3,17-dione were visualised, eluted and counted as for the acetylated fractions. A flow diagram for the method is given in Fig.II - 2.

K. Miscellaneous Methods.

(i) Colourimetric estimation of heparin.

The assay employed was based on that of the Bitter and Muir (1962) modification of the method of Dische (1947) and measures the reaction of carbazole with uronic acid residues.

Reagents.

(a) Sulphuric acid reagent was made from 0.025M

disodium tetraborate.10H₂O dissolved in sulphuric acid of specific gravity 1.84.

(b) Carbazole reagent consisted of 0.125% carbazole in absolute ethanol or methanol and was stable for 12 weeks in the dark at 4°C.

(c) Standards of glucuronolactone and heparin in the range 4-100µg/ml were prepared by dilution in distilled water saturated with benzoic acid. Wherever possible unknown samples were also made up in benzoic acid saturated water.

Method.

Sulphuric acid reagent (3ml) was placed in stoppered test tubes, and cooled to 4°C. Sample or standard (0.5ml) was carefully layered on top of the acid, the tubes stoppered and shaken gently with constant cooling, to mix the two phases. At no time did the temperature of the mixture exceed room temperature. The tubes were heated for 10min. in a vigorously boiling water bath, and cooled again to room temperature. The carbazole reagent (0.1ml) was layered on top, the tubes shaken and heated in the boiling water bath for a further 15min. After cooling again to room temperature the optical density was read at 530nm in a Unicam SP600 spectrophotometer. The optical density of a sulphuric acid blank was consistently below 0.025.

(ii) Protein determination.

Protein concentrations over the range 50-500 μ g were measured by the method of Lowry et al., (1951).

Standard solutions were prepared as in distilled water as follows:-

- A - 2% Na₂CO₃
- B - 2% Na K tartarate
- C - 1% CuSO₄.5H₂O
- D - This was prepared daily from 100ml A + 1ml B + 1ml C.
- E - Folin & Ciocalteu's Phenol Reagent, diluted 1/1 to make it 1N with respect to acid.

Standard protein solutions and all samples were precipitated by standing for at least 30min. after the adding of 10% trichloroacetic acid (TCA) (1ml). The tubes were centrifuged at 1700g for 5min. and inverted to allow the TCA supernatant to drain out. The protein precipitate was dissolved in 1N NaOH (0.5ml) and after 10min. solution D (5ml) was added. After a further 10min. solution E was added and mixed immediately. The optical density at 625nm was read in a Unicam SP600 spectrophotometer after the colour had been allowed to develop for 20min.

CHAPTER III.

CHAPTER IIIHEPARIN AND RELATED COMPOUNDS
IN THE CONTROL OF
PROSTATIC TESTOSTERONE 5 α -REDUCTASE1. INTRODUCTION.

Heparin, a naturally occurring polyanionic mucopolysaccharide commonly used as an anticoagulant, has recently been reported to inhibit steroid 5 α -reductase enzymes in testis (Kizanowski & Troop, 1968), adrenal (Lakatos *et al.*, 1970), liver (Steinberger & Fisher, 1971) and rat prostate (Blaquier & Calandra, 1973). It seemed reasonable, therefore, to examine its role in the control of prostatic testosterone 5 α -reductase especially since heparin is often found associated with connective tissue, which is a major component of the human prostate gland (Schubert & Hammerman, 1968).

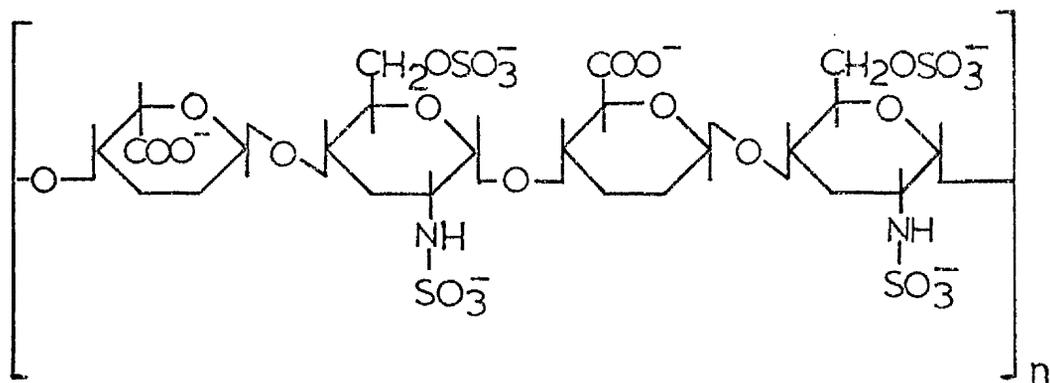
Many studies have been made of the chemical structure of heparin, with investigations of the glycosidic linkage, the nature of the uronic acid component, the sulphur content, the presence of additional groups and the extent of branching. These studies have been reviewed by Ehrlich &

Stivala (1973). Both 1→4 and 1→6 linkages are present with the 1→4 form predominating. The major uronic acid component is D-glucuronic acid, although L-iduronic acid has also been reported. As a result of degradation studies with Flavobacterium heparinium it was concluded that the heparin molecule is composed largely of repeating sequences of (1→4)-linked 4-O-(α-L-idopyranosyluronic acid 2-sulphate)-2-(deoxy-2-sulfamino-α-D-glucopyranosyl-6-sulphate)biose residues (Perlin et al., 1971), although Helting & Lindahl (1971) proposed a structure of repeating tetrasaccharide units each of which contained both glucuronic and iduronic residues (fig. III - 1a). More recently Silva & Dietrich (1975) using a similar enzyme degradation technique have suggested that trisulphated and disulphated disaccharides with the structure shown in fig. III - 1b are linked alternately in a proportion of 3:1. Heparin is polydispersed and molecular weights from 6,000 to 20,000 have been reported. It is usually considered to be a linear molecule although this has not been conclusively proven. Horner (1971) has isolated heparin with a molecular weight of 1.1×10^6 from rat skin and he suggested that this macromolecular heparin is a multichain form which must be depolymerised to become biologically active.

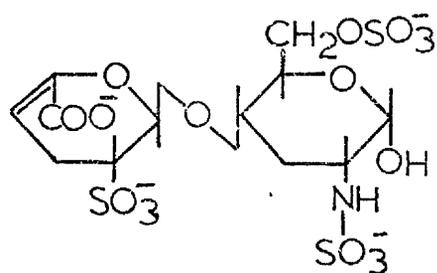
Heparin is distributed widely throughout the body

Fig.III - 1Suggested Forms for the Basic Structure of Heparin.

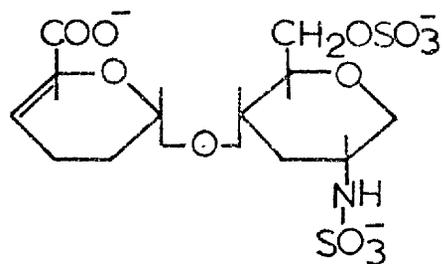
(a) Helting & Lindahl (1971)



(b) Silva & Dietrich (1975)



trisulphated disaccharide



disulphated disaccharide

and has been isolated from the skin of many animals. Although its concentration in blood is small it has been found in significant quantities in kidney and liver (Ehrlich & Stivala, 1973), whilst commercial heparin is prepared from pig intestinal mucosa. There appears to have been no attempt to assess the heparin content of human prostate tissue.

Heparin has been widely studied as an enzyme inhibitor and has been shown to inhibit β -glucuronidase, renin, hyaluronidase, alkaline phosphatase and collagenase (Ehrlich & Stivala, 1973). Whilst it has been suggested that it may be a non-specific inhibitor for some enzymes there is some evidence in certain systems that there is specificity in its action. For example, in rat adrenal, heparin and heparinoid substances reduce the production of aldosterone but do not diminish the synthesis of important glucocorticoids (Glaz & Sugar, 1964).

Recently there has been interest in heparin as an inhibitor of steroid 5α -reduction and it has been shown to be an effective inhibitor in rat liver (Kizanowski & Troop, 1968), adrenal (Lakatos et al., 1970), testis (Steinberger & Fisher, 1971) and prostate (Blaquier & Calandra, 1973). In rat liver homogenates the A ring reductions of androstenedione, progesterone, deoxycortisol and testosterone are inhibited (Krzanowski & Troop, 1968). Cortisone

reduction is also inhibited and may be reversed by the addition of the heparin antagonist protamine sulphate (Troop et al., 1966). In vivo, heparin has been shown to have no effect on the hepatic 5 α -reductase enzyme levels when administered over a 7½ hour period, although plasma from these treated rats could inhibit the enzyme activity when added to the assay mixture (Troop & Biggs, 1965). This is presumably due to heparin in the plasma. However when the NADPH generating system was omitted from the assay a small effect could be seen (Carter et al., 1968). Heparin has also been shown to have an anti-inflammatory effect in intact but not in adrenalectomised rats, and to potentiate the anti-inflammatory effect of cortisol in the adrenalectomised animals (Carter et al., 1968). These workers also showed that the intravascular half-life of [¹⁴C]-corticosterone was significantly prolonged after heparin administration, facts which indicate heparin may influence adrenal steroid metabolism in vivo. Several polysulphated polysaccharides other than heparin have been shown to inhibit cortisone 5 α -reduction by rat liver homogenates (Troop, 1969). This study suggests that the sulphate content of polysaccharides appeared to be important in determining their inhibitory activity. The degree of sulphation of the heparin molecule itself has been

shown to be important in determining its activity as a protein synthesis inhibitor (Waldman et al., 1974).

A well established role exists for heparin as an agent capable of affecting steroid A ring reduction. It was the intention of the work described in this chapter to assess the ability of heparin to inhibit rat and human prostatic testosterone 5 α -reductase in vitro and to extend the finding to investigate a possible role for heparin and related compounds in vivo. In the latter connection it was thought important to attempt an assessment of the heparin content of human hyperplastic glands.

2. RESULTS.

A. Initial Experiments.

The specific activity of prostatic testosterone 5 α -reductase varies considerably from species to species (Gloyna & Wilson, 1969). In particular its specific activity in human hyperplastic tissue is considerably greater than in rat tissue. Moreover, within a single species there is also marked fluctuation in the observed enzyme levels not only between glands, but also between different regions of the same gland. For these reasons it is often difficult to select the correct enzyme concentration to use in an individual incubation and the initial experiments described were designed to select homogenate dilutions for normal rat and hyperplastic human prostate tissue that would consistently yield testosterone conversions that could be measured accurately but were not excessive. Having arrived upon a suitable enzyme concentration other parameters of the assay were examined to ensure that it was fully optimised.

Using the assay procedure described in Chapter II, homogenates of fresh human hyperplastic prostate tissue were prepared over the range 0.5-5% prior to measuring the 5 α -reduction of 2ng of [1,2-³H]-testosterone. The results from two separate experiments are recorded in Table III - 1. It is

Table III - 1

The Effect of Enzyme Dilution on the Conversion of Testosterone to 5 α -Dihydrotestosterone using Fresh Human Prostatic Homogenate.

| Enzyme dilution (g tissue/ ml buffer) | Specific Activity (pmoles/hr/ mgprotein) | % conversion of 2ngTestosterone to DHT |
|---|--|--|
| <u>Prostate A</u> | | |
| 1/20 | 5.4 | 95.4 |
| 1/60 | 9.2 | 47.6 |
| 1/100 | 47.4 | 26.8 |
| 1/200 | 29.0 | 13.5 |
| <u>Prostate B</u> | | |
| 1/20 | 15.7 | 95.4 |
| 1/60 | 27.6 | 45.8 |
| 1/100 | 25.2 | 24.3 |
| 1/200 | 33.2 | 15.0 |

apparent that whilst the specific activities of the two preparations differed, the percentages of testosterone converted into DHT were similar and an homogenate of 1g tissue per 100ml buffer was selected as the most suitable. Similar experiments with rat tissue revealed that an homogenate of 1g tissue per 20ml of buffer was optimal. These concentrations were employed in all subsequent experiments although minor adjustments were often necessary when using frozen tissue to take into account the deterioration of testosterone 5 α -reductase activity on storage.

Time course experiments were performed to check that only the initial rate of reaction was being measured. It was shown that the production of DHT was almost linear for the initial 20 - 30 mins. the reaction rate slowing down thereafter.

Since the enzyme requires NADPH for its function it is important to ensure an adequate supply of this cofactor. Hence, incubations including NADPH in the range of 0-0.5mM were performed. Enzyme activity increased rapidly from 0 to 0.1mM NADPH, and at greater than 0.25mM NADPH no further increase in activity was obtained (Fig.III-2a) therefore in all subsequent incubations 0.25mM NADPH was used.

It has been reported that rat prostatic testosterone 5 α -reductase activity decreases with

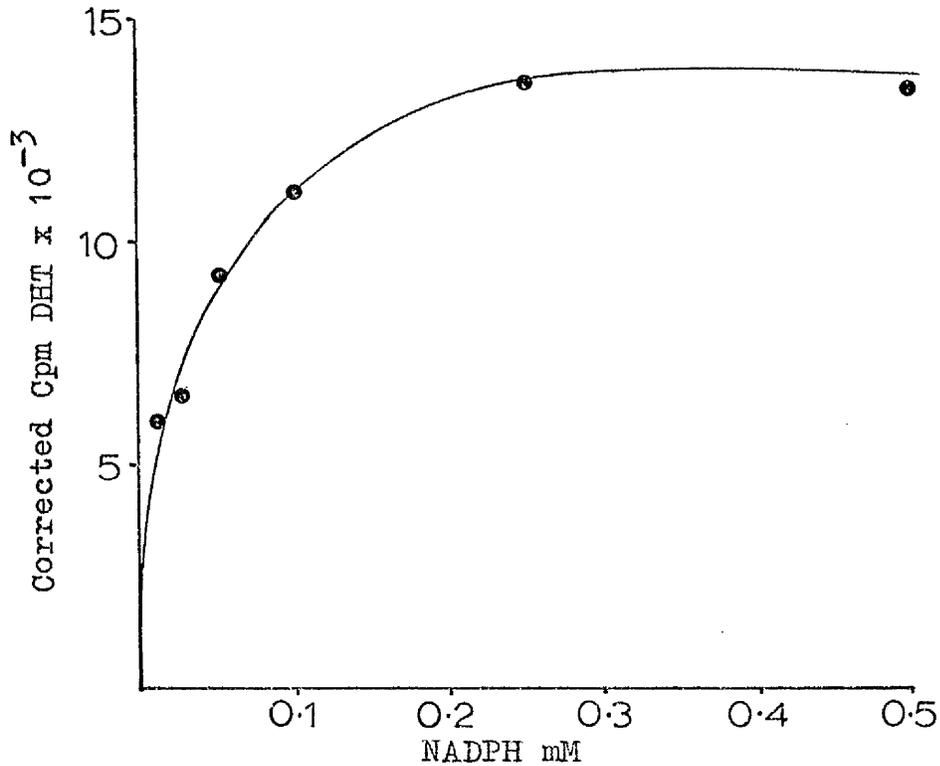
Fig. III-2.

An homogenate preparation of human prostate was incubated for 20 min at 37°C with a substrate concentration of 4.3nM. In (a) the NADPH concentration was varied from 0-0.5mM.

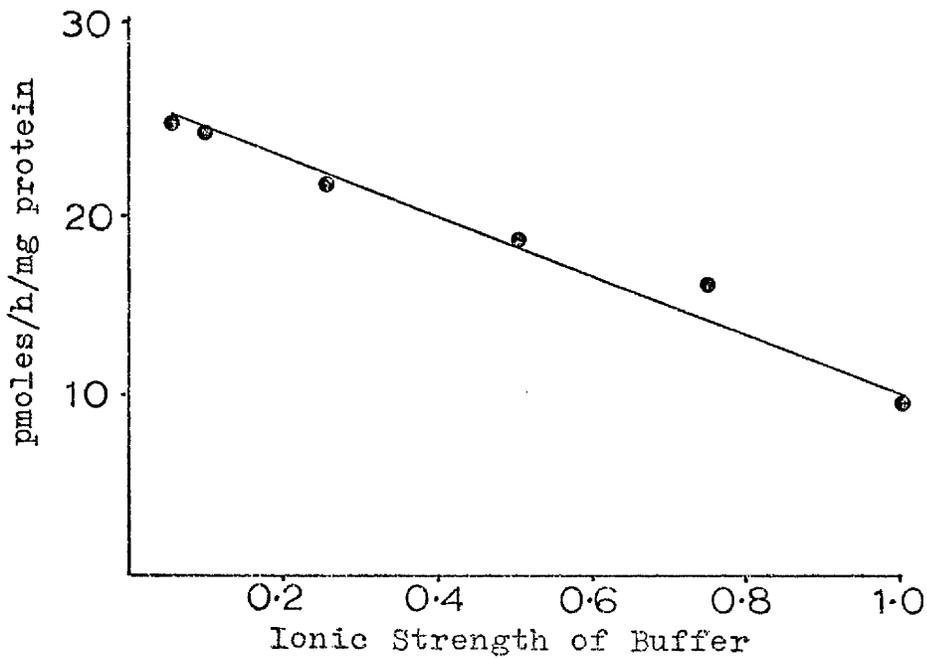
In (b) KCl was added to the buffer to increase the ionic strength.

Fig.III - 2.

- (a) The Effect of NADPH Concentration on Testosterone 5 α -Reductase Activity.



- (b) The Effect of Ionic Strength on Testosterone 5 α -Reductase Activity.

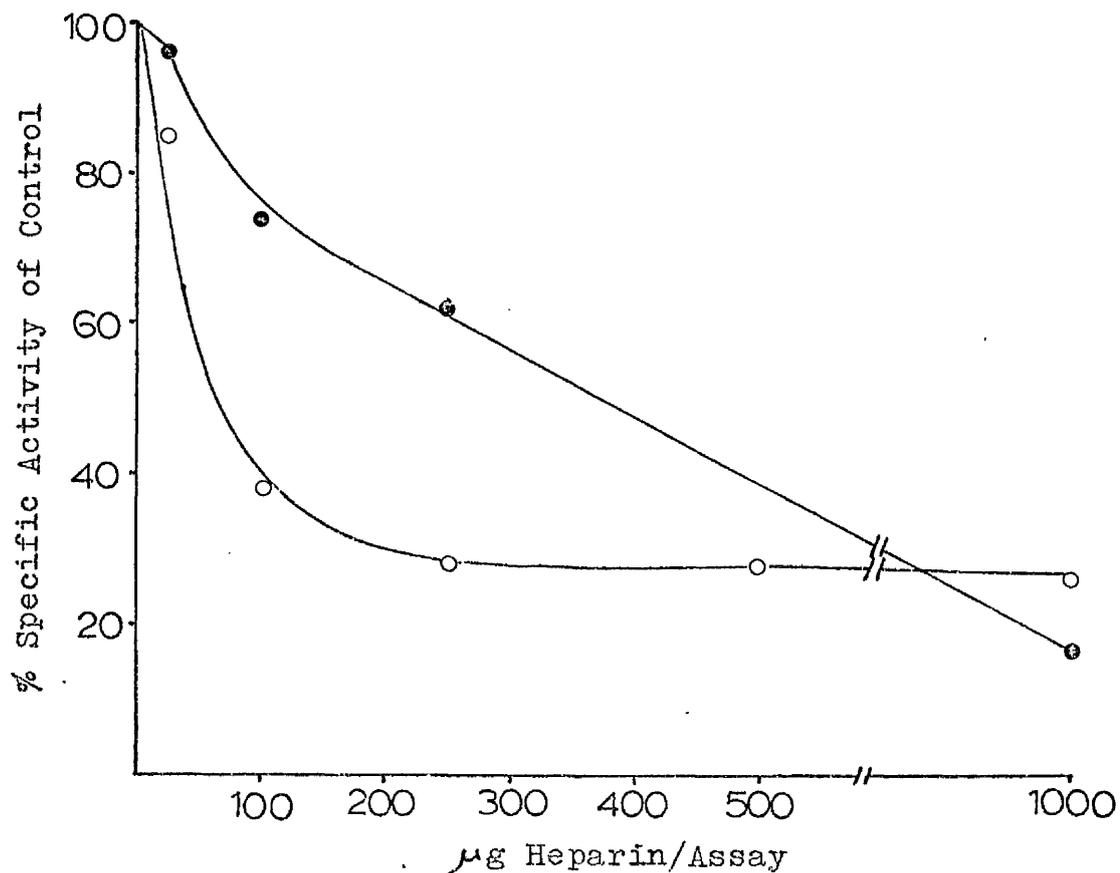


increasing ionic strength (Roy, 1971), hence it was important to establish whether or not the same was true of the human enzyme. Addition of KCl to a Tris/HCl buffer of ionic strength 0.05 caused a linear decrease in activity until at an ionic strength of 1 the activity had dropped by 50% (Fig.III-2b). It is clearly important therefore to keep the ionic strength of the buffer constant. For the experiments presented in this chapter, 0.05M Tris/HCl buffer, with an ionic strength of 0.05, was used.

The precision of the assay must be known in order that valid comparisons can be made between control and experimental incubations. The coefficient of variation of each of eleven experiments with between four and ten control tubes was determined and ranged from 3.8% to 11.0% with a mean of 7.5%.

B. The Effect of Heparin on the In Vitro
5 α -Reduction of Testosterone.

In order to examine the effect of exogenous heparin on the 5 α -reduction of testosterone by prostatic tissue, homogenates were incubated as described in Chapter II in the presence of increasing concentrations of added heparin. The results from two typical experiments, one for rat tissue and the other for hyperplastic human tissue are shown in Fig.III-3. The results are expressed in terms of

Fig.III - 3The Inhibitory Effect of Heparin on Rat and Human Prostatic Testosterone 5 α -Reductase.

● - Rat Prostate Homogenate (1g/20ml)

○ - Human Prostate Homogenate (1g/100ml)

Homogenates of rat or human prostate were incubated with [3 H]-testosterone as described in Methods (p 62) in the presence of heparin over the concentration range 0 - 1000 μ g/assay.

the percentage specific activity of the no heparin control tubes since this overcomes the variability between glands of the absolute specific activity of the testosterone 5 α -reductase. Each point on the graph is the mean of at least two values and for the controls each point is the mean of at least four determinations.

The preliminary experiments just described were performed with an NADPH generating system (0.065mM NADP, 0.65mM glucose-6-phosphate and 3 units glucose-6-phosphate dehydrogenase (SA 360 units/mg protein). Although Troop et al., (1966) claimed that heparin did not affect the rate of NADPH production by glucose-6-phosphate dehydrogenase it was thought advisable to check this point. Accordingly, the enzymatic activity of glucose-6-phosphate dehydrogenase was determined by a standard spectrophotometric method (Lohr & Waller, 1963) over a range of NADP concentrations in the absence of heparin and in the presence of increasing concentrations of polysaccharide. The results presented in Fig.III-4a demonstrate that at high concentrations of heparin inhibition of glucose-6-phosphate dehydrogenase did take place, the Lineweaver & Burk plot (Lineweaver & Burk, 1934) shows that the inhibition follows competitive kinetics. Similar results were obtained for the inhibition of 6-phosphogluconate dehydrogenase,

(a) Heparin concentrations.

- - 0
- - 0.8 mg/ml
- ▲ - 1.6 mg/ml
- △ - 4 mg/ml

(b) NADP concentrations.

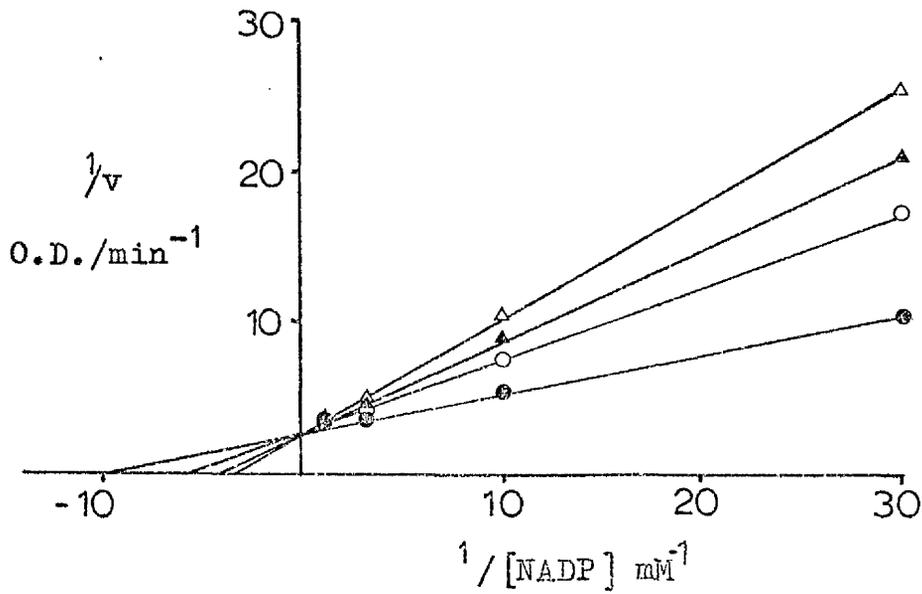
- - 0.033 mM
- - 0.100 mM
- ▲ - 0.330 mM
- △ - 1.000 mM

Glucose-6-phosphate dehydrogenase activity was measured by the method of Lohr & Waller (1963). Enzyme activity was determined over a range of NADP concentrations (0.033-1.0mM) and in the presence of varying heparin concentrations (0-4.0mg/ml).

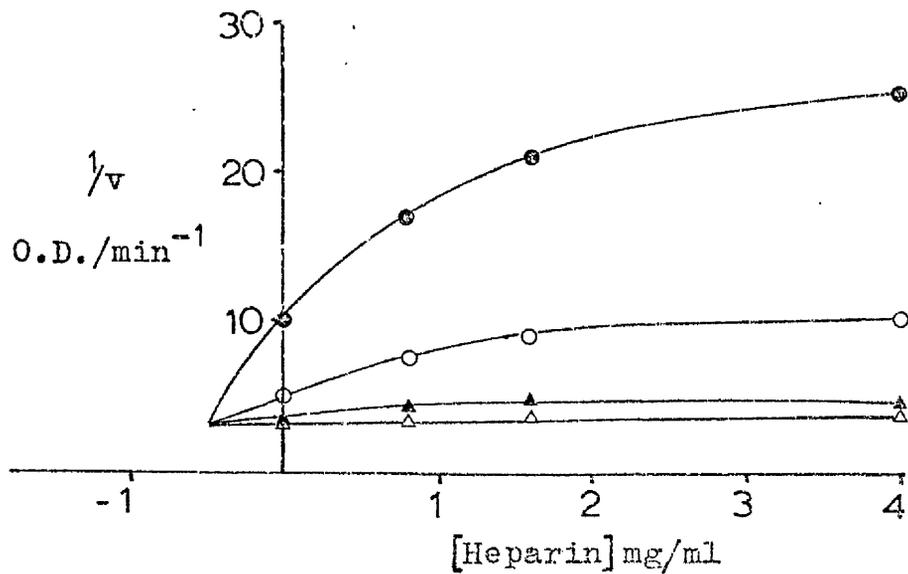
Fig.III - 4

The Effect of Heparin on Glucose-6-Phosphate-Dehydrogenase Activity.

(a) Lineweaver & Burke plot.



(b) $1/v$ vs $[I]$ plot.



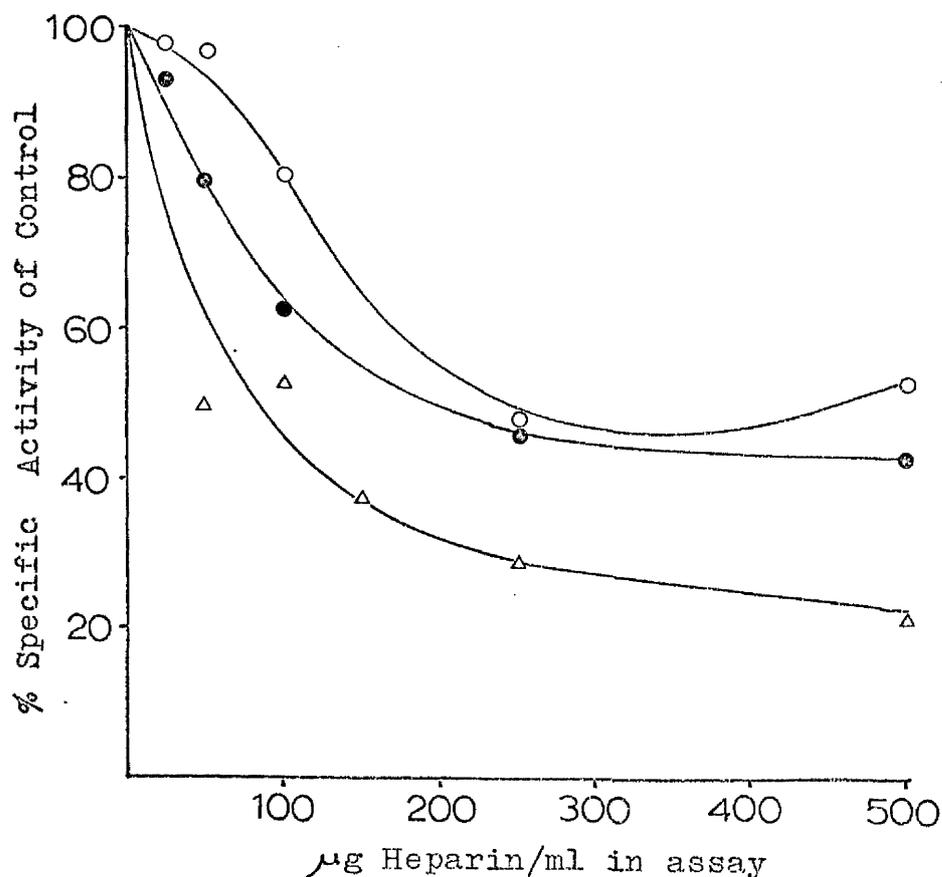
another enzyme commonly used in NADPH generating systems (Hohrst, 1963).

When the data is plotted as $1/V$ vs I the result is a hyperbolic curve (Fig.III-4b). When this shape of curve results it is an indication that an enzyme-inhibitor complex is not formed, but that the inhibitor changes the affinity of the enzyme for the substrate (Mahler & Cordes, 1966).

Whilst the heparin concentrations required to inhibit the production of NADPH were high, it was nevertheless considered undesirable to use a generating system for the studies of the action of heparin on testosterone 5α -reduction in prostatic tissue, and in all subsequent experiments NADPH itself was included in the incubation medium at a final concentration of 0.25mM, which has already been shown to be quite adequate under the assay conditions employed. The inclusion of exogenous NADPH in the incubation medium did not prevent the inhibitory effect of heparin on testosterone 5α -reductase either from normal rat or from hyperplastic human prostate tissue. Fig.III-5 shows the heparin dose response curves from three experiments with human tissue. The extent of heparin inhibition clearly varies from gland to gland and this emphasises the need for including a heparin inhibition curve in all experiments with

Fig. III - 5

Heparin Inhibition of Human Prostatic Testosterone
5 α -Reductase from Different Glands.



- 1g tissue/80ml buffer
- 1g tissue/80ml buffer
- △ 1g tissue/50ml buffer

The variability of heparin inhibition was examined using homogenate preparations from three different human glands. Testosterone 5 α -reductase activity was measured under standard conditions (p 62). In each case there was between 10 and 15% conversion of substrate in control incubations.

potential inhibitors to permit standardisation.

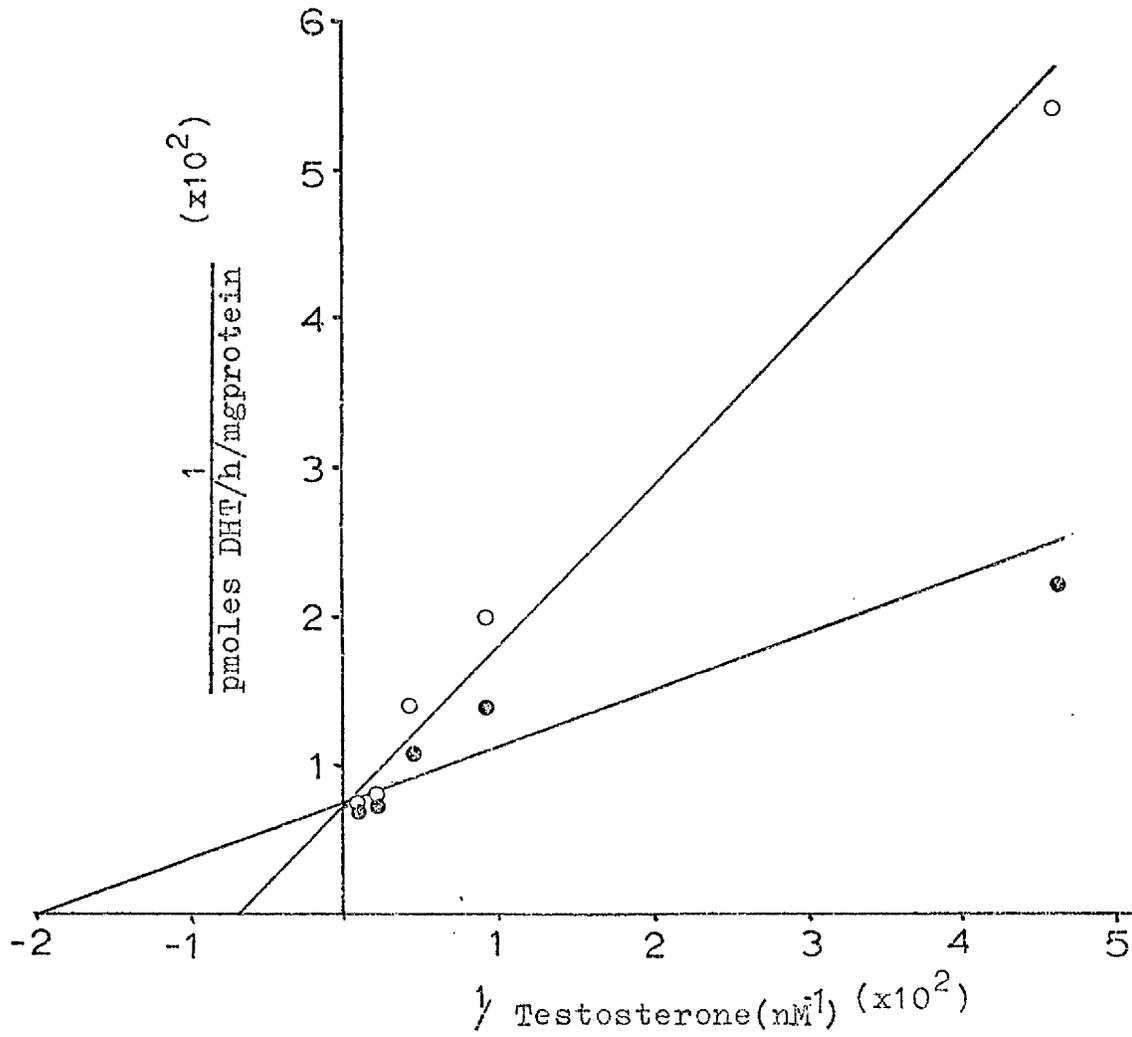
Having established that heparin is an effective in vitro inhibitor of testosterone 5 α -reductase an investigation was planned to determine the nature of the inhibition. Accordingly testosterone 5 α -reductase activity was determined using the standard assay system over a range of testosterone concentrations and in the presence and absence of added heparin. The results recorded in Fig.III-6 are from a human hyperplastic prostate gland and they demonstrate that heparin apparently exhibits competitive kinetics with respect to substrate concentration. It is worthy of note that the apparent Michaelis constant, $-K_m$, obtained for human prostatic testosterone 5 α -reductase from Fig.III-6 is $0.02 \times 10^{-6} M$, a figure that agrees well with that previously published (Wallace, 1975).

