SOME ASPECTS OF STEROID ENDOCRINOLOGY

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Dear Sir,

Submission for the Degree of D.Sc.
Some Aspects of Steroid Endocrinology.

I wish to submit the enclosed thesis for consideration for the degree of D.Sc. These publications have not been submitted in whole, or in part, for any other degree.

I certify that the works in the thesis are my own. In earlier years, much of the work was undertaken by the author. In later years, my work was conducted through my various post-doctoral fellows, postgraduate Ph.D. students or technical staff, often in close association with various clinical colleagues who supplied the tissues for the different forms of analysis. A list of colleagues who have supported the work of the author is given in the thesis and the position or role of these collaborators is stated.

Yours sincerely,

Keith Griffiths.
ABSTRACT

This thesis is concerned with the steroid biochemistry of endocrine-related cancer, particularly that concerned with carcinoma of breast and prostate, although investigations have covered various other topics such as the relationship between structure and function of the cells of the human adrenal cortex, steroid biosynthesis in tissue from ovarian and testicular tumours and a study of the endocrine factors controlling parturition in the sheep.

The adrenal gland studies directed attention to the ability of cells from both zona reticularis and zona fasciculata to synthesise and secrete cortisol and corticosterone. The capacity of testicular tissue from a patient with testicular feminisation syndrome to synthesise testosterone was an interesting observation and the possibility that the particulate material, talc, may be concerned in the aetiology of ovarian cancer will stimulate interest for a number of years.

The observed capacity of breast tissue to metabolise steroids identified the paraendocrine function of a breast cancer. The relationship between oestrogen receptor status, prognosis and the natural history of breast cancer clearly indicated the value of such assays for the management of patients with the disease. The role of prolactin in breast disease and the biochemical action of tamoxifen have also been studied. A major section of this thesis deals with the aetiology and endocrinology of prostatic cancer and the monitoring of patients undergoing treatment for the disease.
SOME ASPECTS OF STEROID ENDOCRINOLOGY

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INTRODUCTION
This Thesis is concerned with various aspects of steroid endocrinology and relates to investigations reported in papers published during the period of time between 1960 and 1983. The work was undertaken in three Centres of research: during one year in the Department of Histochemistry, University of Minnesota USA, five years during the author's term as a lecturer in the Department of Steroid Biochemistry, University of Glasgow and thirdly from 1966 to 1983, after moving to establish the newly built Tenovus Institute for Cancer Research, in the role of Director of Research and Professor of Cancer Research in the Welsh National School of Medicine of the University of Wales.

Five senior scientists have provided the author with tremendous support and encouragement throughout the past three decades. Their attitudes, flair and originality in their approach to research and their fastidious scientific care and discipline made a lasting impression. It is with pride and affection that the author records the privilege of working closely with the late Professor Guy Marrian, Professor of Biochemistry, University of Edinburgh until 1959, Dr. Jim Grant, Department of Steroid Biochemistry, University of Glasgow, Professor Sir Thomas Symington, University of Glasgow, Professor Pat Forrest, University of Edinburgh and Professor David Glick, University of Minnesota, U.S.A.

Some of the earlier investigations were concerned with the functional zonation of the human adrenal cortex and the steroid biosynthetic activity of the two major regions of the cortex, the zona fasciculata and zona reticularis. This work was undertaken in association with Professor Sir Thomas Symington and Dr. J.K. Grant. These studies clearly established that the cells of both these
zones were capable of the synthesis and secretion of the adreno-corticosteroids, cortisol and corticosterone, contrary therefore to the earlier belief that the source of these steroid hormones was exclusively the zona fasciculata. The work did indicate that possibly, because of the cholesterol deposits in the clear cells of the zona fasciculata, this tissue responded more effectively to stimulation by corticotrophin. Although the investigations suggested that 11β-hydroxyandrostenedione was similarly synthesised and secreted by the cells of both cortical zones, later studies demonstrated that the enzyme systems for sulphating dehydroepiandrosterone (DHA) were localised in the zona reticularis, thereby indicating this tissue as the source of adrenal DHA-sulphate secretion. The techniques to specifically localise enzymic activity in the various layers of the adrenal cortex were established after the author's period of study in the Department of Histochemistry, University of Minnesota, where the influence of corticotrophin on the 11β-hydroxylase enzyme system was investigated in association with Professor David Glick. Research activity was also directed to the establishment of procedures to elucidate the biosynthetic pathways for the production of the adrenal steroids and these experiments unequivocally showed that 17α-hydroxyprogrenenolone was the major intermediary steroid in the synthesis of cortisol from pregnenolone. Progesterone, when formed from pregnenolone, was converted to corticosterone and little was hydroxylated at the C-17 position. Some of the earlier experiments, which drew attention to the possibility that adrenal tumour cells may well lose their ability to respond specifically to corticotrophin, were undertaken with human tissue and it appeared that the tissue was to some extent
capable of responding to other pituitary hormones such as prolactin.

During the author's period in the University of Glasgow, the opportunity came to study the capacity to synthesise testosterone, of testicular tissue removed from a young patient with testicular feminisation syndrome. The feminine phenotype of the patient suggested an inability of these cryptorchid testes to secrete the male androgenic steroid. Surprisingly, the tissue was found to be quite effective in vitro in producing testosterone and it was soon subsequently shown, that the concentrations of this steroid in plasma in such patients were of a normal male level directing attention therefore to the possibility of end-organ defects in the action of testosterone. Similar studies to determine the biosynthetic capacity of pathological testicular tissue were then completed whenever possible. The trend of the investigations again centred on attempts to determine the steroid biosynthetic capacity of histologically well identified cell types and certain pioneering studies were established with the late Dr. A.H. Baillie, to develop histochemical techniques to identify and localise steroid metabolising enzymes in the various cells of steroid secreting glands.

In the early 1960's, much of the research in steroid endocrinology centred on determining the pathways by which steroid hormones were secreted and the possible abnormalities of these biosynthetic systems in the cells of tumours such as the androgen secreting ovarian hilus cell tumour, or of 'feminising' ovarian cancers, such as the granulosa cell tumour of the ovary, which produced precocious effects in younger children. Techniques developed by the author to intensively investigate such biosynthetic pathways were applied to
ovarian tumour tissue and then later, to normal ovarian tissue, particularly tissue of the human corpus luteum, to study the synthesis of oestrogens from C₁₉-steroids via their 19-hydroxylated derivatives. The author's first PhD student, Dian Fahmy, was concerned with this programme of work. The ability of such cells to simultaneously produce progesterone as well as oestrogens was not then, easily understood and it seemed reasonable to search for alternative biosynthetic pathways involving precursors other than progesterone, for the synthesis of the C₁₉- and C₁₈-steroids.

At the same time, the presence of certain 'luteinised thecal cells', not unlike the cells of the corpus luteum, were often described in association with malignant ovarian adenocarcinomas. Did such cells contribute synthesised steroid hormone, locally, to promote carcinogenesis or tumour growth? What in fact initiated cancer in the human ovary?

Of particular interest in this respect, was the identification of fine particles of talc embedded in both normal and carcinomatous ovarian tissue removed at surgical operation. Talc, a magnesium silicate, with a chemical composition not unlike the various forms of asbestos believed to be responsible for the initiation of mesotheliomas of lung, was identified by electron microscope microanalysis, using an extraction-replication technique developed in the Institute for the study of biological tissues with Mr. William Henderson, Chief Technical Research Officer in the Electron Microscopy Unit. The requirement for more effective procedures to investigate particulate contamination of human biological tissues was seen as a prerequisite to an investigation into such foreign particulate material, present in carcinoma of lung and stomach, that might be
considered environmental carcinogens. Whether talc can initiate carcinogenesis in the ovary remains to be established. Certainly talc can migrate to the ovary after being placed within the cervix of the female rat and sub-bursal insertion of talc into the ovary of the animal will induce, within the surface epithelium of the ovary, small areas of papillary change which might be considered early preneoplasia, but care has been taken not to irresponsibly interpret this data.

It is generally accepted that malignant ovarian tumours originate from the surface germinal epithelium and occur most frequently during the peri-menopausal and early postmenopausal years, the incidence being higher in nulliparous women. Such epidemiological data might suggest a hormonal influence on the development of these rare, yet most aggressive tumours and studies of a young American postgraduate student, Tom Hamilton, complementary to the talc investigation, were established to investigate the steroid receptor characteristics of ovarian cancer and the hormonal responsive nature of the cells of the surface germinal epithelium. Evidence was obtained for the presence of androgen, oestrogen and progesterone receptor proteins and a suggestion also that glucocorticoid receptors were present. The close relationship between these receptor profiles and those seen in breast cancer certainly direct attention to the potential of endocrine treatment as part of the overall therapeutic strategy for the clinical management of ovarian cancer and to the possibility that androgens may be implicated in its aetiology.

The potentially harmful effects of talc on a highly differentiated tissue such as the ovary with its inter-related cell types and cyclical changes of secretory activity should not be ignored.
Little is really known about the aetiology of ovarian carcinoma and any lead, established on sound data, should be followed.

In 1968, the interest of the author in steroid biochemistry and the arrival in Cardiff of the newly appointee to the Chair of Obstetrics and Gynaecology, Professor Alec Turnbull, resulted in a very natural and exciting research association directed to the investigation of the endocrine factors concerned with the initiation of parturition. Since ethical considerations prevented the development of reasonable investigations in late human pregnancy, the study centred on the metabolism of steroids by the pregnant sheep, its foetus and placenta. A grant from the Wellcome Trust supported the programme and Dr. Colin Pierrepoint and the late Dr. Anne Anderson completed the senior research team.

The interest of the group had been stimulated by previous studies of Dr. Liggins in New Zealand, showing that parturition could be induced prematurely in the sheep by the continuous infusion for several days of either cortisol or corticotrophin into the lamb in utero, thereby tending to implicate foetal adrenal synthesis and secretion of corticosteroids in the initiation of parturition.

The studies of the group centred on the steroid metabolising enzyme systems of the maturing sheep foetus and placenta. Incubations of steroids in vitro were conducted with foetal and maternal placental tissue, foetal liver and adrenal glands removed from foetuses in the normal course of gestation and from lambs in whom premature parturition had been induced by the intra-peritoneal infusion of Synacthen in utero. Little was then known about steroid hormone biosynthesis in foetal or adult ovine adrenal glands and
this formed the subject of a PhD thesis eventually submitted by Dr. Anderson. Biosynthetic pathways for cortisol production were studied. Evidence was obtained for a marked increase in 11β-hydroxylase activity in the final few days of gestation and the results quickly indicated that there were few similarities to the concept of a human foetoplacental unit described by Egon Diczfalusy. For example, neither the foetal nor adult adrenal tissue possessed steroid sulphokinase activity allowing the formation of DHA sulphate, so closely involved in oestrogen synthesis in the human foetoplacental unit. On the other hand, epitestosterone featured as a metabolite from incubations of precursor C19-steroids with placental tissue as did oestradiol-17α and various oestrogen sulphates, particularly oestrone sulphate. The human placenta however appeared to lack the capacity to synthesise steroid sulphates.

The activities of the group stimulated interest at that period of time. Considerable information accumulated from the research programme and work in this field of endocrinology was continued by Dr. Anderson when she and Professor Turnbull moved on to Oxford.

In 1966, when the author was appointed Director of Research at the newly completed Tenovus Institute, it was very reasonable and appropriate that the expertise and experience of steroid endocrinologists should be directed to the study of cancer of the breast and prostate gland, two major causes of death from cancer.

The principal clinical collaborator with the author on the prostatic cancer programme has been Mr. Brian Peeling, Head of Urology, St. Woolos Hospital, Newport, South Wales. Dr. Colin Pierrepont was also associated with much of the work and many
postgraduate students have been concerned with the studies, two in particular Maureen Harper and Peter Davies, who now work as post-doctoral senior research scientists in the Institute.

The androgen dependent nature of prostatic cancer has been long established and its treatment by antiandrogen therapy, such as castration or diethylstilboestrol (DES) administration well accepted, following the pioneering studies of Charles Huggins in the early 1940s. It was only realised in the late 1960s and in the early part of 1970, however, that testosterone was effectively metabolised by the male accessory sex organs such as the prostate, and that 5α-dihydrotestosterone (5α-DHT) was probably the principal, active androgen within the cell, controlling its growth and function. This stemmed from work of various research groups, including the author's, in which Maureen Harper played a major role. 5α-DHT was further metabolised to various 5α-androstanediols and work was directed to the possible biochemical effects within the cell of these various metabolites of testosterone. It was interesting to speculate that the metabolites may indeed have specific roles to play in eliciting the androgenic response and preliminary studies on the effects, in vitro, of these steroids on prostatic enzyme systems such as the DNA nucleotidyltransferase (DNA-polymerase) supported such a concept. This approach was followed a year or so later by Peter Davies, in investigations directed to the effects in vitro of steroids on prostatic DNA-dependent RNA-polymerase.

It was clearly possible that the prostate gland itself could induce its own diseased state by transformation of steroids presented to it in the plasma, some of which could be of adrenal origin. Abnormalities of the metabolic pattern of testosterone metabolism
could lead to changes in the intracellular concentrations of the various metabolites with consequent varying effects on the processes concerned with growth. High resolution selected ion monitoring provided valuable data on the concentrations of these testosterone metabolites in prostatic tissues and use was made of a model system, the BIO 87.2 strain of Golden Hamster, which spontaneously develops benign prostatic hypertrophy, to investigate the relationship of such changes to disease progression. These studies tended to suggest that abnormal levels of testosterone metabolites in dysfunctional prostatic tissue may be a consequence of, rather than a cause of the condition.

There was uncertainty at this time about the effects of DES on the pituitary-testicular-prostatic axis. It was not completely understood whether the principal effect was on the pituitary, thereby suppressing LH secretion with a consequent decrease in production of testosterone by the testis, or if the therapeutic effect was essentially based on a direct action on either testis or prostate. Work was directed to this problem and to the potential of other stilboestrol analogues that could equally well have an effective anti-androgenic action, but with less oestrogenticity, considered by many urologists to be a major clinical problem of DES treatment and the cause of cardiovascular difficulties.

The inhibitory effects of DES and various other stilboestrol analogues on the isolated prostatic DNA-polymerase and DNA-dependent RNA-polymerase enzyme systems were of interest. It was also interesting that administration of these compounds affected the metabolism of testosterone by prostatic tissue, work of another young PhD student from Thailand, Varapan Danutra. She was also able
to demonstrate that DES directly affected the testis, influencing the enzymes concerned with steroid biosynthesis and the subsequent secretion of testosterone, prior to the observed suppression of LH output by the pituitary.

The fundamental knowledge of the biochemical mechanisms by which the androgens control the growth and development of the prostate gland is clearly the essential underpinning to any understanding of the basic cellular abnormalities associated with neoplasia of the organ. Preliminary studies with Peter Davies centred on to the development of a reconstituted system in vitro to study the effects of testosterone and its metabolites on DNA-dependent RNA-polymerase prepared from prostatic nuclear preparations. Direct stimulation of the enzymic activity by 5α-DHT in the presence of prostatic cytosol, directed attention to the requirement for the androgen-receptor complex to influence and stimulate transcriptional activity. This stimulation was dependent on the presence of a specific native prostatic chromatin template. The degree of stimulation, by steroid-receptor complexes, of the nucleolar or extranucleolar RNA polymerase activity was studied under varying ionic conditions. Studies on the effect of the acidic receptor complex on the extent of prostatic template availability and the relative specificity of the action of human and rat prostatic receptor complexes, the latter indicating structural similarities of receptor proteins from different species, followed in natural order.

Although prostatic tissue from the rat provided the basis for these studies, in vitro, on the interaction of the steroid-receptor complex with the genome and the factors that control gene expression, attempts were consistently made to show that mechanisms analogous to
those in the animal species existed in human prostatic tissue, particularly that from patients with benign prostatic hypertrophy, the aetiology of which was often related to high intracellular 5α-DHT. Despite difficulties, procedures for studying human prostatic preparations were established, leading the way to a better understanding of the influence of the androgen-receptor complex on the genome of normal and diseased human tissue. As part of the rat study, procedures were also developed to study occupied and unoccupied androgen receptor sites, both nuclear and cytoplasmic, techniques which were used for a more complete assessment of the androgenic status of human prostatic tissues.

Whereas endocrine therapy, either castration or the administration of an oestrogen such as DES was generally accepted in the late 1960's as the most effective primary treatment for advanced carcinoma of the prostate, the role of the androgens, or other plasma hormones, in the aetiology of the disease was little understood. Similarly, little was known about the endocrinology of the disease during treatment. Although the majority of patients initially responded, inevitably they relapsed as the disease recurred, or progressed, and the secondary production of androgens by the adrenal gland was often considered the reason. Adrenalectomy or hypophysectomy at this late stage in the disease process, however, rarely produced an effective clinical response.

In association with clinical colleagues, but particularly, Mr. Brian Peeling, the concentration of various hormones were measured, by newly developed radioimmunoassays, in plasma of patients taken when they presented with prostatic cancer. Furthermore, changes in these hormone profiles were monitored during treatment in
relation to the progression of the disease. This investigation was to some extent the stimulus to the development of the British Prostate Study Group as other urologists in the United Kingdom joined the study.

Many interesting observations were made. No relationship was found between primary tumour staging (T category) and hormone levels. No progressive relationship was seen as the disease developed from T1 to T4, although some evidence emerged from multivariate analysis that growth hormone levels were higher in patients with metastatic disease. More data, recently analysed, however, now indicates that pre-treatment concentrations of testosterone and LH in plasma, which relate to the histological grading of the tumour, also correlate with survival of the patient, thereby allowing the establishment of a prognostic index for the selection of high risk patients. This index is now being used in a prostatic cancer trial in South Wales where the 'high risk' patients are being identified for more aggressive therapy and the low risk then undergo castration, or receive the I.C.I. LH-RH analogue, 118630, a compound extensively studied by the Institute prior to its clinical trials.

Little if any relationship was found between plasma levels of spermine or spermidine, considered potentially useful tumour markers, and tumour stage or metastatic status of patients with carcinoma of the prostate.

Honvan, DES-diphosphate was given in massive doses in the early 1970's resulting in a decrease in plasma testosterone concentration, but elevation of prolactin and GH levels. Experimental studies had earlier implicated both hormones in influencing to some extent, prostatic growth and a certain amount of evidence had been
obtained to suggest that prolactin may have a role in the synthesis of $C_{19}$-steroids by the adrenal gland. The use of high doses of the DES derivative seemed contraindicated.

A section of this thesis is however concerned with certain studies on the relationship between prolactin and the prostate gland, the Institute being possibly the first laboratory in Europe to develop a radioimmunoassay for human prolactin.

The co-operative studies of the British Prostate Study Group quickly indicated that 1 mg DES (t.d.s.) was an acceptable dose, sufficient to lower plasma testosterone concentrations to castration levels and for a while, was used as a major form of therapy by the Group, although subcapsular orchidectomy is now considered the treatment of choice after checking its effectiveness in lowering plasma testosterone levels.

Following castration, or DES treatment, the plasma testosterone concentration reached values of approximately 60 mg %, and this level of testosterone was shown by stimulation and suppression tests, to be of adrenal origin. It seemed reasonable to suggest that anti-adrenal therapy, prednisolone treatment or administration of an antiandrogen, to either remove the potential effect of the adrenal androgen on the prostatic tissue, or block its action, might form part of the primary treatment. No evidence was obtained that disease progression could be related to increased adrenal activity during the period of treatment and it was clear that the disease progressed principally due to the cancer cells, whose growth was independent of hormonal influence. It was interesting however, and important to report, that the development of a radioimmunoassay for DES allowed a study which indicated that in certain patients, 3 mg
DES daily may not completely suppress pituitary and testicular activity and castration levels of plasma testosterone may not be realised in these men. Lack of response to therapy is not therefore due to the hormonal independence of the carcinoma and it suggests that regular monitoring of plasma or salivary levels of testosterone are necessary in these patients.

In relation to this, was the realisation that in monitoring the levels of various hormones in plasma of patients being treated for relatively long periods of time, good quality control of assay procedures was required if comparison of data was to be made throughout the investigation. With an associate, Doug Wilson, together with Professor Kenneth Kemp and his colleagues in the Department of Mathematical Statistics and Operational Research, University College, Cardiff this resulted in the development of a new approach to quality control of hormone assays and the work from the study comprises a section of this thesis.

Equally of interest has been the development of immunoassays for steroids in saliva. Although previously considered to be possible, no real attention had been paid to the potential of such salivary assays for the assessment of the endocrine status of individuals. New and sensitive assays had to be developed. It was found that the concentration of steroid in saliva relates to the free, non-protein bound steroid levels of plasma which is of course the moiety considered the biological active hormone in plasma. This clearly offers a new dimension to endocrine investigations. Furthermore, despite previously established concepts, steroid concentrations in saliva do not change with change in salivary flow rate. This aspect of our work will over the next
few years make an immense impact in endocrinology and in studies on endocrine cancer. Much is due to the enthusiasm and drive of my colleague, Dian Fahmy, who together with our younger colleagues and PhD students, Richard Walker, Asli and Atilla Turkes and Graham Read, has done so well in promoting and developing this important, new aspect of endocrinology.

Perhaps the most challenging aspect of the author's investigations has been related to carcinoma of the breast, and our attempts to increase our understanding of this most difficult disease. Early work in the Institute in the late 1960's tended to be dominated by work emanating from another centre for cancer research in the United Kingdom, the Imperial Cancer Research Fund. There, Bulbrook and his colleagues described a "discriminant function", a mathematical concept of sorts, which tended to suggest that it might be possible to identify women who may be at risk of developing breast cancer. Work of the author's team, with his colleague Professor Pat Forrest, was directed to testing in another Centre, the potential value of the urinary analyses which Bulbrook found so effective in London. The adrenal androgens, particularly DHA sulphate appeared to be implicated in the aetiology of breast cancer and the determination of plasma levels of this steroid in patients with the disease seemed reasonable. Concurrently with John Adams, working in Australia, the hypothesis was tested that breast tumour tissue could have a paraendocrine function and metabolise $C_{19}$-steroids to oestrogens, or to other $C_{19}$-steroids, which may have an intracellular role in controlling the growth promoting action of oestradiol-17$\beta$. Androstene-3$\beta,17$-$\beta$-diol, for example, was shown to translocate the oestrogen receptor in the
dimethylbenzanthracene (DMBA) induced mammary tumour of rats suggesting a role for this metabolite of DHA sulphate in controlling the level of cytosolic receptor available for the promotion of an oestrogenic effect. Later, mass spectrometry, using high resolution selected ion monitoring was used to determine the levels of DHA-sulphate and its metabolites in breast tumour tissue.

As was previously indicated, the Institute was one of the first laboratories pioneering the development of radioimmunoassays for plasma protein hormones. Arthur Boyns, a colleague in the Institute in the early 1970's, worked hard in developing a radioimmunoassay for plasma prolactin which was extensively used in co-operative studies with Professor Forrest and his colleagues, and later with Professor Sellwood and his group, to attempt to identify the role of prolactin in human breast cancer. Little data of real interest emerged, although it was shown that certain women with benign breast disease had elevated levels of plasma prolactin a finding which consequently led to the assessment of bromocryptine therapy for the treatment of this condition. There is still a great deal to learn about the relationship of prolactin to the aetiology of breast disease and work in the Institute continues along these lines.

The Institute was one of the first laboratories in the United Kingdom to receive samples of an ICI compound, ICI 46,474, considered to have some potential as an oral contraceptive, for further biochemical studies. The late Dr. Arthur Walpole of ICI Ltd. did however, wonder if ICI 46,474 affected the DMBA-induced mammary cancer of rats. Bob Nicholson, then a Ph student, now a senior colleague in the Institute, devoted considerable effort in studying
the biochemical effects of this compound, soon to be called Tamoxifen and now used world-wide as an antioestrogen for the treatment of breast cancer. Surprisingly then, Tamoxifen was shown not only to prevent oestrogen binding to its receptors, but it also translocated the oestrogen receptor to the nucleus, promoted RNA synthesis and generally was seen to have various oestrogenic effects. A major contribution was made in ascertaining the mode of action of Tamoxifen in the control of breast cancer growth. At the same time, the interesting effect of Tamoxifen of increasing plasma levels of oestradiol-17β when administered to premenopausal women was described.

Perhaps one of the most valuable associations was developed with Roger Blamey, a young surgeon studying for a higher degree in the Institute in the late 1960's who subsequently became Professor of Surgery in the University of Nottingham. This resulted in the Tenovus-Nottingham study on the natural history of human breast cancer and the value of the determination of steroid receptor content of primary breast tumours in relation to disease prognosis and other clinical factors considered relevant. Many American scientists including Elwood Jensen and Bill McGuire directed their attention to the clinical value of oestrogen and progesterone receptor concentrations in metastatic breast tissue. The Institute-Nottingham study was initiated in 1973 and directed to the potential prognostic value of receptor analyses in primary breast tumour tissue, their clinical use for deciding on appropriate therapy once the disease had recurred and their relationship to survival. These investigations were particularly successful, an immense amount of data has been derived from the study clearly suggesting the possi-
bility that a prognostic index might be used in the management of
the disease.

All patients in Nottingham were seen in a specialised Breast
Cancer Clinic supervised by one senior surgeon, Professor Blamey.
The clinical value of the receptor analysis of primary tumours
removed from patients presenting at one of 15 clinics of surgeons
managing breast cancer in a major city, Liverpool, was also assessed.
This study was established with the cooperation of Professor R.
Shields and his colleagues in the University of Liverpool and
again provided information of interest in the evaluation of receptor
data available from such a source. The potential of fluorescein
labelled steroids for the localisation of receptor in sections of
breast tumour tissue was also assessed and the development of
techniques for the isolation and purification of oestrogen receptor
protein, work of a PhD student, Nabeel Al-Nuaimi from Iraq, is
hoped will ultimately lead to an immunoassay for this clinically
valuable analysis.

The most innovative concept emerged from an association with a
most inspiring colleague, Dr. Hugh Simpson, who was a student with
the author in the University of Edinburgh and is now Reader in the
Department of Pathology, University of Glasgow. It was becoming
very obvious that breast cancer generally presents as a systemic
disease and that more effort was required to identify the breast
at risk, or at least preneoplasia or early cancer, long before
the disease is clinically manifest as a palpable lump. The
concept of the "chronobra" was developed jointly between Glasgow
University and the Institute. Essentially, the surface temperature
of the breast was measured by heat sensors built into the
'chronobra', but not at one period of time as with thermography, but regularly at short intervals of time, possibly every 3 minutes for up to 72 hours. Variations in the rhythmic parameters of the temperature changes with time were found which indicated that areas of the breast where cancer was present might be identified. Considerably more sophisticated electronics are required before such a device will see clinical or routine service, but the concept was tested and found correct, that accurately determined breast temperature rhythms are capable of identifying abnormality, which might ultimately allow the 'preneoplastic breast' to be found and such women subjected to close clinical monitoring and care.
I. INVESTIGATIONS RELATING TO CANCER OF THE PROSTATE.
1. INVESTIGATIONS RELATING TO CANCER OF THE PROSTATE.

A. Steroid hormone action in prostatic tissue.

(1) The effect of steroids on deoxyribonucleic acid polymerase activity.
Biochem. J. (1967), 105, 6c-7c.

(2) The inhibition of deoxyribonucleic acid nucleotidyltransferase by stilboestrol derivatives.
A. R. Fahmy and K. Griffiths.

(3) The effect of some stilboestrol compounds on deoxyribonucleic acid polymerase from human and canine prostatic tissue.

(4) The effect of some stilboestrol compounds on DNA polymerase from human prostatic tissue.

(5) The effect of prostatic metabolites of testosterone and other substances on the isolated deoxyribonucleic acid polymerase of the canine prostate.

(6) Hormonal effects in vitro on prostatic ribonucleic acid polymerase.
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P. Davies and K. Griffiths.

(8) Studies on the effect of androgens on prostatic DNA polymerase.
M. E. Harper, T. Jones and K. Griffiths.
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(9) Effect in vitro of 5α-dihydrotestosterone-protein receptor complexes on prostatic RNA polymerase.
P. Davies and K. Griffiths.


B. Studies on the metabolism of steroids by prostatic tissue


Calculation and statistical assessment of kinetic data from infusion experiments.
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J. Endocrinol. (1976), 68, 25-26P.

Endogenous androgen concentrations in the dorsal and ventral prostate glands of golden hamsters with benign prostatic tumours.

Tissue androgen concentrations in golden hamsters with benign prostatic tumours.

Plasma androgen concentrations and prostatic 3α/β-hydroxysteroid oxidoreductase activities in golden hamsters with benign prostatic tumours.

Proliferation of human prostatic epithelial cells in culture: aspects of identification.
C. Prolactin and the prostate

(36) An effect of prolactin on prostatic adenylate cyclase activity.

(37) Prolactin studies with the prostate.

(38) The effects of certain stilboestrol analogues on plasma prolactin and testosterone concentrations in the rat.

(39) The effect of certain stilboestrol analogues on plasma prolactin and testosterone in the rat.

(40) Effects of 2-Br-a-Ergocryptine (CB 154) on plasma hormones and accessory sex organs in the male rat.