It was considered possible that heparin may also interfere with cofactor binding to the testosterone 5 α -reductase. Accordingly, this was tested, and in the case of the enzyme from the rat prostate heparin can be shown to exhibit competitive type kinetics with respect to the NADPH cofactor.

The experiments just described have demonstrated that heparin, a polyanionic substance, is capable of inhibiting prostatic testosterone 5 α -reductase. The same enzyme has previously been shown to be

Fig.III - 6

Lineweaver & Burk Plot showing Heparin Inhibition of Human Prostatic Testosterone 5 α -Reductase.



● - No Heparin

○ - 500 μ g Heparin/assay

inhibited by a number of divalent cations, including zinc (Wallace & Grant, 1975). Since the prostate is a rich source of zinc (Mann, 1964) it was considered of interest to examine the activity of testosterone 5 α -reductase in the presence of both heparin and zinc in order to determine whether or not the presence of zinc could reduce the effectiveness of heparin as an inhibitor. Additionally, the activity of the enzyme was assessed in the presence of both heparin and protamine sulphate since the latter substance is well established as an antagonist of the blood anti-coagulation properties of heparin (Ehrlich & Stivala, 1973).

The results of this combined study are shown in Fig.III-7a for the human enzyme and in Fig.III-7b for the rat enzyme. In both cases the heparin induced inhibition of the enzyme was partially reversed by the addition of an equal mass of protamine sulphate. This may have been due to precipitation of a heparin-protamine sulphate complex since assay tubes containing this mixture had a cloudy appearance. By contrast the addition of increasing concentrations of zinc to the incubation media failed to reverse the heparin inhibition, and in the human system, at least, the presence of both heparin and $5 \times 10^{-6} \text{M}$ zinc induced a slightly greater inhibition than observed from

Key(a) Human

- O = no additions
 H = 100 μ g Heparin
 H + P = 100 μ g Heparin + 100 μ g Protamine Sulphate
 H + Z₁ = 100 μ g Heparin + 5 x 10⁻⁸M ZnCl₂
 H + Z₂ = 100 μ g Heparin + 5 x 10⁻⁷M ZnCl₂
 H + Z₃ = 100 μ g Heparin + 5 x 10⁻⁶M ZnCl₂
 Z₂ = 5 x 10⁻⁷M ZnCl₂

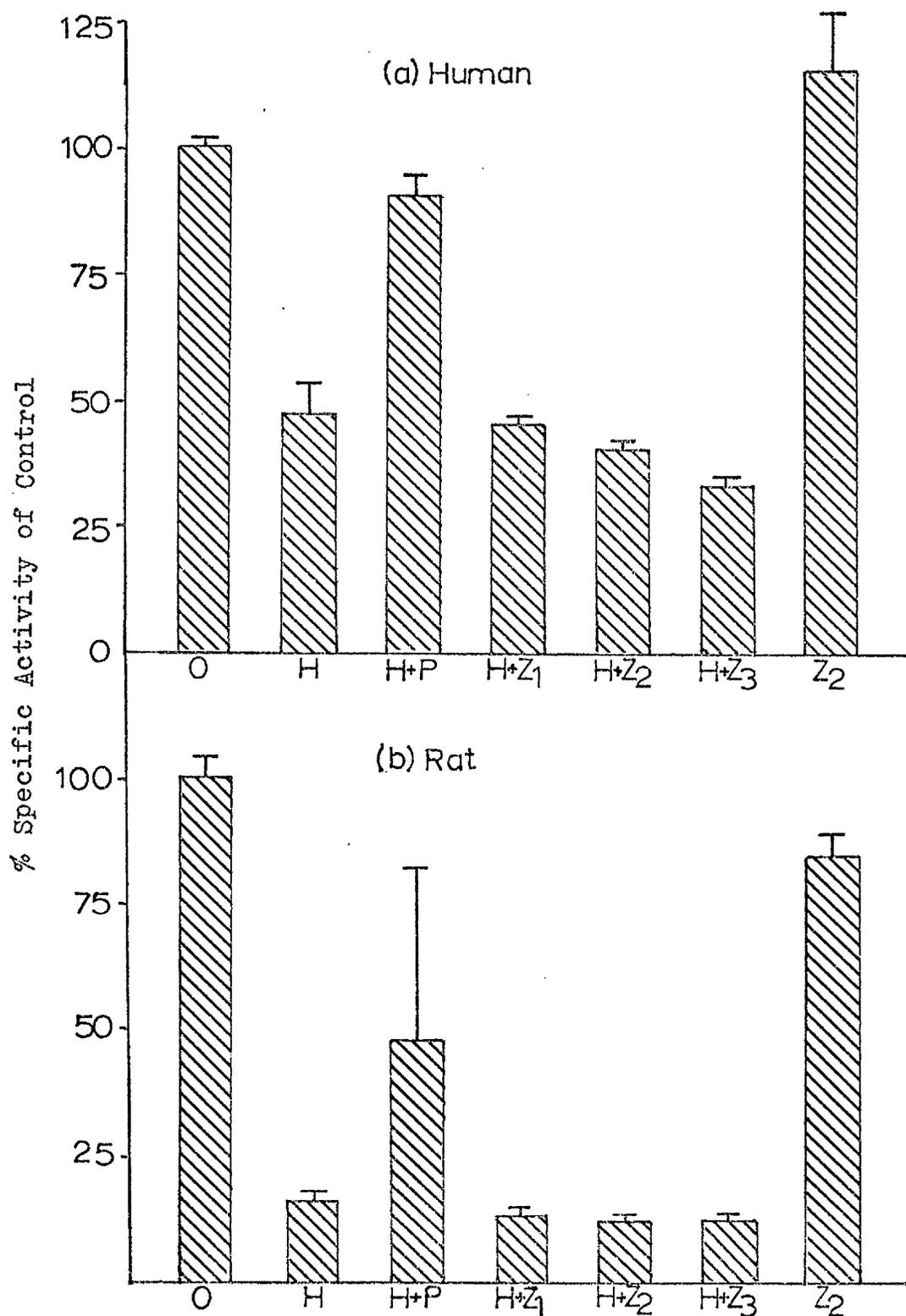
(b) Rat

- O = no additions
 H = 2mg Heparin
 H + P = 2mg Heparin + 2mg Protamine Sulphate
 H + Z₁ = 2mg Heparin + 5 x 10⁻⁸M ZnCl₂
 H + Z₂ = 2mg Heparin + 5 x 10⁻⁷M ZnCl₂
 H + Z₃ = 2mg Heparin + 5 x 10⁻⁶M ZnCl₂
 Z₂ = 5 x 10⁻⁷M ZnCl₂

The conversion of testosterone to DHT was measured under standard conditions with homogenate preparations. Heparin, zinc and protamine sulphate were included either singly or in the combinations indicated above.

Fig.III - 7

The Effect of Various Compounds on Prostatic
Testosterone 5 α -Reductase.



heparin alone. The concentrations of zinc used in this study were chosen very carefully since it has previously been observed that a concentration of $5 \times 10^{-7} \text{M}$ zinc is a mild stimulator of the human prostatic testosterone 5α -reductase (Grant et al., 1971) whilst at $5 \times 10^{-5} \text{M}$ the same metal is a potent inhibitor of the enzyme system (Wallace & Grant, 1975). In the present experiments it is worthy of note that $5 \times 10^{-7} \text{M}$ zinc alone had little effect on either the rat or human enzyme systems.

C. The Effects of Selected Monosaccharides, Polysaccharides and Polyanionic Substances on Prostatic Testosterone 5α -Reductase.

The consistent inhibition of prostatic testosterone 5α -reductase by heparin prompted the question of the specificity of the effect. Consequently it was decided to carry out a study of the effectiveness of other sulphated polysaccharides as inhibitors of testosterone 5α -reductase. Moreover a number of monosaccharides were screened for inhibitory activity as were a number of selected substances other than polysaccharides. It was hoped that the information gleaned from this investigation would provide evidence as to the structural requirements of an effective non-steroidal inhibitor of prostatic testosterone 5α -reductase.

The compounds tested were obtained in the purest forms available and, because of the uncertainty of the molecular weights of many of them, were added to incubations by weight rather than by molarity. A list of the main features of the polysaccharides studied is given in Table III -2.

It is apparent from Figs.III-8a & b that the monosaccharides glucose-6-SO₄ and D-glucuronic acid caused no inhibition of the testosterone 5 α -reductase of human hyperplastic prostate nor did polyglutamate. N-acetyl glucosamine, chondroitin sulphate and the synthetic compound Ro1-8307 had only very small inhibitory effects. The other polysaccharides tested all showed considerable inhibition of the enzyme and on a strictly mass basis the relative potencies of the compounds were as follows:-

Ro2-7528 > Ro2-7059 > Ro2-3053 >

Hyaluronic acid > Heparin

The results displayed in Figs.III-8a & b were obtained from a series of experiments and whilst heparin was included in each experiment as an internal standard it was considered important to confirm the ranking of inhibitory ability in a single incubation. Therefore, all the polysaccharides which had shown any ability to inhibit human hyperplastic prostatic testosterone 5 α -reductase were reassessed at a single concentration (500 μ g/tube). The results shown in

Table III - 2

Composition of Polymeric Compounds tested for Inhibition of Testosterone 5 α -Reductase.

| <u>Polysaccharide</u> | <u>Repeating Unit</u> | <u>Linkages</u> | <u>Sulphur Content</u> | <u>Molecular Weight</u> |
|-----------------------|--|--|------------------------|-------------------------|
| Chondroitin Sulphate | D-glucuronic acid; N-acetyl-D-glucosamine 4 or 6 sulphate | β , 1 \rightarrow 3 β , 1 \rightarrow 4 | 6.3% | 15,000 |
| Heparin | 4-O-(α -L-idopyranosyluronic acid-2-sulphate)-2-(deoxy-2-sulphamino- α -D-glucuranosyl-6-sulphate)biase | α , 1 \rightarrow 4 | 10-14% | 12,000 - 20,000 |
| Hyaluronic Acid | D-glucuronic acid; N-acetyl glucosamine | β , 1 \rightarrow 3 β , 1 \rightarrow 4 | <0.05% | 200,000 - 500,000 |
| Ro1 - 8307 | N-formylchitosan polysulphuric acid | - | 14-16% | 5,000 |
| Ro2 - 3053 | Sulphated galachironic acid methyl ester methyl glycoside | - | 15.7% | 4,000 - 5,000 |
| Ro2 - 7059 | Sulphated cycloheptaamylose | - | 18% | 3,000 |
| Ro2 - 7528 | Sulphated cyclohexaamylose | - | 18% | 15,000 |
| Polyglutamate | L-glutamic acid | - | - | 15,000 |

Key.

- (a)
- Heparin
 - Hyaluronic Acid
 - ▲ N-Acetyl Glucosamine
 - △ Glucuronic Acid
 - Glucose-6-Sulphate
 - Polyglutamate

- (b)
- Ro2 - 7528
 - Ro1 - 8307
 - ▲ Ro2 - 7509
 - △ Ro2 - 3053
 - Chondroitin Sulphate

Homogenate enzyme preparations were incubated under standard conditions with the various test substances listed above in the concentration range 0 - 500 μ g/assay.

Fig.III - 8

The Effect of Selected Saccharides and Polyanions
on Human Prostatic Testosterone 5 α -Reductase.

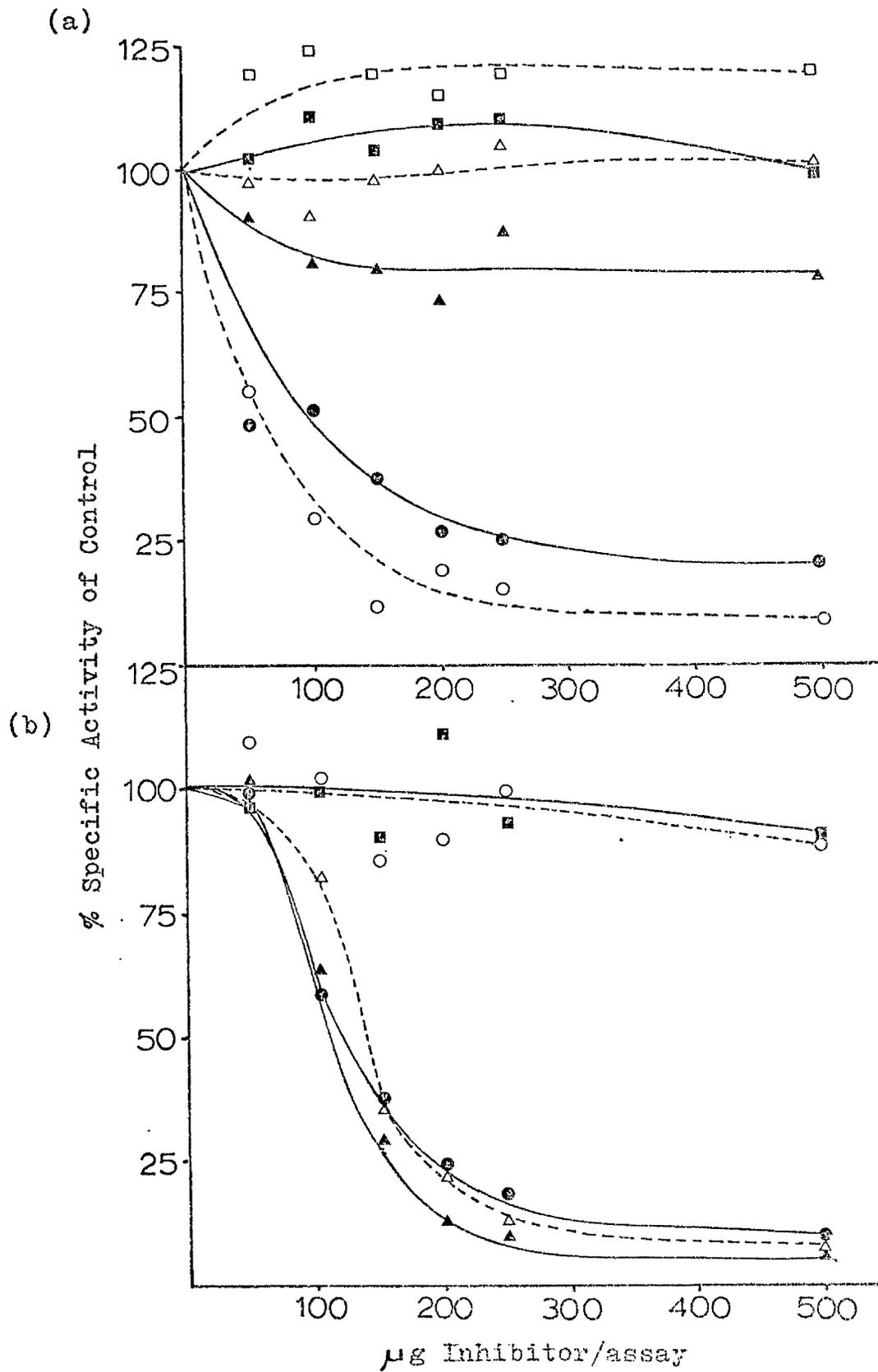


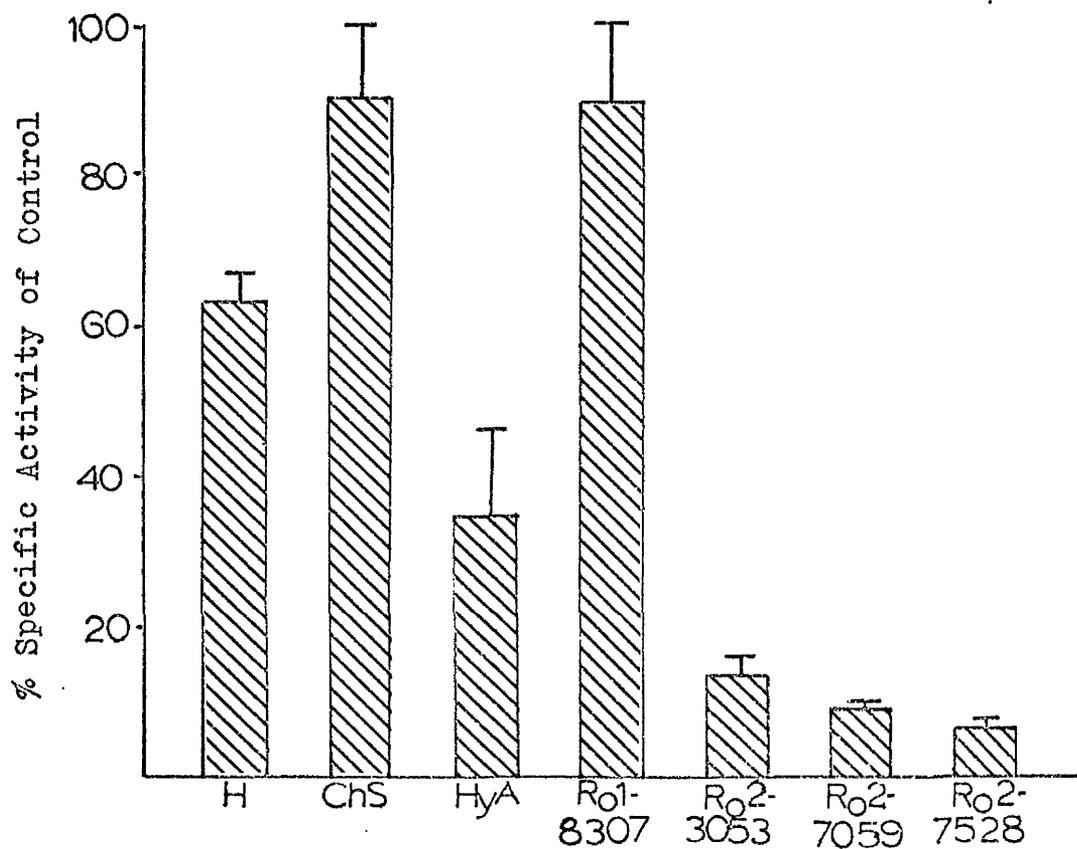
Fig.III-9 demonstrate that the ranking previously obtained could be confirmed.

When similar experiments were performed with these compounds using rat prostatic 5α -reductase, the same trends were seen as with the human enzyme (Beastall, G.H., unpublished data).

D. Prostatic Testosterone 5α -Reductase Following the In Vivo Administration of Heparin to Rats.

In order to assess whether heparin may have a physiological role in the control of prostatic testosterone 5α -reductase it is important to ascertain whether the molecule is capable of entering the gland from the circulation. Whilst it may seem unlikely that a molecule as large as heparin could be taken up by prostatic tissue, Gláz & Sugar (1964) demonstrated that the in vivo administration of heparin and heparinoid substances decreased the production of aldosterone by the adrenals, and Carter et al., (1968) observed a reduction in adrenal 5α -reductase activity following a similar administration regime. The experiment described below was adapted from that of the latter authors.

Male adult Sprague Dawley rats weighing 300 - 400g were divided into three groups. The first group (S) were given intraperitoneal injections of saline at three 12h intervals and were sacrificed by

Fig.III - 9Polysaccharide Inhibition of Human Prostatic
Testosterone 5 α -Reductase.

Key:

- H - Heparin
- ChS - Chondroitin Sulphate
- HyA - Hyaluronic Acid

The standard testosterone 5 α -reductase assay conditions were employed to test the inhibitory effect of selected polysaccharides at a concentration of 500 μ g/assay.

cervical dislocation one hour after the last injection. The second group (L) and the third group (H) were given intraperitoneal injections of 2500U and 12500U of heparin respectively, according to the same timetable. Not all the animals survived this injection schedule, one of the low dose group and four of the high dose group dying prior to completion of the experiment from internal haemorrhage, presumably resulting from the anti-coagulant properties of the administered heparin.

The ventral prostates of all the surviving rats were removed immediately following sacrifice and kept on ice. They were dissected free of membranes, weighed, minced into 3mm cubes and washed with ice-cold saline. Each mince was transferred to a conical flask containing 5ml of Krebs Ringer Bicarbonate buffer pH 7.4 to which had been added 50ng (10^5 cpm) of $[1,2-^3\text{H}]$ -testosterone. The flasks were incubated at 37°C for 30min. in a shaking water-bath. The reaction was terminated by the addition of ethanol (5ml), the lipids were extracted and the activity of the testosterone 5α -reductase determined in the usual manner and expressed as pmoles testosterone converted per hour per prostate.

The results are expressed in Table III - 3, and it is apparent that the control group S exhibited a mean enzymic activity equivalent to a 40% conversion

Table III - 3.

Rat Prostatic Testosterone 5 α -Reductase Activity
Following the In Vivo Administration of Heparin.

| Animal Group | N | Mean Prostate weight(g) \pm SD | Testosterone 5 α -Reductase (pmol/h) \pm SD |
|---------------|---|----------------------------------|--|
| Control (S) | 8 | 0.59 \pm 0.11 | 144 \pm 16.3 |
| Low Dose (L) | 7 | 0.43 \pm 0.07 * | 139 \pm 22 |
| High Dose (H) | 4 | 0.45 \pm 0.04 † | 134 \pm 14 |

Control - 0.5ml Saline per injection.

Low Dose - 2500 U Heparin⁺ in 0.5ml saline per injection.

High Dose - 12500 U Heparin in 0.5ml saline per injection.

* p < 0.005

† p < 0.025

p refers to difference between control and appropriate test as determined by Students t test.

+ USP units

of the added substrate. The testosterone 5α -reductase levels observed in the two test groups I and H were not significantly different from those of the control group and it is apparent that under the conditions of this experiment it was not possible to demonstrate the uptake of heparin from the circulation by prostatic tissue.

It is of note that the mean prostatic weight of the control group S was significantly higher than for the two test groups, ($p < 0.005$; $p < 0.025$ respectively) a fact that was obvious from a brief examination of the glands at the time of their removal, the prostates of heparinised animals having a dehydrated appearance. Nevertheless this reduction of prostate weight was not accompanied by a reduction in testosterone 5α -reductase activity.

E. Preliminary Investigation of the Endogenous Heparin-like Content of Benign Hyperplastic Human Prostate.

The previous section suggested that heparin is unlikely to enter the rat prostate gland from the blood stream. It is reasonable, therefore, to assume that compounds related to heparin would also be unable to gain entry into the tissue and that the human prostate is likely to present the same barrier as the rat gland. Under these

circumstances it must be postulated that any in vivo role for heparin or related compounds in the control of prostatic testosterone 5 α -reductase would arise as a result of synthesis of the inhibitor within the gland itself. Since there appears to be no reliable report of the heparin content of prostate tissue a preliminary investigation was initiated and a tissue extract obtained, which was tested both for heparin content and for its ability to inhibit testosterone 5 α -reductase.

Heparin was quantitated by the method of Bitter & Muir (1962) which measures the reaction of carbazole with uronic acid residues. However, this method could not be applied to whole tissue preparations since certain components present produced charring which interfered in the colourimetric determination. Experimentation revealed that neither DNA, RNA or protein were responsible for the charring but both sucrose and glucose at concentrations of 100 μ g per assay tube produced gross discolouration.

Most of the procedures published on the extraction of heparin from animal tissues are based on the work of Charles & Scott (1933, a & b) which was developed for preparative rather than analytical purposes, using large quantities (Kg) of tissue, relatively rich in heparin. However,

methods for the extraction of heparin from smaller quantities of tissue have been reported, and two of these were investigated in turn. In order to assess the efficiency of each extraction procedure a known amount of [^{35}S]-heparin was added immediately following homogenisation.

The first method was adapted from that of Freeman et al., (1957). A sample of tissue (5g) was homogenised with 5ml cold water, and immediately poured into 5 volumes of acetone to remove lipids. After standing for a few minutes the suspension was centrifuged and the supernatant discarded. The sediment was suspended in 10ml of 0.5M NH_4Cl buffer, pH8.5 and heated in a boiling waterbath for 15min. The suspension was transferred to dialysis tubing together with one millilitre of a 50% glycerol solution containing 100mg of purified trypsin. The mixture was dialysed against 0.5 litres of the 0.5M NH_4Cl buffer for 48h at 37° . A second 1ml aliquot of the trypsin solution was added, the buffer reservoir renewed and dialysis continued for a further 48hr. Finally the contents of the dialysis sac were dialysed against running tap water overnight. The contents of the sac were transferred to a 50ml centrifuge tube, NaCl was added to a concentration of 1% and the solution boiled for 15min. in order to inactivate and coagulate the trypsin. The mixture

was cooled to room temperature, centrifuged at 1000g for 5min. and the supernatant decanted into 5 volumes of acetone. After standing for 30min. the mixture was recentrifuged at 1000g for 5min. and the supernatant discarded. The sediment was dissolved in a small volume of 1% NaCl and 5 vol. of methanol were added. After standing for a further 30min. the solution was centrifuged at 1000g for 5min., the supernatant discarded, the tubes inverted and drained for several minutes, and finally dried in a vacuum oven. The residue was dissolved in 2ml distilled water. Approximately 50% of the added [^{35}S]-heparin was recovered and 3mg of heparin-like material was extracted from 5g of tissue as estimated by the colourimetric assay described in Chapter II. However the extract still contained a considerable amount of protein (Table III - 4).

Recoveries of [^{35}S]-heparin from standard protein solutions of bovine albumin (BSA) were found to be low (< 1%), much of the heparin being lost with the protein removed in the earlier stages of the purification, and the acetone precipitation step also caused large losses.

An attempt was made to improve the method by the use of 10% trichloroacetic acid (TCA) to precipitate the protein, leaving the heparin in solution. TCA could then be removed from the

Table III - 4

Composition of Final Heparin Extracts of Human
Hyperplastic Prostate Tissue.

| Method of Extraction | Amount of Tissue Extracted | Protein Content | Estimated Heparin content | |
|--------------------------------------|----------------------------|-----------------|---------------------------|-----------|
| | | | Carbazole | Reductase |
| Freeman <u>et al.</u> , (1957) | 5g | 9.6mg | 2.5mg | 0.32mg |
| Horner (1971) | 20g | 0.6mg | 2mg | 2mg |

supernatant by extracting with diethyl ether. This process did not affect the potency of heparin in the 5 α -reductase assay but it also did not improve the recovery of heparin from BSA or increase the yield from prostatic extracts.

The second approach to the problem used the method of Horner (1971) which is based on the precipitation of heparin from a protein digest with cetyl pyridinium chloride (CPC).

Human prostatic tissue (20g) was chopped into 0.5cm cubes and exhaustively extracted firstly with two separate 250ml volumes of acetone then 1 litre of petroleum spirit (bpt 40-60 $^{\circ}$ C) using a soxhlet apparatus. The product was first air dried, then vacuum dried, yielding an average of 2.5g of dried defatted tissue. This was suspended in 50ml of 0.1MTris/HCl+0.01M CaCl_2 , pH8.0 containing toluene 0.1ml. Pronase (20mg) was added and the mixture incubated at 40 $^{\circ}$ for 48h with intermittent mixing. After digestion NaCl was added to a concentration of 1.0M, the gross solids were removed by vacuum filtration and washed with 1.0M NaCl at 40 $^{\circ}$ C, prior to refiltration. The two filtrates were combined for treatment with cetyl pyridinium chloride (CPC).

The slightly cloudy filtrate was diluted with water to a NaCl concentration of 0.80M and solid Na_2SO_4 was added to a concentration of 0.05M.

The solution was warmed to 35° and CPC added in a ratio of 1g/100ml filtrate. One gram of Hyflo Supercel was stirred in before filtering through a Millipore filter (15ml with Whatman G.F./C 25mm filter discs). The resulting precipitate was extracted with four 5ml portions of warm (40°C) 2.0M NaCl. Each extract was allowed to stand for 10min. then recovered by centrifuging for 10min. at 200g at room temperature. The combined extracts were mixed with 2 volumes of ethanol, 1 volume H₂O then 2 volumes of ethanol. After standing overnight at 4°, the precipitate was centrifuged for 30min. at 500g at room temperature, dissolved in 2.0M NaCl and precipitated again with ethanol as above. The precipitate was washed with ethanol and vacuum dried. Using Horner's method, 75% of added [³⁵S]-heparin was recovered from a standard protein solution although the recovery from prostatic homogenates was lower at 35%. A yield of 2mg of heparin-like material was obtained from 20g of human hyperplastic prostate as estimated by colourimetry, and in contrast to the previous method the extract was essentially free from contaminating protein (Table III - 4).

The extracts obtained by both methods were tested in the 5 α -reductase assay and were shown to cause inhibition when added to the assay tubes (Table III - 4). The correlation between this

estimate of heparin action and the colourimetric estimate was especially good with the material extracted by Horner's method.

3. DISCUSSION.

The assay for prostatic testosterone 5 α -reductase was successfully validated and the within batch precision of the method established. No attempt was made to assess the between batch precision of the method since each preparation of enzyme came from a different source of tissue. Instead, each experiment was carefully designed so that sufficient controls were included to enable a conclusion to be reached without the need for inter assay comparison.

It is interesting that the ionic strength of the reaction mixture had such a profound effect on the activity of human prostatic testosterone 5 α -reductase. Such an observation has been made previously for rat prostatic testosterone 5 α -reductase (Roy, 1971). In this study ionised substances were being assessed as potential inhibitors and hence it was clearly of paramount importance to standardise upon an ionic strength for use throughout the study.

Using the standardised assay system it was apparent that heparin is an effective in vitro inhibitor of the testosterone 5 α -reductase enzymes of both rat and human prostate tissue. These results extend the isolated observation of Blaquier & Calandra (1973) that a high concentration of heparin can inhibit rat prostatic testosterone 5 α -reductase.

High concentrations of heparin were demonstrated to affect the reduction of NADP by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, but the effect of the polysaccharide on the reduction of testosterone was shown to be independent of the provision of an adequate supply of cofactor.

The mechanism of the heparin inhibition of testosterone 5 α -reductase was investigated and competition was apparently demonstrated between heparin and both the substrate and the cofactor. It is perhaps surprising that heparin should produce such kinetics when it is structurally unrelated to either testosterone or NADPH, but similar effects of heparin have been observed in the production of angiotensin by the kidney enzyme renin (Sealey et al., 1967). Furthermore the investigations described in this chapter have demonstrated that heparin also apparently exerts competitive kinetics with respect to the reduction of NADP by both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

A clue to this apparent anomaly may be obtained from the $\frac{1}{V}$ vs I plots for the two NADPH generating systems since the results suggest that no enzyme-inhibitor complex is formed but rather that the heparin changes the affinity of the enzymes for the NADP substrate (Mahler & Cordes, 1966). Since

heparin contains many sulphated residues it is a highly charged molecule and it is not unreasonable to suppose that it would bind to positively charged groups within a protein molecule. It is possible, therefore, that the observed effects of heparin result from a non-specific binding of the polysaccharide to basic amino acids adjacent to the substrate and cofactor binding sites of the enzymes under study. In the case of the prostatic testosterone 5 α -reductase the enzyme has not yet been obtained in a semi-purified form so it is likely to be many years before the three-dimensional structure of the active site of the enzyme is elucidated.

The pronounced effect of ionic strength on the prostatic testosterone 5 α -reductase activity cannot be altogether ignored since the addition of heparin to incubations would undoubtedly increase the ionic strength of the medium. However it is unlikely that such an effect would depend upon the concentrations of substrate and cofactor, and one would therefore have expected different kinetics if ionic strength was playing a major part in the inhibition process.

Heparin is not a specific inhibitor of prostatic testosterone 5 α -reductase and in addition to the effects on the enzymes previously mentioned it has

been demonstrated to inhibit β -glucuronidase, RNA polymerase, fumarase, alkaline phosphatase, collagenase, elastase and serum protease (Ehrlich & Stivala, 1973). Such a spectrum of action would support the mechanism of action outlined above and would certainly suggest that attention be given to these effects before employing heparin therapy. However, heparin is not a universal enzyme inhibitor having no effect on staphylococcal coagulase (Fumarola, 1969) and actually stimulating other enzymes such as pepsinogen (Horowitz et al., 1970) and diamine oxidase (Hansson & Thysell, 1970).

The acidic nature of heparin means that in addition to binding to basic proteins it should be capable of binding other cationic substances. In this context the physiological action of histamine[Ⓢ] is reduced by heparin (Tidball, 1967) and heparin inhibits calcification in vitro presumably by binding calcium ions (Harris et al., 1969). Finally, although the role of heparin in anticoagulant therapy is now thought to result primarily from its interaction with the protein factors associated with the conversion of prothrombin to thrombin, it is not yet possible to discount a role for heparin in preventing the provision of the calcium ions essential for the coagulation process (Ehrlich & Stivala, 1973).

The prostate is an organ which contains high

levels of cations, and in particular zinc, which is found in higher levels here than elsewhere in the body (Mann, 1964). Since zinc is known to have a strong inhibitory effect on the 5 α -reductase (Wallace, 1975) it did not seem unreasonable to postulate that there could be an ionic interaction between the zinc and heparin, which could have bearing on the control of the enzyme. However, it was not possible to demonstrate that added zinc affected the in vitro inhibition of the testosterone 5 α -reductase of either human or rat prostate, although this simple approach to a complex topic does not necessarily exclude all possibility of an ionic interaction in vivo. It is worthy of note that protamine sulphate, which is used to reverse the anticoagulation properties of heparin (Ehrlich & Stivala, 1973), was also capable of reversing the effects of heparin on prostatic testosterone 5 α -reductase, again presumably as a result of an ionic interaction.

As heparin is a large complex molecule with many functional groups, it was thought relevant to establish whether the overall structure in general or any one of the groups in particular was responsible for the 5 α -reductase inhibition. Thus a number of related monosaccharides and polysaccharides containing various functional groups

were tested in the assay system. Of the monosaccharides tested only N-acetyl glucosamine showed any inhibition over the wide range tested. Although all the monosaccharide units of heparin are not commercially available, those that make up hyaluronic acid were tested, and whilst the individual sugar units had only a small effect on the enzyme, when combined together as hyaluronic acid a potent inhibitor of testosterone 5 α -reductase resulted. The finding that hyaluronic acid inhibits the prostatic 5 α -reductase differs from a similar study of cortisone reduction in rat liver conducted by Troop (1969) who found no inhibition with this carbohydrate, even at concentrations greater than those used in the present study. Other polysaccharides studied by Troop, Ro2-3053, Ro2-7509, Ro2-7528, heparin and chondroitin sulphate, showed similar trends to those reported here, but appeared to be less potent in the inhibition of cortisone reduction; and Ro1-8307 which was only marginally effective in the prostatic system showed as much potency as Ro2-3053 in rat liver. Ro1-8307 was also shown to be as effective as heparin in decreasing aldosterone production in the rat adrenal (Gláz & Sugar, 1964).

The degree of sulphation alone does not seem to determine the extent of inhibition of testosterone

5 α -reductase as hyaluronic acid with a low sulphur content (< 0.05%) was potent, whilst Chondroitin sulphate and Ro1-8307, with 6.3% and 15% sulphur respectively, showed little effect. In a Krebs ascites cell-free system heparin was shown to be a potent inhibitor of rabbit globin mRNA translation (Waldman et al., 1974). This group examined the effect of sulphate content by progressively desulphating the molecule with mild acid, and testing for anticoagulant activity and protein synthesis inhibition. Anticoagulant activity was reduced by 50% within one hour, but at least eight hours of treatment were required before 50% of the protein synthesis inhibition was lost. These experiments indicate that sulphate content is important, but to varying degrees in different systems.

Before speculating about the physiological implications of the data presented it was necessary to establish the presence of heparin in prostatic tissue. In normal individuals the plasma heparin levels are almost undetectable (Ehrlich & Stivala, 1973) and it seems unlikely therefore that any heparin in the prostate would be manufactured in another site and transported. Further evidence against the concentration of heparin within the prostate gland from the blood is shown in the in vivo experiments, where the intraperitoneal

injections of high doses of heparin did not reduce prostatic 5 α -reductase activities. Whilst it seems reasonable to assume that a molecule as large as heparin would have difficulty in crossing the cell membrane, there are reports of in vitro systems which claim this can occur (Costachel et al., 1964). If heparin is not transported into the gland one is left with the alternative that the tissue itself must synthesise it. This is quite a reasonable hypothesis as heparin is commonly associated with connective tissue (Schubert & Hamerman, 1968) in the form of glycoproteins in the mast cells. The simplest way to test for the presence of heparin in the prostate is to isolate an appropriate fraction from the tissue, and to test for both heparin content and testosterone 5 α -reductase inhibitory properties. The results presented suggest that human hyperplastic prostate does possess some uronic acid containing molecules which show mild 5 α -reductase inhibition, and are present in quantities which correlate with reported levels of heparin in other tissues. No attempt was made to further identify the uronic acid residues within prostatic tissue since such a major project seemed outwith the scope of this thesis.

The fact that heparin-like compounds are found in whole tissue extracts does not mean that they will be located within the cell where they can influence

the testosterone 5 α -reductase. Evidence to support or contradict this hypothesis would be most difficult to produce since the amount of tissue which would be required to obtain measurable amounts of heparin after subcellular fractionation would be beyond the limits of practicable experimentation. At present, therefore, it is not possible to do more than speculate as to the role heparin and related compounds may play in the aetiology of human benign prostatic hyperplasia. The lack of specificity of the described effects argues against a central role for heparin in controlling the activity of prostatic testosterone 5 α -reductase although a decline in tissue concentrations of the polysaccharide may contribute to the removal of a brake on the enzyme and so encourage greater 5 α -dihydrotestosterone formation which in turn could promote hyperplasia.

For heparin to be of any value in the treatment of prostatic hyperplasia, in vivo administration should result in a lower testosterone 5 α -reductase activity. Gláz & Sugar (1964) reported in vivo inhibition of aldosterone production both with heparin and heparinoid compounds, and Carter et al., (1968) reported that in vivo administration of heparin inhibited cortisone A ring reduction by intact cells in liver slices from female rats. The in vivo administration of heparin reported here was

based on the regime of Carter et al., (1968) but no similar inhibition in prostatic slices was observed. Moreover, the survival rate among the treated rats was low, indicating that lower doses of heparin would have been necessary in further experiments, and in this context perhaps a lower dose administered over a longer time period would have shown some effect.

Even if heparin were freely accessible to prostatic testosterone 5α -reductase it is still unlikely that it would be used in the treatment of benign prostatic hyperplasia since its multiplicity of physiological action would produce undesirable side effects.

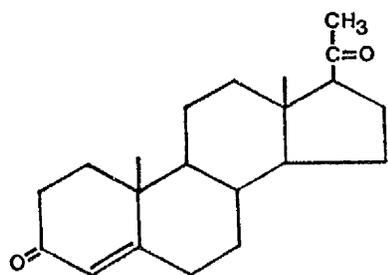
In conclusion, heparin and related compounds have been shown to be effective in vitro inhibitors of the testosterone 5α -reductase of both rat and human prostatic tissue, although the inhibition appears to be lacking in specificity. Whilst prostatic tissue seems unlikely to concentrate heparin from the circulation it may well be capable of synthesising significant quantities of related substances and it remains to be established whether these compounds can in any way influence the onset of benign prostatic hyperplasia.

CHAPTER IV.

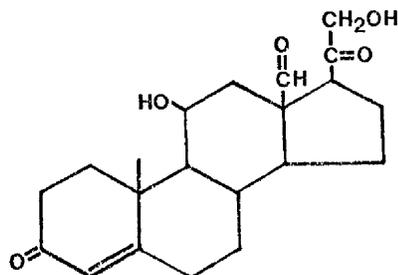
CHAPTER IV.THE INTERACTION OF SPIRONOLACTONE METABOLITES
WITH PROSTATIC TESTOSTERONE 5 α -REDUCTASE.1. INTRODUCTION.

The spironolactones are a group of substances widely used in clinical practice as aldosterone antagonists. Their action is achieved by competition between the spironolactones and aldosterone for the mineralocorticoid binding sites in the renal tubules (Farnestil, 1968), and the reduction in aldosterone induced effects produces Na⁺ loss and promotes K⁺ retention. Spironolactone itself, the form in which the drug is most widely administered, is a steroid with a basic androstane skeleton (Fig.IV - 1). Following absorption spironolactone is rapidly metabolised, mainly to aldadiene (canrenone) and to canrenoate (Fig.IV - 1)(Sadée et al., 1973), which exist together in the plasma in equilibrium. Relatively large doses of spironolactone (e.g. 200mg) are necessary to inhibit the physiological effects of 1mg of aldosterone.

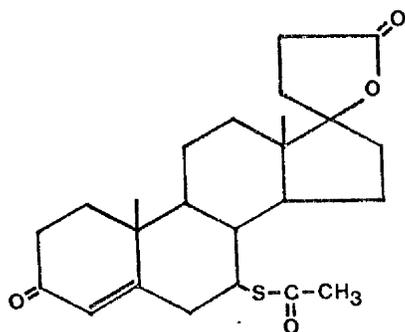
Recently, interest has been stimulated in the possible antiandrogenic side effects of spironolactone

Fig.IV - 1.Steroid Structures.

(a) Progesterone
4-pregnene-3,20-dione

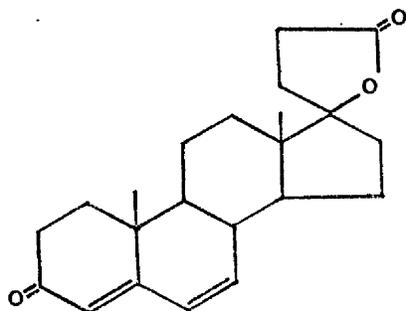


(b) Aldosterone
11 β ,21-dihydroxy-3-oxo-4-pregnen-18-al

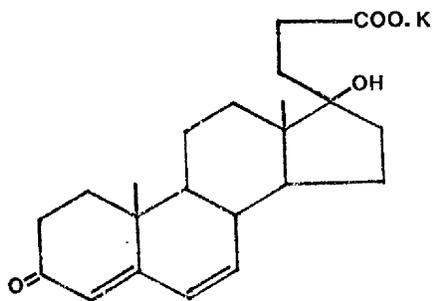


(c) Spironolactone

17-hydroxy-7-mercapto-3-oxo-17 α -4-pregnene-21-carboxylic acid- γ -lactone,7-acetate



(d) Aldadiene (Canrenone)
17-hydroxy-3-oxo-17 α -4,6-pregnadiene-21-carboxylic acid- γ -lactone



(e) K. Canrenoate
17-hydroxy-3-oxo-17 α -4,6-pregnadiene-21-carboxylic acid, monopotassium salt

and its metabolites following the observation of gynaecomastia, menstrual irregularities and impotence in patients receiving oral spironolactone (Dymling et al., 1972). Moreover, Basinger & Gittes (1974) noted the atrophic appearance of rat seminal vesicles and prostates, but not adrenals, in spironolactone treated animals. Histologically these glands were shown to have low columnar epithelium, a decrease in cytoplasmic to nuclear ratio and a loss of epithelial involutions. Similarly, spironolactone has been demonstrated to inhibit the survival of cultured human prostatic tissue (Castro & Sellwood, 1974).

The antiandrogenic effects of spironolactone may in part be due to an inhibition in androgen production and in part to a blocking of the action of androgens. Thus, in rat testis, the drug has been shown to decrease the formation of testosterone from progesterone as a result of inhibition of the cytochrome P450 dependent steroid 17α -hydroxylase (Menard et al., 1974), and a significant rise in plasma concentrations of progesterone and 17α -hydroxy progesterone have been reported following the administration of 400mg/day spironolactone to healthy men (Stripp et al., 1975), which is consistent with loss of P450 dependent 17α -hydroxylase and desmolase activity. Several studies have been carried out to assess the effect of spironolactones on the binding

of DHT to the prostatic androgen receptor and it is now well established that there is a marked inhibition of binding of DHT to both cytoplasmic and nuclear receptors in the presence of those compounds. Thus Bonne & Raynaud (1974) showed potent effects on DHT binding for spironolactone itself and for aldadiene but not for potassium canrenoate.

The effects of spironolactones on the activity of testosterone 5α -reductase are far less well defined, for whilst Corvol et al., (1975) have shown no effects of spironolactone or potassium canrenoate on enzyme activity the inhibitory role of aldadiene does not seem to have been assessed. This omission is perhaps surprising in view of both the quantitatively significant role of aldadiene and the structural features that make it potentially the most likely inhibitor of testosterone 5α -reductase. In this study the effects of the two principal spironolactone metabolites, aldadiene and potassium canrenoate, have been examined with the human prostatic testosterone 5α -reductase enzyme system.

2. EXPERIMENTAL AND RESULTS.

A. Interaction of Aldadiene and Potassium Canrenoate with Testosterone 5 α -reductase.

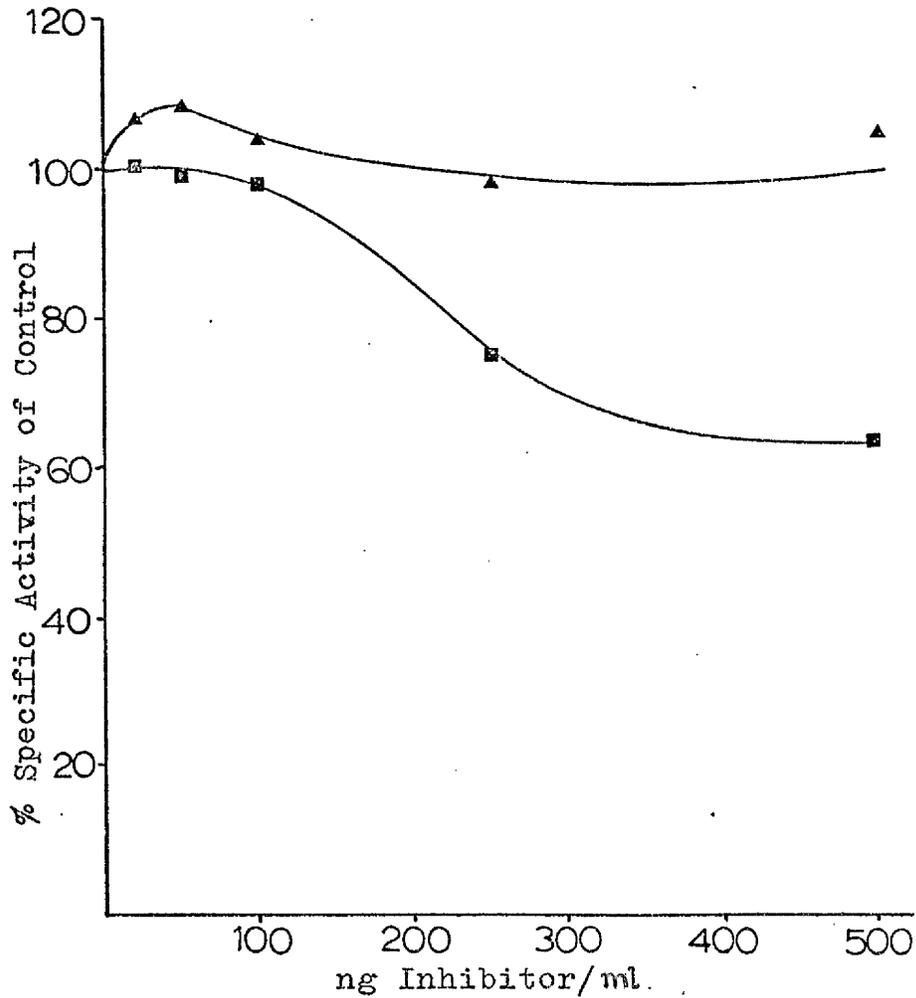
An oral dose of 100mg spironolactone is a standard regime which produces a peak plasma aldadiene concentration of 250ng/ml (Searle, personal communication). Accordingly, the concentration ranges in the following experiments were based on this plasma concentration. Fig.IV - 2 shows the effect of adding either aldadiene or potassium canrenoate to the standard testosterone 5 α -reductase assay described in Chapter II. Aldadiene had a significant inhibitory effect on the enzyme activity at 250 and 500ng/ml. Potassium canrenoate on the other hand would appear to have had no effect over this concentration range.

B. The Purity of Aldadiene.

The purity of the added aldadiene was checked by thin layer chromatography. Aldadiene (100 μ g) was spotted on to Silica TLC plates and developed in a solvent system of benzene/ethylacetate:6/4. The aldadiene had an Rf of 0.19 in this system and no other spots were visualised under UV light at either 264nm or 350nm. Scanning a solution of the aldadiene preparation on a Unicam SP800 spectrophotometer from 200 - 450nm showed the compound

Fig.IV -- 2.

Inhibition of Testosterone 5 α -Reductase by
Aldadiene and Canrenoate.



The spironolactone metabolites, aldadiene and canrenoate, were tested over the concentration range 0-500ng/ml for their ability to inhibit the standard testosterone 5 α -reductase assay described on page 62.

▲ - Canrenoate

■ - Aldadiene

2ng testosterone substrate added
per assay.

to have a single absorption maximum at 283nm - which is characteristic of the conjugated double bond system in the A and B rings of aldadiene. The single absorption maximum and single spot on the chromatogram would indicate that there were no significant contaminating impurities and that the inhibition of the testosterone 5 α -reductase was due to aldadiene itself.

C. Kinetics of Aldadiene Inhibition of Testosterone 5 α -Reductase Using Homogenate Preparations.

In order to assess the type of inhibition exerted on the enzyme by aldadiene, homogenate enzyme preparations were incubated at varying substrate concentrations in the presence or absence of aldadiene (250ng). At this concentration, however, aldadiene is nearing the limit of its solubility in water, and the presence of the steroid in the homogenate preparations led to consistently imprecise determinations and the need for a more homogeneous enzyme preparation was indicated.

A microsomal preparation was considered most suitable for the purpose. Nuclear preparations were ruled out both on the grounds of the difficulties in removing contaminating connective tissue fragments, and because of low yields (Wallace, 1975).

D. Preparation of Microsomes and Validation of the Method.

The method used was based on that of Wallace (1975) and is detailed in Chapter II. Human hyperplastic prostatic tissue was thoroughly homogenised and centrifuged at 15,000g for 10min. to precipitate whole cells, mitochondria and nuclei. The low spin supernatant was then recentrifuged at 105,000g for 1h to precipitate the majority of the microsome fragments. The high spin precipitate was suspended in TKM + sucrose buffer prior to incubation. The previously described variability in specific activity of human prostatic testosterone 5 α -reductase was particularly evident in these microsomal preparations and great care was required to ensure that optimal enzyme activity was added to incubation tubes. As a general approximation the microsomes from 1 gram of human prostatic tissue were suspended in 5ml of buffer and 500 μ l of this solution was used in each assay tube.

Table IV - 1 shows the results of the distribution of testosterone 5 α -reductase during the preparation of microsomes from two different preparations, together with the percentage conversion of testosterone substrate obtained in the assay system with each cellular component. It is apparent that the high speed pellet contained a higher specific enzyme

Table IV-1.

Homogenates of human prostate were centrifuged at 15,000 g for 10 min (low spin) followed by 105,000 g for 60 min (high spin). The corresponding pellets were resuspended as indicated and testosterone 5 α -reductase activity determined in all fractions using the method described in Chapter II.

Table IV - 1Distribution of Testosterone 5 α -Reductase Activity
in Different Preparative Fractions.

| Prep. and vol. | <u>PROSTATE A</u> | | <u>PROSTATE B</u> | |
|-------------------------------|-------------------------------|---------|-------------------------------|---------|
| | SA pmoles/h/ mg protein | % Conv. | SA pmoles/h/ mg protein | % Conv. |
| Whole Homog. 250 μ l | 17.3 | 26.3 | 10.2 | 26.1 |
| Low spin pel. 100 μ l | 36.4 | 33.5 | 21.0 | 24.9 |
| 250 μ l | 24.4 | 59.9 | 20.0 | 46.5 |
| 500 μ l | 16.1 | 77.0 | 8.13 | 55.4 |
| High spin pel. 100 μ l | 39.1 | 20.6 | 9.2 | 2.0 |
| 250 μ l | 35.5 | 40.4 | 12.7 | 5.8 |
| 500 μ l | 25.6 | 66.4 | 11.6 | 11.4 |
| High spin sup. 500 μ l | 0.72 | 2.7 | 0.37 | 2.2 |

PROSTATE A

10g tissue homogenised in 100ml TKM + Sucrose
 Low spin Pellet suspended in 25ml TKM + Sucrose
 High spin Pellet suspended in 15ml TKM + Sucrose

PROSTATE B

10g tissue homogenised in 100ml TKM + Sucrose
 Low spin Pellet suspended in 50ml TKM + Sucrose
 High spin Pellet suspended in 100ml TKM + Sucrose

activity than the homogenate, although this specific activity was submaximal if the percentage conversion of substrate was permitted to exceed 50%.

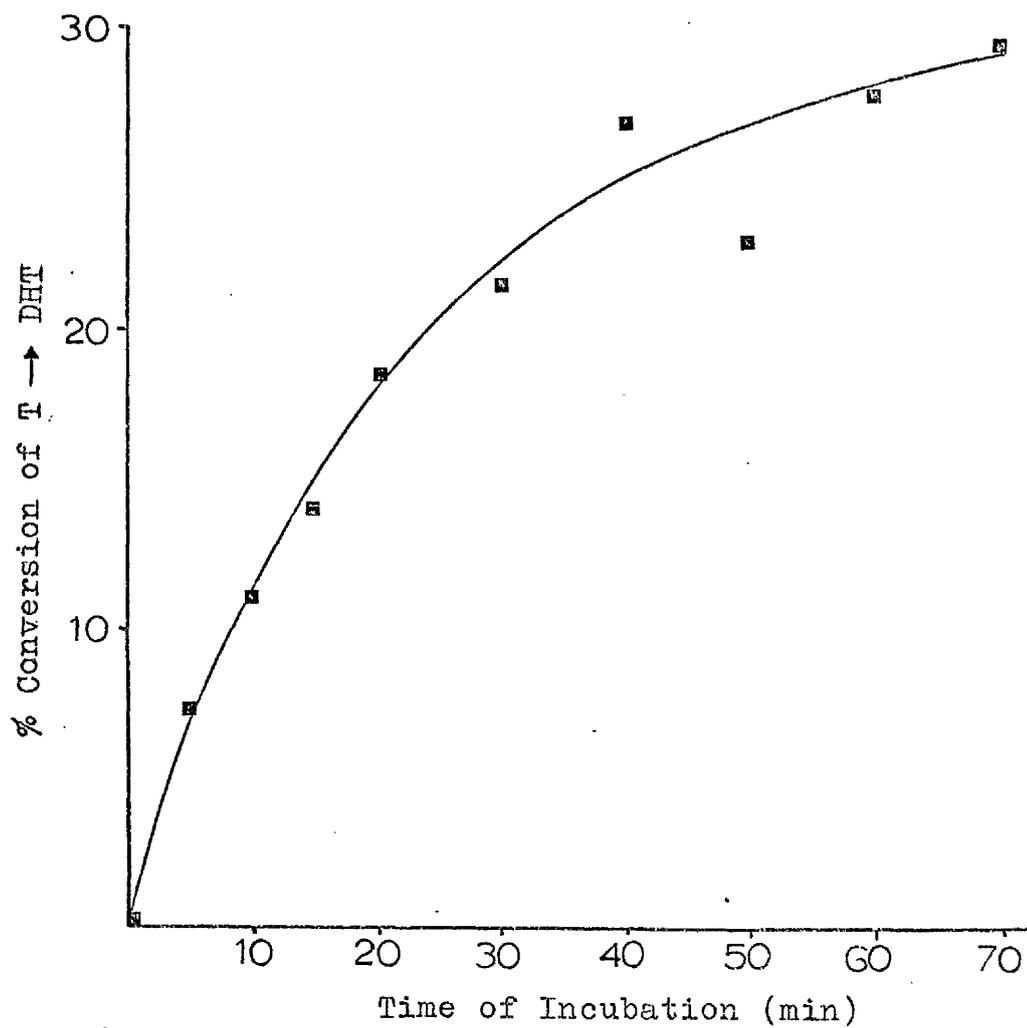
A time course for the incubation of 2ng testosterone with an optimal amount of microsomal preparation showed that the reaction was approximately linear for the first 20 minutes, thereafter the activity decreased (Fig.IV - 3). This indicates that in the standard assay, with a 20min. incubation period, velocities similar to initial reaction velocities were being measured. For each time point over 95% of the total radioactivity at the end of the incubation could be accounted for by [^3H]-testosterone and [^3H]-DHT. No [^3H]-5 α -androstane-3 α ,17 β -diol was detected.

E. Kinetics of Aldadiene Inhibition of Testosterone 5 α -Reductase using Microsome Preparations.

The use of microsome preparations allowed a marked improvement in the precision of the enzyme assay, with the mean coefficient of variation improving from 23.4% for the homogenate assay to 5.6% for the microsome assay, as calculated from three determinations at five different substrate concentrations. Double reciprocal plots were repeated using the microsome preparations. However, whilst the precision had improved considerably, it

Fig.IV - 3.

Time Course for Testosterone 5 α -Reductase using
a Microsome Preparation.



Microsomes from 10g tissue were made up to 50ml in TKM + Sucrose buffer and 500 μ l added per assay.

was still not possible to distinguish the type of kinetics exhibited by aldadiene (Fig.IV - 4). Extending the range of substrate concentrations and increasing the inhibitor concentrations would be possible ways of overcoming this problem, but would necessitate increases of inhibitor concentrations beyond the levels of solubility of aldadiene in the solutions used.

F. Metabolism of [1,2-³H]-Aldadiene.

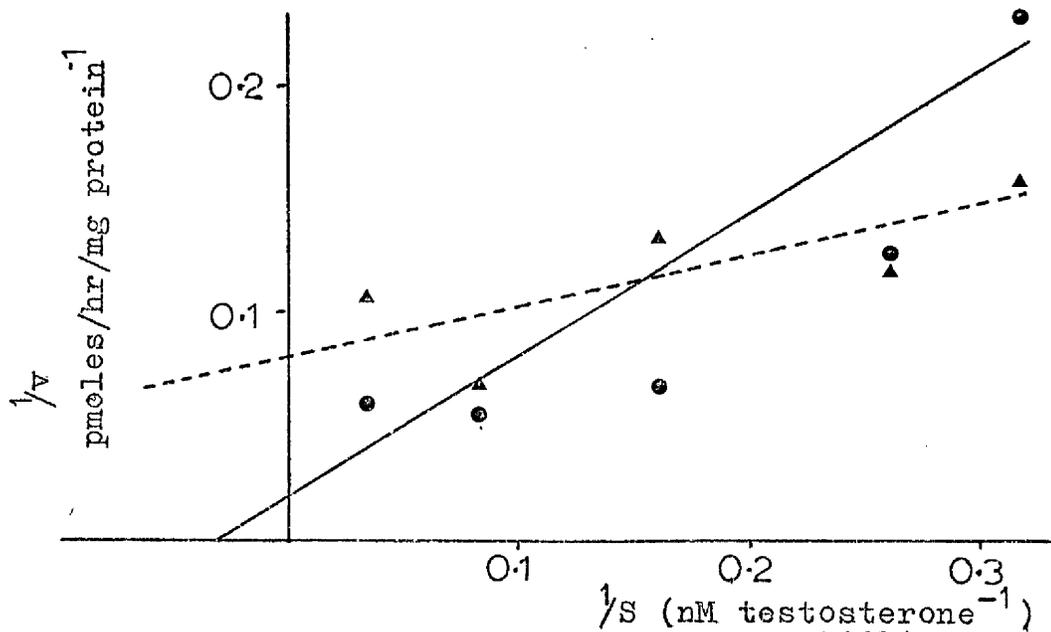
It was considered possible that aldadiene could be metabolised by the testosterone 5 α -reductase, thus accounting for its inhibitory properties on the enzyme. Accordingly, incubations were set up to test this hypothesis.

A standard homogenate enzyme preparation was incubated for 0, 15, 30 and 60min. with [1,2-³H]-aldadiene (5ng, 500,000cpm) and 4mM NADPH. The reaction was terminated with ethanol (2ml) and the steroids extracted with chloroform, evaporated under nitrogen and spotted on paper chromatography strips. The strips were run in the Bush B system and after radioscanning only a single peak could be detected, with an Rf value of 0.6. This peak corresponded to aldadiene itself and it was therefore concluded that no significant metabolism of this steroid had taken place.

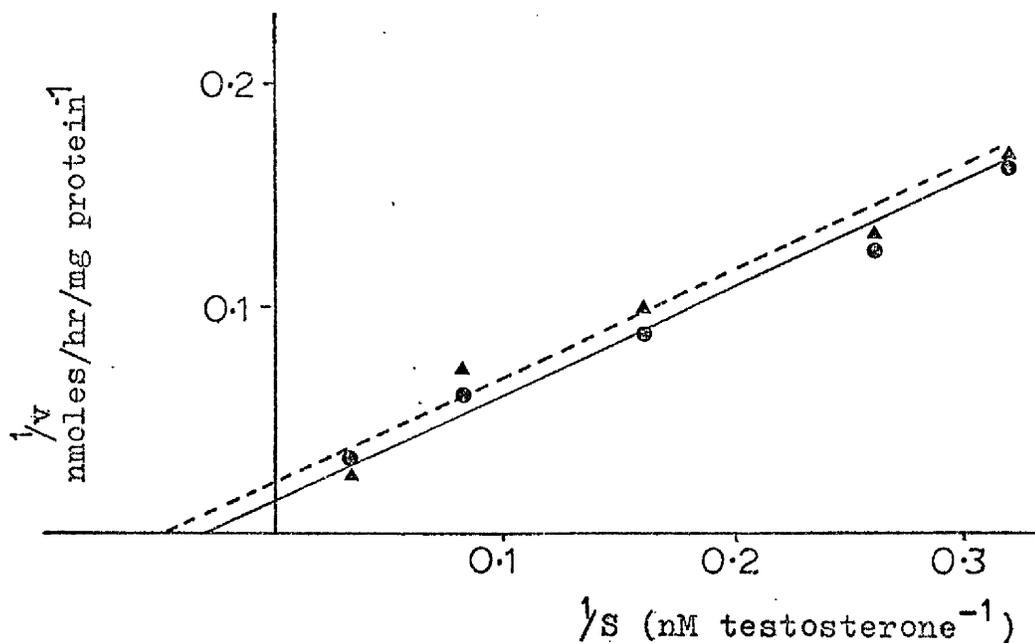
Fig.IV - 4

Double Reciprocal Plots of 5 α -Reductase Inhibition by Aldadiene.

(a) Homogenate preparation.



(b) Microsome preparation.



Key: ● No Aldadiene
 ▲ + 250ng Aldadiene

Each point is the mean of triplicate incubations.

3. DISCUSSION.

The spironolactone metabolites, aldadiene and canrenoate were tested in the standard human prostatic 5 α -reductase assay system for possible inhibition properties. At concentrations similar to those found in the plasma of spironolactone treated patients, aldadiene caused a small but significant inhibition of testosterone reduction. Canrenoate showed no inhibition at either this or higher concentrations. The inhibitory properties of aldadiene could not be attributed to impurities in the steroid preparation.

The lack of effect of canrenoate is consistent with the literature report of Corvol et al., (1975) for the rat prostate system. The effectiveness of aldadiene as an inhibitor of testosterone 5 α -reductase probably results from the presence of the lactone ring which may be required for binding to the surface of the enzyme. A similar requirement for the lactone ring has been shown by Bonne & Raynaud (1974) for binding to the prostatic DHT receptor and the varying effects of spironolactone metabolites as inhibitors of adrenal steroid hydroxylation have similarly been attributed to structural differences (Cheng et al., 1976).

Considerable difficulty was experienced in determining the type of inhibition exerted on the testosterone 5 α -reductase by aldadiene. This

difficulty was in part due to the poor solubility of aldadiene and in part to relatively small degree of inhibition being studied. Although the use of a microsomal preparation of 5 α -reductase significantly improved the precision of the enzyme assay it did not permit conclusive evidence as to the nature of the inhibition, although the failure of the enzyme system to metabolise aldadiene suggests that simple competitive kinetics are unlikely.

Spironolactones would thus seem to have three possible sites of action as antiandrogens - inhibition of androgen synthesis, inhibition of androgen metabolism and competition for the DHT receptor site. It is impossible to predict from a simple study of this nature the relative importance of these three sites of action in vivo although it is not unreasonable to suppose that the small inhibitory effects of aldadiene on testosterone 5 α -reduction are unlikely to be of major importance in deciding the antiandrogenicity of spironolactone preparations.

CHAPTER V.

CHAPTER V.THE INVESTIGATION OF AN ISOTOPE EFFECT IN
THE CHEMICAL AND BIOLOGICAL METABOLISM OF
17 α -TRITIATED ANDROGENS.1. INTRODUCTION.

The following study was initiated as a result of a casual observation made during the validation of the experimental model used in the simultaneous superfusion of human prostate slices with [17 α -³H]-testosterone and [1,2-³H]-DHT. Following the chromatographic separation of the testosterone and DHT fractions from the tissue it was noted that not all the radioactivity in the DHT fraction was converted into 5 α -androstane-3,17-dione by chromic acid oxidation. Further experimentation revealed that this resistance to dehydrogenation resulted from the presence of the 17 α -³H atom and it was decided to investigate this isotope effect in greater detail.

Isotope effects arise because of the varying energy required to break isotopically different bonds, and in general, the greater the mass of the isotope the higher the activation energy of the bond.

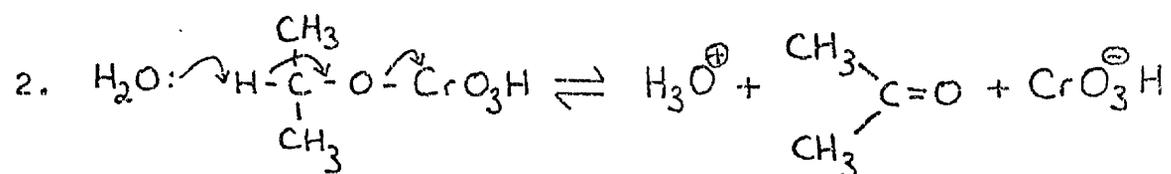
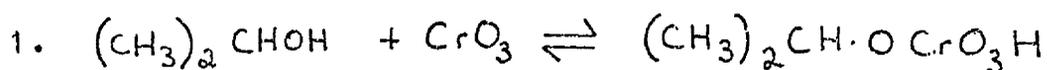
Primary isotope effects are usually most apparent since the isotopic bond is directly involved in the rate limiting step. Secondary isotope effects do not involve the isotopic bond but may result from the close proximity of the isotope (Evans, 1974).

Isotope effects are particularly common in reactions involving hydrogen isotopes since there is a greater proportional difference in the masses of protium, deuterium and tritium than for any other atomic species. Accordingly protium reactions have been reported to be five to eight times as fast as deuterium whilst tritium isotope effects may be twice as large as those of deuterium (Morrison and Boyd, 1966).

Isotopic labelling results in a different vibration frequency in the bond holding the labelled atom to the molecule, and hence a difference in the susceptibility of that bond to breakage. Such a change may affect the participation of the labelled species not only in chemical reactions, but also in enzymatic reactions where binding of the substrate to the enzyme, and hence the Michaelis constant, may be affected. Therefore it does not necessarily follow that tritium labelled substrates behave in an identical manner to their non-radioactive counterparts - an essential assumption in any radiotracer study.

Great care must be taken before an isotope effect can be established since there are several artifacts which can apparently bring about differential metabolism of isotopically different substrates. Uncertainty about the specific activity of a substance is often the cause of unexpected results, as is a lack of knowledge of the stereospecific nature of the labelled species. Tritium atoms are particularly labile and this may result in low reaction rates, whilst intramolecular migration of tritium can produce very confusing data.