(41) Studies on subcellular zinc distribution in relation to hormone levels in rat prostatic tissue using the electron microscope microanalyser EMMA.

(42) Prolactin and the prostate.
In 'Normal and Abnormal Growth of the Prostate',

(43) The effect of 2-bromo-a-ergocryptine (CB 154) administration on the hormone levels, organ weights, prostatic morphology and zinc concentrations in the male rat.

(44) Immunocytochemical staining obtained with a rat prolactin antiserum in various rat tissues.
Protein hormones and prostatic cancer.  

The immunocytochemical detection of protein hormones in human prostatic tissues.  

The immunocytochemical detection of growth hormone and prolactin in human prostatic tissues.  
D. Some clinical aspects of prostatic disease.

(48) Steroids and the prostate.
W.B. Peeling and K. Griffiths.

(49) Plasma prolactin, GH, LH, FSH, TSH and testosterone during treatment of prostatic carcinoma with oestrogens.

(50) The adrenal cortex and prostatic cancer.
W.B. Peeling and K. Griffiths.

(51) Plasma steroid and protein hormone concentrations in patients with prostatic carcinoma, before and during oestrogen therapy.

(52) The effect of ACTH on plasma testosterone and androstenedione concentrations in patients with prostatic carcinoma.

(53) Plasma spermine concentrations of patients with benign and malignant tumours of the breast or prostate.

(54) Prostatic cancer.
W.B. Peeling and K. Griffiths.

The British Prostate Study Group.

(56) The Etiology and endocrinology of prostatic cancer.
In 'Endocrinology of Cancer' Vol. II.


II. STUDIES CONCERNED WITH CANCER OF THE OVARY
A. Steroid biosynthesis by ovarian tissue.

(66) Steroid biosynthesis 'in vitro' by granulosa-theca tumour tissue.
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(IV) STEROID HORMONE BIOSYNTHESIS IN THE TESTIS

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I. INVESTIGATIONS RELATING TO CANCER OF THE PROSTATE.

A. STEROID HORMONE ACTION IN PROSTATIC TISSUE.
The Effect of Steroids on Deoxyribonucleic Acid Polymerase Activity

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(Received 17 July 1967)

The effects of steroid hormones on metabolic processes concerned with growth and cell division have not been fully investigated. Although investigations concerned with the effects of steroid hormones on RNA polymerase and RNA synthesis have been reported (Liao & Williams-Ashman, 1966; Wilson, 1962; Hamilton, 1964), their effect on DNA synthesis has received little attention. It was therefore decided to investigate the possible regulatory action of steroid hormones on enzyme systems associated with cell division. The stimulation in vitro of the DNA nucleotidyltransferase (EC 2.7.7.7) by certain oestrogens is now described.

The enzyme system studied was isolated from calf thymus tissue by using the semi-purified P2 fraction described by Shepherd & Keir (1966). The enzyme (100-250 μg. of protein) was incubated at 37° for 60 min. with 50μmole of [3H]thymidine triphosphate (specific activity 20 x 10^6 disintegrations/min./μmole) in 250 μl. of a medium containing 5μmole of tris-HCl buffer, pH 7.5, 15μmole of KCl, 100μmole of EDTA, 1μmole of MgCl2, 2μmole of 2-mercaptoethanol, 50μmole each of deoxy-ATP, deoxy-CTP and deoxy-GTP together with 100μg. of thermally denatured DNA. Either 5μl. of ethanol or steroid dissolved in ethanol to provide a final concentration of 40μM in the incubation medium was added. This amount of ethanol had no effect on the enzyme activity. Protein content of the enzyme was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The following steroids were studied: 3-hydroxyoestra-1,3,5(10)-triene-17-one (oestrone), oestra-1,3,5(10)-trien-3,17β-diol (oestradiol-17β), oestra-1,3,5(10)-trien-3,17α-diol (oestradiol-17α), oestra-1,3,5(10)-trien-3,16β,17β-triol (oestradiol-17β), oestra-1,3,5(10)-trien-3,16β,17α-triol (16-epi oestradiol), 17β-hydroxyandrost-4-en-3-one (testosterone), 11β,17α,21-trihydroxyprog-4-en-3,20-diones (cortisol) and the 3-methyl ether of oestradiol-17β.

Samples (50 μl.) were withdrawn at zero time and after 60 min. placed on fibre-glass disks (Whatman GF/C), washed in ice-cold 5% (w/v) trichloroacetic acid and dried with ethanol. The disks were placed in vials containing 10ml. of scintillation liquid [473ml. of toluene, 25ml. of Hyamine hydroxide, 100mg. of 1,4-bis-(5-phenyloxazol-2-y1)benzene and 2g. of 2,5-diphenyloxazole] and heated for 10 min. at 57°. The radioactivity was measured with a Nuclear-Chicago model 6860 liquid-scintillation spectrometer.

Evidence for the stimulation of DNA nucleotidyltransferase in vitro by oestradiol-17β, oestradiol-17α, oestradiol and 16-epi oestradiol is presented in Table 1. The results show that, whereas oestrone has no effect, oestradiol-17β, oestradiol-17α, oestradiol and 16-epi oestradiol caused a small but significant increase in activity. Neither testosterone nor cortisol had any effect on DNA polymerase, although these two steroid hormones produce a

<table>
<thead>
<tr>
<th>Steroid added</th>
<th>DNA polymerase activity (counts/min. /mg. of enzyme protein)</th>
<th>Activation (mean of four separate experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.50±3540</td>
<td>Mean activation (%)</td>
</tr>
<tr>
<td>Oestrone</td>
<td>8.52±3380</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6.100±2550</td>
<td></td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>7.5200±2650</td>
<td>14</td>
</tr>
<tr>
<td>None</td>
<td>1.27980±3460</td>
<td></td>
</tr>
<tr>
<td>Oestradiol-17α</td>
<td>1.45260±4820</td>
<td>13.5</td>
</tr>
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<td>None</td>
<td>1.13900±2040</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>1.20560±3830</td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>2.04610±8320</td>
<td>8.6</td>
</tr>
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<td>Oestradiol-17β</td>
<td>2.22300±2200</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>8.5245±3540</td>
<td></td>
</tr>
<tr>
<td>Oestradiol-17α</td>
<td>7.9860±1820</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6.5265±1675</td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>6.1890±1000</td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td>6.1650±1700</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Evidence for the stimulation in vitro of DNA polymerase by oestrogens

DNA polymerase activities are given as means±s.e.m. for one experiment comprising three incubations with duplicate counting. P values are based on Fisher's t test. The values for mean activation are the averages of four separate experiments.
marked increase in the activity of RNA polymerase in vitro (Lukács & Sekeris, 1967).

Although it has been recognized for a number of years that certain mammalian tissues do not maintain their normal rate of growth and function without the continued presence of small amounts of oestrogenic hormone, little is known about the mechanism of hormone dependence. The experiments in vivo of Jensen et al. (1966) have elegantly demonstrated the selective uptake of certain oestrogens by various hormone-responsive tissues such as uterus, vagina and mammary carcinoma. Other studies (King, Cowan & Inman, 1965) are concerned with intracellular localization of oestrogens. It is noteworthy in relation to the studies now reported that, although there is a selective uptake of oestradiol-17β by these target organs, there was no special affinity for oestrone (Jensen et al. 1966). There was, however, some affinity for oestradiol, and both oestrogens with affinity for these receptor sites caused some stimulation in vitro of DNA polymerase. The stimulation by oestradiol-17α and 16-epioestriol is also noteworthy. The lack of activity in vitro of the 3-methyl ether of oestradiol-17β suggests that the 3-hydroxyl group is required for stimulatory effect.

The manner in which each hormone affects the metabolism of certain types of cells to produce a specific physiological effect is a major unsolved problem, and, despite the small activation of DNA polymerase in vitro by oestrogenic hormone, its significance in the promotion of cell division in vivo in hormone-dependent tissues may be considerable.

The authors are grateful to the Tenovus organization for their great financial support and to Dr K. Wong for the statistical analysis on the computer.

The Inhibition of Deoxyribonucleic Acid Nucleotidyltransferase by Stilboestrol Derivatives

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(Received 19 February 1968)

The inhibition by diethylstilboestrol of DNA nucleotidyltransferase isolated from calf thymus was studied. The inhibition exercised by diethylstilboestrol appears to obey competitive kinetics with respect to DNA primer. The activities of both replicative and terminal enzymes were affected to the same extent. There was no evidence of binding between DNA and diethylstilboestrol. A comparative study of the inhibitory effects of some stilboestrol derivatives is presented. The alkyl substitution in the $\alpha\alpha'$-positions seems to alter the inhibitory effect of these compounds: dimethylstilboestrol was more inhibitory than stilbene, and diethylstilboestrol was more inhibitory than dimethylstilboestrol. Hexoestrol, in which the $\alpha\alpha'$-ethylenic linkage is saturated, was the most effective inhibitor.

The androgen-dependence of human prostatic cancer was established by the classical investigations by Huggins & Hodges (1941). Treatment of prostatic cancer by anti-androgen therapy such as castration or diethylstilboestrol administration followed from these experiments. The oestrogen-dependent nature of breast carcinoma has also resulted in extensive trials of anti-oestrogenic compounds such as 17$\beta$-hydroxy-2$\alpha$-methyl-5$\alpha$-androstan-3-one (Blackburn & Childs, 1959). Studies on the molecular basis of steroid action must therefore provide valuable information on the biochemical control mechanisms associated with hormone-dependent tumours. Though investigations concerned with the effects of steroid hormones on RNA polymerase and RNA synthesis have been reported (Liao & Williams-Ashman, 1962; Wilson, 1962; Hamilton, 1964), little attention has been directed towards the hormonal control of DNA synthesis. However, Fahmy, Griffiths, Mahler & Williams (1967) have shown that certain oestrogens stimulate in vitro the isolated DNA polymerase enzyme system (DNA nucleotidyltransferase, EC 2.7.7.7) prepared from calf thymus gland. Since diethylstilboestrol inhibits the oestrogen-stimulated mitotic activity of mouse vagina (Emmens, Cox & Martin, 1962), it was therefore decided to investigate the effect of this compound and those with related structures on the DNA polymerase enzyme system. It is noteworthy that Mangan, Neal & Williams (1967) have reported that diethylstilboestrol inhibited both RNA polymerase activity and the incorporation of amino acids by ribonucleoprotein particles isolated from prostatic tissue.

MATERIALS AND METHODS

Chemicals. Deoxy-GTP, deoxy-CTP, deoxy-ATP and $[^3H]$TTP* (specific radioactivity 2-0$\times$10$^6$ counts/min./mole) were obtained from Schwarz BioResearch Inc., New York, N.Y., U.S.A. Non-radioactive TTP was purchased from Sigma (London) Chemical Co., London, S.W. 6. Calf thymus DNA and all other A.R. reagents were supplied by British Drug Houses Ltd., Poole, Dorset. The dimethylstilboestrol was a gift from Dr L. Martin, Imperial Cancer Research Fund, London, W.C. 2, and the dieneoestrol was provided by Dr O. Morton, Medical Director, E.D.H. (Research) Ltd., London, W. 1. Hexoestrol, diethylstilboestrol and stilbene were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Enzyme studies. The enzyme preparation used in this investigation was the semi-purified F2 fraction isolated from calf thymus by the procedure of Shepherd & Keir (1966). The enzyme was stored in sealed 2-0ml. ampoules at $-20^\circ$. The [3]$^H$TTP was made up in water and diluted with non-labelled TTP to provide a specific radioactivity of $10^6\times10^{-12}$ 12 $\times$ 10$^6$ counts/min./mole. The enzyme (200-250 $\mu$g of protein) was incubated at 37$^\circ$ for 60min. (unless otherwise stated) with $50\mu$moles of [3]$^H$TTP in 255 $\mu$l. of a medium containing 3 $\mu$moles of tris-HCl buffer, pH7.5, 15 $\mu$moles of KCl, 100 $\mu$moles of EDTA, 1 $\mu$mole of MgCl$_2$, 2 $\mu$moles of 2-mercaptoethanol and 50 $\mu$g. (unless stated otherwise) of thermally denatured DNA. For the determination of the

* Abbreviations: TTP, thymidine 5'-triphosphate; TMP, thymidine 5'-monophosphate.
replicative enzyme activity, the medium contained 50 mmol of each of deoxy-ATP, deoxy-CTP and deoxy-GTP. These triphosphates were omitted from the terminal enzyme assay. Either 5 µl of ethanol or one of the compounds under investigation in this volume of ethanol was added to the reaction mixture.

Protein content of the enzyme preparation was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Radioactivity measurement. Samples (50 µl) were withdrawn at zero time and after 60 min. placed on fibre-glass disks (Whatman GF/C), washed in ice-cold 5% (w/v) trichloroacetic acid and dried with ethanol. The disks were placed in vials containing 10 ml of scintillation liquid [473 ml of toluene, 25 ml of Hyamine hydroxide, 100 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 2 g. of 1,5-diphenyloxazole] and heated for 10 min. at 57°. The radioactivity was measured with a Nuclear-Chicago model 6860 liquid-scintillation spectrometer.

RESULTS

Inhibition of DNA polymerase by diethylstilboestrol. The relationship between DNA polymerase activity (expressed as mmol of [3H]TMP incorporated/mg. of protein) and the concentration of diethylstilboestrol in the incubation medium is shown in Fig. 1. A linear relationship was observed between 2 and 6 µg. of diethylstilboestrol. Insolubility of diethylstilboestrol occurs at concentrations above 15 µg./reaction mixture.

Inhibition of DNA polymerase activity by diethylstilboestrol at various DNA primer concentrations. The activity of DNA polymerase was determined at various concentrations of DNA primer, with and without the presence of diethylstilboestrol (2-5 µg. in the reaction medium). The results are shown in Fig. 2. A small amount of [3H]TMP (0.3 mmol/mg. of protein) was incorporated at zero concentration of DNA primer. This was not removed by subsequent washings with ice-cold 5% trichloroacetic acid and could be due to enzyme contamination with DNA from thymus tissue. This was taken into account in the results illustrated in Fig. 2 by subtracting this amount from all the data presented in Fig. 2.

The data of Fig. 2 were redrawn as a double-reciprocal plot (Lineweaver & Burk, 1934) shown in Fig. 3. Linear plots were obtained for the control and diethylstilboestrol-inhibited assays. The two plots intersected on the vertical axis, suggesting that diethylstilboestrol exerts its effect by competitively inhibiting the enzyme.

Inhibition of DNA polymerase activity by compounds related to diethylstilboestrol. Table 1 summarizes the results of the comparisons made between the compounds investigated. The compounds were each added in 5 µl. of ethanol to provide a final concentration of 40 µM in the incubation medium.