The existence of isotope effects in the oxidation of alcohols has been established for many years. As early as 1949 Westheimer and Nicolaides compared the rates of chromic acid oxidation of propan-2-ol and 2-deutero-propan-2-ol. Their demonstration of a slower oxidation of the deuterated species was consistent with the mechanism below, in which there is the initial formation of a chromic acid ester followed by the rate-determining abstraction of the carbinol proton by an elimination process:



A similar mechanism has been confirmed for the chromic acid oxidation of the steroid alcohols 5 α -cholestane-3 α -ol (Elie ℓ et al., 1965) and 5 α -pregnane-11 β -ol (Roček et al., 1962) and in both cases the mechanism was confirmed by the use of deuterated species and the demonstration of an isotope effect.

Isotope effects have also been observed and used in enzyme reactions. Fisher and his colleagues (1953) used 1,1-dideuteroethanol to demonstrate a direct transfer of hydrogen from ethanol to NAD in the reaction catalysed by alcohol dehydrogenase, and Talalay and his colleagues (1955) used 17 α -deutero-17 β -hydroxy-4-androstene-3-one to show a direct transfer of hydrogen between testosterone and position 4 of NAD in the reaction catalysed by 17 β -hydroxysteroid-dehydrogenase. Both these authors discussed the occurrence of isotope effects during their studies.

Thus, although an isotope effect has not been specifically reported for either the chemical or biological oxidation of 17 α -tritiated androgens there seems little reason why such a phenomenon should not exist and accordingly it was decided to further investigate the observation made during the validation of the superfusion system.

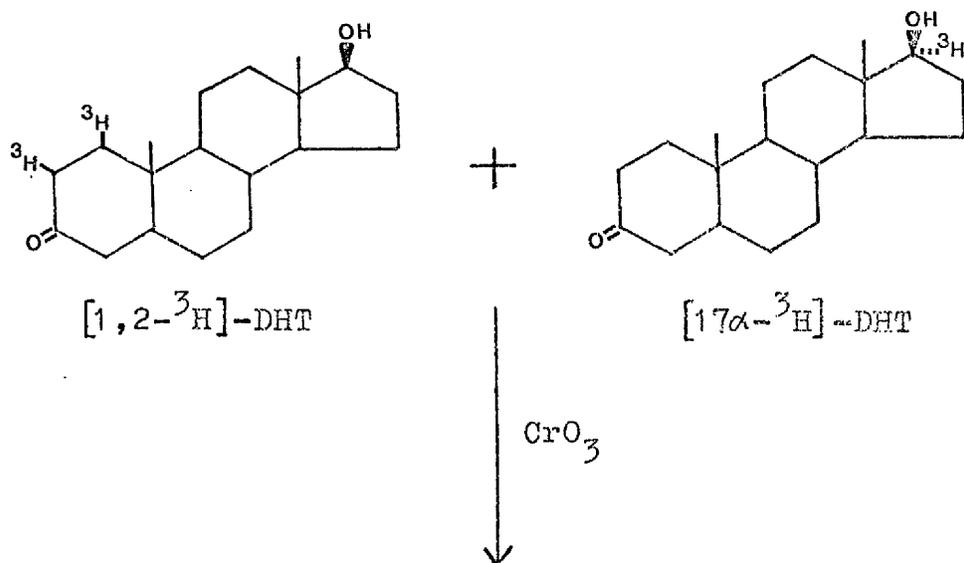
2. RESULTS.

A. Initial Observations.

When human prostatic tissue is superfused simultaneously with $[17\alpha^3\text{H}]$ -testosterone and $[1,2-^3\text{H}]$ -DHT the resulting mixture of steroids extracted from the tissue and perfusate includes $[17\alpha^3\text{H}]$ -testosterone, $[17\alpha^3\text{H}]$ -DHT and $[1,2-^3\text{H}]$ -DHT. Testosterone and DHT can be readily separated by chromatographic methods. However, the $[17\alpha^3\text{H}]$ -DHT and $[1,2-^3\text{H}]$ -DHT require further processing before the radioactivity due to the different isotopically labelled compounds can be distinguished. The separation is facilitated by oxidation of the steroids with CrO_3 in glacial acetic acid, a procedure which results in the production of 5α -androstane-3,17-dione from DHT, (and 4-androstene-3,17-dione from testosterone). This oxidation involves the conversion of the 17β -hydroxyl group into a 17-keto group with the loss of the 17α -hydrogen atom. Thus in a mixture of $[17\alpha-^3\text{H}]$ - and $[1,2-^3\text{H}]$ -DHT the steroid formerly labelled at the 17α position becomes non-radioactive following oxidation, leaving the only radioactivity in the 5α -androstane-3,17-dione as that originating from $[1,2-^3\text{H}]$ -DHT. Provided correction is made for procedural losses, it is thus possible to determine the fraction of $[17\alpha-^3\text{H}]$ -DHT in the original mixture. (Fig. V - 1).

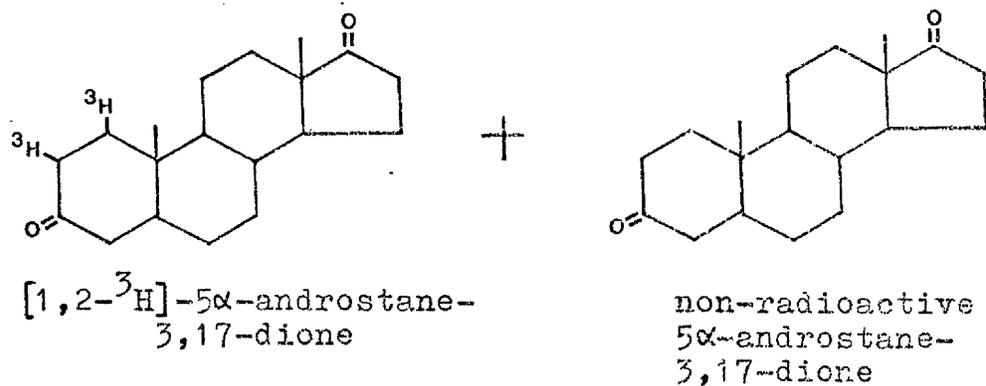
Fig.V -- 1The Chromic Acid Oxidation of [1,2-³H]-DHT and [17 α -³H]-DHT.DHT Mixture

Total Radioactivity = A cpm/mol.

5 α -Androstane-3,17-dione Mixture.

Total Radioactivity = B cpm/mol.

(Corrected for procedural losses)

Calculation: $[17\alpha-^3\text{H}]\text{-DHT} = A - B \text{ cpm/mol.}$

During the validation of the superfusion system the chromic acid oxidation step was examined. The DHT fraction from the paper chromatograms (see Chapter II), containing $[1,2-^3\text{H}]\text{-DHT}$ and $[17\alpha-^3\text{H}]\text{-DHT}$ from the tissue and added $[4-^{14}\text{C}]\text{-DHT}$, was oxidised as described in Chapter II, and the products subjected to silica gel TLC. Bands corresponding to DHT and $5\alpha\text{-androstane-3,17-dione}$ were eluted from the plates and radioassayed. As expected, greater than 95% of the ^{14}C isotope was recovered in the $5\alpha\text{-androstane-3,17-dione}$ fraction with negligible ^{14}C being found in the DHT fraction. Conversely, approximately 30% of the applied ^3H remained in the DHT band. It was apparent, therefore, that during the oxidation step ^{14}C and ^3H species of DHT were being oxidised at different rates. Since this step is of key importance in the validation of the superfusion work up further studies were undertaken.

B. Systematic Study of the Oxidation of Testosterone.

A systematic study of the chromic acid oxidation step was carried out using different isotopically labelled species of testosterone. Testosterone was chosen rather than DHT since large quantities of pure $[17\alpha-^3\text{H}]\text{-DHT}$ were unavailable and could not be prepared readily without risking randomisation of

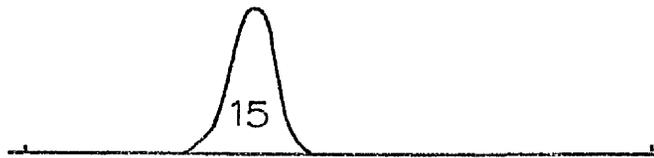
the tritium atoms. The product of chromic acid oxidation of testosterone is 4-androstene-3,17-dione which can be separated from the substrate in the same TLC system used for the resolution of DHT and 5 α -androstane-3,17-dione (Chapter II - System 2).

Approximately equal masses (50 μ g) of [4-¹⁴C]-testosterone, [1,2-³H]-testosterone and [17 α -³H]-testosterone were oxidised in the usual manner either alone or in combination. The oxidation products were subjected to TLC and the developed plates scanned using the Panax Radio-chromatogram Scanner. The results obtained are displayed in Figure V - 2 and whilst results from the Panax are only semi-quantitative it is nevertheless apparent that a considerable proportion of the [17 α -³H]-testosterone remained whereas both the other radioactive species were apparently quantitatively converted in 4-androstene-3,17-dione.

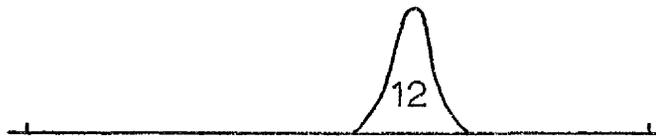
In order to demonstrate that the ³H atom could be removed by oxidation of [17 α -³H]-testosterone, variable reaction conditions were employed. A fixed substrate mixture of [17 α -³H]-testosterone (10⁵cpm), [4-¹⁴C]-testosterone (1.1x10³cpm) and unlabelled testosterone (200 μ g) was oxidised at either 20°C, 37°C or 60°C for either 30min. or 60min. The products were extracted in the usual manner and divided into two fractions. One half of the extract

Figure V-2. Panax Radiochromatogram Scans of the Chromic Acid Oxidation Products of Testosterone.

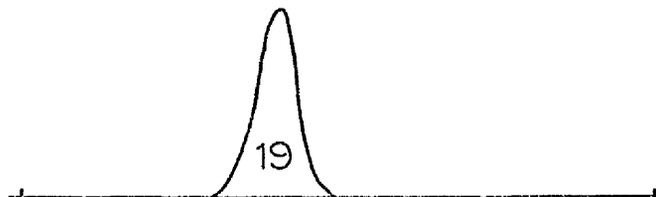
1. [1,2-³H]-T



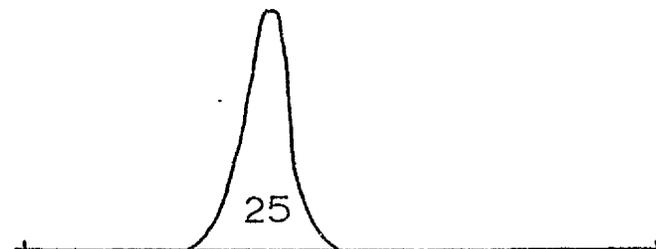
2. [17 α -³H]-T



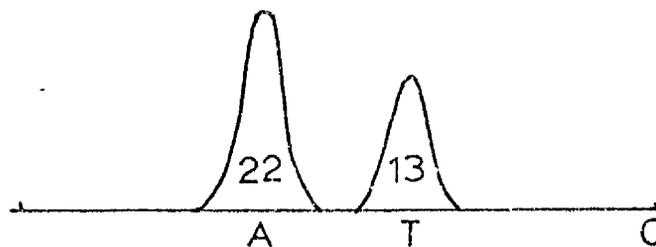
3. [4-¹⁴C]-T



4. [1,2-³H]-T + [4-¹⁴C]-T



5. [17 α -³H]-T + [4-¹⁴C]-T



O = origin

T = testosterone

A = 4-androstene-3,17-dione

Silica TLC plates in a solvent system 2.

Figures in peaks refer to peak heights in cm.

was counted directly and the $^3\text{H}/^{14}\text{C}$ ratio determined whilst the second half of the extract was chromatographed and the plates scanned as described previously.

The results shown in Table V-1 demonstrate that almost quantitative removal of the $17\alpha\text{-}^3\text{H}$ atom occurs in the oxidation performed at 37°C for 60min. By contrast, only 23% of the tritium is lost under the normal oxidation conditions of 20°C for 30min. This can be increased substantially for the same time period by increasing the reaction temperature.

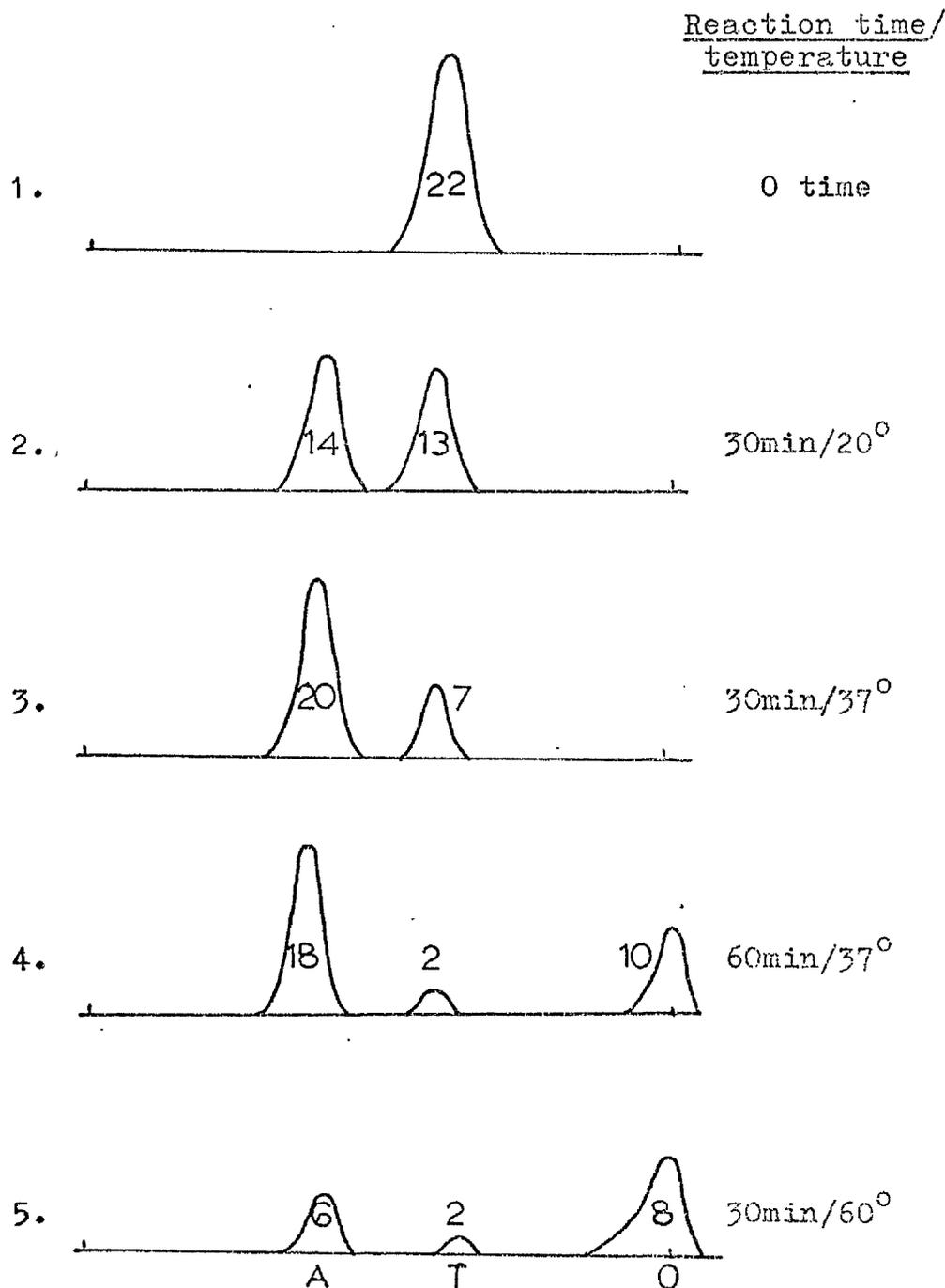
The developed TLC plates were examined under ultraviolet light before radioscanning. In no plate was a band visible in the testosterone region whereas an intense 4-androstene-3,17-dione band was seen in all extracts. An additional band was observed at the origin of the plate containing the products of the 60°C oxidation. The results of the radioscanning shown in Figure V-3 confirm the $^3\text{H}/^{14}\text{C}$ data in that progressively less radioactivity remains in the testosterone region as the reaction temperature is increased. The scan from the oxidation carried out at 37°C for 60min. confirms the almost total loss of the $[17\alpha\text{-}^3\text{H}]$ -testosterone. The small peaks of radioactivity observed at the origin of scans 4 and 5 presumably represent oxidation products arising from the opening of the

Table V - 1 $^3\text{H}/^{14}\text{C}$ Ratios Following the Chromic AcidOxidation of a Mixture of $[17\alpha\text{-}^3\text{H}]$ -Testosterone and $[4\text{-}^{14}\text{C}]$ -Testosterone.

| Oxidation Temperature ($^{\circ}\text{C}$) | Time of Oxidation (min) | $^3\text{H}/^{14}\text{C}$ of Product | % Loss of ^3H |
|--|-------------------------|---------------------------------------|------------------------|
| 20 | 0 | 9.08 | 0 |
| 20 | 30 | 7.01 | 23 |
| 37 | 30 | 3.19 | 65 |
| 37 | 60 | 0.17 | 98 |
| 60 | 30 | 1.74 | 81 |

Figure V - 3

Panax Radiochromatogram Scans of the Products of
Chromic Acid Oxidation of a Mixture of [17 α -³H]-
and [4-¹⁴C]-Testosterone.



Q = origin T = testosterone

A = 4-androstene-3,17-dione

Silica TLC plates in solvent system 2.

Figures refer to peak heights in cm.

steroid nucleus - particularly since scintillation counting revealed these products contained only the ^{14}C -isotope.

C. The Purity of $[17\alpha\text{-}^3\text{H}]$ -Testosterone.

The previous section has shown that $[17\alpha\text{-}^3\text{H}]$ -testosterone is apparently oxidised more slowly than either $[4\text{-}^{14}\text{C}]$ -testosterone or $[1,2\text{-}^3\text{H}]$ -testosterone. Before concluding that an isotope effect is responsible, it is necessary to establish that the $[17\alpha\text{-}^3\text{H}]$ -testosterone used in this study did not contain contaminants of an equal polarity that might co-chromatograph with testosterone in the TLC system used.

(i) Assessment of purity by paper chromatography.

An aliquot of $[17\alpha\text{-}^3\text{H}]$ -testosterone was chromatographed on paper strips using the Bush B solvent system (Chapter II). Radio scanning of these strips showed a single peak of radioactivity corresponding to testosterone.

(ii) Assessment of purity by thin layer chromatography.

The $[17\alpha\text{-}^3\text{H}]$ -testosterone was chromatographed using Silica TLC plates and a solvent system of $\text{CHCl}_3/\text{Acetone}:7/1$. When radioassayed again only a single band of radioactivity was detected in a position corresponding to testosterone.

(iii) Assessment of purity by recrystallisation.

An aliquot of $[17\alpha\text{-}^3\text{H}]$ -testosterone was taken, ^{14}C and unlabelled testosterone were added, and the solution crystallised from hexane/acetone as described in Chapter II. Samples of the original solution, the crystals and the mother liquor were taken, dried, and the tritium to carbon-14 ratio measured by scintillation counting. The resulting ratios were 9.62, 9.45 and 9.53 respectively, indicating that the $[17\alpha\text{-}^3\text{H}]$ -testosterone appeared to have a purity > 95%.

(iv) Assessment of purity by derivative formation.

Derivative formation is a method commonly used in chemistry for the identification of compounds and the determination of their purity.

Acetylation of $[17\alpha\text{-}^3\text{H}]$ -testosterone, $[4\text{-}^{14}\text{C}]$ -testosterone and a mixture of these two isotopes was carried out as detailed in Chapter II, followed by chromatography on Alumina TLC plates in solvent system - 1. In all cases only a single band of radioactivity was detected on the plates in a position corresponding to testosterone acetate. No radioactivity remained at the origin or was found in the band corresponding to testosterone. The $^3\text{H}/^{14}\text{C}$ ratio of the testosterone acetate product (13.4) was almost identical to that of the substrate mixture (13.6).

D. Assessment of the Stereochemical Purity of
[17 α -³H]-Testosterone.

The previous four paragraphs have established that the [17 α -³H]-testosterone used in this study was essentially free of non-testosterone contaminants. It remained to be established however, that the tritium atom within the substrate was located exclusively in the 17 α - position.

The results of section B. demonstrate that using prolonged oxidation conditions it was possible to remove 98% of tritium from the [17 α -³H]-testosterone substrate. The only carbon atom of testosterone which could carry tritium and behave in this manner during a chromic acid oxidation yielding 4-androstene-3,17-dione as product, is carbon atom 17. Tritium in the 17-hydroxyl position would exchange with the hydrogen of the solvents and would be lost on acetylation. As this does not occur here it seems reasonable to conclude that the tritium is in the 17-carbon position. It is possible that the [17 α -³H]-testosterone (17 β -hydroxy-4-androstene-3-one) species could be contaminated by [17 β -³H]-epitestosterone (17 α -hydroxy-4-androstene-3-one). Whilst testosterone and epitestosterone would not be expected to co-crystallise it was considered desirable to obtain chromatographic proof concerning epitestosterone contamination of the [17 α -³H]-

testosterone substrate.

An aliquot of the substrate was acetylated and the products of acetylation applied to alumina TLC plates which were developed continuously for 18h with cyclohexane/ethylacetate (9/1). Elution and radioassay of the bands corresponding to testosterone acetate (4.5cm from the origin) and epitestosterone acetate (6.5cm from the origin) revealed that only the former band contained any appreciable radioactivity.

It must be concluded from these studies that the $[17\alpha\text{-}^3\text{H}]$ -testosterone used for chromic acid oxidation was greater than 95% pure and that the presence of impurities could not therefore be responsible for the apparent resistance to oxidation of the labelled species.

E. Time Course of the Chromic Acid Oxidation of Isotopically Labelled Species of Testosterone.

In Sections A and B semi-quantitative data was produced to suggest that $[17\alpha\text{-}^3\text{H}]$ -testosterone is oxidised more slowly by chromic acid than is $[1,2\text{-}^3\text{H}]$ -testosterone. Having established the purity of the $[17\alpha\text{-}^3\text{H}]$ -testosterone species in Sections C and D, it was considered important to obtain quantitative evidence as to the differing rates of oxidation of the two tritiated testosterone

substrates.

Accordingly two substrates mixtures were prepared:

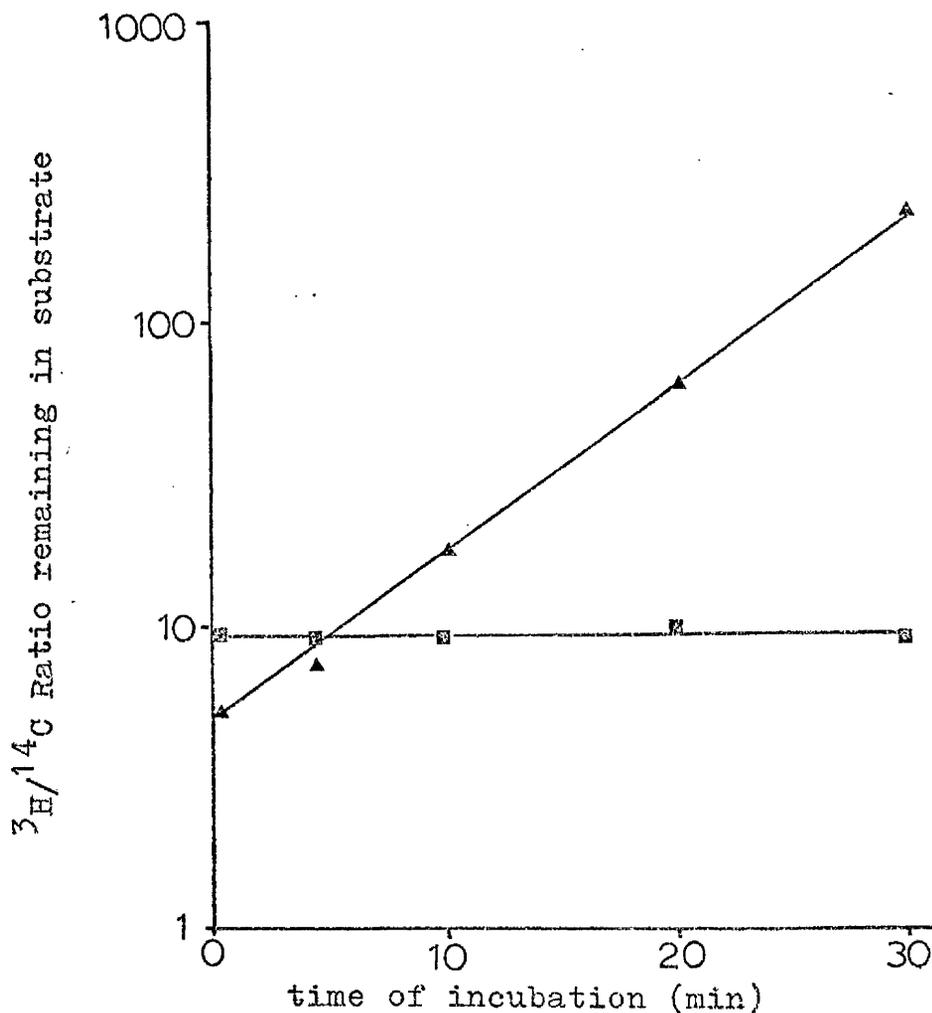
- (a) $[1,2-^3\text{H}]$ -testosterone (10^5 cpm), $[4-^{14}\text{C}]$ -testosterone (10^4 cpm), unlabelled testosterone ($200\ \mu\text{g}$).
- (b) $[17\alpha-^3\text{H}]$ -testosterone (5×10^4 cpm), $[4-^{14}\text{C}]$ -testosterone (10^4 cpm), unlabelled testosterone ($200\ \mu\text{g}$).

These substrate mixtures were simultaneously oxidised at 20°C using a standard concentration of chromic acid. The reaction was stopped at intervals over the time period 0-30min. and at each time point the reaction products were extracted and examined by TLC. The $^3\text{H}/^{14}\text{C}$ ratio of the testosterone band was assessed at each time point and a time-course plotted for the oxidation of both substrate mixtures.

Figure V-4 reveals that for mixture (a) containing $[1,2-^3\text{H}]$ -testosterone the $^3\text{H}/^{14}\text{C}$ ratio does not change with time even though at 30min. less than 1% of the original testosterone remains. Hence the rates of oxidation of $[1,2-^3\text{H}]$ -testosterone and $[4-^{14}\text{C}]$ -testosterone are identical. By contrast the $^3\text{H}/^{14}\text{C}$ ratio for mixture (b) increases markedly as a function of time and by consideration of the slope of the line and the percentage conversion of $[4-^{14}\text{C}]$ -testosterone it is apparent that

Fig.V - 4.

Time Course of Chromic Acid Oxidation of
Testosterone Isotopes.



- - substrate (a) $[1,2-^3\text{H}]$ -testosterone + $[4-^{14}\text{C}]$ -testosterone
- ▲ - substrate (b) $[17\alpha-^3\text{H}]$ -testosterone + $[4-^{14}\text{C}]$ -testosterone

Testosterone substrate mixtures (200 μg) were oxidised with chromic acid as described on page 57, for 0-30min. The $^3\text{H}/^{14}\text{C}$ ratio of the remaining testosterone was determined at each time point.

[17 α -³H]-testosterone is oxidised only 15% as quickly as the carbon labelled species.

Acetylation of the eluted testosterone bands followed by TLC resulted in no change in any of the ³H/¹⁴C ratios.

F. The Enzymic Metabolism of [17 α -³H]-Testosterone.

The demonstration of an isotope effect in the chemical oxidation of [17 α -³H]-testosterone raises the question whether a similar effect can be observed in a biological system. Hence the metabolism of [17 α -³H]-testosterone by two different enzyme systems was examined. In the first system [17 α -³H]-testosterone was oxidised in a manner analagous to the chromic acid technique by the 17 β -hydroxysteroid dehydrogenase of Pseudomonas testosteroni. In the second system [17 α -³H]-testosterone was used as substrate for the 5 α -reductase enzyme of human hyperplastic prostate.

(i) Metabolism of [17 α -³H]-testosterone by the 17 β -hydroxysteroid dehydrogenase of Pseudomonas testosteroni.

Prior to examining the time course of testosterone metabolism by 17 β -hydroxysteroid dehydrogenase it was necessary to optimise the concentration of enzyme used. Accordingly enzyme dilutions over the range ¹/100 - ¹/10,000 (0.5ml)

were incubated with [4-¹⁴C]-testosterone (50ng, 10⁵cpm) for 10min at 37°C in 1.5ml pyrophosphate buffer containing NAD (0.5mM). The products of the incubation were extracted and chromatographed on silica gel TLC plates using solvent system 2. The percentage conversion of testosterone into 4-androstene-3,17-dione was assessed for each dilution of enzyme and it is apparent from Table V-2 that a 1/4,000 dilution of the extract of Pseudomonas testosteronei yielded an optimal conversion of 30%.

Using an enzyme dilution of 1/4,000, a time course was carried out, of testosterone oxidation by 17β-hydroxysteroid dehydrogenase.

Two testosterone substrates were employed:

- (a) [4-¹⁴C]-testosterone(10⁴cpm) +
[1,2-³H]-testosterone(6x10⁴cpm) - 50ng/tube
- (b) [4-¹⁴C]-testosterone(10⁴cpm) +
[17α-³H]-testosterone(9x10⁴cpm) - 50ng/tube

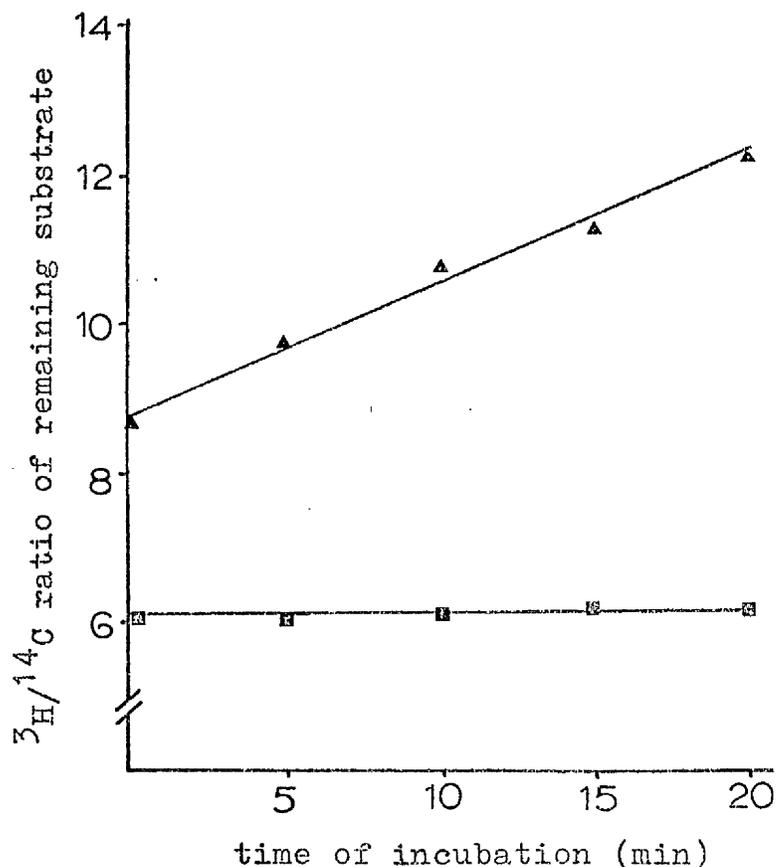
and the extent of metabolism assessed over the period 0 - 20min. The ³H/¹⁴C ratio of the testosterone fraction was determined at each time point. It can be seen from Figure V-5 that the ³H/¹⁴C ratio of substrate (a) was independent of time indicating that [4-¹⁴C]-testosterone and [1,2-³H]-testosterone are metabolised at the same rate, whilst the increase in ³H/¹⁴C ratio of substrate (b) must be interpreted as a resistance to oxidation of [17α-³H]-testosterone.

Table V - 2.Metabolism of Testosterone by Different EnzymeConcentrations of the 17 β -HydroxysteroidDehydrogenase of Pseudomonas Testosteroni.

| <u>Enzyme</u> <u>Dilution</u> | <u>Percentage Conversion of</u> <u>[4-¹⁴C]-testosterone into</u> <u>4-androstene-3,17-dione</u> |
|----------------------------------|--|
| 1/100 | 99 |
| 1/1000 | 90 |
| 1/4000 | 30 |
| 1/10,000 | 5 |

Fig.V - 5.

Time Course of Oxidation of Testosterone Isotopes
by the 17β -Hydroxy-Steroid Dehydrogenase from
P. Testosteroni.



- - substrate (a) $[1,2-^3\text{H}]$ -testosterone +
 $[4-^{14}\text{C}]$ -testosterone
- ▲ - substrate (b) $[17\alpha-^3\text{H}]$ -testosterone +
 $[4-^{14}\text{C}]$ -testosterone

Testosterone substrate mixtures (50ng) were oxidised using the 17β -hydroxysteroid dehydrogenase from *P.testosteroni* as described on page 65. The $^3\text{H}/^{14}\text{C}$ ratio of the remaining substrate was determined following 0-20min incubations.

Consequently, the isotope effect has been confirmed in an enzymic oxidation as well as in a chemical oxidation. The $[4-^{14}\text{C}]$ -testosterone was metabolised approximately twice as rapidly as the $[17\alpha-^3\text{H}]$ -testosterone.

(ii) Metabolism of $[17\alpha-^3\text{H}]$ -testosterone by human prostatic testosterone 5α -reductase.

By analogy with the 17β -hydroxysteroid dehydrogenase experiment a time course of testosterone metabolism was performed with homogenates of human hyperplastic prostate tissue. The same two testosterone substrates (a) and (b) (25ng/tube) were incubated with a 5% homogenate of prostate (0.5ml) in the presence of 0.5mM NADPH over a time period of 0-30min. The reaction was terminated and the products extracted and separated according to the standard assay procedure described in Chapter II. The $^3\text{H}/^{14}\text{C}$ ratio was determined at each time point both for the testosterone substrate and the DHT product. The results in Table V-3 demonstrate that the $^3\text{H}/^{14}\text{C}$ ratios of both testosterone and DHT are constant as a function of time both for substrate (a) and for substrate (b). Clearly $[17\alpha-^3\text{H}]$ -testosterone can be reduced as rapidly as either $[4-^{14}\text{C}]$ -testosterone or $[1,2-^3\text{H}]$ -testosterone.

Table V - 3

Time Course of Testosterone Metabolism by the
5 α -Reductase of Human Hyperplastic Prostate Tissue.

| Time of Incubation (min) | Isotopes in Substrate | $^3\text{H}/^{14}\text{C}$ Ratio | |
|--------------------------|---|----------------------------------|------|
| | | T | DHT |
| 0 | (a) [1,2- ^3H] + [4- ^{14}C] | 13.4 | - |
| 5 | " | 13.4 | 10.2 |
| 10 | " | 14.2 | 11.2 |
| 20 | " | 14.4 | 10.5 |
| 30 | " | 13.5 | 10.4 |
| 0 | (b) [17 α - ^3H] + [4- ^{14}C] | 12.0 | - |
| 5 | " | 12.1 | 11.8 |
| 10 | " | 12.7 | 12.0 |
| 20 | " | 12.6 | 12.4 |
| 30 | " | 12.4 | 12.5 |

3. DISCUSSION.

It is clear from the experiments described in this chapter that $[17\alpha\text{-}^3\text{H}]$ -testosterone is less readily oxidised than $[1,2\text{-}^3\text{H}]$ -testosterone and that the resistance to oxidation can be observed in both chemical and biological systems. By contrast the 5α -reduction of $[17\alpha\text{-}^3\text{H}]$ -testosterone proceeds at the same rate as that of $[1,2\text{-}^3\text{H}]$ -testosterone.

The cause of the impaired oxidation of $[17\alpha\text{-}^3\text{H}]$ -testosterone would seem to be due to the tritium atom itself, since the substrate was shown to be free of contaminants and the tritium atom was clearly located only in the 17α -position. The larger tritium atom results in a higher activation energy of the carbon-tritium bond than the carbon-hydrogen bond and thus renders it more resistant to breakage. In the biological system it is also possible that the tritium atom interferes with the normal binding of the substrate to the active site of the 17β -hydroxysteroid dehydrogenase. Such an interference in binding would exhibit itself as a reduction in reaction rate.

Whilst the experiments described were not specifically designed to quantitate the relative rates of oxidation of 17α -protium and 17α -tritium atoms an estimate of the $k^{\text{H}}/k^{\text{T}}$ could be obtained. For the chromic acid oxidation the $k^{\text{H}}/k^{\text{T}}$ was

approximately 7 whilst for the 17β -hydroxysteroid dehydrogenase reaction the k_H/k_T was approximately 2. The magnitude of the ratio in the chromic acid reaction agrees well with those published for the oxidation of $[3\beta-^2\text{H}]-5\alpha$ -cholestan- 3α -ol (ElieI et al., 1965) and of $[11\alpha-^2\text{H}]-5\alpha$ -pregnane- 11β -ol (Roček et al., 1962) by the same reagents. The smaller effect in the enzyme catalysed oxidation may be explained by the fact that the enzyme lowers the activation energy necessary for the reaction and stabilises the transition state, thus making the differences in bond energy of less importance in determining the overall reaction rate.

The isotope effects described for $[17\alpha-^3\text{H}]-$ testosterone are presumably equally applicable to the oxidation at carbon atom 17 of $[17\alpha-^3\text{H}]-\text{DHT}$ and it seems most likely therefore, that the initial observation of $[^3\text{H}]-\text{DHT}$ remaining following chromic acid oxidation can be attributed to the presence of $[17\alpha-^3\text{H}]-\text{DHT}$ which was more resistant to oxidation than either the $[1,2-^3\text{H}]-\text{DHT}$ or the $[4-^{14}\text{C}]-\text{DHT}$.

The apparent absence of an isotope effect in the 5α -reduction of the tritiated testosterone species was to be expected, since the tritium atoms of both substrates tested were remote from the region of the molecule being reduced, and thus unlikely to play any part in the reaction itself. Under these

circumstances any isotope effect could only be of the secondary type and hence of minimal importance.

The implications of the observed isotope effect need careful consideration. In the superfusion system being validated the isotope effect is unlikely to bring about any significant discrepancies. In the prostatic tissue itself the predominant route of testosterone metabolism is via the 5α -reductase enzyme. Beastall, (1975) and Malathi & Gurpide (1977, b) have estimated that in the superfusion system the reductive pathway is greater than 50 times as active as the 17β -hydroxysteroid dehydrogenase pathway. Consequently, small differences in the minor transformation of testosterone into 4-androstene-3, 17-dione are unlikely to affect the calculation of the parameters used in the study. Clearly an entirely different picture would be produced in a biological system having considerable 17β -hydroxysteroid dehydrogenase activity, and it would be completely invalid to use $[17\alpha\text{-}^3\text{H}]\text{-testosterone}$ in any quantitative study of testosterone metabolism in such a tissue.

The isotope effect first presented itself during the work up procedure following superfusion. However, the presence of the isotope effect in the chromic acid oxidation of $[17\alpha\text{-}^3\text{H}]\text{-DHT}$ does not alter the values of the derived parameters since the

oxidation step was included specifically to remove all tritium atoms from the 17 α position and it does not matter whether this removal is achieved by oxidation or by a lack of reaction since only the radioactivity associated with the 5 α -androstane-3, 17-dione product is used in subsequent calculations.

The observation of an isotope effect in such a simple metabolic study stresses the need for validation of the system being used. It is commonplace to assume that tritiated precursors behave in an identical manner to their unlabelled counterparts, but it is very rare for investigators to take the trouble to show that this is the case. In this particular study sufficient experimentation was performed to establish that an isotope effect existed, but further investigation of the effect was considered beyond the scope of the project.

CHAPTER VI.

CHAPTER VI.THE INTERACTION OF PROGESTERONE
AND RELATED COMPOUNDS WITH
PROSTATIC TESTOSTERONE 5 α -REDUCTASE.1. INTRODUCTION.

Antiandrogens have figured prominently in the pharmacological approach to the treatment of benign prostatic hyperplasia and prostatic carcinoma. Thus, the following steroids have all been claimed to relieve, at least partially, some of the symptoms of these common conditions; cyproterone acetate (Geller et al., 1975; Scott & Wade, 1969), medrogestone (Rangno et al., 1971; Paulson & Kane, 1975), flutamide [SCH 13521] (Caine et al., 1975), 17 α -hydroxyprogesterone caproate and chloramidone acetate (Geller et al., 1969), spironolactone (Castro et al., 1971) and estrogens (Wendel et al., 1972).

Whilst the mode of action of these substances has not always been established it is generally accepted that they act at one or more of three possible levels; the hypothalamic-pituitary axis to reduce gonadotrophic production, the testes to

prevent androgen production, and the prostate to prevent androgen metabolism and action. Thus both estrogens (Sherins & Janick, 1976) and medrogestone (6,17-dimethylpregna-4,6-diene-3,20-dione) (Paulson & Kane, 1975) have been shown to reduce circulating FSH and LH levels, medrogestone has been demonstrated to reduce testosterone production in the testes (Paulson & Kane, 1975) and many of the active agents are thought to act within the prostate either to reduce testosterone metabolism or to prevent DHT binding to the receptor (King & Mainwaring, 1974).

Significantly, many of the agents thought to be acting within the prostate itself also give rise to progestational side effects such as impotence and gynaecomastia, and it is not surprising to discover that many of these steroids are structurally closely related to progesterone. The use of such substances as pharmacological agents raises the question of a possible role for progesterone and related compounds in the aetiology of benign prostatic hyperplasia since such steroids are part of the normal spectrum of hormones found in adult plasma and changes either in their production or metabolic clearance rates, as a function of age, could well alter the intracellular concentrations of key steroid metabolites within the prostate gland.

Progesterone, itself, has been shown to inhibit

the conversion of testosterone to DHT in the human prostate gland (Jenkins & McCaffery, 1974; Tan et al., 1974; Morfin et al., 1975) and has been suggested for use as an antiandrogen (Mauvais-Jarvis, 1974). However, whilst progesterone has been shown to be an inhibitor of the 5 α -reductase, little information is available regarding the mechanism of the progesterone inhibition in the human gland, or the structural components of the progesterone molecule necessary for interaction with the enzyme. Hence it was proposed that a study of this type should be undertaken, using the previously described assay system to test the kinetics of inhibition and the relative potencies of several structurally related and naturally occurring steroids.

In vitro effects cannot be directly extrapolated with confidence into the in vivo situation, and it was decided therefore, that the progestin with the most potent 5 α -reductase inhibiting properties should be investigated further, using an experimental design which produces conditions more closely resembling those encountered in vivo. Hence a study of the entry, metabolism, retention and release of progesterone from prostatic tissue was undertaken using an in vitro superfusion system in the hope that it would provide useful information about the interaction of potential inhibitors of the prostatic

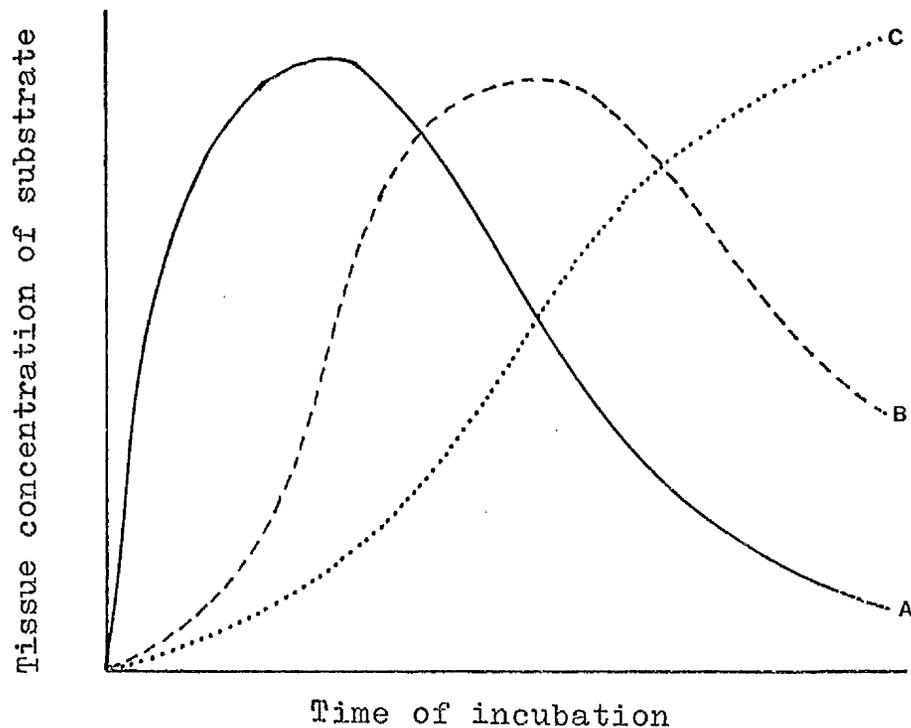
testosterone 5 α -reductase.

Many of the details specific to the superfusion of the human prostate gland have already been described in full elsewhere (Gurpide & Welch, 1969; Giorgi et al., 1971, 1973). Superfusion offers a major advantage over static incubation methods for studying steroid metabolism, for with the former technique it is possible to work under steady state conditions, both for the substrate and for the various products. This may be explained by reference to Fig.VI - 1, which considers the time courses of the simple metabolic reaction $A \rightarrow B \rightarrow C$ when studied by a static incubation (a) and by tissue superfusion (b). In the static incubation the tissue takes up the substrate A and converts it to B which in turn is converted to C. As there is only a finite amount of substrate A the tissue concentration of A reaches a peak before falling again, to zero in the case of complete metabolism. The time course for B is similar to that of A, the time of the peak being later, whilst that of C rises progressively until in the case of complete metabolism it accounts for the total product level. During superfusion on the other hand the substrate A in the tissue is being constantly replaced from the medium and a situation will eventually be reached where a steady state exists between the entry of A into the tissue and

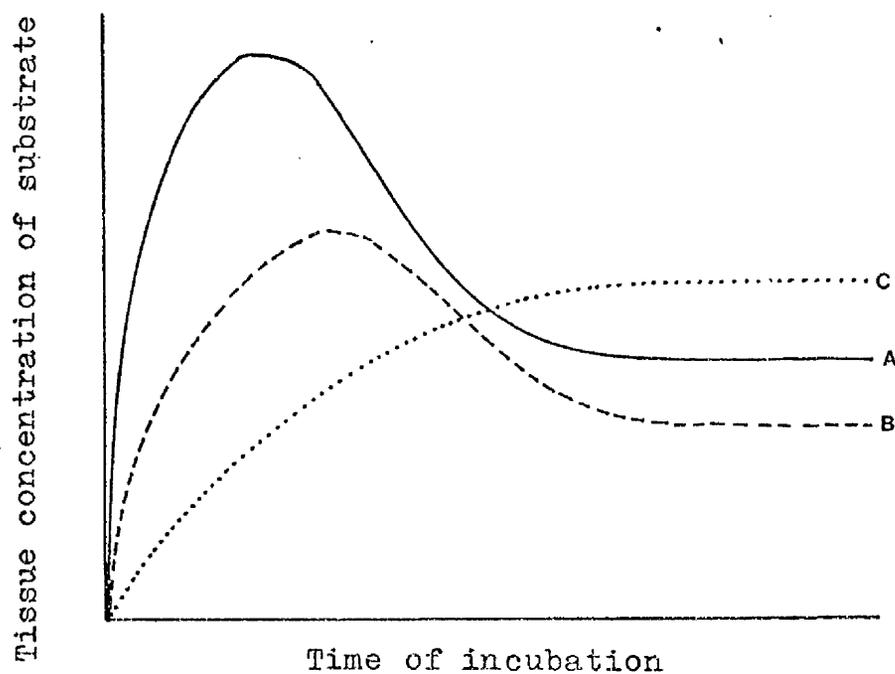
Fig.VI - 1

Time Courses for the Reaction $A \rightarrow B \rightarrow C$ when Studied
by Static Incubation and Tissue Superfusion.

(a) Static Incubation.



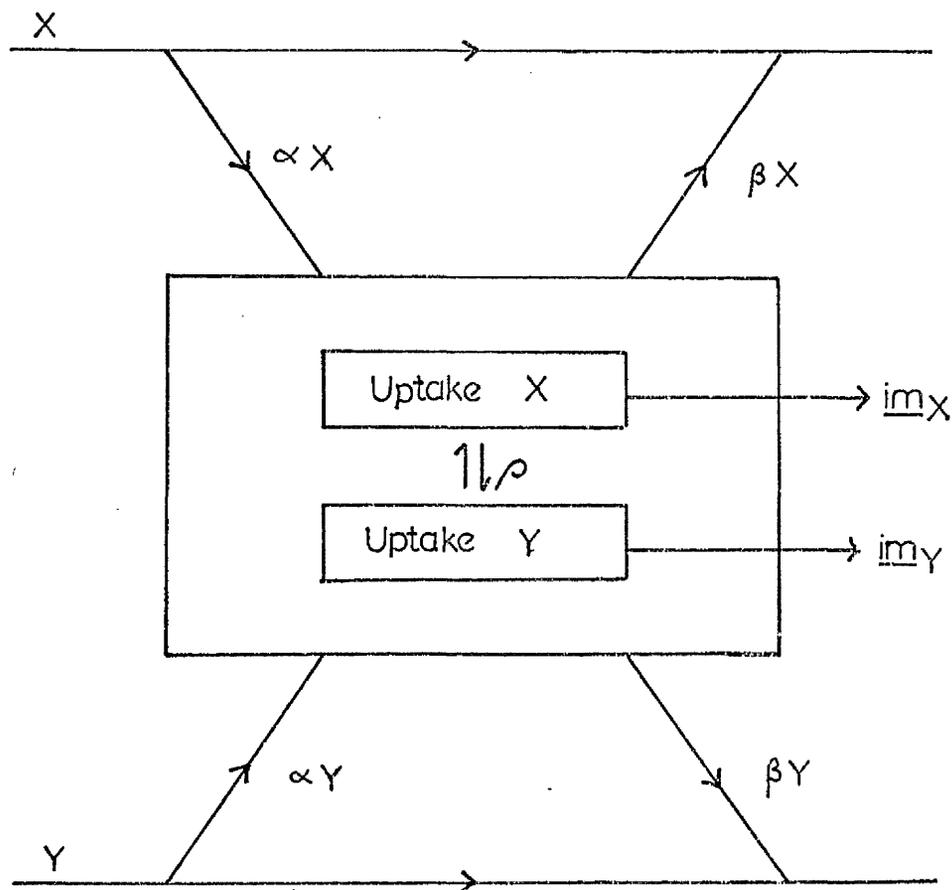
(b) Tissue Superfusion.



the metabolism and release of A. A similar argument can be applied to B and C and thus it is theoretically possible to achieve a time point when A, B and C are all at the steady state.

In vitro superfusion techniques may be used for the simultaneous measurement of the entry of steroids into the tissue, the metabolism of steroids within the tissue, the release of the steroids and their metabolites back into the medium, and the retention of the steroids and their metabolites within the tissue. This is illustrated in Fig.VI - 2. Two different but metabolically related steroids may be superfused simultaneously, and it is apparent that the maximum information can only be obtained from the system if there is some way of distinguishing those steroids derived from X from those produced from Y. The simplest way for this to be achieved would be by using different radioactive labels for X and Y, such as [^3H]-X and [^{14}C]-Y. However, because of the difficulty in synthesising [^{14}C]-labelled steroids with high specific radioactivity this would necessitate the undesirable use of steroid concentrations in excess of those encountered physiologically. This problem has been overcome by the use of two different [^3H]-labelled steroids, one labelled conventionally on carbon positions 1 and 2, and the other with the tritium

Fig.VI - 2.

Parameters Measured by Superfusion.

- α = entry of steroids into tissue.
 β = release of steroids from tissue.
 \underline{im} = irreversible metabolites of steroids.
 ρ = metabolism of $X \rightarrow Y$ or $Y \rightarrow X$

introduced at carbon atom 17. For studies with testosterone and related steroids the latter tritium atom can be removed by a simple chemical oxidation step, thus enabling the source of the steroid under study to be elucidated.

The existence of the steady state during superfusion offers the advantage that the mathematics of the system are much simplified and thus also the quantitation of the previously mentioned parameters.

2. RESULTS.

A. The Inhibition of Testosterone 5 α -Reductase by Progesterone and Other C21 Steroids.

Several groups of workers have shown that testosterone 5 α -reductase can be inhibited by steroids related to progesterone (Frederiksen & Wilson, 1971; Tan et al., 1974). However no systematic study has been performed for human prostatic testosterone 5 α -reductase and no attempt has been made to correlate the structural features of a potential inhibitor with its actual effectiveness.

Accordingly the testosterone 5 α -reductase activity of human hyperplastic prostate was determined by the standard method in the absence of added inhibitor and in the presence of up to 2 μ moles/litre of the steroids listed in Fig.VI - 3. The results of a single typical experiment are recorded in Fig.VI - 4. It is apparent that progesterone itself was the most potent inhibitor of testosterone 5 α -reductase and the inhibitory capacity of the added steroid decreased with the number of hydroxyl groups attached to the steroid nucleus. Moreover, the position of the additional hydroxyl group also exerts a profound effect upon the ability of the steroid to inhibit testosterone 5 α -reductase, with the 11 β -hydroxyl having a greater effect than the 17 α -hydroxyl group which in turn had a greater effect

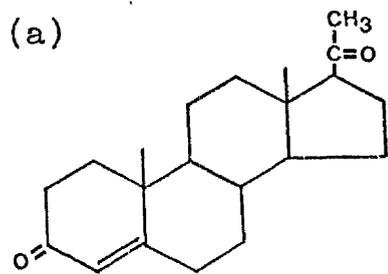
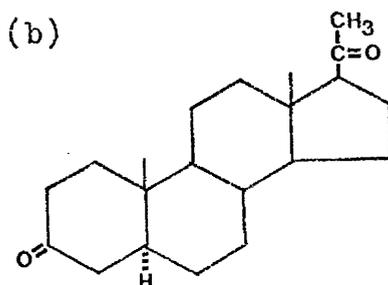
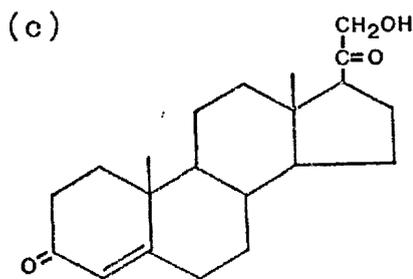
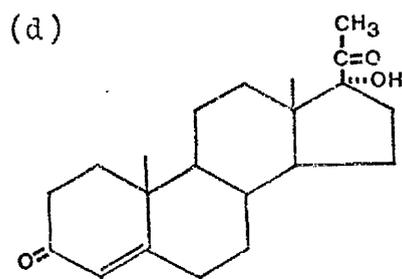
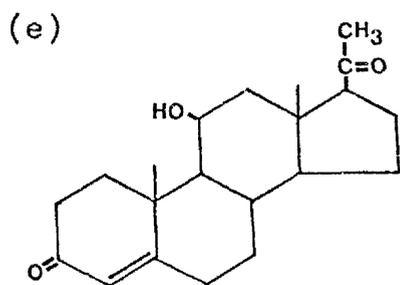
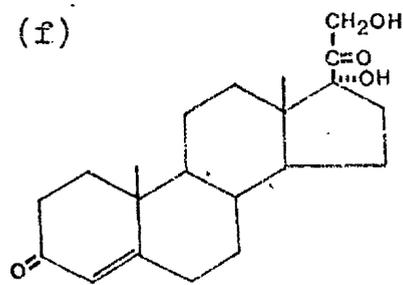
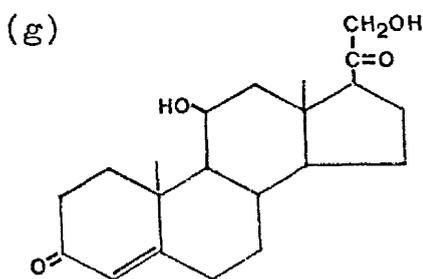
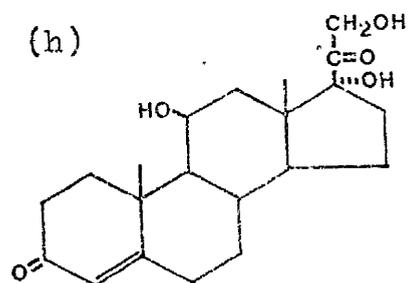
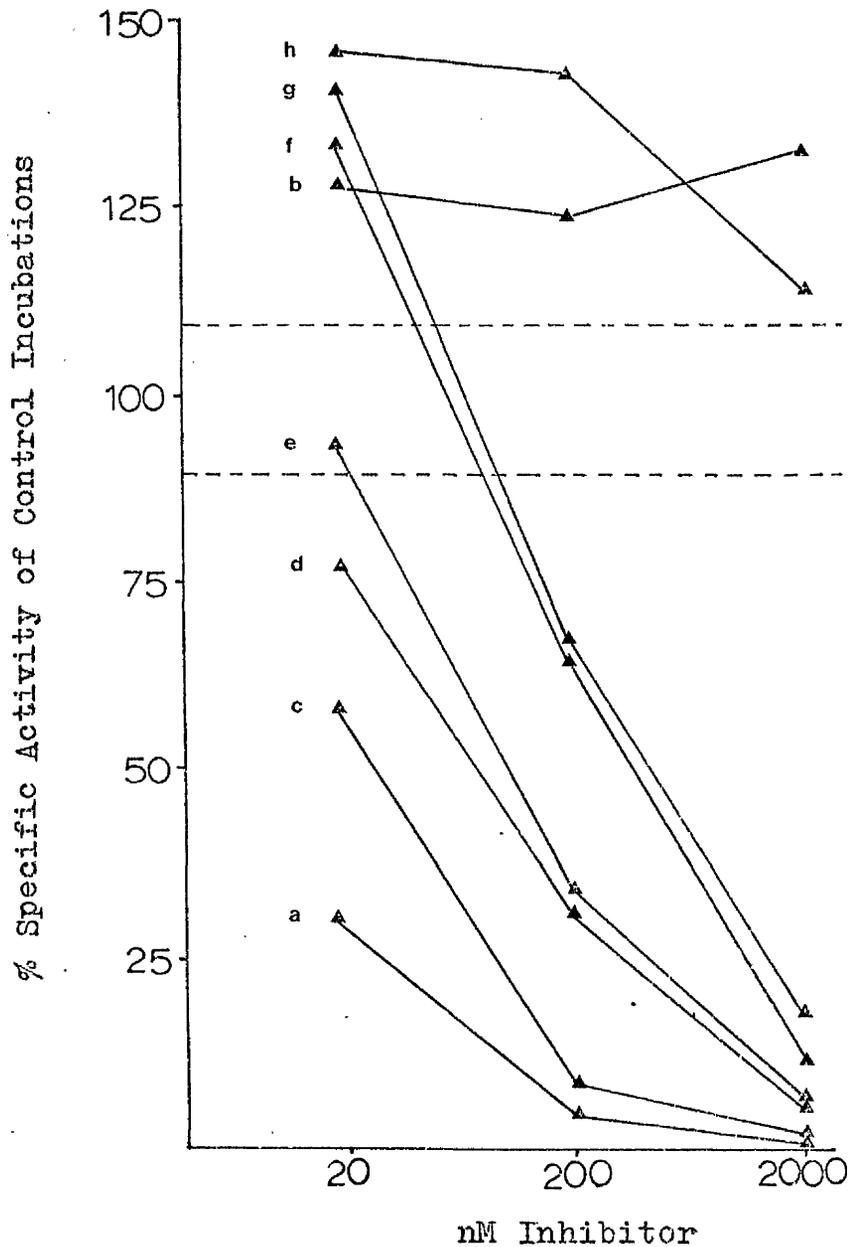
Fig. VI -- 3.Progestins Examined as Possible Inhibitors of the
Testosterone 5 α -Reductase Activity of Human Prostate.4-pregnene-3,20-dione
(progesterone)5 α -pregnane-3,20-dione21-hydroxy-4-pregnene-
3,20-dione17 α -hydroxy-4-pregnene-
3,20-dione11 β -hydroxy-4-pregnene-
3,20-dione17 α ,21-dihydroxy-4-pregnene-
3,20-dione11 β ,21-dihydroxy-4-pregnene-
3,20-dione11 β ,17 α ,21-trihydroxy-4-pregnene-
3,20-dione

Fig.VI - 4.

The Inhibition of Prostatic Testosterone 5 α -Reductase
by Naturally Occurring Progestins.



Key: Inhibitors were added in the concentrations indicated. Identification of inhibition a \rightarrow h is given in Fig.VI - 3 on the facing page. Dotted lines denote one standard deviation of the control incubations. The standard homogenate assay (p 62) with 4.3nM testosterone substrate was used.

than the 21-hydroxy group. Neither cortisol (11 β ,17 α , 21-trihydroxy-progesterone) nor the 5 α -reduced product of progesterone (5 α -pregnane-3,20-dione) had any significant inhibitory effect on testosterone 5 α -reductase activity.

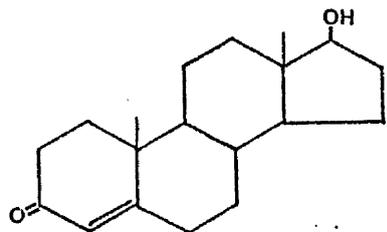
B. The Inhibition of Testosterone 5 α -Reductase by Naturally Occurring Androgens.

In view of the observed marked inhibition of testosterone 5 α -reductase by progesterone, it was considered important to compare these results with the effects of adding androgens, at similar concentrations, to the system. Accordingly the androgens listed in Fig.IV - 5 were assessed for their ability to inhibit the prostatic testosterone 5 α -reductase preparation. For comparative purposes testosterone itself was included, the concentration of additional testosterone being treated separately from that of the radioactive substrate. It is apparent from Fig.VI - 6 that the effect of the added testosterone was similar to that of progesterone at equimolar concentrations although when compared in a single experiment the progesterone was a marginally more potent inhibitor.

The presence of a ketone group on carbon atom 17 in 4-androstene-3,17-dione rather than the 17 hydroxyl group in testosterone only marginally

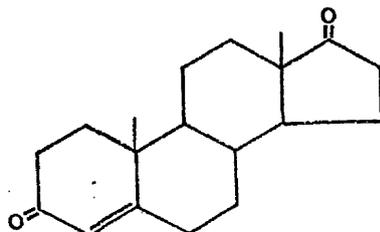
Fig. VI - 5Androgens Examined as Possible Inhibitors of the
Testosterone 5 α -Reductase Activity of Human Prostate.

(a)



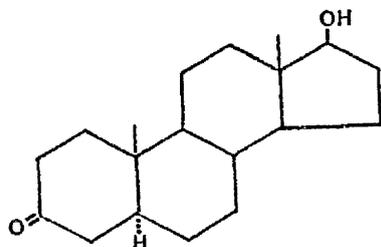
17 β -hydroxy-4-
androsten-3-one.
(Testosterone)

(b)



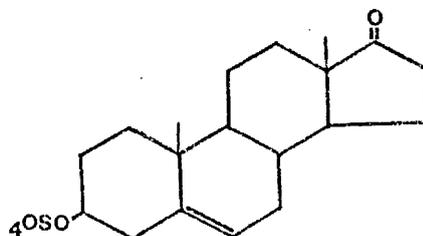
4-androstene-3,17-
dione

(c)



17 β -hydroxy-5 α -
androstan-3-one
(DHT)

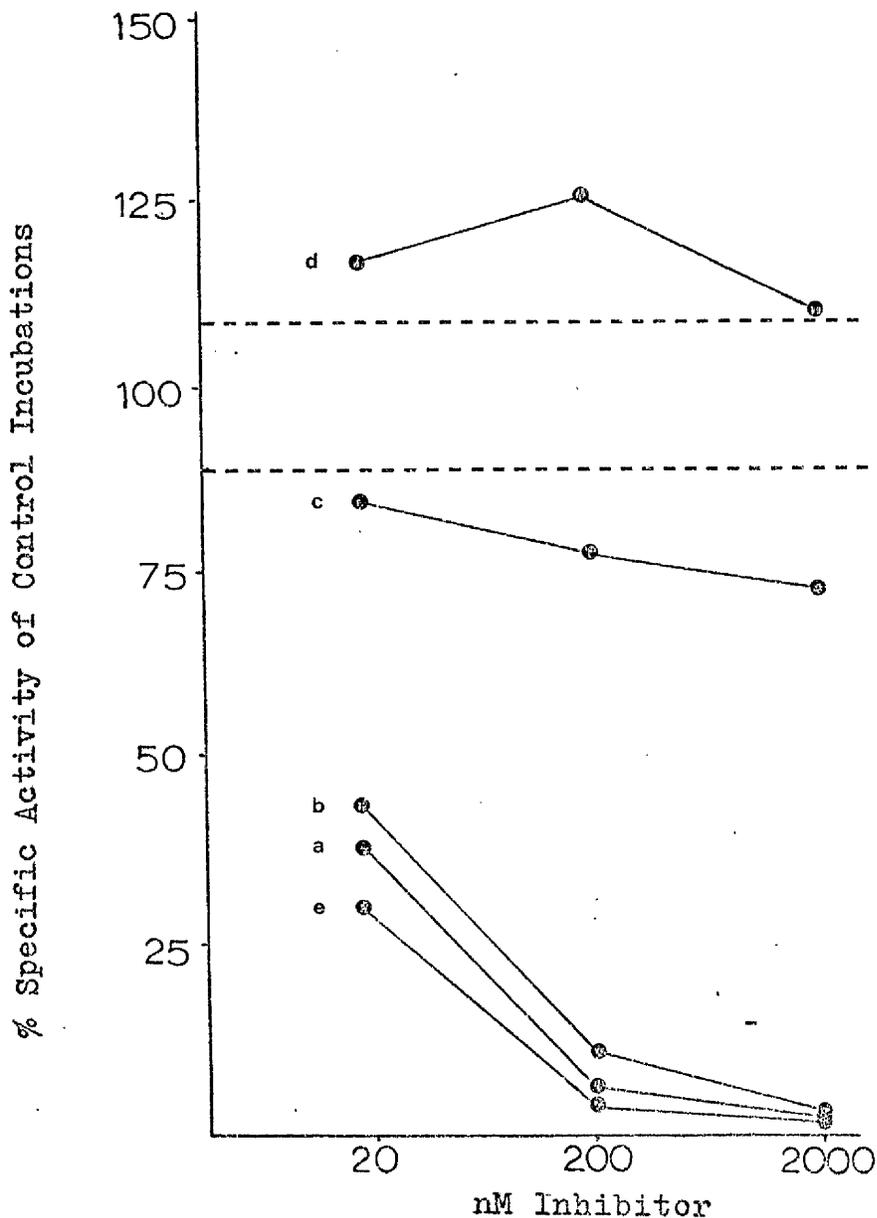
(d)



3 β -hydroxy-5-
androsten-17-one
sulphate

Fig.VI - 6.

The Inhibition of the 5 α -Reduction of [1,2- 3 H]-Testosterone
by Naturally Occurring Androgens.



Key. Inhibitors were added in the concentrations indicated. Identification of a→d is given in Fig.VI - 5 on facing page, and e refers to progesterone. Dotted line denotes one standard deviation of the control incubations. The standard homogenate assay (p 62) with 4.3nM testosterone substrate was used.

reduced the inhibitory properties of the steroid which is not unexpected as 4-androstene-3,17-dione is also a substrate for the rat ventral prostatic enzyme (Frederiksen & Wilson, 1971). Compared to testosterone and 4-androstene-3,17-dione, DHT does not have a great effect, indicating that product inhibition is probably of little importance in this enzyme system. The effect of DHT is similar to that of 5 α -pregnane-3,20-dione (Fig.VI - 4) and the absence of the 4-5 double bond presumably reduces the possible interaction of the steroid with the enzyme system.

It has been suggested that steroids of adrenal origin may play a part in the control of prostatic function (Harper et al., 1974), and for this reason dehydroepiandrosterone sulphate (DHAS) was also considered for possible inhibitory actions. The lack of effect over the concentration range studied (Fig.VI - 6) may possibly be explained either by the presence of the bulky sulphate group at carbon atom 3 or by the 5-6 double bond.