![Fig. 1. Inhibition of DNA polymerase activity by diethylstilboestrol. The reaction mixture was prepared according to the method of Fahmy et al. (1967). A 50 µg. sample of thermally denatured DNA (calf thymus), 200 µg. of protein of a partially purified DNA polymerase isolated from calf thymus, [3H]TTP (specific radioactivity 10×10^6 counts/min./µmole) and the indicated amounts of diethylstilboestrol were added. Incubation was at 37° for 2 hr.](image1)

![Fig. 2. Inhibition of DNA polymerase activity by diethylstilboestrol at various concentrations of DNA primer. The assay conditions were as described in Fig. 1 with the following exceptions: thermally denatured DNA was included at the concentrations shown and the specific radioactivity of [3H]TTP was 12×10^6 counts/min./µmole. Incubation was at 37° for 2 hr. O, Control incubation without diethylstilboestrol; •, incubation with 2.5 µg. of diethylstilboestrol added.](image2)
the presence of two distinct enzymes. The replicative enzyme gives optimum activity with the four triphosphates. The terminal enzyme utilizes only one triphosphate (Keir & Smith, 1963), extensively incorporating TMP only into the terminal positions of the DNA primer (Keir, Shepherd & Hay, 1963). The inhibitory effect of diethylstilboestrol on both enzymes over a 3 hr. period is illustrated in Fig. 4. The activity of the terminal enzyme in the preparation was 15–20% of the activity of the replicative system. Both enzymes were inhibited to the same extent by diethylstilboestrol. The concentration of ethanol used in these experiments had no effect on the activity of either the replicative or terminal enzymes.

**DISCUSSION**

The present investigation clearly demonstrates the inhibition in vitro by diethylstilboestrol of the activity of DNA polymerase isolated from calf thymus gland. The degree of inhibition varied from one enzyme preparation to another, as observed by Keir, Omura & Shepherd (1963) when they reported inhibition of DNA polymerase activity by actinomycin D. It is noteworthy that Mangan et al. (1967) demonstrated the inhibition by diethylstilboestrol of the RNA polymerase activity of nuclei isolated from rat prostate. Since both DNA polymerase and RNA polymerase utilize DNA as a primer in the system, it might be suggested that diethylstilboestrol exercises its inhibitory action either by interference with the DNA primer that is common to both enzymes, or by interfering with the binding of DNA to the active site on the enzymes. Preliminary observations, not reported, suggest that the u.v.-absorption characteristics of the DNA are not altered by the presence of diethylstilboestrol. Binding may not therefore occur, though actinomycin D exerts its inhibitory effect by binding to the guanine residues of the DNA primer (Reich, Goldberg & Rabinowitz, 1962; Goldberg, Rabinowitz & Reich, 1962).

The introduction of oestrogen administration for the treatment of carcinoma of the prostate (Huggins, Scott & Hodges, 1941) was based on experiments showing that the growth of prostatic cancer was androgen-dependent and that oestrogens antagonized the androgenic action. It is not completely

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**Table 1. Relation between chemical structure and degree of inhibition**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stilbene</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>4,4':Dihydroxystilbene</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Dienoestrol</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Hexoestrol</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>38 ± 2</td>
</tr>
</tbody>
</table>
The relationship between the structure of the stilboestrol derivative and the degree of inhibition of DNA polymerase (Table 1) is of interest. The observation that diethylstilboestrol more efficiently inhibits the system than dimethylnitroestrol indicates that the groups attached to the central ethylene linkage are effective in the binding of inhibitor to enzyme. Though the p-hydroxyl groups may also aid this binding, it has been shown that stilbene inhibited DNA polymerase nearly as effectively as did dihydroxystilbene. Hexoestrol, the reduced form of diethylstilboestrol, showed the greatest inhibitory action of the compounds studied.

Diethylstilboestrol has therefore been shown to inhibit the activity of the DNA polymerase enzyme system isolated from calf thymus. Though actinomycin also inhibits this system, the effectiveness of diethylstilboestrol as an anti-androgenic compound may be due at least in part to its selective uptake by prostatic tissue. It is noteworthy that 17β-hydroxy-2α-methyl-5α-androstane-3-one, which has been used as an anti-oestrogen in breast cancer and prevents the uptake of oestrogen by human breast tissue (Deshpande, Jensen, Bulbrook, Berne & Ellis, 1967), also inhibits the DNA polymerase by 10% (A. R. Fahmy & K. Griffiths, unpublished work). Hexoestrol, the most effective of the inhibitors studied, is also concentrated by hormone-responsive human breast tumours to a greater extent than by the unresponsive type of tumour (Folea, Glascock & Irvine, 1961).

The authors are grateful to the Tenovus Organisation for their generous financial support. The technical assistance of Miss J. Williams is appreciated.

REFERENCES


The Effect of some Stilboestrol Compounds on Deoxyribonucleic Acid Polymerase from Human and Canine Prostatic Tissue

By M. E. Harper, C. G. Pierrepont, and K. Griffiths (Tenovus Institute for Cancer Research, Cardiff), A. R. Fahmy (Velindre Memorial Centre for Cancer Research, Cardiff), and R. W. Blamey (Royal United Hospitals, Bath)

The androgen-dependence of human prostatic cancer and its treatment by anti-androgen therapy is well established (Huggins, Scott & Hodges, 1941).

Studies on the action of steroid hormones provide valuable information about the control of hormone-dependent tumours. There are numerous reports concerned with the effects of steroid hormones on RNA polymerase and RNA synthesis, but little attention has been directed to their effect on DNA synthesis. Fahmy, Griffiths, Mahler & Williams (1967) showed that certain oestrogens stimulated in vitro the DNA polymerase prepared from calf thymus. Fahmy & Griffiths (1968) also showed inhibition of this same enzyme system with diethylstilboestrol and some of its analogues.

The present study reports the effects in vitro of steroids on DNA polymerase prepared from normal dog prostatic tissue and from human hyperplastic and neoplastic prostatic tissue. The enzymes were prepared as described by Shepherd & Keir (1966). The effect in vitro of steroids on DNA polymerase was determined as described by Fahmy & Griffiths (1968).

The activity of the DNA polymerase, measured in terms of nmoles of [3H]thymidine 5'-monophosphate incorporated into DNA/mg. of protein, was inhibited by the addition of 10-2nmoles of diethylstilboestrol. The degree of inhibition was in range 22–57% with enzymes prepared from seven human hyperplastic prostate specimens, 35–56% with preparations from neoplastic prostatic tissue and 36–43% with enzyme systems isolated from normal dog prostatic tissue. Equimolar concentrations of oestradiol-17β and the disulphate and diphasphate of diethylstilboestrol had approx. 20% of the inhibitory capacity of diethylstilboestrol. Diethylhydrostilboestrol, synthesized by modification of the method of Dodds, Golberg, Lawson & Robinson (1939), was nearly as effective an inhibitor as diethylstilboestrol with the human and dog enzyme systems. Only slight inhibition (<7%) was found with the cis and trans isomers of dibutylstilboestrol. It was observed that testosterone and 17β-hydroxy-5a-androstan-3-one stimulated the DNA polymerase isolated from certain of the human hyperplastic prostatic tissues, whereas testosterone was shown to have no effect on the DNA polymerase prepared from calf thymus tissue.

The authors are grateful to the Tenovus Organization for their generous financial support.


THE EFFECT OF SOME STILBOESTROL COMPOUNDS ON DNA POLYMERASE FROM HUMAN PROSTATIC TISSUE

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Received November 1, 1969

ABSTRACT

The inhibition by diethylstilboestrol of DNA nucleotidyltransferase isolated from human hyperplastic and neoplastic prostatic tissue has been studied. Whereas diethylstilboestrol consistently inhibited the enzyme, oestradiol-17β often had little or no effect. Dihydrodi-butylstilboestrol, the synthesis of which is described, was also found to consistently inhibit the DNA polymerase, which contrasted with the lack of effect observed with the cis and trans isomers of dibutylstilboestrol. Two of the enzyme preparations derived from hyperplastic prostates were stimulated by testosterone and 5α-dihydrotestosterone.

INTRODUCTION

Earlier studies of Fahmy, Griffiths, Mahler and Williams(1) showed that certain natural oestrogens stimulated in vitro the DNA polymerase enzyme system (DNA nucleotidyltransferase, EC2.7.7.7.) prepared from calf thymus gland. More recently, it was also shown by Fahmy and Griffiths (2) that diethylstilboestrol and some of its analogues inhibited the activity of this enzyme system. Since the androgen dependent nature of human prostatic cancer is well known and its treatment by diethylstilboestrol well established (3), it was decided to investigate the effects of diethylstilboestrol and other
steroid hormones on the DNA polymerase enzyme system prepared from these tissues. At the same time, the effect on these enzyme preparations of a newly synthesized dihydrodibutylstilboestrol was studied. A preliminary report of some of these experiments has been presented (4).

**MATERIALS AND METHODS**

**Chemicals** Deoxy-GTP, deoxy-CTP, deoxy-ATP and \(^{3}\text{H}\)thymidine 5'-triphosphate (TTP, specific activity 5.0 c/m-mole) were obtained from Schwarz BioResearch Inc., New York, N.Y., U.S.A. Non-radioactive TTP was purchased from Sigma (London) Chemical Co., London, S.W.6. Calf thymus DNA and all other A.R. reagents were supplied by British Drug Houses, Ltd., Poole, Dorset. Diethylstilboestrol deoxy-p-anisoin, and n-butylbromide were obtained from Koch-Light Laboratories, Ltd., Colnbrook, Bucks., England. Diethylstilboestrol diphosphate (Honvan) was a gift from Ward Blenkinsop and Co. Ltd., Wembley, London.

**Tissues** Prostatic tissue was cooled to 0°C after removal and retained at this temperature until processed in the laboratory 60 mins. later.

**Enzyme studies** The DNA polymerase preparations studied were those of the semi-purified F2-fraction isolated by the procedure of Shepherd and Keir (5). Enzyme preparations were stored in sealed vials at -20°C. The enzyme (100-250 μg, protein) was incubated at 37°C for 2 hr. with 50 μmole of \(^{3}\text{H}\)TTP (specific activity 10 × 10^6 counts/min/μmole) in 255 μl. medium containing 5 μmole tris HCl buffer, pH 7.5, 15 μmole KCl, 100 μmole EDTA, 1 μmole MgCl₂, 2 μmole 2-mercaptoethanol, and 50 μmole each of deoxy-GTP, deoxy-CTP and deoxy-ATP together with 50 μg. thermally denatured DNA. Either 5 μl. ethanol, or compounds in this volume of ethanol, were added to the reaction mixture. Protein content of the enzyme was determined by the method of Lowry et al (6).

**Radioactivity determination** Samples (50 μl.) were withdrawn at zero time and after 2 hr. placed on fiberglass disks (Whatman GF/C), washed in ice-cold 5% (w/v) trichloroacetic acid and dried with ethanol. The disks were placed in vials containing 10 ml. scintillation liquid (473 ml. toluene, 25 ml. hyamine hydroxide, 100 mg. 1,4-bis (5-phenyloxazol-2-yl) benzene, and 2 g. of 2,5-diphenyloxazole) and heated for 10 min. at 57°C. The radioactivity was measured with a Nuclear-Chicago
Model 6860 liquid scintillation spectrometer.

Chemical synthesis of Dibutylstilboestrol The method of Dodds et al. (7) for the preparation of alkyl substituted stilboestrols was adopted with some modifications. The outline of the procedure was as follows (Fig. 1).

Stage (i) Deoxy-p-anisoin was alkylated with n-butylbromide and sodium ethoxide, and (Stage ii) the product brought into reaction with a Grignard reagent (C₄H₉MgBr). The resulting carbinol was then dehydrated using PBr₃ in CHCl₃ (Stage iii) with the formation of dibutylstilboestrol dimethylether. Dodds et al. (7) effected demethylation of the ether by using alcoholic KOH in a constantly rotating autoclave at 200-210°C (max. pressure approximately 15 atmospheres) for 24 hr. Moore (8) however, demethylated the ether by heating in alcoholic KOH in a sealed bomb at 210°C for 20 hr. Unsuccessful attempts to demethylate the ether with aluminium bromide were made (7). However, it has now been shown in these investigations that demethylation of the ether can be effected with boron tribromide (BBr₃).

Dibutylstilboestrol dimethylether (5 g.) was dissolved in 150 ml. dichloromethane and slowly added to a cold solution of BBr₃ (3.5 ml.) in 125 ml. dichloromethane and left overnight at room temperature. A CaCl₂ filled drying tube protected the reaction mixture from moisture. The mixture was then shaken with water to hydrolyse excess BBr₃ and boron complexes and the phenolic product collected by extraction into ether. The solvent was evaporated in a rotary film evaporator. The oily product was purified by distillation under vacuum and the fraction, distilling at 192-196°C, 0.18 mm Hg., was collected. The yield was 3.2 g. of a pale yellow oil.

The purity of the compound was checked by chromatography on a thin layer of Merck silica gel G in the solvent system petroleum ether (60-80°C) 75: acetone 25 (v/v) (system A). The principal component, (Rf. 0.28) had a faint pink fluorescence in UV-light (240 m\(\lambda\)) changing within 45 secs. exposure to yellow. A pale blue fluorescing material (Rf. 0.68) was identified as the dibutylstilboestrol dimethylether.

The oil was further purified by column chromatography on silica gel (100-200 mesh). The column (15 cm. length, 1 cm. diam.) was packed with silica gel (6 g.) prepared as a slurry with petroleum ether (60-80°C). The column was equilibrated and washed with this solvent. The oil (0.2 g.) adsorbed on silica gel and suspended in petroleum ether,
Fig 1

\[
\begin{align*}
\text{HCO} & \text{-} \text{OCH}_3 \\
\text{HCO} & \text{-} \text{C} \text{H} \\
\text{HCO} & \text{-} \text{C} \text{H} \\
\text{HCO} & \text{-} \text{C} \text{H} \\
\end{align*}
\]

Deoxy-p-anisoin

\[
\begin{align*}
\text{HCO} & \text{-} \text{OCH}_3 \\
\text{HCO} & \text{-} \text{CH} \\
\text{HCO} & \text{-} \text{CH} \\
\text{HCO} & \text{-} \text{CH} \\
\end{align*}
\]

\(\alpha\)-n-Butyl deoxyanisoin

\[
\begin{align*}
\text{HCO} & \text{-} \text{OCH}_3 \\
\text{HCO} & \text{-} \text{CH} \\
\text{HCO} & \text{-} \text{CH} \\
\text{HCO} & \text{-} \text{CH} \\
\end{align*}
\]

4,4'-dimethoxy-\(\alpha,\beta\)-di-n-butylstilbene

\[
\begin{align*}
\text{HCO} & \text{-} \text{OCH}_3 \\
\text{HCO} & \text{-} \text{CH} \\
\text{HCO} & \text{-} \text{CH} \\
\text{HCO} & \text{-} \text{CH} \\
\end{align*}
\]

Dibutylstilboestrol dimethylether
was layered carefully onto the column. Elution was carried out with a stepwise gradient elution with increasing concentrations of acetone in petroleum ether 60-80°C. The material appearing as a yellow band when viewed with UV-light, was eluted with 10% acetone in petroleum spirit. This material gave one spot on thin layer chromatography (Rf. 0.28) in solvent system A and analysis indicated C, 81.2%; H, 8.8% (C22H28O2 - C, 81.5%; H, 8.6%).