C. The Kinetics of Progesterone Inhibition of Testosterone 5 α -Reductase.

The preceding experiments have shown that of the compounds studied progesterone is the most effective inhibitor of the testosterone 5 α -reductase of human

hyperplastic prostate and for this reason it was chosen for further study. The first requirement was to confirm the type of kinetics exhibited by the enzyme when subjected to progesterone inhibition of testosterone metabolism.

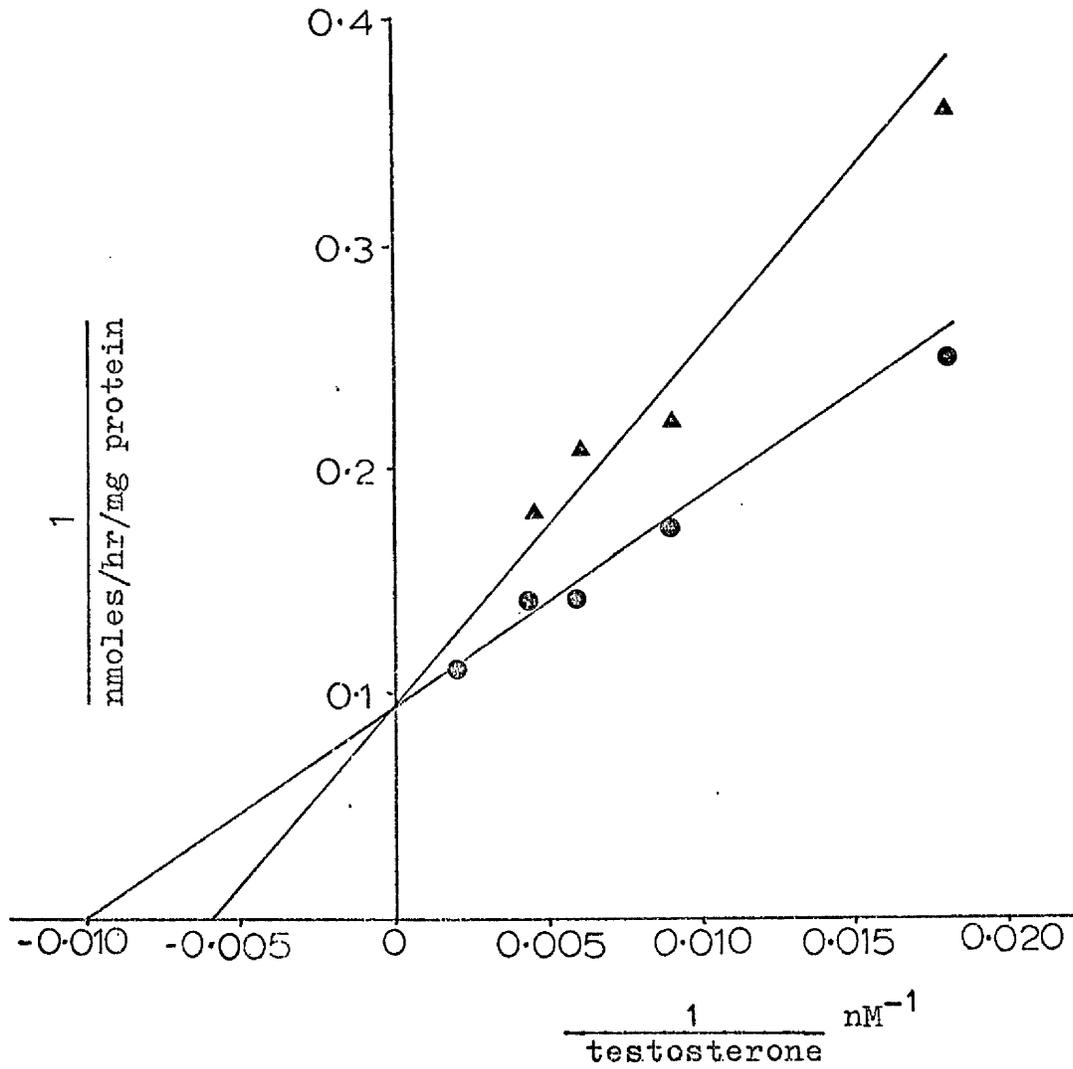
A microsomal enzyme preparation (Chapter II) was incubated with increasing levels of testosterone in the presence and absence of 40nM progesterone and the results obtained were analysed by double reciprocal plots (Michaelis & Menten, 1913). It is apparent from Fig.VI - 7 that V_{max} is not altered by the addition of progesterone, whereas K_m is increased, indicating that the inhibition is of the competitive type with respect to substrate. The following parameters were determined from the double reciprocal plots:-

| | | |
|-----------|---|--------------------------|
| V_{max} | - | 1.25 nmoles/hr/mgprotein |
| K_m | - | $10^{-7}M$ |
| K_{mi} | - | $1.67 \times 10^{-7}M$ |

D. The Effect of Progesterone on Testosterone Metabolism in Superfused Human Prostatic Tissue.
In vitro incubations of the testosterone 5α -reductase enzyme preparations with progesterone have shown this compound to be a potent competitive inhibitor of the enzyme under the conditions used. However, extrapolation to the in vivo situation must

Fig.VI - 7.

The Inhibition, by Progesterone, of Testosterone
5 α -Reductase from a Prostatic Microsome Preparation.



Key: ● = no added progesterone
 ▲ = + 40nM progesterone

be treated with great caution, since in the in vitro enzyme preparation, one is far removed from the situation where the physiological parameters of molecular entry into the cell, cellular compartmentation and cofactor balance, play an important part in determining the potency of biological compounds. A closer approximation to the in vivo situation is possible by using tissue culture or tissue superfusion methods. For the enzyme system under examination in this study, in vitro superfusion of thin tissue slices was the method of choice. As previously stated this system allows the simultaneous calculation of entry, metabolism, retention and release of the steroids under study. The validation and calculation of parameters and observations obtained with this system are presented below.

(i) A Time Course for the Uptake of Testosterone, DHT and Progesterone.

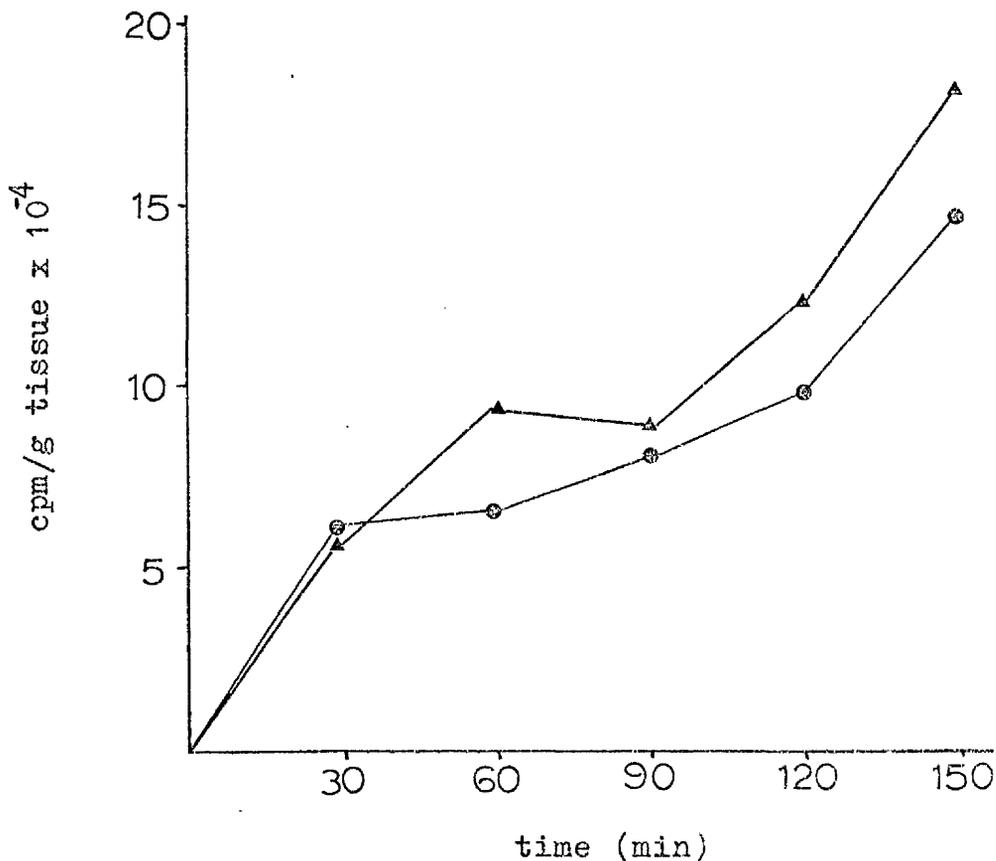
The tissue was prepared as detailed in Chapter II and placed in an open superfusion chamber. For testosterone and DHT uptake studies the tissue was perfused with buffer containing 5,000cpm [17α - ^3H]-testosterone/ml and 5,000cpm [$1,2$ - ^3H]-DHT/ml. Aliquots of tissue were removed at intervals and analysed together with the perfusate and buffer. Radioactivity in the tissue increased until about

60 min whereafter there was a leveling off until 90 - 120min. indicating that the steady state had been reached. After this time the tissue began to deteriorate and radioactivity in the tissue increased again (Fig.VI - 8). When the metabolic products of testosterone were separated, it was apparent that the majority of testosterone taken up by the tissue had been metabolised to DHT.

Progesterone uptake studies were carried out at 5ng/ml and 50ng/ml of steroid. In each case 10,000cpm [1,2,6,7-³H]-progesterone/ml were used together with unlabelled testosterone (0.8ng/ml) and DHT (0.12ng/ml). Repeated experiments indicated that the steady state was approached between 60 - 120min. of superfusion. Typical examples of these superfusions are shown in Fig.VI - 9. It is perhaps worthy of note that human prostatic tissue has a large capacity for the uptake of progesterone since the amount of radioactive steroid in the tissue did not decrease greatly with the increasing concentrations of superfused non-radioactive progesterone.

Having established that the steady state is approached between 60 and 90min. of superfusion for all three steroids, this time period was used in further experiments for the calculation of the parameters of entry, uptake, release and metabolism.

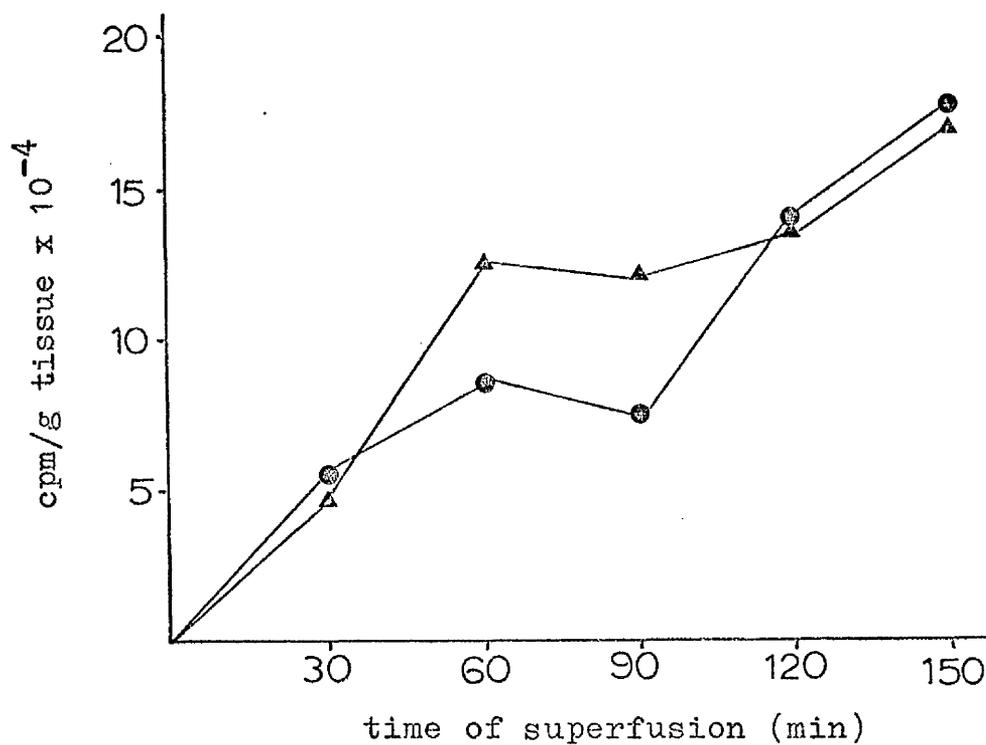
Fig. VI - 8.

Time Course for the Uptake of Testosterone and DHT.

Slices of human prostate were superfused in an open chamber with buffer containing [$^{17}\alpha$ - ^3H]-testosterone (5,000cpm/ml) and [1,2 - ^3H]-DHT (5,000cpm/ml). Aliquots of tissue (100mg) were removed at the time intervals shown and assayed for radioactivity.

- — Prostate A
- ▲ — Prostate B

Fig.VI - 9.

Time Course for the Uptake of Progesterone.

Slices of human prostate were superfused in an open chamber with buffer containing testosterone (0.8ng/ml), DHT (0.12ng/ml) and [1,2,6,7-³H]-progesterone (either 5ng or 50ng/ml). Aliquots of tissue (100mg) were removed at the time intervals shown and assayed for total radioactivity.

- ▲ 5ng/ml Progesterone
- 50ng/ml Progesterone

(ii) The Parameters Calculated from the Superfusion of Testosterone and DHT and the Errors in their Measurement.

(a) Calculation of the parameters.

By measurement of $[17\alpha\text{-}^3\text{H}]$ -testosterone, $[17\alpha\text{-}^3\text{H}]$ -DHT and $[1,2\text{-}^3\text{H}]$ -DHT in the buffer, perfusate and tissue, all the information required for the calculation of the parameters of entry, retention, release and metabolism of these two metabolically related steroids is available. Inclusion of $[^{14}\text{C}]$ -testosterone and $[^{14}\text{C}]$ -DHT before extraction of the above steroids permits the correction for procedural losses to be made. Verification of the model was made by Giorgi et al., (1971, 1974) and is illustrated in Fig.VI - 10.

The basic assumption necessary for the use of a superfusion experiment to calculate the various parameters, is that at the steady state an equal fraction of steroid enters (α) and leaves the tissue. Thus for DHT

$$\alpha_{\text{DHT}} = \frac{im_{\text{DHT}}}{C_{\text{DHT}}} + \beta_{\text{DHT}} \quad \dots\dots (1)$$

where $\frac{im_{\text{DHT}}}{C_{\text{DHT}}}$ is the fraction of steroid leaving as metabolites and β_{DHT} is the fraction of steroid released unchanged from the tissue. The difference in concentration (cpm/ml) between the superfusing buffer and the collected perfusate, divided by the concentration in the superfusing buffer gives us $\frac{im_{\text{DHT}}}{C_{\text{DHT}}}$.

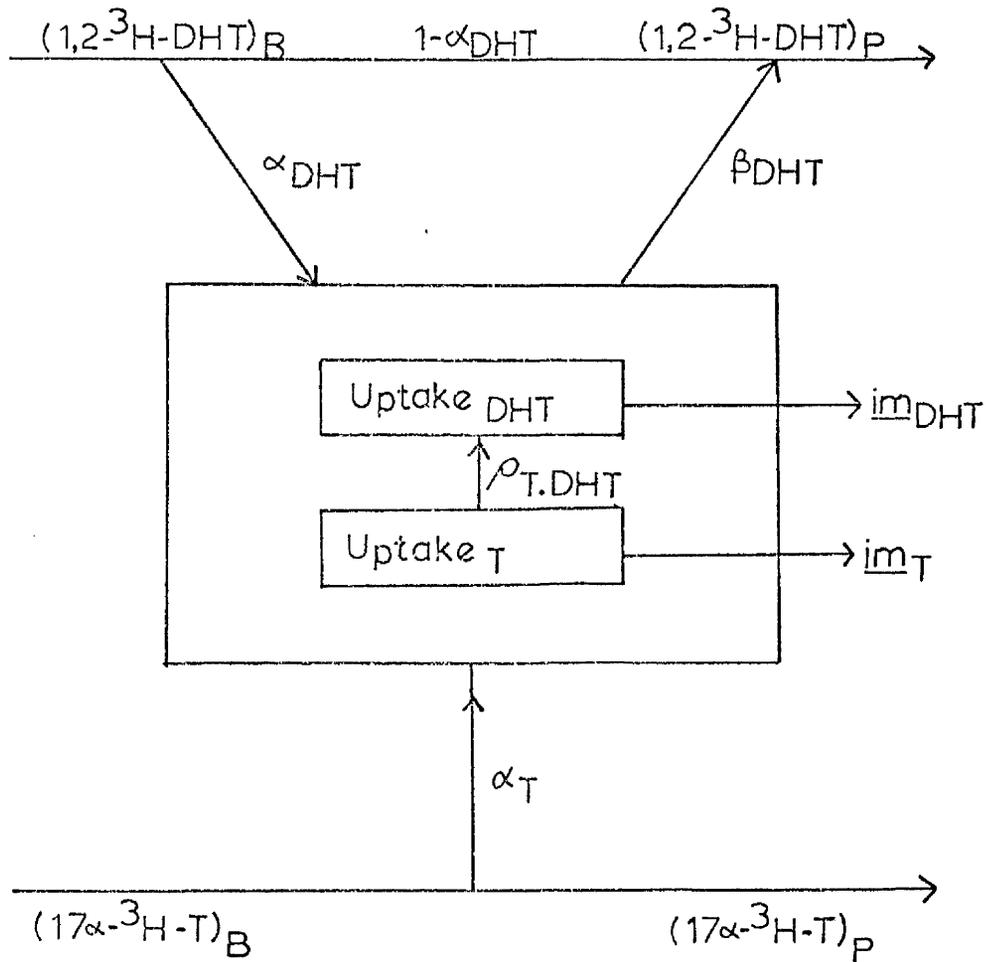
Key:

T and DHT indicate testosterone and 5 α -dihydrotestosterone respectively; brackets () indicate the concentration of steroids in cpm/ml followed by the suffixes B, P and t, which refer to perfusing buffer, collected perfusate and tissue respectively. (It is assumed that 1g tissue \equiv 1ml buffer).

The fraction of superfused steroid entering the tissue is denoted by α ; β is the fraction of superfused steroid released back into the medium and $\rho_{T.DHT}$ is an index of the fraction of testosterone entering the tissue which is converted to DHT, whilst \underline{im} is the fraction of superfused steroid irreversibly metabolised. The concentration of steroid in the tissue is expressed in ng/gtissue, and "uptake" is an expression of the ratio of the steroid within the tissue to that in the superfusing buffer.

Fig.VI - 10.

The Parameters Measured in Human Prostatic Tissue after Superfusion with Testosterone and DHT.



$$\frac{im_{DHT}}{DHT} = \frac{(1,2-^3H-DHT)_B - (1,2-^3H-DHT)_p}{(1,2-^3H-DHT)_B} \dots\dots (2)$$

The testosterone entering the tissue is in part metabolised to DHT, and it is assumed that this will be in a common pool with the DHT entering the tissue from the buffer. Therefore any DHT leaving the tissue will be made up of both $[17\alpha-^3H]$ -DHT and $[1,2-^3H]$ -DHT in the same proportion as that of the tissue. Thus we can also calculate

$$\beta_{DHT} = \frac{\left\{ \frac{(1,2-^3H)_{DHT}}{(17\alpha-^3H)_{DHT}} \right\}_t \times (17\alpha-^3H-DHT)_p}{(1,2-^3H-DHT)_B} \dots\dots (3)$$

This in turn means that α_{DHT} can also be calculated from equation (1).

These calculations can also be applied to testosterone. It has been shown in experiments by Giorgi et al., (1972) that testosterone is not released in measurable amounts from the tissue and it is thus permissible to let $\beta_T = 0$.

The uptake, or ratio of the concentration of steroid in the tissue compared to that in the buffer, is another parameter of interest and is defined by equation (4). It is calculated from the cpm/ml of buffer and cpm/g of tissue, where it is assumed that 1g tissue is equivalent to 1ml buffer.

$$\text{Uptake}_{\text{DHT}} = \frac{(1,2\text{-}^3\text{H-DHT})_t}{(1,2\text{-}^3\text{H-DHT})_B} \dots (4)$$

The same equation applies equally for $[17\alpha\text{-}^3\text{H}]$ -testosterone.

Also of importance is the concentration of steroid derived both from the buffer and intracellular formation which is retained in the tissue

$$\text{retention}_T = \frac{(17\alpha\text{-}^3\text{H-T})_t}{\text{Sp.Act.}[17\alpha\text{-}^3\text{H}]\text{-T}} \dots (5)$$

$$\text{retention}_{\text{DHT}} = \frac{(1,2\text{-}^3\text{H-DHT})_t}{\text{Sp.Act.}[1,2\text{-}^3\text{H}]\text{-DHT}} + \frac{(17\alpha\text{-}^3\text{H-DHT})_t}{\text{Sp.Act.}[17\alpha\text{-}^3\text{H}]\text{-T}} \dots (6)$$

where Sp.Act. is the specific radioactivity of the superfused steroids (cpm/ng).

When two metabolically related compounds are being superfused, it is of interest to know the fraction of the entering steroid which is being converted to the other.

Thus the conversion factor for testosterone to DHT is

$$f_{T.DHT} = \frac{\alpha_{\text{DHT}} \times \frac{(1,2\text{-}^3\text{H-DHT})_B}{(17\alpha\text{-}^3\text{H-T})_B}}{\alpha_T \times \left[\frac{(1,2\text{-}^3\text{H})_{\text{DHT}}}{(17\alpha\text{-}^3\text{H})} \right]_t} \dots (7)$$

Further parameters, such as the rate of formation of metabolites, and intracellular clearance rates can also be calculated, but in the experiments presented here these were not determined.

- (b) The errors involved in measurement of the superfusion parameters.

Macroscopic examination of hyperplastic human prostate reveals a nodular appearance. These nodules vary in size, and have a paler and tougher texture, and may have different biochemical potential than the surrounding tissue. For this reason it was of vital importance to ensure that the division of tissue between the separate superfusion chambers was as systematic as possible. This was achieved most easily by using one petrie dish for each superfusion chamber, and placing the slices into each dish in rotation, before weighing and transferring the slices to the superfusion chamber. In order to make valid comparisons between control and experimental superfusions it is necessary to know the variations obtained between control superfusions themselves. Since a maximum of three chambers only can be superfused at any one time duplicate lines cannot be run during all experiments. It was therefore necessary to perform a specific experiment to test the precision.

Three identical chambers were set up, each containing 500mg of tissue slices, and these were perfused for 90min. with buffer containing $[17\alpha\text{-}^3\text{H}]$ -testosterone and $[1,2\text{-}^3\text{H}]$ -DHT. The 60 -- 90min. perfusate, the tissue and a sample of

buffer from each line were collected and processed as detailed in Chapter II, and the parameters calculated for each chamber. The results of these superfusions are expressed in Table VI- 1a and b.

The coefficient of variation (CV) of each of the steroid fractions from the three separate lines was less than 10% for all except the tissue $[17\alpha-^3\text{H}]$ -testosterone and the perfusate $[17\alpha-^3\text{H}]$ -DHT. The large variation in these two cases can be accounted for by the relatively small levels of radioactivity present in these fractions. This variation is also reflected in the calculated parameters in Table VI-1b and the values of % CV obtained may be used as a basis in deciding the possible significance of effect of additional compounds used. It can be seen in Table VI-1a that there are relatively few counts in the tissue testosterone fraction compared to the amount of $[17\alpha-^3\text{H}]$ -DHT in the tissue, indicating that the majority of the testosterone has been metabolised to DHT. This is reflected in the high value of $\rho_{\text{T.DHT}}$ in Table VI-1b. The entry (α) of testosterone and of DHT is similar. The release (β) of DHT is very small, and contributes in a large part to the error in other calculations which depend upon it, for example α_{DHT} and $\rho_{\text{T.DHT}}$. However, the value of all these parameters are within the range reported by Giorgi et al., (1971, 1973, 1974).

Table VI - 1.

The Errors Involved in the Measurement of the Superfusion Parameters.

(a) The Radioactivity in Separate Assayed Fractions.

| Fraction Assayed | Corrected cpm/g tissue or /ml buffer | | |
|------------------|---|---------------------------|------------------------------------|
| | [17 α - ³ H]-Testosterone | [1,2- ³ H]-DHT | [17 α - ³ H]-DHT |
| Tissue | 2190 \pm 384 (17.6) | 12308 \pm 662 (5.4) | 15274 \pm 1836 (12.0) |
| Perfusate | 6315 \pm 288 (4.6) | 4851 \pm 412 (8.5) | 37 \pm 36 (98) |
| Buffer | 7635 \pm 119 (1.6) | 6117 \pm 366 (6.0) | 0 |

(b) The Calculated Parameters.

| Parameter | Testosterone | DHT |
|----------------|------------------------|-------------------------|
| \bar{u} | | 0.187 \pm 0.069 (37) |
| β | - | 0.005 \pm 0.006 (103) |
| α | 0.172 \pm 0.038 (22) | 0.191 \pm 0.075 (39) |
| C_i/C_o | 0.286 \pm 0.050 (18) | 2.062 \pm 0.111 (5) |
| $(C)_t$ | 0.156 \pm 0.027 (17) | 1.200 \pm 0.129 (11) |
| $\rho_{T.DHT}$ | 1.038 \pm 0.229 (22) | |

Figures in brackets refer to % Coefficient of Variation.

As mentioned previously tissue varies greatly from one prostate to another and this is reflected in a relatively large variation in many of the calculated parameters. The parameters obtained from a series of superfusions with six prostates is shown in Table VI - 2.

(iii) The Effect of Added Progesterone on the Parameters of Testosterone and DHT Metabolism.

Progesterone has been shown to be a potent inhibitor of the testosterone 5 α -reductase, causing almost 50% inhibition of the enzyme at equimolar concentrations to the testosterone substrate. Hence it was decided to examine the effects of superfused progesterone on the parameters of testosterone and DHT metabolism.

In initial experiments 5ngprogesterone/ml buffer was added along with the standard amount of radioactive testosterone and DHT. This is approximately twenty times the concentration of progesterone normally found in male plasma (Vermeulen & Verdonck, 1976) and is almost five times greater than the concentration of testosterone in the superfusion buffer. No consistent significant effect on either testosterone or DHT entry, uptake, metabolism or release was observed during four superfusions at this progesterone concentration (Table VI - 3). Superfusions with further 10 fold increases of

Table VI - 2.

Comparison of Parameters from Six Different Prostates.

| Prostate | α_T | C_i / C_oT | $(CT)_t$ | \ln_{DHT} | β_{DHT} | α_{DHT} | C_i / C_oDHT | $(C_{DHT})_t$ | $\rho_{T.DHT}$ |
|----------|------------|--------------|----------|-------------|---------------|----------------|----------------|---------------|----------------|
| 1 | 0.340 | 0.832 | 0.375 | 0.320 | 0.126 | 0.477 | 5.634 | 3.223 | 1.497 |
| 2 | 0.498 | 0.582 | 0.232 | 0.384 | 0.076 | 0.461 | 7.771 | 6.924 | 0.782 |
| 3 | 0.250 | 0.567 | 0.241 | 0.233 | 0.068 | 0.302 | 3.254 | 1.236 | 0.877 |
| 4 | 0.100 | 0.266 | 0.102 | 0.100 | 0.007 | 0.108 | 1.841 | 1.198 | 1.636 |
| 5 | 0.081 | 0 | 0 | 0.099 | 0.060 | 0.160 | 0.80 | 0.647 | 2.972 |
| 6 | 0.101 | 0.131 | 0.757 | 0.114 | 0.036 | 0.151 | 1.982 | 16.844 | 2.145 |

Table VI - 3.

The Effect of Progesterone at Different Concentrations on the Parameters Obtained by Simultaneous Superfusion with Testosterone and DHT.

| Progesterone Concentration | α_T | C_i/C_{oT} | $(C_T)_t$ | $\rho_{T.DHT}$ |
|----------------------------|------------------|-------------------|------------------|------------------|
| 0 | 0.340 | 0.382 | 0.375 | 1.497 |
| 5ng/ml | 0.286 (-12) | † 1.257 (+29) | † 0.567 (+28) | † 2.262 (+29) |
| 0 | 0.498 | 0.582 | 0.232 | 0.782 |
| 5ng/ml | 0.482 (+2) | 0.669 (+10) | 0.275 (+12) | 0.745 (-3) |
| 0 | 0.081 | 0 | 0 | 2.972 |
| 5ng/ml | * 0.240 (+70) | 0.049 (+) | 0.019 (+) | 2.622 (-9) |
| 0 | 0.250 | 0.567 | 0.241 | 0.877 |
| 5ng/ml | † 0.364 (+26) | 0.700 (+15) | 0.298 (+15) | 0.824 (-4) |
| 50ng/ml | 0.221 (-9) | 0.680 (+13) | 0.289 (+13) | 0.972 (+7) |
| 0 | 0.100 | 0.266 | 0.102 | 1.636 |
| 500ng/ml | 0.086 (-11) | * 0.675 (+61) | * 0.259 (+61) | * 0.709 (-56) |
| 0 | 0.100 | 0.131 | 0.757 | 1.241 |
| 5µg/ml | 0.078 (-16) | * 0.746 (+99) | * 4.30 (+38) | * 0.339 (-80) |
| 5µgT/ml | * 0.029 (-78) | * 0.890 (+105) | * 5.131 (105) | † 0.754 (-34) |
| S.D. of test. | (± 22) | (± 18) | (± 17) | (± 22) |

* values outwith 2 S.D.

† values outwith 1 S.D.

| \bar{im}_{DHT} | β_{DHT} | α_{DHT} | C_i/C_{oDHT} | $(C_{DHT})_t$ |
|------------------|-------------------|------------------|------------------|------------------|
| 0.320 | 0.126 | 0.447 | 5.634 | 3.223 |
| 0.186 (-37) | 0.047 (-65) | † 0.234 (-44) | *4.313 (-19) | *5.628 (+38) |
| 0.384 | 0.076 | 0.461 | 7.771 | 6.924 |
| 0.361 (-4) | 0.061 (-15) | 0.423 (-6) | † 8.757 (+8) | *3.584 (-46) |
| 0.099 | 0.060 | 0.160 | 0.980 | 0.647 |
| 0.304 (+72) | 0.142 (+57) | † 0.446 (+67) | *1.925 (+46) | *1.191 (+42) |
| 0.233 | 0.068 | 0.302 | 3.254 | 1.236 |
| 0.282 (+13) | 0.121 (+40) | 0.404 (+20) | *4.227 (+18) | † 1.633 (+20) |
| 0.225 (-2) | 0.106 (+31) | 0.332 (+7) | † 5.034 (+30) | *1.744 (+24) |
| 0.100 | 0.007 | 0.108 | 1.841 | 1.198 |
| 0.103 (+2) | 0 (-) | 0.076 (-25) | *2.421 (+19) | † 0.901 (-20) |
| 0.038 | 0.023 | 0.061 | 1.265 | 14.858 |
| 0.052 (+64) | 0.008 (-65) | 0.061 (=) | *0.663 (-50) | *1.551 (-115) |
| 0.030 (-16) | † 0.002 (-118) | 0.033 (-42) | *0.693 (-41) | *2.653 (-98) |
| (± 37) | (± 103) | (± 39) | (± 5) | (± 11) |

Figures in brackets refer to
% Coefficient of Variation.

progesterone (50ng/ml, 500ng/ml and 5 g/ml) were also performed. Very little effect was seen with the 50ng/ml progesterone, but when 500ng/ml and 5 μ g/ml progesterone were included in the buffer results that might be expected from progesterone inhibition of testosterone 5 α -reductase in the tissue slices were seen (Table VI - 3). There was a very significant decrease in $\rho_{T.DHT}$ when 5 μ g/ml of progesterone was added, suggestive of a significant inhibition of the enzyme; a similar though less marked effect was seen with 500ng progesterone/ml. At lower progesterone concentrations no significant decrease in $\rho_{T.DHT}$ was observed.

In a single experiment 5 μ g testosterone/ml was added to one superfusion chamber as a further control. In this experiment the 5 μ g testosterone was treated purely as a potential inhibitor for comparison with progesterone and only the concentration of the superfused radioactive testosterone was used in the calculation of the parameters. It is clear from Table VI - 3 that this concentration of testosterone lowers the apparent $\rho_{T.DHT}$ value significantly, although the reduction in this parameter is not as great as is seen with the same concentration of progesterone.

With a reduced conversion of testosterone into

DHT in the tissue following progesterone administration, one could reasonably expect to see an increase in the concentration of testosterone in the tissue. This is in fact the case as $(C_T)_t$, the concentration of testosterone in the tissue, shows a significant increase in each case where a decrease in $\rho_{T.DHT}$ occurs, i.e. at higher progesterone concentrations the concentration of DHT in the tissue, $(C_{DHT})_t$, also appears to fall in correlation with the fall in $\rho_{T.DHT}$ and rise in $(C_T)_t$. However the variations of this parameter at lower concentrations of progesterone make interpretation difficult.

In conclusion, the results presented in Table VI - 3 suggest that progesterone is indeed exerting some effect on testosterone metabolism in prostatic tissue, but that the effect is much less marked than the homogenate enzyme studies had suggested.

E. Uptake and Retention of Progesterone, Testosterone and Cortisol within the Prostate.

In Section D it was shown that progesterone inhibited testosterone 5 α -reductase in prostate tissue slices, but that the extent of this inhibition was considerably less than was predicted from the inhibition of the enzyme by progesterone in an homogenate. In the homogenate 50% inhibition was

caused by equimolar concentrations of progesterone and testosterone substrate, whereas in the superfusion a 500 fold excess of progesterone was required before any effect could be seen. The reasons for this lack of inhibition are not obvious, but lack of entry of progesterone into the cells was considered, although it has been shown that in vivo progesterone is taken up by human prostatic cells in preference to testosterone (Orestano et al., 1975) and this might also be expected to occur in vitro.

Prostatic tissue contains within it a large quantity of plasma proteins, which are not totally washed out of the tissue even after as much as 2 hours of superfusion (Cowan et al., 1976). It could therefore be argued that much of the uptake and retention of the superfused steroids previously discussed was due to binding to these plasma proteins rather than to specific intracellular receptors. One of the major plasma steroid binding proteins is cortisol-binding globulin (CBG) which binds not only cortisol but also other steroid hormones. Progesterone is bound to CBG with high affinity, but the binding of testosterone is much less avid (Doe & Seal, 1963), a fact which could account in part for the previous observations of progesterone uptake without the expected effect on the testosterone 5 α -reductase: that is, uptake of

progesterone by the extracellular protein components of the tissue rather than entry into the prostatic cells themselves. In order to test this hypothesis superfusions were performed with [1,2,6,7-³H]-progesterone or [1,2,6,7-³H]-cortisol and [4-¹⁴C]-testosterone. This regime allowed simultaneous radioassaying of the steroids, without separation of all the possible metabolites involved, since only the entry and retention of testosterone derived, progesterone derived or cortisol derived radioactivity was being considered.

The following experiments were performed with Krebs-Ringer Bicarbonate buffer containing approximately 6,000cpm each of [³H]-progesterone or [³H]-cortisol and [¹⁴C]-testosterone. Non-radioactive progesterone or cortisol was added to compensate for the different specific radioactivities of the steroids, and thus enable them to be perfused at near equimolar concentrations.

After slicing and weighing, the tissue was rinsed in buffer and placed in open superfusion chambers. During uptake experiments the same buffer was perfused throughout, and samples of tissue were removed at 20min. intervals. Retention studies were performed by perfusing with buffer containing the radioactive steroids for 75min., after which the tissue was removed from the chamber, washed with

buffer (3 x 5ml) and transferred to a fresh chamber which was then perfused with buffer containing no steroid. Tissue slices were then removed at 20min. intervals and tissue from uptake and retention studies were then treated in the same manner. After the tissue had been removed from the superfusion chamber it was rinsed in ice cold buffer(3 x 3ml) blotted free of excess fluid, weighed, finely minced and placed in glass scintillation vials along with 6ml non-aqueous scintillator. The vials were stored overnight at 4°C to allow the steroids to be extracted from the tissue. The scintillator was transferred to fresh vials, leaving behind all the tissue, and the radioactivity measured in a liquid scintillation spectrophotometer. All values were corrected for spillover between channels and efficiency, and expressed as concentration steroid/g tissue.

Perfusates (1ml) were counted in aqueous scintillator (10ml) and again all values were corrected for spillover between channels and efficiency, and were expressed as concentration of steroid/ml buffer.

(i) Uptake and Retention of Testosterone and Cortisol.

The tissue slices were superfused with buffer containing [4-¹⁴C]-testosterone (0.12nmoles/ml) and [1,2,6,7-³H]-cortisol (0.16nmoles/ml) which gave a

cortisol/testosterone ratio (C/T) of 1.35. There was a rapid uptake of testosterone, and by 80min. the concentration of testosterone in the tissue was tenfold greater than in that of buffer ($C_i/C_o = 10.0$). The concentration of cortisol in the tissue did not differ significantly from that of the buffer ($C_i/C_o = 0.9$). This difference in uptake resulted in a fall in C/T from 0.22 at 20min. to 0.12 at 160min. as illustrated in Fig.VI - 11.

Wash out experiments indicate a preferential retention of radioactivity derived from testosterone by the tissue, the tissue cortisol concentration being extremely small by the end of the washout period.

The results presented would indicate that there is a different specificity and mechanism for the uptake and retention of cortisol and testosterone by the prostatic tissue, and supports the theory that cortisol is bound extracellularly to CBG and other plasma proteins, whereas testosterone is bound intracellularly to high affinity proteins or receptors, probably as the metabolite DHT.

(ii) Uptake and Retention of Testosterone and Progesterone.

In this study the perfusing buffer had a steroid concentration of 0.19nmoles progesterone/ml and 0.09nmoles testosterone/ml giving a progesterone/

Fig.VI-11.

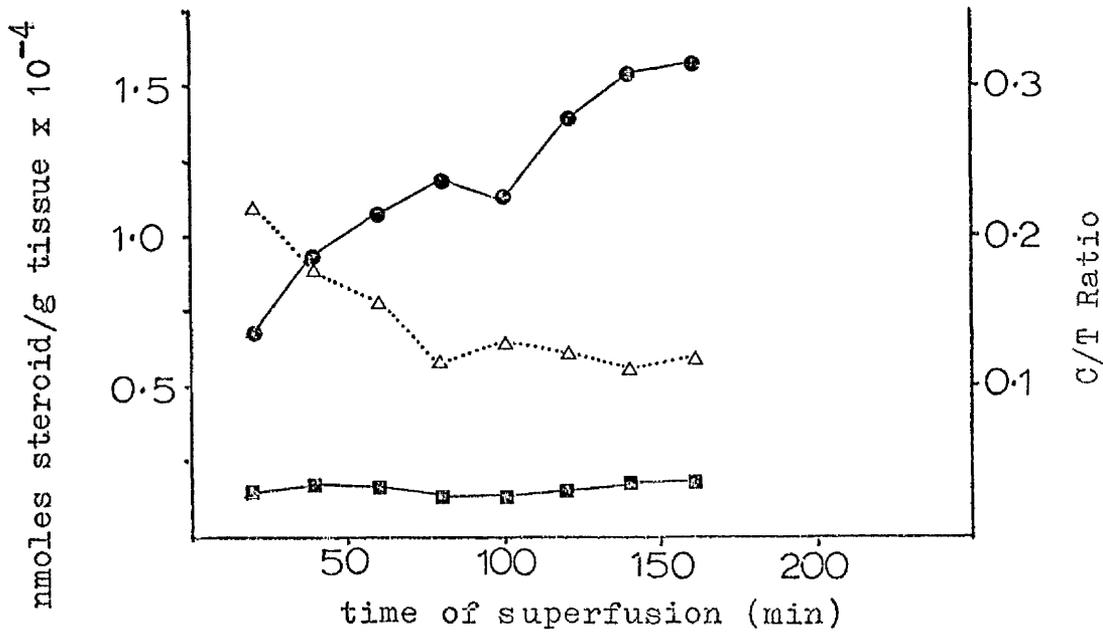
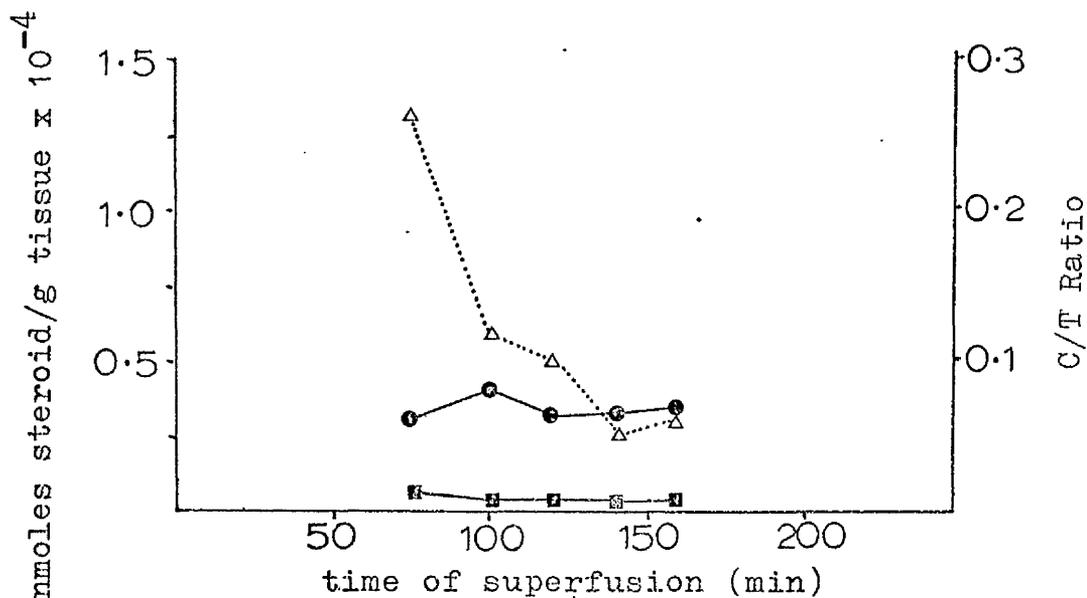
Tissue slices of human prostate were superfused with buffer containing [4-¹⁴C]-testosterone (0.12nmoles/ml) and [1,2,6,7-³H]-cortisol (0.16nmoles/ml).

During uptake studies aliquots of tissue were removed at intervals and total ³H and ¹⁴C radioactivity determined.

Tissue for retention studies was superfused as above for 75min then transferred to a fresh chamber and superfused with buffer containing no steroids for the remaining period.

Fig. VI - 11.

Uptake and Retention of Testosterone and Cortisol
by Human Hyperplastic Prostate.

(a) Uptake.(b) Retention.

Key.

- - Cortisol
- - Testosterone
- △ ... Ratio C/T

testosterone ratio (P/T) of 2.2. It can be clearly seen in Fig.VI - 12 that there was a rapid uptake of both progesterone and testosterone, and by 80min. when the steady state was reached C_i/C_{o_T} was 11.0 and C_i/C_{o_P} was 12.0. Over the period of superfusion the concentration of "progesterone" in the tissue increased four fold from 0.07nmoles/g at 20min. to 0.29nmoles/g at 160min., where as the "testosterone" concentration rose only two fold from 0.05nmoles/g to 0.1nmole/g and resulted in a change in tissue P/T from 1.4 to 2.8. This may be a reflection of preferential uptake of progesterone, but care must be taken in interpreting this since the tissue P/T was essentially the same as that of the perfusing buffer from 60 to 120min. when the steady state is approached.

Although there appears to be specific retention of both steroids within the tissue, it would appear that progesterone is retained in preference to testosterone since tissue P/T increases markedly in the washout experiment.

The pattern of uptake and retention of progesterone resembles that of testosterone rather than cortisol and would indicate that the binding protein involved is not predominantly CBG, but more probably an intracellular component, binding

Fig. VI-12.

Tissue slices of human prostate were superfused with buffer containing [4-¹⁴C]-testosterone (0.09nmoles/ml) and [1,2,6,7-³H]-progesterone (0.19nmples/ml).

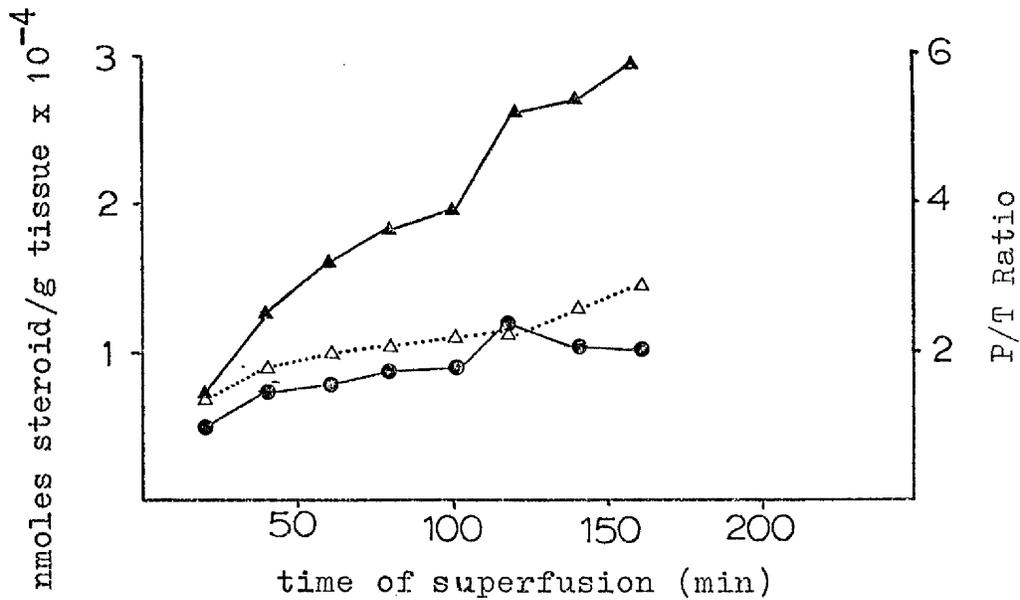
During uptake studies aliquits of tissue were removed at intervals and total ³H and ¹⁴C radioactivity determined.

Tissue for retention studies was superfused as above for 75min then transferred to a fresh chamber and superfused with buffer containing no steroids for the remaining period.

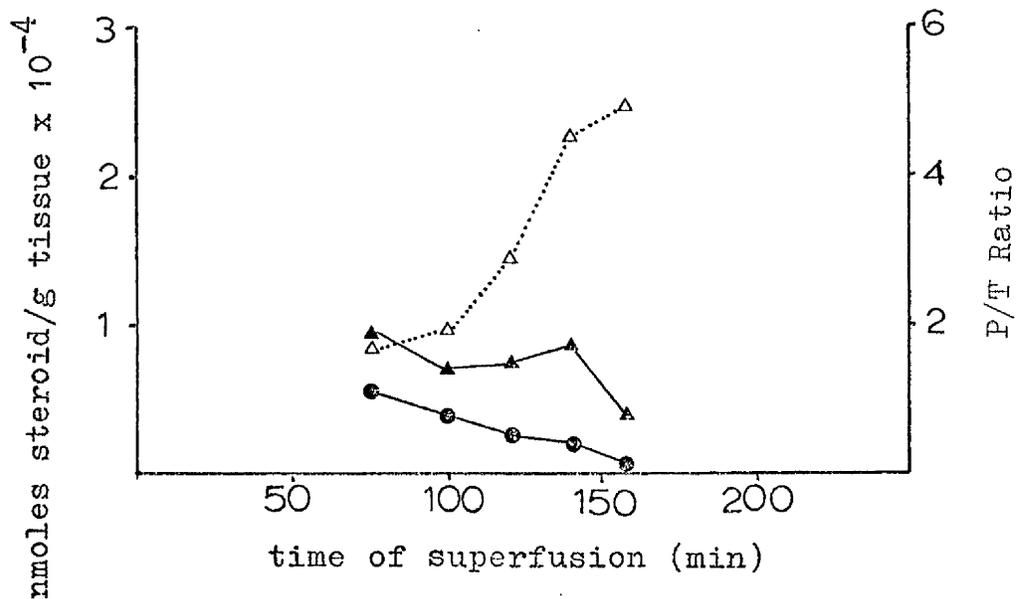
Fig.VI - 12.

Uptake and Retention of Testosterone and Progesterone by Human Hyperplastic Prostate.

(a) Uptake.



(b) Retention.



Key.

- ▲ - Progesterone
- - Testosterone
- △ ... Ratio P/T

progesterone or one of its metabolites.

If progesterone is taken up by the tissue into intracellular location, an explanation other than lack of entry into the cells must be sought to explain the lack of effect of progesterone on testosterone metabolism at the lower superfusion concentrations.

F. The Metabolism of Progesterone within Human Hyperplastic Prostate Cells.

The previous section has shown that progesterone is taken up and retained by prostatic tissue slices in a manner which can best be explained by intracellular entry and binding. More conclusive evidence of the intracellular location of progesterone could be obtained if the progesterone was metabolised by the tissue slices, since this is unlikely to take place extracellularly.

Using human prostatic tissue minces, Morfin et al., (1975) showed that progesterone is metabolised to 5α -pregnane- $3,20$ -dione and 3β -hydroxy- 5α -pregnane- 20 -one. It was considered that an investigation of possible metabolism of progesterone in the tissue slices would provide a simple way of detecting entry of progesterone into the cells and of access to the enzyme system under study.

(i) Preliminary investigations.

Tissue slices were prepared as described in Chapter II and superfused for 90min. with Krebs-Ringer bicarbonate buffer containing $[1,2,6,7-^3\text{H}]$ -progesterone at a concentration of 0.5ng/ml. The tissue, buffer and 60 - 90min. perfusate were extracted in ethylacetate as described in Chapter II. Cold steroid, 100 μg each of progesterone, 5α -pregnane-3,20-dione, 3β -hydroxy- 5α -pregnane-20-one and 3α -hydroxy- 5α -pregnane-20-one were added before extraction. The extracts were dried and spotted on thin layer chromatography plates. Alumina TLC plates and solvent system-1(Cyclohexane/Ethylacetate: 3/1) gave three distinct bands, corresponding to progesterone, 5α -pregnane-3,20-dione, and to 3α -+ 3β -hydroxy- 5α -pregnane-20-one, which were not resolved in this system. Silica TLC plates and a solvent system of chloroform/Methanol:99/1 gave good resolution of the 3α - and 3β -hydroxy-pregnanes, but separation of progesterone from 5α -pregnane-3,20-dione was poor in this system (System 3). The cpm obtained in each fraction are shown in Table VI - 4.

It can be seen that in the tissue there has been considerable metabolism of the progesterone which has entered the tissue mainly to 5α -pregnane-3,20-dione and 3β -hydroxy- 5α -pregnane-20-one. This is in agreement with the findings of Morfin et al., (1975).

Table VI - 4.

Radioactive Products obtained from Prostatic Tissue
after Superfusion with [1,2,6,7-³H]-Progesterone.

| Chromatographic fraction | Tissue (cpm/800mg) | 60-90min Perfusate (cpm/5ml) | Buffer (cpm/5ml) |
|---|--------------------|------------------------------|------------------|
| Progesterone | 1071 | 13297 | 14608 |
| 5 α -pregnane-3,20-dione | 5754 | 809 | 403 |
| 3 α -hydroxy-5 α -pregnane-20-one | 1151 | 93 | 68 |
| 3 β -hydroxy-5 α -pregnane-20-one | 3996 | 122 | 95 |

It is interesting to note that the 3β -isomer predominates over the 3α -isomer in the progesterone metabolism, ($\beta/\alpha:4/1$) which is the opposite case to the metabolism of testosterone where the 3α -hydroxy-androstanediol is the predominant metabolite of DHT. Only a small quantity of the 5α -pregnane- $3,20$ -dione and of the 3 -hydroxy- 5α -pregnane- 20 -ones are present in the perfusate, indicating that the release of these steroids from the tissue is small.

(ii) Time course of progesterone uptake and metabolism.

Human prostatic tissue was sliced and 800mg placed in an open superfusion chamber to permit removal of tissue samples during the period of superfusion. The perfusion buffer contained 10,000 cpm/ml of $[1,2,6,7-^3\text{H}]$ -progesterone and unlabelled progesterone to make the final progesterone concentration either 0.5ng/ml or 5ng/ml. Tissue samples were removed at 30min. intervals and perfusates collected over each of these periods. The tissue, perfusates (5ml) and buffer (5ml) were extracted as previously described after the addition of 100 μ g each of progesterone, 5α -pregnane- $3,20$ -dione and 3β -hydroxy- 5α -pregnane- 20 -one to permit recoveries to be monitored. Solvent system-1 and alumina TLC plates were used, as the three major metabolites are well separated on this system. No attempt was made

to separate the 3α and 3β hydroxy-pregnan-20-ones. Following separation of the steroids and elution from the TLC plates, half of each steroid sample obtained was radioassayed by liquid scintillation spectrophotometry, the rest being retained for gas liquid chromatography to monitor recoveries. A 6foot x 1/4inch column filled with 3% SE30 on gas chrom Q at 210°C gave acceptable separation of the three steroids without excessive retention time. The retention times for progesterone, 5α -pregnane-3,20-dione and 3β -hydroxy- 5α -pregnane-20-one were 12.5min., 9.75min. and 9min. respectively. The amount of steroid injected was related to peak height, and the injection of standard steroid solutions showed this relationship to be linear from 0 to 1,000ng for each of the three steroids measured. The average peak height of at least two injections was used for the calculation of recoveries.

Progesterone was taken up by the tissue and was metabolised to 5α -pregnane-3,20-dione and to 3β -hydroxy- 5α -pregnane-20-one (Fig.VI - 13). More 5α -pregnane-3,20-dione was present in the tissue throughout all time points than either of the other two steroids, and this compound was also released in small quantities from the tissue. The release of the 3 -hydroxy steroids was extremely small and almost indistinguishable from background radioactivity.

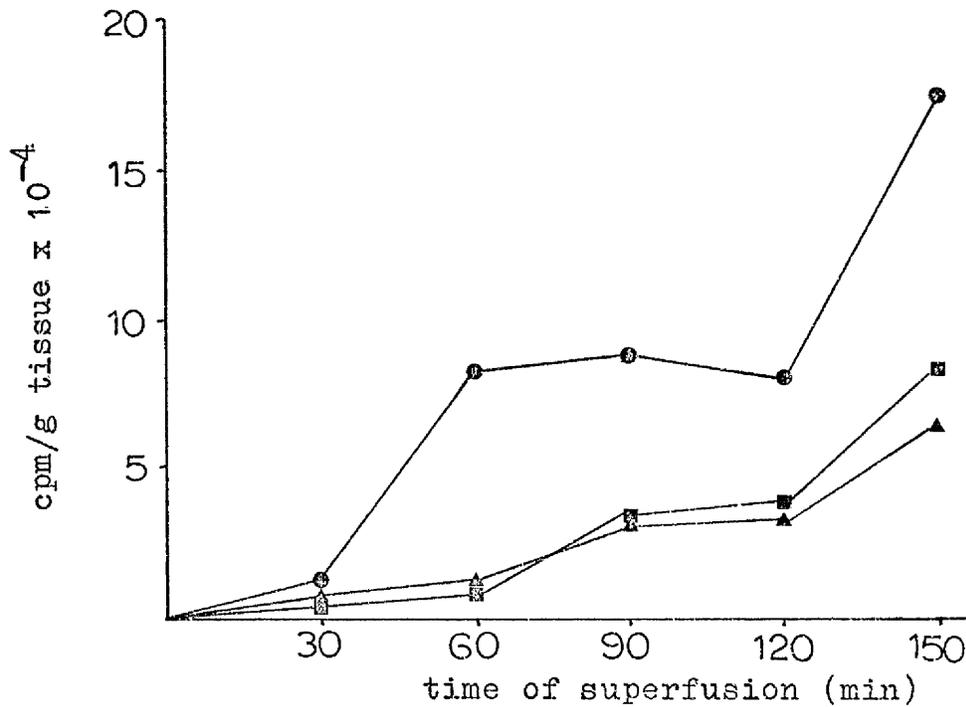
Fig. VI-13.

Tissue slices of human prostate were superfused with buffer containing 10,000cpm/ml [$1,26,7$ - ^3H]-progesterone (either 0.5ng/ml or 5ng/ml). Aliquots of the tissue were removed at 30 min intervals and progesterone metabolites measured as described on p.162.

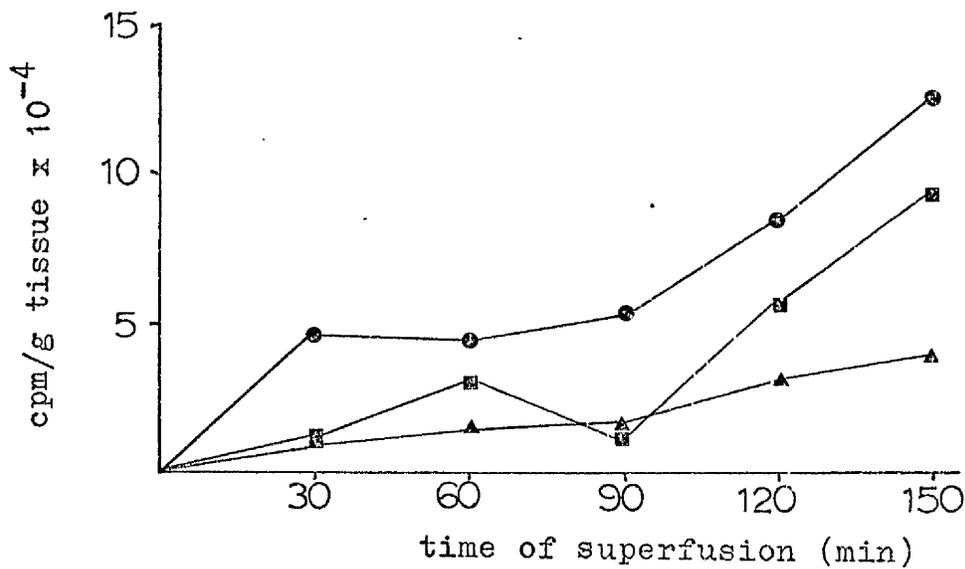
Fig. VI - 13.

The Metabolism of Progesterone by Superfused Human Prostatic Tissue.

(a) 0.5ng progesterone/ml.



(b) 5ng progesterone/ml.



Key.

- Δ - Progesterone
- ◻ - 3-hydroxy-5α-pregnane-20-one
- - 5α-pregnane-3,20-dione

Fig.VI - 13 illustrates the radioactivity in the three steroid fractions found in the tissue during the superfusion at the two progesterone concentrations, 0.5ng/ml and 5ng/ml. The steady state is reached at both progesterone concentrations. At 0.5ng progesterone/ml the steady state extends from 60 - 120min. as judged by the levels of 5α -pregnane-3,20-dione, the progesterone and 3β -hydroxy- 5α -pregnan-20-one plateauing at 90 - 120min. The total radioactivity is however constant at 90 - 120min. When the progesterone is superfused at 5ng/ml the steady state appears to be at 30 - 90min. for all the steroids. Although there is a tenfold difference in the concentration of progesterone in the two superfusion buffers (the cpm/ml is the same in both) the cpm/g in the tissue is depressed by less than 50%, which would indicate not only a large capacity for progesterone uptake, but also for progesterone metabolism by prostatic tissue.

3. DISCUSSION.

Progesterone was shown to be a more effective inhibitor of testosterone 5 α -reductase than testosterone itself. The fact that the A, B and C rings of testosterone and progesterone have the same active groups, means that the geometry of these rings will be essentially identical, and thus, stereospecifically, a good fit in the active site of the enzyme may be achieved. Constituents on the D ring of progesterone and testosterone differ significantly, but it can be argued that the C₁₇ side chain is less important in determining the binding of progesterone in the active site and as progesterone is efficiently metabolised by the enzyme this is probably the case. Another effective inhibitor is 4-androstene-3,17-dione, which is only slightly less potent than testosterone. Here again the difference in structure is small, the 17 β -hydroxyl of testosterone being replaced by a ketone function in 4-androstene-3,17-dione.

The addition of hydroxyl groups to the progesterone nucleus had a varying effect on the potency of these compounds as inhibitors of the testosterone 5 α -reductase. When the graphs were plotted a pattern emerged which indicated that the number and proximity of the hydroxyl group to the A ring was important in determining the potency of

the inhibitors. It would appear that a C₂₁ hydroxyl group has least effect followed by a 17 α -hydroxyl, and that the 11 β -hydroxyl reduces the inhibitor potential to the greatest extent. Combinations of two hydroxyls show the same pattern and when three hydroxyl groups are present, 11 β , 17 α and 21, as in cortisol, there is no inhibition. One can envisage that the addition of these hydroxyls interferes with the binding of the steroid to the active site of the enzyme and that the closer one gets to the A ring where the reduction is taking place the greater the effect on the binding, as distortions in the fit within the active site may be less well tolerated.

Reduction of the Δ^4 double bond of progesterone and testosterone to 5 α -pregnane-3,20-dione and DHT respectively produces ineffective inhibitors, and would also indicate that product inhibition by DHT is not a limiting factor in the enzyme reaction. The kinetics of the inhibition by progesterone would appear to be of the competitive type as could be expected from the closely related molecular structure of progesterone and the substrate, testosterone. This finding confirms the previous observation of Jenkins & McCaffery (1974).

Whilst it would have been interesting to extend this survey from purely naturally occurring compounds to other hydroxylated progestins and known

antiandrogens, it was felt unnecessary in the context of this present project especially since studies of this type have been carried out previously (Tan et al., 1974). Therefore, for the purpose of selecting a natural steroid with potent 5α -reductase inhibiting properties for use with superfused tissue slices, the present studies proved sufficient.

Progesterone was the steroid of choice since it was the most potent of the progestins investigated. It has previously been shown by Orestano et al., (1975) that progesterone and gestonorone caproate were effective inhibitors of prostatic testosterone metabolism. Concentrations of progesterone of only twice that of testosterone caused a decrease in 5α -reduction products by approximately 50%, which is similar to the results presented in the present investigations.

The use of an in vitro superfusion method to simulate the constant flow of material to and from the tissue which is found in vivo, emphasised the danger of direct extrapolation from one model to another. Superfusion with progesterone in the buffer resulted in no detectable decrease in testosterone metabolism until its concentration was in excess of 600 times that of the natural substrate. This is in direct contrast to the situation observed in an homogenate or microsomal enzyme assay, where

equimolar concentrations of the progesterone inhibitor and testosterone substrate resulted in almost 50% decrease in DHT production.

The explanation for this difference of effect in the two systems is not immediately obvious. Uptake experiments indicate that the progesterone has a predominantly intracellular location in the tissue slices after superfusion, since the pattern of progesterone uptake resembled more closely that of testosterone than cortisol. Tissue concentrations of cortisol did not differ significantly from that of the buffer and could probably be accounted for by extracellular binding to plasma proteins such as CBG and albumin. Superfusion with radioactive testosterone and progesterone resulted in at least tenfold increases in tissue radioactivity over that of the medium and would indicate specific transport into intracellular compartments. This uptake of radioactivity by the tissue is in part due to intracellular metabolism of both progesterone and testosterone.

Retention studies also point to an intracellular location for progesterone or its metabolites, since there appears to be specific retention of radioactivity originating from progesterone and testosterone, but not from cortisol. The uptake and retention results also indicate that progesterone is taken up and

retained by the tissue even more avidly than is testosterone. This has previously been implied by Morfin et al., (1975), but further work would be necessary before this could be stated with confidence.

There is rapid metabolism of progesterone by prostatic homogenates and tissue slices. The major products of this metabolism were shown to be 5 α -pregnane-3,20-dione, 3 α -hydroxy-5 β -pregnane-20-one and 3 β -hydroxy-5 α -pregnane-20-one; the ratio of the 3-hydroxy pregnanes being $\beta/\alpha:4/1$. These results are similar to those presented by Morfin et al., (1975), who also reported significant metabolism of progesterone to 5 α -pregnane-3,6,20-trione. Polar metabolites of this type were not investigated in the present study.

A tenfold difference in the specific radioactivity of the superfused [^3H]-progesterone did not result in a parallel difference in the radioactivity extracted from the tissue slices. This can be seen also for testosterone in the work by Giorgi et al., (1972) and indicates a large capacity for uptake, metabolism and retention of both testosterone and progesterone by the prostatic tissue slices. This large metabolic capacity of the tissue is possibly the explanation for the unexpected lack of potency of progesterone in decreasing testosterone metabolism in tissue slices, compared to homogenate and

microsomal incubations.

At 90min. of superfusion the total concentration of steroid in the tissue resulting from testosterone entry is in the region of 10pmoles/g tissue and assuming a value of 100mg protein/g wet tissue, this would give a value of 0.1pmoles steroid/mg tissue. When one considers the value of V_{max} obtained using an homogenate preparation -- approximately 100pmoles/hr/mg protein-- it is apparent that if the same value is true in vivo and that if cofactor concentrations are not limiting, then the capacity for metabolism is far greater than the supply of substrate at the steroid concentrations used in the perfusing buffer. The addition of the lower concentrations of progesterone does not increase the potential substrate concentration significantly and thus it also will be metabolised at the maximum rate. This maximum rate of metabolism of the steroids is reflected in the values of $\rho_{T.DHT}$ obtained, where it is apparent that the majority of the testosterone entering the tissue is converted to DHT. This fact is more easily recognised by examination of the relative concentrations of $[17\alpha\text{-}^3\text{H}]$ -testosterone and $[17\alpha\text{-}^3\text{H}]$ -DHT present in the tissue (see Table VI - 1). Thus it seems reasonable to postulate that only when the combined concentrations of testosterone and progesterone are reaching a level where enzyme

concentration becomes the limiting factor, will any depression in testosterone metabolism due to the presence of progesterone be observed. An analogous conclusion was reached by Baulieu et al., (1975) from their work with rat ventral prostate explants in constant flow cultures. These workers found that when they added the antiandrogen 17 β -ethynyl-4-estrene-3-one (R4144) to the medium there was a decrease in the amount of DHT released into the medium, but that DHT did not decrease in the explants themselves. They therefore suggested that for an anti-5 α -reductase agent to be efficient at lowering the intracellular DHT content it should be almost 100% effective as an enzyme inhibitor.

The implications to be taken from the results presented in this chapter are significant with respect to the mode of action of antiandrogenic compounds. Whilst many antiandrogens appear to inhibit the prostatic testosterone 5 α -reductase, it is probable that this is not their main mode of action, since very high intracellular concentrations would be necessary for any other than those which were bound irreversibly to the active site of the enzyme. Thus for the majority of antiandrogens their major mode of action within the prostate will probably be found to be by competition with DHT for cytoplasmic and nuclear receptors.

CHAPTER VII.

CHAPTER VII

CONCLUSIONS AND SCOPE FOR FUTURE INVESTIGATION.

In the previous chapters several compounds, both steroidal and non-steroidal, have been investigated with respect to their interaction with the testosterone 5 α -reductase from human hyperplastic prostate.

Heparin and several other polysaccharides have been shown to reproducibly inhibit the testosterone 5 α -reductase. Heparin itself exerts this inhibition with competitive kinetics with respect to both substrate and cofactor. The inhibition is achieved by interference with substrate binding rather than by direct competition for the active site. No conclusions could be made in the present study about the part of the molecule responsible for inhibition, since no pattern was seen with change in size, sulphate content or glycoside linkage. Many more synthetic compounds would require testing to elucidate a possible pattern.

Administration of heparin in vivo to male rats produced no change in the enzyme activity within the

prostate. The regime used was the short term administration of high doses of heparin and it may be possible to produce more positive results if a long term regime were employed. This would necessitate the use of much lower doses of the drug to prevent the high mortality observed with the regime described.

The method of Horner (1971) was found to be the more satisfactory of the two methods used for extraction of heparin from prostatic tissue. A uronic acid containing residue was obtained which was able to inhibit the prostatic 5α -reductase. Further studies using thin layer chromatography and a combination of the spectrophotometric methods of Dische (1947) and Bitter & Muir (1962) would permit confirmation of the nature of the uronic acid residue. Identification of this type was not undertaken in the present study, since the larger quantity of prostatic tissue which would be necessary for this, was not readily available.

Investigations on the intra and extra cellular locations of heparin may also prove of value, and could be performed by both tissue fractionation and autoradiographic methods.