On standing in CHCl3 at room temperature white needle shaped crystals separated. These were filtered, recrystallized from CHCl3 (MP 145-146°C) and analysed. The analysis indicated C, 81.6%; H, 9.5%. The material had an Rf of 0.28 on thin layer chromatography in the same solvent system A. Nuclear magnetic resonance studies indicated that this stable crystalline material was the trans-isomer of dibutylstilboestrol. The cis isomer was almost completely converted to the trans form by dissolving the oil in CCl4 containing 0.5% iodine and refluxing for 45 mins. The CCl4 and iodine were distilled and the residue crystallized from CHCl3, this procedure providing a 90% yield.

**Chemical synthesis of dihydrodibutylstilboestrol** Dodds et al (7) hydrogenated diethylstilboestrol in acetone solution using a palladium catalyst. In the present study the dibutylstilboestrol was hydrogenated in acetic acid solution using a platinum catalyst. The crystalline compound that separated was recrystallized twice from benzene, (MP 165-166°C, uncorrected). Analysis indicated C, 80.8%; H, 9.1% (C22H30O2 would be C, 81.0%; H, 9.2%). A MP of 167-168°C for the meso-form of dihydrodibutylstilboestrol has been reported (9).

**Preparation of dipotassium diethylstilboestrol disulphate** This sulphate was prepared by the method of Short and Oxley (10). Diethylstilboestrol (3 g.) and sulphamic acid (7 g.) in 20 ml. pyridine were heated at 100°C with stirring for 20 min. The separated solid was collected and recrystallized from 1N KOH. Subsequent crystallizations were made from hot water, and the white needle-like crystals dried in vacuo and P2O5 at 55°C for three days. Analysis indicated C, 42.4%; H, 3.9%; S, 12.5%; K, 15.3% (C18H18O3S2K2 requires C, 42.8%; H, 3.6%; S, 12.7%; K, 15.5%).

The compound had a Rf. of 0.6 when run on thin layers of silica gel G in the solvent system n-butanol, acetic acid, water: 4, 1, 1 (v/v).
RESULTS

Diethylstilboestrol was found to inhibit the DNA polymerase from both hyperplastic and neoplastic human prostatic tissue. Fig. 2 shows the average degree of inhibition of the enzyme system, and the range of this effect in a number of experiments. On average, there was a 40% inhibition of the enzyme from both types of tissue.

Fig 2
EFFECT OF DIETHYLSTILBOESTROL (DES) ON DNA POLYMERASE ISOLATED FROM PROSTATE TISSUE

The effect of 10 μmoles diethylstilboestrol on the DNA polymerase activity of three neoplastic and seven hyperplastic human prostates, each experiment completed in triplicate. The range for different preparations is shown.
The effects of various stilboestrol analogues on the DNA polymerase is indicated in Table 1, which shows the relative inhibitory capacity of diethylstilboestrol and its reduced product hexoestrol, and also of dibutylstilboestrol and dihydrodibutylstilboestrol. It is interesting to note that whereas diethylstilboestrol and hexoestrol inhibited the enzyme to the same degree, dibutylstilboestrol generally had little effect. However, dihydrodibutylstilboestrol consistently and effectively inhibited the DNA polymerase.

**TABLE 1**

**THE EFFECT OF VARIOUS STILBOESTROL COMPOUNDS ON DNA-POLYMERASE ISOLATED FROM HUMAN PROSTATE TISSUE**

<table>
<thead>
<tr>
<th>DNA polymerase activity (cnts/min/mg of protein)</th>
<th>Gland pathology</th>
<th>% Inhibition of DNA polymerase activity by the compound at 40μM final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diethylstilboestrol</td>
</tr>
<tr>
<td>7,523</td>
<td>Neoplasia</td>
<td>31</td>
</tr>
<tr>
<td>14,611</td>
<td>Neoplasia</td>
<td>45</td>
</tr>
<tr>
<td>8,713</td>
<td>Neoplasia</td>
<td>28</td>
</tr>
<tr>
<td>9,103</td>
<td>Hyperplasia</td>
<td>31</td>
</tr>
<tr>
<td>8,485</td>
<td>Hyperplasia</td>
<td>29</td>
</tr>
</tbody>
</table>

In fig. 3 the activity of this same enzyme was determined at various concentrations of DNA primer, with and without the presence of diethylstilboestrol and dihydrodibutylstilboestrol, (40 μM final concentration of both inhibitors).
A small amount of $[^{3}\text{H}]$ TTP was incorporated at zero concentration of DNA primer. This was not removed by subsequent washings with ice-cold 5% trichloroacetic acid and is probably due to enzyme contamination with DNA from the prostatic tissue. Fig. 4 shows the effect of varying the concentration of inhibitor, both diethylstilboestrol and dihydrodibutylstilboestrol on a DNA polymerase preparation from hyperplastic prostate tissue.

**Fig. 3** Inhibition of DNA polymerase activity by diethylstilboestrol and dihydrodibutylstilboestrol at various concentrations of DNA primer. The assay conditions are as described in methods except that thermally denatured DNA was included at the concentrations shown. Control incubation: • incubation with diethylstilboestrol: △ (40 μM final concentrations), incubation with dihydrodibutylstilboestrol: ○ (40 μM final concentration). **Fig. 4** Inhibition of DNA polymerase activity from hyperplastic prostatic tissue by diethylstilboestrol ○, and by dihydrodibutylstilboestrol added in amounts indicated.
The results shown in table 2 indicate that the phosphate and sulphate esters were less effective inhibitors of the DNA polymerase than the parent compound under these experimental conditions.

**TABLE 2**

THE EFFECT OF PHOSPHATE AND SULPHATE ESTERS OF DIETHYLSTILBOESTROL (DES) ON PROSTATIC DNA POLYMERASE

<table>
<thead>
<tr>
<th>DNA polymerase activity (cnts/min/mg.of protein)</th>
<th>Gland pathology</th>
<th>% Inhibition of DNA polymerase activity by diethylstilboestrol (40μM)</th>
<th>% Inhibition of DNA polymerase activity by diethylstilboestrol diphosphate (40μM)</th>
<th>% Inhibition of DNA polymerase activity by diethylstilboestrol disulphate (40μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,644</td>
<td>Neoplasia</td>
<td>30.7</td>
<td>16.0</td>
<td>-</td>
</tr>
<tr>
<td>7,052</td>
<td>Hyperplasia</td>
<td>51.2</td>
<td>10.5</td>
<td>8.0</td>
</tr>
<tr>
<td>8,614</td>
<td>Hyperplasia</td>
<td>33.0</td>
<td>9.5</td>
<td>7.0</td>
</tr>
<tr>
<td>6,727</td>
<td>Hyperplasia</td>
<td>22.4</td>
<td>6.0</td>
<td>-</td>
</tr>
<tr>
<td>8,485</td>
<td>Hyperplasia</td>
<td>29.5</td>
<td>11.3</td>
<td>9.7</td>
</tr>
</tbody>
</table>

Unlike the diethylstilboestrol, oestradiol-17β did not consistently inhibit the prostatic DNA polymerase (Table 3).

**TABLE 3**

THE EFFECT OF OESTRADIOL-17β ON PROSTATIC DNA POLYMERASE

<table>
<thead>
<tr>
<th>DNA polymerase activity (cnts/min/mg.of protein)</th>
<th>Gland pathology</th>
<th>% Inhibition of DNA polymerase activity by oestradiol-17β, 40μM final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>11389</td>
<td>Hyperplasia</td>
<td>7.9</td>
</tr>
<tr>
<td>7,464</td>
<td>Hyperplasia</td>
<td>13.0</td>
</tr>
<tr>
<td>9,103</td>
<td>Hyperplasia</td>
<td>5.6</td>
</tr>
<tr>
<td>8,644</td>
<td>Neoplasia</td>
<td>0</td>
</tr>
<tr>
<td>14,611</td>
<td>Neoplasia</td>
<td>33.0</td>
</tr>
<tr>
<td>8,713</td>
<td>Neoplasia</td>
<td>2.5</td>
</tr>
</tbody>
</table>
This inhibitory effect of oestradiol-17β on the prostatic DNA polymerase is in contrast to the observed stimulation of the calf thymus enzyme previously described (1). Also of interest in these investigations, was the observed stimulation by 17β-hydroxyandrost-4-en-3-one (testosterone) and 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone) of two enzyme preparations derived from hyperplastic prostate tissue. At a final concentration of 40 μM, testosterone stimulated the enzyme systems by respectively, 14 and 38% and 5α-dihydrotestosterone by 32 and 30%. Neither compound had any effect on the prostatic tumour tissue studied, and there was also no effect on the calf thymus enzyme system.

DISCUSSION

Studies on the molecular basis of steroid action must provide valuable information regarding the biochemical control mechanisms associated with hormone-dependent tumours. It would appear that the observed effects of testosterone in vivo, on the glands of reproduction (11 - 13) are dependent upon the preliminary synthesis of RNA in the cell nuclei of these organs (14 - 17). Liao and Williams-Ashman (18) have also described an increased activity of the RNA polymerase of rat prostate within an hour of testosterone administration to castrated animals. Little attention has however been
directed towards the hormonal control of DNA synthesis in prostatic tissue, although it has been shown (19) that the DNA polymerase of ventral prostate extracts from castrated rats was enhanced after testosterone administration.

The results of the present study demonstrate in vitro, that diethylstilboestrol inhibits the DNA polymerase extracts isolated from human neoplastic and hyperplastic prostatic tissue. Although the degree of inhibition varied from one DNA polymerase preparation to another, as did the inhibition of the calf thymus enzyme by actinomycin-D reported by Keir et al (20), diethylstilboestrol consistently inhibited the prostatic enzyme.

It is noteworthy that Mangan et al (21) demonstrated the inhibition by diethylstilboestrol of the RNA polymerase activity of nuclei isolated from rat prostate. Since DNA and RNA polymerase utilize DNA as a primer, it is possible that diethylstilboestrol exerts its inhibitory action by interfering with the primer or with the binding of the DNA to the active site on the enzymes. These results provide further support for the suggestion that at least part of the effectiveness of diethylstilboestrol in the treatment of carcinoma of the prostate may be due
to its direct biochemical effect on the prostatic tissue. Hexoestrol, the most effective of the inhibitors studied by Fahmy and Griffiths (2) on the calf thymus preparations also consistently inhibited the prostatic enzyme, and it is interesting that hexoestrol is concentrated by hormone responsive human breast tumours to a greater extent than by the unresponsive type of tumour (22).

The earlier observations of Fahmy and Griffiths (2) suggested an interesting relationship between the structure of the stilboestrol analogue and the degree of inhibition of DNA polymerase. The alkyl substitution in the aa'-positions altered the inhibitory effect; the larger the alkyl group, the greater was the degree of inhibition. Hexoestrol with the saturated aa'-ethylenic linkage was the most effective inhibitor.

Because of these results, the cis and trans isomers of dibutylstilboestrol were synthesized, and also the corresponding saturated compound dihydrodibutylstilboestrol. It was of particular interest to observe that in most instances the cis and trans isomers of dibutylstilboestrol had little effect on the prostatic DNA polymerase, whereas generally the reduced compound was equally as effective as diethylstilboestrol. Since dihydrodibutylstilboestrol has only 0.1% of the oestrogenic activity of diethylstilboestrol
(23) it will be of further interest to study its uptake and localisation in prostatic tissue, relative to diethylstilboestrol and also its effect on plasma testosterone and luteinizing hormone with regard to its possible use in the treatment of prostatic cancer. Diethylstilboestrol therapy decreases plasma testosterone and LH in man (24) and also decreases the activity of the testicular enzyme 17β-hydroxysteroid dehydrogenase converting androstenedione to testosterone (25).

The decreased effectiveness of the phosphate and sulphate esters of diethylstilboestrol would suggest that the free p-hydroxyl groups have some role in the inhibitory effect, as does the group substitution on the ethylene linkage (2). The treatment of prostatic carcinoma with diethylstilboestrol diphosphate (Honvan) probably depends upon the high phosphatase activity of the prostate, releasing the diethylstilboestrol within the target tissue.

Although oestradiol-17β did inhibit the enzyme in certain prostatic preparations, it had little or no effect on others. Furthermore, it was of interest to observe that oestradiol-17β inhibited the prostatic enzyme whereas previously (1) it was consistently found to stimulate the DNA polymerase from the calf thymus. Also of interest was the marked stimulation by both testosterone and 5α-dihydrotestosterone on the activity of two DNA polymerase enzymes.
prepared from human hyperplastic prostatic tissue. These androgens did not stimulate all preparations from hyperplastic tissue, and had little or no effect on the enzyme isolated from prostatic tumour tissue, or on the calf thymus enzyme. Further investigations on the relationship between these observations and the histology of the tissue are continuing.

Studies are also in progress to further purify these prostate enzyme systems to eliminate the possibility that the variability in some of the hormone effects is due to the presence of contaminating nucleases.

The authors wish to acknowledge the generous financial support of the Tenovus Organisation in Cardiff, Wales.

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Short Communications

The Effect of Prostatic Metabolites of Testosterone and other Substances on the Isolated Deoxyribonucleic Acid Polymerase of the Canine Prostate

By M. E. Harper, C. G. Pierrepont, A. R. Fahmy* and K. Griffiths
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(Received 15 July 1970)

The activity of DNA polymerase (DNA nucleotidytransferase, EC 2.7.7.7) in androgen target-organ has not been widely studied. Earlier investigations in this laboratory (Fahmy, Griffiths, Mahler & Williams, 1967; Fahmy & Griffiths, 1968) showed that the isolated DNA polymerase from calf thymus tissue was responsive to various steroid hormones and suggested that further studies concerned with the hormone-responsiveness of a similar enzyme system from prostate tissue could be particularly germane to the understanding of the mechanism of action of these hormones. In this respect it was of note that testosterone and 5a-dihydrotestosterone (17β-hydroxy-5a-androstan-3-one) stimulated DNA polymerase isolated from human hypertrophic prostatic tissue (Harper, Fahmy, Pierrepont & Griffiths, 1970a) and, if this observation truly reflects the state in vivo, it would seem that this system of investigation could be of value in screening substances with regard to their clinical effectiveness in controlling prostatic dysfunction. This communication now describes further experiments on canine prostatic DNA polymerase.