The antialdosterone agent, spironolactone, had previously been shown to have significant anti-androgenic effects on the rat ventral prostate

(Basinger & Gittes, 1974) and more specifically that spironolactone and its metabolic products, aldadiene and canrenoate, bind to the prostatic androgen receptor (Bonne & Raynaud, 1974). The present study confirms that canrenoate has no significant inhibitory effect on the testosterone 5α -reductase, but shows that aldadiene is capable of inhibiting the enzyme to an extent of 40% at a concentration of 250 times that of the substrate. Whilst there is a high concentration of aldadiene in plasma during spironolactone treatment, it is unlikely in view of the findings in Chapter VI that significant inhibition of testosterone metabolism will take place in vivo. Therefore, one must conclude that any antiandrogenic effect observed is likely to result from competition for the androgen receptor between DHT and the spironolactones, or to lowered androgen production by the testis. This latter possibility deserves further investigation, for whilst it has been shown that spironolactone inhibits the cytochrome P₄₅₀ dependent steroid 17α -hydroxylase, and desmolase activity, it is possible that the drug may also act at the pituitary level altering pituitary hormone production, thus in part accounting for the gynecomastia, impotence and menstrual irregularities observed as side effects of this drug.

A deviation was made from the main line of investigation when an isotope effect was suspected during the oxidation of $[17\alpha\text{-}^3\text{H}]\text{-DHT}$ with chromic acid. Further investigations of this isotope effect were made using $[17\alpha\text{-}^3\text{H}]\text{-testosterone}$, since this had been the starting material for the $[17\alpha\text{-}^3\text{H}]\text{-DHT}$ and was more readily available. The isotopic purity of the compound was established and an isotope effect confirmed when the labelled steroid was oxidised with either chromic acid or by 17β hydroxysteroid dehydrogenase from Pseudomonas Testosteroni. Since no isotope effect was seen when the $[17\alpha\text{-}^3\text{H}]\text{-testosterone}$ was reduced by the prostatic testosterone 5α -reductase and since the conversion of testosterone to $4\text{-androstene-}3,17\text{-dione}$ is very small compared to the conversion of testosterone to DHT in the prostate, it was decided that the observed isotope effect would not invalidate the use of $[17\alpha\text{-}^3\text{H}]\text{-steroids}$ in the superfusion system.

The demonstration of this isotope effect emphasises the dangers of using isotopically labelled species in biochemistry for tracer work. Isotope effects themselves can be made use of in determining reaction mechanisms, and one could envisage the use of variously labelled substrates and cofactors in further investigations of the mechanism of the 5α -reductase reaction.

A group of naturally occurring steroids were tested for inhibitory properties. The main difference between the pregnane series of compounds considered was the number and position of the hydroxyl groups. A pattern emerged from the study which indicated that the number and proximity of the hydroxyl groups to the A ring influenced the degree of inhibition seen. The 5α -reduced compounds 5α -pregnane- $3,20$ -dione and DHT caused little inhibition even at the highest concentrations tested, indicating that direct product inhibition is not likely to occur with this enzyme. The most potent inhibitors were shown to be progesterone and 4-androstene- $3,17$ -dione which were as potent as testosterone itself in decreasing the radioactivity in the DHT product.

Progesterone was chosen as a test inhibitor for use in the superfusion system. This system uses thin tissue slices and is designed to approximate more closely the in vivo environment than does a test tube incubation with a tissue homogenate. The results obtained with this system were quite different to those of homogenate incubations. Progesterone did not cause any decreased production of DHT until it was over 500 times in excess of the substrate testosterone. This lack of effect could not be accounted for by lack of entry of progesterone into the cells, since the steroid was taken up and retained

by the tissue, as well as being rapidly metabolised to 5α -pregnane-3,20-dione, 3α -hydroxy- 5α -pregnan-20-one and 3β -hydroxy- 5α -pregnan-20-one.

The most satisfactory explanation for the lack of inhibition by progesterone in this system, lies in the concentration and activity of the 5α -reductase enzyme itself. The maximum velocity of the enzyme in an homogenate incubation was shown to be 13 nmoles/hr/g tissue, which indicates that a high substrate concentration is required before the enzyme itself becomes the limiting factor in determining the reaction rate. Since progesterone is a competitive inhibitor and substrate, it does not alter V_{max} for testosterone, thus one will not see an alteration in DHT production until the combined concentration of testosterone and progesterone exceeds the metabolic capacity of the enzyme present. The implications from these results are that inhibitors of testosterone 5α -reductase will have to be present in very high concentrations to influence the intracellular production of DHT. It seems likely, therefore, that the plasma concentration of progesterone and the majority of other antiandrogenic compounds used to date are not sufficiently high to affect DHT production by the prostate, and that their method of action is most probably due to competition with DHT for the androgen receptor with the gland.

Progesterone was specifically taken up, metabolised and retained by prostatic tissue. Indeed the data of Chapter VI suggested that progesterone may be taken up and retained in preference to testosterone. In support of the retention of progesterone being specific, Asselin et al., (1976) and Cowan et al., (1977) have shown the presence of a progesterone receptor-like protein in cytosol from human benign prostatic hyperplastic tissue. One may speculate about the physiological significance of its presence, whether it is a progesterone receptor per se or an atypical androgen receptor.

In view of the significant metabolism of progesterone by the prostatic tissue it may also be important to determine the binding of 5α -pregnane-3,20-dione to these receptor proteins.

It is unclear as yet whether progesterone has a role in the normal functioning of the prostate. However, since progesterone enters the tissue, is metabolised by the tissue, and appears to be specifically bound within the hyperplastic prostate, it is not unreasonable to postulate some function for this steroid, and it remains to be determined whether this function is part of the normal gland per se, or merely a reflection of altered capabilities in the hyperplastic gland.

In vivo and in vitro work by Geller et al., (1976)

has shown that megestrol acetate administered in vivo caused a substantial fall in testosterone 5 α -reductase when measured in vitro. Similar results were obtained by Tan et al., (1974) using medrogestone. A fall in endogenous tissue DHT concentration and lack of [3 H]-DHT binding to a cytosol receptor protein after megestrol acetate administration was also reported by Geller et al., (1976). It is unclear from these studies, however, whether the fall in testosterone 5 α -reductase activity is due to inhibition of the enzyme by endogenous antiandrogen, or whether it is a result of a lower enzyme concentration as a direct consequence of antiandrogenic displacement of DHT from the receptor, the latter being the more probable in view of the results presented here. It would be of interest to test this hypothesis using the method of tissue superfusion in parallel to homogenate incubations, as was done in the present study for progesterone.

In conclusion, it is clear that because prostatic testosterone 5 α -reductase has a large metabolic capacity it is unlikely that attempts to control this enzyme alone will significantly alter the course of benign prostatic hyperplasia. Therefore, for the future, we must look toward antiandrogens without the many side effects of those in present use, and yet which will specifically interfere with DHT-receptor interaction.

LIST OF REFERENCES

LIST OF REFERENCES

- Anderson, K.M. & Liao, S. (1968) *Nature*, 219, 277-279.
- Asselin, J., Labrie, F., Gourden, Y., Bonne, C. & Raynaud, J-P. (1976) *Steroids*, 28, 499-459.
- Baker, H.W.G., Jefferson, L.S. & Bardin, C.W. (1977) 58th Ann. Meeting of the Endocrine Society, p 95.
- Basinger, G.T. & Gittes, R.F. (1974) *J.Urol.*, 111, 77-80.
- Baulieu, E.E., Lasnitzki, I. & Robel, P. (1968) *Nature*, 219, 1155.
- Baulieu, E.E., Le Goascone, C., Groyer, A., Feyer-Cabanes, T. & Robel, P. (1975) *Vitamines & Hormones*, 33, 1-38.
- Beastall, G.H. (1975) *Biochemical Society Transactions*, 55th Meeting, 3, 677-680.
- Becher, H., Kaufmann, J., Klosterhalfen, H. & Voigt, K.D. (1972) *Acta Endocrinol. (Kbh)*, 71, 589-599.
- Bird, C.E., Green, R.N., Calandra, R.S., Connolly, J.G. & Clark, A.F. (1971) *Acta Endocrinol (Kbh)*, 67, 733-739.
- Bitter, T. & Muir, H.M. (1962) *anal. Biochem.*, 4, 330-334.
- Bjorkem, I. & Holmberg, I. (1973) *Eur. J. Biochem.*, 33, 364-367.
- Blandy, J.P. (1976) *Urology (Blandy J.P. Ed.) Vol. II*, pp 859-913, Blackwell Scientific Publications, Oxford.
- Blaquier, J.A. & Calandra, R.S. (1973) *Endocrinology*, 93, 51-60.
- Bonne, C. & Raynaud, J-P. (1973) *Biochimie*, 55, 227-229.
- Bonne, C. & Raynaud, J-P. (1974) *Mol. Cell Endocrinol.*, 2, 59-67.
- Briggs, M.H. & Briggs, M. (1973) *J. Clin. Endocrinol. Metab.*, 36, 600-604.
- Bruchovsky, N. (1971) *Endocrinology*, 89, 1212-1222.

- Bruchovsky, N. & Craven, S. (1975) *Biochem. Biophys. Res. Commun.*, 62, 837-843.
- Bruchovsky, N., Rennie, P.S. & Vanson, A. (1975) *Biochim. Biophys. Acta*, 394, 248-266.
- Bruchovsky, N. & Wilson, J.D. (1968,a) *J. Biol. Chem.*, 243, 5953-5960.
- Bruchovsky, N. & Wilson, J.D. (1968,b) *J. Biol. Chem.*, 243, 2012-2021.
- Bush, I.E. (1952) *Biochem. J.*, 50, 370-378.
- Bush, I.E. (1961) *The Chromatography of Steroids*, (a) p 358, (b) p 363, Pergamon Press.
- Caine, M., Perlberg, S. & Gordon, R. (1975) *J. Urol.*, 114, 564-568.
- Caputo, M.J., Hosty, T.A. & Melton, H.E. (1973) *Metabolism*, 22, 623-625.
- Carter, J.R., Hagen, A.A., Biggs, J.T. & Troop, R.C. (1968) *Metabolism*, 17, 352-359.
- Castro, J.E., Griffiths, H.J. & Edwards, D.E. (1971) *Brit. J. Surg.*, 58, 485-489.
- Castro, J.E. & Sellwood, R.A. (1974) *J. Pathol.*, 113, 217-222.
- Chamberlain, J., Jaqarinec, N. & Ofner, P. (1966) *Biochem. J.*, 99, 610-616.
- Chandler, J.A., Harper, M.E., Blundell, G.K. & Morton, M.S. (1975) *J. Endocrinol.*, 65, 34 P.
- Charles, A.F. & Scott, D.A. (1933, a) *J. Biol. Chem.*, 102, 425-429.
- Charles, A.F. & Scott, D.A. (1933, b) *J. Biol. Chem.*, 102, 437-448.
- Cheng, S.C., Suzuki, K., Sadee, W. & Harding, B.W. (1976) *Endocrinology*, 99, 1097-1116.
- Chisholm, G.D. (1970) *In Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer*, (K. Griffiths & C.G. Pierrepoint, Eds.) pp 119-125.

Cohen, P.P. (1957) In Manometric Techniques
(W.W. Umbreit, R.H. Burns & J.F. Stauffer, Eds.)
p 149-150, Burgess Publishing Co.,
Minneapolis, Minn.

Corvol, P., Michand, A., Menard, J., Freifeld, M. &
Mahondeau, J. (1975) J. Endocrinol., 97,
52-58.

Costachel, O., Fadei, L. & Nachtigal, M. (1964)
Exp. Cell. Res., 34, 542-547.

Cowan, R.A., Cowan, S.K. & Grant, J.K. (1977)
J. Endocrinol., 74, 281-289.

Cowan, R.A., Cowan, S.K., Grant, J.K. & Elder, H.Y.
(1976) J. Endocrinol., 71, 121-131.

Cowan, R.A., Cowan, S.K., Grant, J.K. & Elder, H.Y.
(1977) J. Endocrinol., 74, 111-120.

Craven, S., Lesser, B. & Bruchovsky, N., (1974)
Endocrinology, 95, 1177-1180.

David, K., Dirgemanse, E., Freu, J. & Laqueur, E.
(1935) Z. Physiol. Chem., 233, 281-282.

Davidson, S.J. & Talalay, P. (1966) J. Biol. Chem.,
241. 906-915.

Dische, Z. (1947) J. Biol. Chem., 167, 189-198.

Doe, R.P. & Seal, U.S. (1963) J. Clin. Invest. 42, 929.

Duncan, J.F. & Cook G.B. (1968) Isotopes in
Chemistry, p 137, Clarendon Press, Oxford.

Dymling, J-F., Nilsson, K.O. & Hokfelt, B. (1972)
Acta Endocrinol. (Kbh)., 70, 104-112.

Ehrlich, J. & Stivala, S.S. (1973) J. Pharm. Sci.,
62, 517-544.

Elieil, E.L., Allinger, N.L., Angyal, S.J. & Morrison,
G.A. (1965) Conformational Analysis, p 271,
Interscience, New York.

Evans, E.A. (1974) Tritium and its Compounds,
pp 654-657, Butterworths, London.

Evans, C.R. & Pierrepoint, C.G. (1975) J. Endocrinol.,
64, 539-548.

- Fanestil, D.D. (1968) *Biochem. Pharmacol.*, 17, 2240-2242.
- Fang, S., Anderson, K.M. & Liao, S. (1969) *J. Biol. Chem.*, 244, 6584-6595.
- Fang, S. & Liao, S. (1969) *Mol. Pharmacol.*, 4, 428-431.
- Farnsworth, W.E. (1970) Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer, (Griffiths, K. & Pierrepont, C.G., Eds.) pp 3-15.
- Farnsworth, W.E. (1973) *Steroids*, 21, 647-664.
- Fingehut, B. & Veenema, R.J. (1967) *J. Urol.*, 97, 508-517.
- Fisher, H.F., Conn, E.E., Vennesland, B. & Westheimer, F.H. (1953) *J. Biol. Chem.*, 202, 687-697.
- Franks, L.M. (1954) *Ann. R. Coll. Surg. Eng.*, 14, 92.
- Franks, L.M. (1974) The Treatment of Prostatic Hypertrophy and Neoplasia (Castrol, J.E., Ed.) p 1-26, Medical and Technical Publishing Co.Ltd., U.K.
- Frederiksen, D.W. & Wilson, J.D. (1971) *J. Biol. Chem.*, 246, 2584-2593.
- Freeman, L. (1964) *Am. J. Cardiol.*, 4, 3-7.
- Freeman, L., Posthuma, R., Gordon, L. & Marx, W. (1957) *Arch. Biochem. Biophys.*, 70, 159-177.
- Fumarola, D. (1969) *BoU Ist. Sieroter, Milan*, 48, 1-23.
- Geller, J., Albert, J., Geller, S., Lopez, D., Cantor, T. & Yen, S. (1976) *J. Clin. Endocrinol. Metab.*, 43, 1000-1008.
- Geller, J., Albert, J., Lopez, D., Geller, S. & Niwagama, G. (1976) *J. Clin. Endocrinol. Metab.*, 43, 686-688.
- Geller, J., Angrist, A., Nakao, K. & Newman, H. (1969) *J. Am. Med. Assoc.*, 210, 1421-1427.
- Geller, J., Fishman, J. & Canfor, T.L. (1975) *J. Steroid Biochem.*, 6, 837-843.

- Giorgi, E.P. & Moses, T.F. (1975) J. Endocrinol., 65, 279-280.
- Giorgi, E.P., Moses, T.F., Grant, J.K., Scott, R. & Sinclair, J. (1974) Mol. Cell. Endocrinol., 1, 271-284.
- Giorgi, E.P., Shirley, I.M., Grant, J.K. & Stewart, J.C. (1973) Biochem.J., 132, 465-474.
- Giorgi, E.P., Stewart, J.C., Grant, J.K. & Scott, R. (1971) Biochem. J. 123, 41-55.
- Giorgi, E.P., Stewart, J.C., Grant, J.K. & Shirley, I.M. (1972) Biochem. J., 126, 107-121.
- Gláz, E. & Sugar, K. (1964) Endocrinology, 74, 159-164.
- Gloyna, R.E. & Wilson, J.D. (1969) J. Clin. Endocrinol. Metab., 29, 970-977.
- Gordon, G.G., Altman, K., Southren, A.L. & Olivo, J. (1971) J. Clin. Endocrinol. Metab., 32, 457-461.
- Gorski, J. & Morgan, M.S. (1967) Biochem. Biophys. Acta, 149, 282-287.
- Grant, J.K. & Giorgi, E.P. (1972) J. Steroid Biochem., 3, 471.
- Grant, J.K., Minguell, J., Taylor, P. & Weiss, M. (1971) Biochem. J., 125, 21 P.
- Grayhack, J.T. (1963) Natl. Cancer Inst. Monog., 12, 189-200.
- Griffiths, K., Harper, M.E., Groom, M.A., Pike, A.W., Fahmy, A.R. & Pierrepoint, C.G. (1970) In: Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer. (K. Griffiths & C.G. Pierrepoint, Eds.) pp 88-94.
- Gupta, C. & Bloch, E. (1976) Endocrinology, 99, 389-399.
- Gurpide, E. & Welch, M. (1969) J. Biol. Chem., 244, 5159-5169.
- Gustafsson, J.A. & Pousette, A., (1974) Biochem. J., 142, 273-277.
- Gutman, A.B. & Gutman, E.B. (1938,a) J. Clin. Invest., 17, 473-478.
- Gutman, A.E. & Gutman, E.B. (1938,b) Proc. Soc. Exp. Biol., N.Y., 39, 529-532.

- Habib, F.K., Hammond, G.L., Stitch, S.R. & Dawson, J.B.
(1975) *J. Endocrinol.*, 65, 34 P.
- Habib, F.K. & Stitch, S.R. (1975) *Acta Endocrinol.*
[Suppl.] (Kbh), 199, 129.
- Hajj, Y.J.A. (1972) *Steroids*, 20, 215-222.
- Hansson, R. & ThySELL, H. (1970) *Acta Physiol.*
Scand., 78, 539-546.
- Hansson, V., Tveter, K.J. & Attramadal, A. (1971)
Acta Endocrinol., (Kbh), 67, 384-392.
- Harper, M.E., Pierrepoint, C.G., Fahmy, A.R. &
Griffiths, K. (1971) *J. Endocrinol.*, 49,
213-223.
- Harper, M.E., Pike, A., Peeling, W.B. & Griffiths, K.
(1974) *J. Endocrinol.*, 60, 117-125.
- Harris, A.F., Cotty, V.F., Barnett, L. & Seiman, A.
(1969) *Arch. Int. Pharmacodyn. Ther.*, 181,
489-498.
- Heathcote, J.G. & Washington, R.J. (1973) *J.*
Endocrinol., 58, 421-423.
- Heckel, N.J. (1944) *J. Clin. Endocrinol.*, 4, 166-170.
- Helting, T. & Lindahl, U. (1971) *J. Biol. Chem.*,
246, 5442-5447.
- Hohrst, H-J. (1963) *Methods of Enzyme Analysis*
(H-U Bergmeyer, Ed.) pp 134-138, Academic
Press, New York & London.
- Horner, A.A. (1971) *J. Biol. Chem.*, 246, 231-239.
- Horowitz, M.I., Palmer, T. & Glass, G.B.J. (1970)
Proc. Soc. Exp. Biol. Med., 133, 853-857.
- Horst, H-J., Dennis, M., Kaufman, J. & Voigt, K.D.
(1975) *Acta Endocrinol.*, (Kbh), 79, 394-402.
- Huggins, C. (1945) *Physiol. Rev.*, 25, 281-295.
- Huggins, C. (1947) *Harvey Lect.*, 42, 148-193.
- Huggins, C. & Hodges, C.V. (1941) *Cancer Res.*, 1,
293-297.
- Huggins, C., Scott, W.W. & Hodges, C.V. (1941)
J. Urol., 46, 997-1006.

Humphrey, G.F. & Mann, T. (1948) *Nature*, 161, 352-353.

Humphrey, G.F. & Mann, T. (1949) *Biochem. J.*, 44, 97-105.

Hunter, J. (1786) *Observations on Certain Parts of the Animal Oeconomy*, London.

Imperato-McGinley, J., Guerrero, I., Gautier, T. & Peterson, R. (1974) *Science*, 186, 1213-1215.

Inano, H., Hayashi, S. & Tamaoki, B. (1977) *J. Steroid Biochem.*, 8, 41-46.

Jacobi, G.H. & Wilson, J.D. (1976) *Endocrinology*, 99, 602-610.

Jenkins, J.S. & McCaffery, V.M. (1974) *J. Endocrinol.*, 63, 517-526.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. & De Sombre, E.R. (1968) *Proc. Nat. Acad. Sci. U.S.A.*, 59, 632-638.

Johansson, R. (1976) *Acta Endocrinol. (Kbh.)*, 81, 398-408.

Kerr, W.K., Keresteci, A.G. & Mayoh, H. (1960) *Cancer*, 13, 550-554.

King, R.J.B. & Mainwaring, W.I.P. (1974) *Steroid Cell Interactions*, Butterworths, London.

Krzanowski, P.T. & Troop, R.C. (1968) *Experientia*, 24, 225-226.

Krieg, M., Horst, H-J. & Sterba, M-L. (1975) *J. Endocrinol.*, 64, 529-538.

Krieg, M. & Voigt, K.D. (1976) *J. Steroid Biochem.*, 7, 1005-1012.

Lakatos, K., Koref, O., Sseczek, K., Hollo, I. & Fur, L. (1970) *Acta Endocrinol. (Kbh.)*, 65, 731-736.

Lardy, H.A. & Phillips, P.H. (1945) *Arch. Biochem.*, 6, 53-61.

Lasnitzki, I. (1970) In: *Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer*, (K.Griffiths & C.G. Pierrepoint, Eds.) pp 68-73.

Lasnitzki, I. & Franklin, H.R. (1974) *J. Endocrinol.*, 64, 289-297.

- Lawrence, A.M. & Landau, R.L. (1965) *Endocrinology*, 77, 1119-1125.
- Leake, R. (1976) *Trends in Biochemical Sciences*, 1, 137-139.
- Lee, D.K.H., Yound, J.C., Tamura, Y., Patterson, D.C., Bird, C.E. & Clark, A.F. (1974) *Can. J. Biochem.*, 51, 735-740.
- Lesser, B. & Bruchovsky, N. (1973) *Biochim. Biophys. Acta*, 308, 426-437.
- Levy, G., Marchut, M., Baulieu, E.E. & Robel, P. (1974) *Steroids*, 23, 291-300.
- Lewis, M.H. & Moffat, D.B. (1975) *J. Reprod. Fertil.*, 42, 497-502.
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.*, 56, 658-666.
- Lohr, G.W. & Waller, H.D. (1963) *Methods of Enzyme Analysis* (H-U. Bergmeyer, Ed.) pp 744-751, Academic Press, New York & London.
- Lowry, O.H., Rosenburgh, N.J., Farr, A.L. & Randell R.J. (1951) *J. Biol. Chem.*, 193, 265-275.
- Lundquist, F. (1949) *Acta Physiol. Scand.*, 19, [Suppl.] 66.
- McGuire, J.S., Hollis, V.W. & Tomkins, G.M. (1960) *J. Biol. Chem.*, 235, 3112-3122.
- McGuire, J.S. & Tomkins, G.M. (1960) *J. Biol. Chem.*, 235, 1634-1638.
- Mahler, H.R. & Cordes, E.H. (1966) *Biological Chemistry*, pp 250-256, Harper & Row, New York, Evanston, London.
- Mainwaring, W.I.P. (1970) *Biochem. Biophys. Res. Commun.*, 40, 192-198.
- Mainwaring, W.I.P. & Peterkin, B.M. (1971) *Biochem. J.*, 125, 285-295.
- Malathi, K. & Gurpride, E. (1977,a) *J. Steroid Biochem.*, 8, 141-145.
- Malathi, K. & Gurpride, E. (1977,b) *J. Steroid Biochem.*, 8, 743-746.

Mann, T. (1954) Proc. R. Soc. Lond. Biol., 142, 21-32.

Mann, T. (1964) The Biochemistry of Semen and the Male Reproductive Tract, Methuen & Co., London.

Marberger, H., Segal, S.J. & Flocks R.H. (1957) J. Urol., 78, 287-293.

Mauvaus-Jarvis, P., Kuttan, F. & Baudot, N. (1974) J. Clin. Endocrinol. Metab., 38, 142-147.

Mawson, C.A. & Fisher, M.I. (1952) Can. J. Med. Sci., 30, 336-339.

Menard, R.H., Bartter, F.C. & Gillette, J.R. (1976) Arch. Biochem. Biophys., 173, 395-402.

Menard, R.H. & Martin, H.F. (1974) Life Sciences, 15, 1639-1648.

Menard, R.H., Stripp, B. & Gillette, J.R. (1974) Endocrinology, 94, 1628-1636.

Mercier-Bodard, C., Marchut, M., Perrot, M., Picard, M-T., Baulieu, E.E. & Robel, P. (1976) J.Clin.Endocrinol.Metab., 43, 374-386.

Michaelis, L. & Menten, M.I. (1913) Biochem. Z., 49, 333-369.

Moore, R.J. & Wilson, J.D. (1972) J. Biol. Chem., 247, 958-967.

Moore, R.J. & Wilson, J.D. (1973) Endocrinology, 93, 518-592.

Moore, R.J. & Wilson, J.D. (1974) Biochemistry, 13, 450-455.

*

Morfin, R.F., Aliapoulos, M.A., Bennet, A.H., Harrison, J.H. & Ofner, P. (1970) Hormonal Steroids. Proc. 3rd Int. Congr. Hormonal Steroids, (James, V.H.T. & Martin L., Eds.) pp 337-345, Excerpta-Medica., Amsterdam.

Morfin, R.F., Berovici, J-P., Charles, J.F. & Flock, H.H. (1975) J. Steroid Biochem., 6, 1347-1352.

Morrison, R.T. & Boyd, R.N. (1966) Organic Chemistry, 2nd edn., pp 354-355, Allyn & Bacon, Inc., Boston.

Murphy, B.E.P. (1968) Can. J. Biochem., 46, 299-302.

* Moore, R.J. & Wilson, J.D. (1976) J. Biol. Chem., 251, 5895-5900.

- Negro-Vilar, A., Sadd, W.A. & McCann, S.M. (1977) *Endocrinology*, 100, 729-737.
- Newsam, J.E. & Thomson, D. (1974) *A Companion to Medical Studies*, Vol. 3 (R. Passmore & J.S. Robson, Eds.) Blackwell Scientific Publications.
- Nozu, K. & Tamaoki, B-I. (1973) *Acta Endocrinol.*, (Kbh), 73, 585-598.
- Nozu, K. & Tamaoki, B-I. (1974) *Acta Endocrinol.*, (Kbh), 76, 608-624.
- O'Malley, B.W. & Schrader, W.T. (1976) *Scientific American*, Vol. 234, No.2, p 32-43.
- Orestano, F., Altwein, J.E., Knapstein, P. & Bandhouer, K. (1975) *J. Steroid Biochem.*, 6, 845-851.
- Paulson, D.F. & Kane, R.D. (1975) *J. Urol.*, 113, 811-815.
- Pearlman, W.H. & Pearlman, M.R.J. (1961) *J. Biol. Chem.*, 236, 1321-1327.
- Perlin, A.S., Machie, D.M. & Dietrich, C.P. (1971) *Carbohydr. Res.*, 18, 185-194.
- Pézarid, A. (1911) *C.R. Acad. Sc. Paris*, 153, 1027-1029.
- Pierrepont, C.G. & Davies, P. (1973) *J. Reprod. Fertil.*, 35, 149-152.
- Pierrepont, C.G., Davies, P., Millington, D. & John, B. (1975) *J. Reprod. Fertil.*, 43, 293-303.
- Price, D. (1963) *Natl. Cancer Inst. Monog.*, 12, 1-28.
- Rangno, R.E., McLeod, P.J., Rendy, J. & Ogilvie, R.I. (1971) *Clin. Pharmacol. Ther.*, 12, 658-665.
- Reed, M.J. & Stitch, S.R. (1973) *J. Endocrinol.*, 58, 405-419.
- Rennie, P. & Bruchofsky, N. (1973) *J. Biol. Chem.*, 248, 3288-3297.
- Robel, P., Lasnitzki, I. & Baulieu, E.E. (1971) *Biochimie*, 53, 81-96.
- Robel, P., Corpéchet, C. & Baulieu, E.E. (1973) *FEBS Letters*, 33, 218-220.

- Roček, J., Westheimer, F.H., Eschenmoser, A.,
Moldovanyi, L. & Schreiber, J. (1962)
Helvetica Chimica Acta, 45, 2554-2567.
- Roy, A.B. (1971) Biochimie, 53, 1031-1040.
- Sadée, W., Dagcioglu, M. & Schroder, R. (1973)
J. Pharmacol. Exp. Ther., 185, 686-695.
- Sanborn, T.A., Kowle, R.L. & Sallach, H.J. (1975)
Endocrinology, 97, 1000-1007.
- Schmidt, H., Noack, I. & Voigt, K.D. (1972)
Acta Endocrinol., (Kbh), 69, 165-173.
- Schmidt, H., Giba-Tziampini, O., Rotteck, G.V. &
Voigt, K.D. (1973) Acta Endocrinol., (Kbh),
73, 599-611.
- Schubert, M. & Hamerman, D. (1968) A Primer on
Connective Tissue Biochemistry, Lea & Fabiger,
Philadelphia.
- Scott, W.W. (1963) Natl. Cancer Inst. Monog., 12,
111-130.
- Scott, W.W. & Wade, J.C. (1969) J. Urol., 101, 81-85.
- Sealey, J.E., Gerten, J.N., Ledingham, J.G.G. &
Larach, J.H. (1967) J. Clin. Endocrinol.,
27, 699-705.
- Shain, S.A. & Boesel, R.W. (1975) J. Steroid Biochem.,
6, 43-50.
- Shain, S.A., Boesel, R.W. & Axelrod, L.R. (1975)
Arch. Biochem. Biophys., 167, 247-263.
- Sherins, R.J. & Janick, J.J. (1976) 58th Annual
Meeting of the Endocrine Society, Abs. 54, p 83.
- Shimazaki, J., Ohki, Y., Kurihara, H., Furuya, N. &
Shida, K. (1973) Endocrinol. Jpn., 20,
489-496.
- Shuttelworth, K.E.D. & Blandy, J.P. (1976) Urology,
Vol. II, p 926-953. (J.P. Blandy, Ed.)
Blackwell Scientific Publications, Oxford.
- Siiteri, P.K. & Wilson, J.D. (1970) J. Clin. Invest.,
49, 1737-1745.

- Sliteri, P.K. & Wilson, J.D. (1974) J. Clin. Endocrinol. Metab., 38, 113-125.
- Silva, M.E. & Dietrich, C.P. (1975) J. Biol. Chem., 250, 6841-6846.
- Sinowatz, F. & Pierrepont, C.G. (1977) J. Endocrinol., 72, 53-58.
- Skinner, R.W.S., Pozderac, R.V., Counsell, R.E. & Weinhold, P.A. (1975) Steroids, 25, 189-202.
- Steinberger, E. & Fisher, M. (1971) Endocrinology, 89, 679-684.
- Stripp, B., Taylor, A.A., Bartter, F.C., Gillette, J.R., Loriaux, D.L., Easley, R. & Menard, R.H. (1975) J. Clin. Endocrinol. Metab., 41, 777-781.
- Struthers, G. (1975) Unpublished data.
- Sullivan, J.N. & Strott, C.A. (1973) J. Biol. Chem., 248, 3202-3208.
- Talalay, P., Loewus, F.A., Vennesland, B. (1955) J. Biol. Chem., 212, 801-809.
- Tan, S.Y., Antonipillai, I. & Pearson Murphy, B.E. (1974) J. Clin. Endocrinol. Metab., 39, 936-941.
- Taurog, J.D., Moore, R.J. & Wilson, J.D. (1975) Biochemistry, 14, 810-817.
- Tidball, M.E. (1967) Am. J. Physiol., 213, 407-410.
- Tisell, L-E. (1970) Acta Endocrinol.,(Kbh), 64, 637-655.
- Tomkins, G.M. (1957) J. Biol. Chem., 225, 13-24.
- Troop, R.C. (1969) Eur. J. Pharmacol., 7, 80-84.
- Troop, R.C. & Biggs, J.T. (1965) Metabolism, 14, 867-872.
- Troop, R.C., Krzanowski, P.T. & Biggs, J.T. (1966) Metabolism, 15, 542-547.
- Tullner, W. (1963) Natl. Cancer Inst. Monog., 12, 211-224.
- Turner, C.D. & Bagnara, J.T. (1971) General Endocrinology (5th edition) p 439-489, W.B. Saunders Company, Philadelphia, London, Toronto.

- Verhoeven, G. & DeMoore, P. (1971) *Endocrinology*, 89, 842-846.
- Verhoeven, G., Lamberigts, G. & DeMoore, P. (1974) *J. Steroid Biochem.*, 5, 93-100.
- Vermeulen, A. & Verdonck, L. (1972) *J. Steroid Biochem.*, 3, 421-426.
- Vermeulen, A. & Verdonck, L. (1976) *J. Steroid Biochem.*, 7, 1-10.
- Waldman, A.A., Marx, G. & Goldstein, J. (1974) *Biochim. Biophys. Acta*, 343, 324-329.
- Wallace, A.M. (1975) *Androgen Metabolism in the Human Prostate Gland. Ph. D. Thesis. (Univ. of Glasgow.)*
- Wallace, A.M. & Grant, J.K. (1975) *Transactions of the Biochemical Society*, 540-542.
- Walsh, P.C. & Siiteri, P.K. (1975) *J. Urol.*, 114, 254-256.
- Warwick, R. & Williams, P.L. (1973) *Gray's Anatomy (35th edition)* pp 1348-1350, Longmans, London.
- Watkinson, J.M., Delory, G.E., King, E.J. & Haddow, A. (1944) *Br. Med. J.*, 2, 492-495.
- Wendel, E.F., Brannen, G.E., Putong, P.B. & Groylaek, J.T. (1972) *J. Urol.*, 108, 116-119.
- Westheimer, F.H. & Nicolaidis, N. (1949) *J. Am. Chem. Soc.*, 71, 25-28.
- Wilson, J.D. (1972) *N. Engl. J. Med.*, 287, 1284-1291.
- Wilson, E.M. & French, F.S. (1976) *J. Biol. Chem.*, 251, 5620-5629.
- Wilson, J.D. & Gloyna, R.E. (1970) *Recent Prog. Horm. Res.*, 26, 309-336.
- Wilson, J.D. & Lasnitzki, I. (1971) *Endocrinology*, 89, 859-868.
- Wurtman, R.J. & Jensen, E.V. (1968) *Science*, 159, 1261.