Clinically normal prostate glands were removed from two 2-year-old healthy dogs and one 16-year-old dog. A further grossly enlarged and cystic gland was taken from an animal showing signs of bowel obstruction. The glands weighed 8.4, 12.3, 11.3 and 72.0g respectively. Tissues were prepared as described by Shepherd & Keir (1966) and the semi-purified F₂ fraction was isolated. Enzyme preparations were stored in sealed vials at -20°C before use. The enzyme (100-250μg of protein) was incubated at 37°C for 2h with 50nmol of [3H]-thymidine triphosphate (specific radioactivity 10×10⁶e.p.m./μmol) in 25μl of medium containing 5μmol of tris-HCl buffer, pH 7.5, 15μmol of KCl, 100nmol of EDTA, 1μmol of MgCl₂, 2μmol of 2-mercaptoethanol and 50nmol each of deoxy-GTP, deoxy-CTP and deoxy-ATP together with 50μg of thermally denatured DNA. Either 5μl of ethanol, or a compound in this volume of ethanol, was added to the reaction mixture. Protein content of the enzyme was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The results of this study are shown in Table 1.

A great deal of literature has accumulated on steroid metabolism by the human prostate (reviewed by Ofner, 1969), but a recent study (Harper, Pierrepont, Fahmy & Griffiths, 1970b) was concerned primarily with the canine prostate. The 'prostatic metabolites' listed in Table 1 are those shown to be formed by these same canine prostate glands when incubated with [7α-3H]testosterone. Only three steroids stimulated the DNA polymerase activity, namely testosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17α-diol. Noteworthy was the lack of effect of 5α-dihydrotestosterone on the enzyme system, in the light of experiments showing this steroid to be preferentially retained by rat prostatic nuclei either after testosterone administration in vivo (Bruchovsky & Wilson, 1968) or after incubation of the tissue with labelled testosterone (Anderson & Liao, 1968). Similar experiments with canine prostate tissue (Harper et al. 1970b) indicated a selective accumulation of 5α-androstane-3α,17β-diol in the nucleus, and this, in association with the observed effect of the diol on the canine DNA polymerase, might suggest this steroid is an androgenic metabolite of testosterone in the dog. Whether it plays a more effective androgenic role in the canine prostate than does 5α-dihydrotestosterone, or testosterone itself, can be decided by further study. Relevant, however, are the experiments of Baulieu, Lasnitzki & Robel (1968a,b), showing that certain 5α-androstane- 4β, 16α-diol as well as 5α-dihydrotestosterone stimulate cell division and induce epithelial hyperplasia in explants of rat prostate maintained in tissue culture.

The DNA polymerase isolated from the prostate of the old dog showed little response to steroids, although it was inhibited by the stilboestrol derivatives. It should be emphasized that dihydrodi-butylstilboestrol generally shows a capacity equal to diethylstilboestrol in inhibiting isolated DNA polymerase enzyme systems (Harper et al. 1970a).
Table 1. Effect of various compounds on canine prostatic DNA polymerase

(S), Stimulation; (I), Inhibition. 0, No effect; —, not investigated.

<table>
<thead>
<tr>
<th>Compounds added to incubation (final concn. 40 μM)</th>
<th>First young prostate</th>
<th>Second young prostate</th>
<th>Old prostate</th>
<th>Hypertrophied prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>33.6 (S)</td>
<td>23.6 (S)</td>
<td>3.0 (S)</td>
<td>0</td>
</tr>
<tr>
<td>Prostatic metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>12.8 (S)</td>
<td>10.6 (S)</td>
<td>0</td>
<td>15.1 (S)</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>3 (I)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Testosterone sulphate</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Epistandrosterone</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6β-Hydroxyandrostenedione</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Other compounds tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androst-4-ene-3β,17β-diol</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5β-Dihydrotestosterone</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>1.3 (I)</td>
<td>1.9 (I)</td>
<td>0</td>
<td>1.2 (I)</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>28 (I)</td>
<td>25 (I)</td>
<td>12.1 (I)</td>
<td>1.2 (I)</td>
</tr>
<tr>
<td>Hexoestrol</td>
<td>25 (I)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dibutylylboestrol</td>
<td>11 (I)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dihydro dibutylylboestrol</td>
<td>26 (I)</td>
<td>—</td>
<td>15.1 (I)</td>
<td>—</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DIPA</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chlormadinone acetate</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17α-Ethynylestrone-3β,17β-diol</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17α-Ethynylestradiol-17β</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

and, with its decreased oestrogenicity, might be of value in the treatment of prostatic hypertrophy and neoplasia.

The authors wish to record their appreciation of the generous financial support from the Tenovus Organisation. C.G.P. is also supported by a research grant from the Wellcome Trust. They are also grateful to Mr Adrian Pike for his excellent technical assistance.

Hormonal Effects in vitro on Prostatic Ribonucleic Acid Polymerase

By P. Davies,* A. R. Fahmy,*‡ C. G. Pierrepoint‡ and K. Griffiths‡

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In recent years considerable interest has been directed towards the mechanism by which androgenic steroids control the growth and development of the prostate gland. In particular, the relationship between testosterone (17β-hydroxyandrost-4-en-3-one) and its reduced metabolite 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) within the prostate has been the subject of intensive research. 5α-Dihydrotestosterone was implicated in androgen action when it was found to be localized in the nuclei of the rat ventral prostate (Bruchovsky & Wilson, 1968a,b; Anderson & Liao, 1968). Further, Baulieu et al. (1968a,b) indicated that 5α-dihydrotestosterone had a stimulatory effect on cell division and secretion in rat prostatic explants maintained in culture. Since it is well established that enhancement of RNA polymerase activity is one of the earlier androgenic effects in the prostate gland, it is noteworthy that Alfheim & Unhjem (1971) failed to observe a direct effect of 5α-dihydrotestosterone on the activity of this enzyme system in homogenates, slices or nuclear preparations of rat ventral prostate in vitro.

Further studies on the effects of testosterone and its metabolites on the DNA-dependent RNA polymerase (nucleotide triphosphate–RNA nucleotidytransferase, EC 2.7.7.6) of nuclei prepared from rat and dog prostatic tissue are now reported. Observations on the inhibitory effect of various stilboestrol derivatives on this enzyme system are also described.

Materials and methods

Prostatic tissue from 12 Sprague-Dawley rats, aged 8–12 weeks, was pooled for each experiment and the nuclear preparation obtained by the method of Mainwaring (1969). In all preparative media, however, MgCl₂ was used instead of CaCl₂. Nuclei from the prostates of young dogs were prepared as described by Groom et al. (1971). The procedure of Mangan et al. (1967) was used to determine the activity of the DNA-dependent RNA polymerase in the nuclear preparations.

The enzyme preparation (equivalent to 60–100 µg of DNA) in 0.25 M sucrose–1.0 mM-MgCl₂ was incubated at 37°C for 15 min with 125 pmol of [α-3H]ATP (specific radioactivity 40 Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.) in 500 µl of a medium containing the following constituents: 60 µmol of tris-HCl buffer, pH 8.1, 3 µmol of NaF, 2.5 µmol of 2-mercaptoethanol, 15 µmol of KCl, 2.5 µmol of MgCl₂ and 300 nmol each of CTP, UTP and GTP. Either 50 µl of ethanol, or one of the steroids or stilboestrol derivatives under investigation in this volume of ethanol to give a final concentration of 40 µM, was added to the reaction mixture. DNA of the enzyme preparation was determined by the method of Burton (1956) as modified by Giles & Myers (1965).

Incubations were terminated by the addition of 5 ml of 10% (w/v) trichloroacetic acid. Precipitates were washed three times with cold 5% (w/v) trichloroacetic acid, once with cold ethanol and once more with cold 5% trichloroacetic acid. Acid-insoluble material was dissolved in 4 drops of Hyamine 10X (British Drug Houses Ltd., Poole, Dorset, U.K.) and kept overnight at 37°C, and 10 ml of scintillation liquid (Bray, 1960) was added. Radioactivity was measured in a Nuclear-Chicago model 6860 liquid-scintillation spectrometer. The incorporation of [14C]ATP into RNA was determined in terms of d.p.m./15 min per mg of DNA.

Results and discussion

The effects of the various compounds on the activity of the DNA-dependent RNA polymerase are shown in Table 1. Stimulation of the enzyme system from both rat and dog prostatic tissue was observed on addition of 5α-dihydrotestosterone and to a smaller degree with testosterone. The effect of testosterone could, however, be due to its transformation by the nuclear 5α-reductase enzyme system. The stimulatory effect of 5α-dihydrotestosterone is of interest, again suggesting that this steroid plays a major role in controlling the growth and function of the prostate. The results also support the findings obtained by Bashirelahi et al. (1969) on the effects of testosterone and 5α-dihydrotestosterone in vitro on the RNA polymerase enzyme system and contrast with the reported inhibitory effect of 5-hydroxysterone on the RNA polymerase of lysed thymus nuclei (Abraham & Sekeres, 1971).

Also noteworthy was the observed stimulation of the rat prostate RNA polymerase by both 5α-androstane-3α,17β-diol and also 5α-androstane-3β,17β-diol. These diols are metabolites of testosterone in the rat prostate (Griffiths et al., 1970), and
Table 1. Effect of incubation of rat and dog prostatic nuclei with various steroids and stilboesterol derivatives on the nuclear RNA polymerase activity

Nuclear preparations were incubated for 15 min at 37°C in an assay medium containing these substances at a final concentration of 40 µM. Duplicate assays were carried out in each case. The incorporation of [8-¹⁴C]ATP into acid-insoluble material was determined and expressed as d.p.m./15 min per mg of DNA. Increases or decreases in incorporation are expressed as percentages of the value obtained in the absence of added steroid or stilboesterol derivative. Values followed by an asterisk (*) indicate inhibition, those without indicate stimulation. Dashes show that the compound was not studied in that particular experiment; the zeros indicate that there was no effect on the enzyme.

<table>
<thead>
<tr>
<th>Compound (40µM) added to enzyme assay system</th>
<th>Rat prostatic nuclear prep.</th>
<th>Canine prostatic nuclear prep.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>3</td>
<td>3.7</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td>Epitestosterone (17α-hydroxyandrost-4-en-3-one)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5α-Dihydroepitestosterone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5α-Androstane-3α,17α-diol</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>5α-Androstane-3β,17α-diol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Androst-5-ene-3β,17β-diol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Androst-4-ene-3,17-dione</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3α-Hydroxy-5α-androstan-17-one</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dibutylstilboestrol</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dibutyldihydrostilboestrol</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

It is reported that the 3β,17β-diol stimulated the growth and activity of rat ventral prostatic tissue in culture (Baulieu et al., 1968a,b). It is noteworthy that, in contrast with the rat prostate enzyme system, the 3α,17β-diol had no effect on the activity of the dog RNA polymerase. However, the 5α-androstane-3α,17α-diol stimulated the dog RNA polymerase and not the rat enzyme, corresponding to the results of previous investigations (Harper et al., 1970), which showed that of the four 5α-androstane-diol epimers only the 3α,17α-diol stimulated the DNA polymerase enzyme system isolated from the canine prostate. Together with experiments of Farnsworth (1970), indicating that the 3α,17β-diol controls the activity of adenosine triphosphatase in the prostate gland, these studies suggest that a number of the metabolites of testosterone may be concerned in eliciting the androgenic response in the prostate, and control of this pattern of metabolites may be necessary to promote normal growth and function. Imbalance of this pattern might lead to prostatic neoplasia, and high concentrations of 5α-dihydrotestosterone have been reported in benign hypertrophic prostates (Siiteri & Wilson, 1970).

It should be noted that, in contrast with the studies carried out by Bashirelahi et al. (1969), no stimulation of the RNA polymerase was obtained with steroid concentrations below 40 µM. However, at steroid concentrations of 4 µM 5α-dihydrotestosterone and the 3α,17β- and 3β,17β-diols stimulated the enzyme system of rat prostatic nuclei when cytosol (remaining after nuclear sedimentation) was also added (Table 2). In similar experiments with the canine nuclear preparation, 5α-dihydrotestosterone and the 3α,17α- and 3β,17β-diols stimulated at the 4 µM concentration in the presence of cytosol. No stimulation was achieved with the 3β,17α-diol or any of the other steroids tested on rat or dog nuclear preparations, with or without cytosol, at concentrations of steroid up to 40 mM. The possibility that cytoplasmic...
Table 2. Effect of incubation of rat and dog prostatic nuclei with various steroids on the nuclear RNA polymerase activity in the presence and in the absence of a cytosol preparation

Nuclear preparations were incubated for 15 min at 37°C in an assay medium containing steroid (4 μM) in the presence and in the absence of 250 μl of cytosol. Cytosol was obtained by centrifugation (at 100,000 g for 60 min) of the supernatant after removal of the nuclear pellet for RNA polymerase preparation. Incorporation of [8-14C]ATP into acid-insoluble material was determined as d.p.m./15 min per mg of DNA. Accordingly 250 d.p.m. is equivalent to the incorporation of 1 pmol of ATP into RNA.

<table>
<thead>
<tr>
<th>Steroid (4 μM) added to enzyme assay system</th>
<th>Rat prostatic nuclei</th>
<th>Canine prostatic nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Cytosol +Cytosol</td>
<td>-Cytosol +Cytosol</td>
</tr>
<tr>
<td>None</td>
<td>8930 8893</td>
<td>10572 10284</td>
</tr>
<tr>
<td>Testosterone</td>
<td>8856 8860</td>
<td>10532 10461</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>9142 9123</td>
<td>9687 10451</td>
</tr>
<tr>
<td>5α-Androstan-3α,17α-diol</td>
<td>9104 9090</td>
<td>10270 10070</td>
</tr>
<tr>
<td>5α-Androstan-3β,17α-diol</td>
<td>8963 11135</td>
<td>10016 14490</td>
</tr>
<tr>
<td>5α-Androstan-3α,17β-diol</td>
<td>8874 8804</td>
<td>10732 14604</td>
</tr>
<tr>
<td>5α-Androstan-3β,17β-diol</td>
<td>8813 8632</td>
<td>9188 14768</td>
</tr>
<tr>
<td></td>
<td>8735 8851</td>
<td>11401 10502</td>
</tr>
<tr>
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<tr>
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<tr>
<td></td>
<td>8767 16050</td>
<td>11272 16735</td>
</tr>
</tbody>
</table>

proteins concerned with steroid binding are involved in the observed stimulatory effects of the steroid on the RNA polymerase activity is worthy of further study.

Experiments carried out by Harper et al. (1970) indicated that diethylstilbestrol inhibited the activity of DNA polymerase isolated from human hyperplastic and neoplastic prostate tissue. At the same time the newly synthesized dibutylhydro-stilbestrol was shown to produce the same inhibitory effect. It is of interest therefore that both diethylstilbestrol and dibutylhydroxistilbestrol were shown to inhibit the RNA polymerase enzyme from rat and dog prostate tissue (Table 1), together with oestradiol-17β and other stilboestrol derivatives.

The authors are grateful to the Tenovus Organization for their generous financial support.

Bruchovsky, N. & Wilson, J. D. (1968b) J. Biol. Chem. 243, 5953–5960
STIMULATION IN VITRO OF PROSTATIC RIBONUCLEIC ACID POLYMERASE BY 5α-DIHYDROTESTOSTERONE-RECEPTOR COMPLEXES

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The Welsh National School of Medicine,
Heath, Cardiff

Received May 4, 1973

SUMMARY In a reconstituted in vitro system, stimulation of RNA polymerase activities by 5α-DHT-receptor complexes prepared from prostatic supernatant and nuclear fractions has been observed. Stimulation of the nucleolar enzyme rather than the nucleoplasmic enzyme was noted. Higher levels of stimulation were observed in the presence of native chromatin as template than when purified exogenous DNA was used. The involvement of chromatin-associated proteins in the system was apparent.

Investigations in many research centres have indicated that protein receptors are essential factors in transmitting the steroid-hormone effect in cells of responsive tissues. Retention of the steroid hormone in the nucleus is dependent upon (a) an interaction with a specific cytoplasmic receptor protein (1-4) then (b) a close association in the nucleus with chromosomal sites composed of DNA and acidic protein (3,5-8). The present investigation is concerned with the effects of 17β-hydroxy-5α-androstan-3-one (5α-DHT) on the prostate and the reconstitution of an in vitro system which will enable 5α-DHT-receptor complexes formed in the prostate to bring about stimulation of the activity of prostatic DNA-dependent RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6). This involved the purification of the several cellular components from prostate tissue.
MATERIALS AND METHODS

All preparative procedures were performed on ventral prostate tissue excised from 8-12 week old male Sprague-Dawley rats 48 hr after bilateral castration. Nuclei were purified by the method of Mainwaring (9) using 1 mM MgCl₂ instead of CaCl₂ in all media and 2.2 M sucrose for the final centrifugation. Published procedures were used for the preparation of nucleoplasm and nucleoplasmic RNA polymerase (10), nucleoli, chromatin, nuclear and nucleolar RNA polymerase (11), for the selective removal of chromatin-associated proteins (8,12) and preparation of prostatic DNA (13). Labelling of cytoplasmic receptors with [1α,2α-³H₂]5α-DHT (specific radioactivity 47 Ci/mmol: The Radiochemical Centre, Amersham, Bucks. U.K.) and selective precipitation of receptors was achieved using the method of Mainwaring & Peterken (8) and nuclear [1α,2α-³H₂]5α-DHT-receptor complex was prepared by incubation of equal volumes of purified nuclei (100-150 μg of DNA) and labelled cytosol (0.4 pmol radioactive 5α-DHT per ml of mixture) at 37°C for 30 min. The nuclei were spun out, washed extensively and extracted with 0.4 M KCl at 0°C for 30 min. The supernatant from centrifugation for 30 min at 100,000 g contained labelled nuclear receptors. Ionic strength of receptor-complexes could be decreased by passage through columns of Sephadex G-25. Patterns of labelling of cytoplasm and nuclear extract corresponded to those described by others(8). The cytoplasm yielded two steroid-protein complexes, of sedimentation coefficient 8S and 3S, and nuclei yielded one complex, of sedimentation coefficient approx. 4.5S.
RNA polymerase activities were estimated in 500 µl of a medium containing 60 µmol tris-HCl buffer, pH 8.1, 2.5 µmol MgCl₂ or 1.5 µmol MnCl₂, 15 µmol KCl, 200 nmol dithiothreitol, 300 nmol NaF, 300 nmol each of ATP, GTP and CTP, 20 nmol carrier UTP and 125 pmol [U-¹⁴C]UTP (specific radioactivity 514 mCi/mmol, The Radiochemical Centre, U.K.). Assays containing MnCl₂ also contained (NH₄)₂SO₄ (0.4 M). DNA template was added in 50 µl and enzyme in 200 µl medium. In experiments on intact nuclei and nucleoli, DNA template and polymerase were replaced by 250 µl of nuclear (50-100 µg of DNA) or nucleolar (20-50 µg of DNA) suspension. 5α-DHT-receptor complexes were added at a final radioactive 5α-DHT concentration of 250 pM. Control systems contained equal quantities of protein-receptor not equilibrated with 5α-DHT. Neither receptor protein nor 5α-DHT alone affected purified RNA polymerase activity. Enzyme reactions (15 min at 37°C) were terminated by the addition of 2 ml of 10% (w/v) CCl₃ COOH - 1 mM Na₄P₂O₇ and acid-insoluble material prepared for measurement of incorporated radioactivity as previously described (14). After correction for controls in the absence of DNA, the incorporation of [¹⁴C]UMP into RNA was determined as pmol of [¹⁴C]UMP incorporated per unit of DNA.

RESULTS AND DISCUSSION

Incubation of intact nuclei and nucleoli in the RNA polymerase assay system containing cytoplasmic or nuclear 5α-DHT-receptor complexes resulted in an increased incorporation of [¹⁴C]UMP into acid-insoluble material (Table 1). The stimu-
Table 1  Effect of $[\text{H}]5\alpha$-DHT-Receptor Complexes on RNA Polymerase Activity

| Preparation                  | Exogenous template added | % Increase in incorporation of $[14\text{C}]\text{UMP}$ in the presence of Cytoplasmic receptors '8S' | | Nuclear receptor '4.5S' |
|------------------------------|--------------------------|--------------------------------------------------------------------------------------------------|------------------------|--------------------------|------------------------|
| (a) Nuclei                    | -                        | 107.0 ± 3.69                                                                                     | 115.2 ± 7.29          | 53.5 ± 1.73              |
| (b) Nucleoli                  | -                        | 50.5 ± 13.56                                                                                     | 77.5 ± 18.69          | 72.1 ± 2.67              |
| (c) Enzyme solubilised from whole nuclei |                     | 14.4 ± 1.96                                                                                     | 10.1 ± 3.01           | 28.6 ± 2.15              |
|                              | Calf thymus DNA          | 158.2 ± 19.90                                                                                   | 91.9 ± 16.02          | 40.7 ± 4.69              |
|                              | Prostatic nuclear chromatin | 116.3 ± 13.78                                                                                   | 85.8 ± 17.85          | 58.1 ± 4.86              |
|                              | Liver chromatin          | 6.2 ± 3.26                                                                                      | 8.1 ± 2.91            | 3.1 ± 2.64               |

Enzyme preparations were incubated with $5\alpha$-DHT-receptor complexes at a $5\alpha$-DHT concentration of 0.25 pmol/ml (based on radioactivity) or with an equal amount (based on protein) of '5$\alpha$-DHT-free' receptor. Incubations with solubilised enzyme included one of several added templates. Increases in the rate of incorporation of $[14\text{C}]\text{UMP}$ into acid-insoluble material are expressed as percentages above those values observed in the presence of protein only. Values are the average of at least four determinations ± the standard deviations.
Table 2  Effect of 5α-DHT-protein receptor complexes on prostatic RNA polymerases

<table>
<thead>
<tr>
<th>Conditions of assay</th>
<th>Template</th>
<th>% increase in enzyme activity in the presence of '8S'</th>
<th>'4.5S'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleolar Enzyme</strong> (Form I)</td>
<td>Calf thymus DNA</td>
<td>11.8 ± 2.86</td>
<td>26.5 ± 5.47</td>
</tr>
<tr>
<td>Mg$^{2+}$/low salt</td>
<td>Prostatic nuclear chromatin</td>
<td>96.6 ± 6.12</td>
<td>39.0 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>Prostatic nucleolar chromatin</td>
<td>143.5 ± 10.59</td>
<td>50.4 ± 9.27</td>
</tr>
<tr>
<td></td>
<td>Liver chromatin</td>
<td>4.5 ± 0.81</td>
<td>2.9 ± 1.30</td>
</tr>
<tr>
<td><strong>Mn$^{2+}$/high salt</strong></td>
<td>Calf thymus DNA</td>
<td>0</td>
<td>10.6 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>Prostatic nuclear chromatin</td>
<td>5.7 ± 1.30</td>
<td>10.6 ± 1.58</td>
</tr>
<tr>
<td><strong>Nucleoplasmic Enzyme</strong> (Form II)</td>
<td>Calf thymus DNA</td>
<td>2.0 ± 1.89</td>
<td>7.8 ± 1.49</td>
</tr>
<tr>
<td>Mg$^{2+}$/low salt</td>
<td>Prostatic nuclear chromatin</td>
<td>12.8 ± 4.73</td>
<td>11.2 ± 7.04</td>
</tr>
<tr>
<td></td>
<td>Liver chromatin</td>
<td>8.0 ± 5.68</td>
<td>0</td>
</tr>
<tr>
<td><strong>Mn$^{2+}$/high salt</strong></td>
<td>Calf thymus DNA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Prostatic nuclear chromatin</td>
<td>0</td>
<td>16.3 ± 4.47</td>
</tr>
</tbody>
</table>

Nucleolar and nucleoplasmic RNA polymerases solubilised from the respective subnuclear fractions were incubated with various templates and 5α-DHT-receptor complexes (0.25 pmol radioactive 5α-DHT/ml) in assay mixtures containing either MgCl$_2$ (5 mM) and a low salt concentration (0.03 M KCl) or MnCl$_2$ (3 mM) and at high ionic strength 0.4 M (NH$_4$)$_2$SO$_4$. Increases in activity expressed as % increases over values observed in the presence of an equal concentration of 5α-DHT-free receptor protein. Values are the means of 4 or more experiments ± the standard deviation.
lation by both cytoplasmic complexes may suggest the possession of structural similarities (15). The various complexes also stimulated the activity of RNA polymerase solubilised from nuclei (Table 2). The degree of stimulation observed in the presence of purified calf thymus DNA or liver chromatin was slight compared to that observed when prostatic nuclear or nucleolar chromatin were used as template in the system. Nuclear RNA polymerase transcribed native chromatin with approx. 40% of the efficiency with which it transcribed calf thymus DNA. Increases in enzyme activity were not so marked however in the case of the nuclear complex. These results support the concept that specificity of binding of steroid-receptor complexes resides in tissue chromatin (16).

The degree of stimulation of subnuclear forms of prostatic RNA polymerase depended not only on the DNA template provided but also on the intranuclear source of the enzyme and the ionic conditions employed (Table 2). Ionic conditions of assay are known to influence the type of RNA synthesized (17,18). Nucleolar RNA polymerase was preferentially stimulated by 5α-DHT-receptor complexes, especially with Mg$^{2+}$ as activating cation. The nucleoplasmic enzyme, which transcribed prostatic chromatin much more efficiently (40% of DNA) than did the nucleolar enzyme (15% of DNA) was stimulated only slightly in the presence of Mg$^{2+}$ and not at all by cytoplasmic 8S complex in the presence of Mn$^{2+}$/\((\text{NH}_4)_2\text{SO}_4\). The nuclear complex did however stimulate nucleoplasmic enzyme in the presence of prostatic chromatin. These results further suggest that the major RNA product of steroid-
Table 3 Effect of cytoplasmic 5α-DHT-protein receptor complexes on nuclear RNA polymerase in the presence of selectively altered templates

<table>
<thead>
<tr>
<th>Template</th>
<th>pmol[^14]CUMP incorporated in the presence of &quot;control receptor fraction&quot;</th>
<th>pmol[^14]CUMP incorporated in the presence of receptor complex</th>
<th>% age increase in[^14]CUMP incorporation in the presence of receptor complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus DNA</td>
<td>1.12 '8S' 1.22 '3S'</td>
<td>1.29 '8S' 1.30 '3S'</td>
<td>15 '8S' 7 '3S'</td>
</tr>
<tr>
<td>Liver chromatin</td>
<td>0.28 '8S' 0.26 '3S'</td>
<td>0.30 '8S' 0.28 '3S'</td>
<td>6 '8S' 8 '3S'</td>
</tr>
<tr>
<td>Prostatic nuclear chromatin</td>
<td>0.46 '8S' 0.56 '3S'</td>
<td>1.17 '8S' 0.93 '3S'</td>
<td>154 '8S' 66 '3S'</td>
</tr>
<tr>
<td>Histone-deficient prostatic chromatin</td>
<td>0.63 '8S' 0.72 '3S'</td>
<td>1.54 '8S' 1.08 '3S'</td>
<td>144 '8S' 50 '3S'</td>
</tr>
<tr>
<td>Histone and nonhistone-deficient prostatic chromatin</td>
<td>0.71 '8S' 0.76 '3S'</td>
<td>1.62 '8S' 1.13 '3S'</td>
<td>128 '8S' 49 '3S'</td>
</tr>
<tr>
<td>Prostatic DNA</td>
<td>0.89 '8S' 0.75 '3S'</td>
<td>0.97 '8S' 0.86 '3S'</td>
<td>9 '8S' 15 '3S'</td>
</tr>
</tbody>
</table>

RNA polymerase solubilised from prostatic nuclei was incubated in an assay medium containing cytoplasmic 5α-DHT-receptor complexes (0.25 pmol of radioactive 5α-DHT per ml). The template for RNA synthesis varied in composition - calf thymus DNA, liver chromatin, prostatic chromatin intact, deficient of histones, deficient of histone and acidic proteins, and prostatic DNA. Full details are given in the experimental section. The enzyme activity in the presence of the cytoplasmic complexes or an equal quantity of steroid-deficient receptor is expressed as pmol of[^14]CUMP incorporated/µg of template DNA and the percentage increase in activity in the presence of 5α-DHT-receptor complex is specified in each case.
hormone stimulation is of a nucleolar or ribosomal type and that
the production of nucleoplasmic or messenger RNA is a secondary
effect. A system in which chromatin initiation sites recognized
by nucleolar enzyme are available for transcription in a normal
androgenic environment and unavailable after castration may be
hypothesised.

A further insight into the role of chromatin in the system
is shown in Table 3. Stimulation of RNA polymerase by cyto-
plasmic complexes was low in the presence of calf thymus DNA and
liver chromatin, and high in the presence of prostatic chromatin.
Histone removal increased transcription but not the degree of
stimulation by the complexes. Removal of the major part of the
nonhistone-protein again increased transcription but stimulation
remained at the same level. 10% of the chromatin-associated
protein remained at this stage. With protein-free prostatic
DNA as template, transcription was once again higher, but the
levels of stimulation brought about by 5α-DHT-receptor complexes
were low. This suggests that the stimulation of RNA polymerase
activity by the complexes was influenced by nonhistone protein
as was the binding of these complexes to DNA (8). Unlike non-
histone protein (20, 21), histones do not influence tissue-
specificity of chromatin (19). The lack of any effect resulting
from the removal of most of the nonhistone protein confirms that
the major portion of this fraction is not concerned with tissue-
specificity (22, 23) and that this property is conferred by
proteins tightly bound to DNA (24) and only released by phenol
treatment (8).
Our results, therefore, indicate that stimulation of RNA polymerase activity by 5α-DHT-receptor complexes is mainly confined to the nucleolar form of the enzyme in Mg$^{2+}$-low salt conditions and is under the control of acidic chromatin-associated proteins. It is still possible, however, that further controlling factors which exist in vivo will have to be introduced into the system.

ACKNOWLEDGEMENT

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STUDIES ON THE EFFECT OF ANDROGENS ON PROSTATIC DNA POLYMERASE.

Harper, M.E., Jones, T. and Griffiths, K. Tenovus Institute for Cancer Research, Heath, Cardiff, Wales, U.K.

Previous reports from this laboratory described the stimulation in vitro by certain androgens of the activity of DNA polymerase enzyme isolated from human and canine prostatic tissue (Harper, Fahmy, Pierrepoint & Griffiths, 1970). The enzyme isolated from canine prostatic tissue has now been further purified using DEAE-cellulose, phosphocellulose and hydroxyapatite column chromatography. The DNA polymerase was assayed after each purification step in the presence of testosterone, 5α-dihydrotestosterone, various 5α-androstanediols and certain stilboestrol analogues. The stimulatory effect of testosterone and 5α-androstane-3α,17α-diol on the activity of the partially purified preparation was lost after phosphocellulose chromatography. The inhibitory effect of diethylstilboestrol however was observed at all stages of purification. These results will be discussed in relation to studies on the partially purified rat ventral prostate P2-fraction DNA polymerase enzyme which previously had been shown to be uninfluenced by steroids. Cytosol preparation of ventral prostate from 12 week old rats, castrated 24 h. before sacrifice, were subjected to sucrose gradient centrifugation at 105,000 g for 18 h. The fraction containing the 5α-dihydrotestosterone receptor-protein when incubated with the DNA polymerase in the presence of 5α-dihydrotestosterone (1 μM) resulted in the stimulation of enzyme activity.

INFLUENCE OF CYPROTERONE ACETATE ON THE METABOLISM AND THE MODE OF ACTION OF TESTOSTERONE AND 5α-DIHYDROTESTOSTERONE IN TARGET ORGANS OF MALE RATS. x)

H. Schmidt, R. Szalay and K. D. Voigt. Dept. of Clinical Chemistry, 2nd Medical University Clinic, Hamburg, West-Germany.

In continuation of previous studies as well the uptake and the metabolism of cyproterone acetate (CyAc) as its influence on the metabolic fate and the biological action of various androgens have been investigated. 30 min after the intravenous injection of 3H-CyAc a pronounced radioactivity uptake was found in the ventral prostates of adult castrated male rats. Radioactivity readings in seminal vesicles compared well to those obtained in plasma and skeletal muscle. TLC on extracts of target organs revealed that more than 90% of the radioactivity migrated in one fraction (CyAc ?) whereas in extracts from other organs for example from plasma and liver 3 distinct fractions have been obtained. In a second experimental series day 3 castrated adult male rats received 10 min prior to the injection of the tritiated androgens the 13-fold amount (w/w) of cold CyAc. 30 min after the androgen injection the animals were sacrificed. Total radioactivity was counted in target organs, plasma, peripheral muscle, kidney, and liver. By means of TLC in different systems with and without derivative formation the metabolic patterns of the steroids injected have been analyzed. A marked inhibition of androgen uptake in target organs by CyAc has been observed whereas its action on steroid metabolism was less marked. Finally immature castrated male rats were treated with a combination of 5α-DHT and CyAc (0.5 mg/5 mg or 0.5 mg/2.5 mg daily for 3 days intraperitoneally). Control groups were injected either with 5α-DHT (0.5 mg daily for 3 days) or with CyAc (5 mg or 2.5 mg daily for 3 days) or with the solvent only.

By the combined treatment schedule the cell proliferative action of 5α-DHT was completely abolished. The effect of 5α-DHT on cell metabolism was inhibited less effectively by the antiandrogen.


x) Supported by the DFG, Sonderforschungsbereich 34 (Endocrinology).
EFFECT IN VITRO OF 5α-DIHYDROTESTOSTERONE-PROTEIN RECEPTOR COMPLEXES ON PROSTATIC RNA POLYMERASE.

P. Davies and K. Griffiths. Tenovus Institute for Cancer Research and Velindre Memorial Centre, Cardiff, U.K.

The role of steroid-receptor proteins in promoting the action of steroid hormones at the target organ is well established. Although one of the early effects involves an increased activity of nuclear RNA polymerase, the mechanism by which the stimulation is effected is little understood. In a reconstituted in vitro system, the effect of cytoplasmic and nuclear protein-receptor-5α-dihydrotestosterone complexes from rat ventral prostate on RNA polymerase have been studied. RNA polymerase was prepared from rat prostate nuclear and subnuclear fractions by sonication at high salt concentration.

Protein-receptor-5α-dihydrotestosterone complexes from both cytoplasm and nucleus increased the RNA polymerase activity when incubated with intact prostatic nuclear and nucleolar preparations. Both complexes increased the activity of total nuclear solubilised RNA polymerase, although the stimulation was dependent on various factors which may provide some insight into the mechanism of hormone control of RNA synthesis. Maximal stimulation of the nucleolar RNA polymerase was observed in the presence of Mg$^{2+}$ but low salt concentration. Little stimulation of the nucleoplasmic RNA polymerase was achieved especially in the presence of a high salt concentration and Mn$^{2+}$.

The degree of stimulation differed with nuclear and cytoplasmic receptors, and the DNA template available for transcription. Greater enzyme stimulation was obtained with prostatic chromatin as template than with chromatin isolated from other tissue.
Stimulation of Ribonucleic Acid Polymerase Activity in vitro by Prostatic Steroid–Protein Receptor Complexes

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A system has been developed which allows the stimulation in vitro of prostatic RNA polymerase by prostatic 5α-dihydrotestosterone–protein receptor complexes prepared from the tissues of castrated rats. The reconstitution in vitro of such a system necessitates the purification of several subcellular components. Two 5α-dihydrotestosterone–receptor complexes are located in the prostatic soluble supernatant fraction, separable by selective ammonium sulphate fractionation, and one complex can be isolated from the nuclear fraction. In the presence of all these complexes, stimulation of RNA polymerase in intact nuclei and nucleoli was observed. The complexes also increased the activity of the enzyme solubilized from whole nuclei. Greater stimulation of this system was noted in the presence of prostatic chromatin as template, as compared with that observed with calf thymus DNA or liver chromatin as template. The effects of the complexes on subnuclear forms of RNA polymerase, of nucleolar and extranucleolar origin, are also described. RNA polymerase solubilized from nucleoli is more susceptible to stimulation by the 5α-dihydrotestosterone–receptor complexes than is the ‘nucleoplasmic’ enzyme. Stimulation occurs less readily in the presence of Mg²⁺ and at high ionic strength than in the presence of Mg²⁺ and at low ionic strength. Preliminary experiments show that prostatic nucleolar RNA polymerase transcribes prostatic chromatin poorly as compared with the nucleoplasmic enzyme. The observations reported indicate an involvement of non-histone proteins associated with DNA in the process by which stimulation of enzyme activity by the 5α-dihydrotestosterone–receptor complexes is achieved. The implications of these findings in the mechanism of steroid hormone action is considered.

Reports from a number of research centres during the past few years have clearly indicated that protein receptor molecules have an essential role in transmitting the effects of steroid hormones within the cells of responsive tissues. The process involves an interaction between the steroid hormone and a specific receptor protein in the cytoplasm (Jensen et al., 1968; Mainwaring, 1970; Fang & Liao, 1971; O’Malley, 1971). The complex thus formed is transferred to the nucleus, where a close association of the steroid hormone is established with certain chromosomal sites composed of DNA and nuclear acidic protein (Fang & Liao, 1971; Spelsberg et al., 1971a; O’Malley et al., 1971, 1972; Mainwaring & Peterken, 1971). The steroid–receptor complex, rather than the steroid alone, is retained within the nucleus. However, the function of such steroid–receptor complexes within the nucleus and the nature of the effects that the various components of this system may have upon gene transcription are still uncertain.

The present investigation provides some evidence that a steroid–protein receptor complex may be concerned in controlling the activity of the DNA-dependent RNA polymerase (nucleoside triphosphate–RNA nucleotidytransferase; EC.2.7.7.6) from nuclei of rat prostatic tissue. Preliminary studies (Davies et al., 1972) had shown that certain specific steroids were capable of stimulating in vitro the RNA polymerase in purified rat and dog prostatic nuclei. The aim of the investigation now reported was to reconstruct in vivo, from various purified components, a system whereby the 17β-hydroxy-5α-androstan-3-one–receptor complex formed in the prostate could stimulate the activity of the RNA polymerase.

Materials and Methods

Materials

Animals. Adult male Sprague–Dawley rats (8–12 weeks old) were acquired from Charles River Ltd., Margate, Kent, U.K. Animals were castrated by the scrotal route under ether anaesthesia. In all experiments described, ventral prostate tissue used was removed from the animals 48h after bilateral castration.

Chemicals. 17β-Hydroxy-5α-[1α,2α-3H]androstan-3-one (specific radioactivity, 47Ci/mmol) and
[U-14C]UTP (specific radioactivity, 514 mCi/mmoll) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Dithiothreitol, 17β-hydroxy-5α-androstane-3-one, UTP, GTP and CTP (sodium salts) and ATP (disodium salt) were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bovine serum albumin and Escherichia coli (strain K-12) ribonucleic acid polymerase [214 units/mg of protein: suspension in glycerol-Tris-Cl (1:1) buffer, pH 7.9] were supplied by Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, U.K.

Tris (Aristar grade), yeast RNA, Triton X-100 and calf thymus DNA were obtained from BDH Ltd., Poole, Dorset, U.K. Aquasol is a product of the New England Nuclear Corp., and was obtained from Reading, Berks., U.K. Sephadex G-25 was obtained from Pharmacia (U.K.) Ltd., London, U.K. All other substances were of AnalR grade and solutions used were made up in water distilled from all-glass apparatus.

Centrifuges and rotors. All high-speed centrifugation procedures were carried out in the Beckman L65 B preparative ultracentrifuge with either the SW 50 (3 x 5 ml) swinging-bucket rotor (r = 7.3 cm) or the SW 50.1 (6 x 5 ml) swinging-bucket rotor (r = 8.35 cm). Low-speed centrifugation procedures (less than 1000g) were carried out in the MSE Mistral 4L refrigerated centrifuge.

Methods

Preparation of subcellular fractions. All preparative procedures were carried out at temperatures between 0° and 4°C.

Nuclei. Ventral prostate glands were weighed, finely minced with scissors and homogenized in 0.25m-sucrose containing 1mM-MgCl2 in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle with a clearance of 0.15–0.23 mm. Homogenates were filtered through sterile gauze and the filtrate was centrifuged at 800g for 15 min. Pellets were resuspended by gentle homogenization by hand in 2.2m-sucrose containing 1mM-MgCl2 and nuclei purified by centrifugation through this medium at 50000g for 1h. Based on DNA determinations, 60–70% of tissue nuclei were recovered in a purified form. Satisfactory purity was assessed by biochemical and light- and electron-microscopic criteria (Widnell & Tata, 1964).

Nucleoli and nucleoplasm. Purified nuclei were washed once by centrifugation in 0.25m-sucrose and evenly suspended by homogenization by hand in 0.25m-sucrose in 5mM-Tris–HCl buffer, pH 7.4. The suspensions were sonicated for 45s at 20kHz in an MSE 150W ultrasonic disintegrator (medium speed, setting 4; amplitude, 8μm). The sonicate was layered over an equal volume of 0.88m-sucrose and centrifuged at 2500g for 30min. The upper layers, comprising the nucleoplasm, were removed. The nucleoli were purified by repeated resuspension and re-centrifugation at 800g in 0.88m-sucrose (Muramatsu et al., 1963). Percentage disintegration of nuclei was estimated by microscopic examination of specimens stained with Azure C (Ayres, 1948).

Under the conditions described, over 80% of the nuclei were disintegrated. In most nucleolar preparations, nuclear contamination was completely absent. Approx. 15% of nuclear DNA was recovered in the nucleolar preparations. Purified nuclei and nucleoli, if not required for further preparative procedures, were resuspended in 0.25m sucrose–1mM-MgCl2.

Chromatin. This was prepared from purified nuclei or nucleoli by methods similar to those described by Mainwaring et al. (1971) and Mainwaring & Peterken (1971). Nuclei were twice washed by resuspension in 0.25m-sucrose containing 0.2%(w/v) of Triton X-100 followed by centrifugation at 800g for 10min. This removes contaminating basic proteins (Hymer & Kuff, 1964) and allows for reproducible results for the chemical composition of chromatin (Mainwaring & Peterken, 1971). The main procedure consisted of two extractions with 80mM-NaCl–20mM-EDTA, titrated with citrate buffer to pH 6.3, two extractions with 0.35M-NaCl and one extraction with 1.5mM-NaCl in 0.15mM-citrate buffer, pH 7.0. At each successive stage, the chromatin was sedimented by centrifugation at 10000g for 15 min. The final gel-like preparations were suspended in water to a concentration of 20–50μg of DNA/100μl by gentle homogenization by hand in an all-glass homogenizer and dialysed against distilled water. Preliminary chemical analysis of chromatin by the methods of Marushige & Bonner (1966) indicated that 85–95% of the nuclear or nucleolar DNA was recovered in the preparations. The RNA content of chromatin was less than 5% of the DNA content. Chromatin could not be prepared from nucleoplasm, by using the methods described above, with any reproducibility of chemical composition. This presumably was due to the shearing effects of the sonication procedures used to separate nuclei into nucleoli and nucleoplasm. However, chromatin was prepared from liver and spleen nuclei by the method described.

Selective removal of histone and non-histone proteins from prostatic chromatin was accomplished by methods based on those of Mainwaring & Peterken (1971) and Spelsberg et al. (1971b).

Chromatin suspension (2ml) was mixed with 3ml of 2mM-NaCl in 5mM-urea and to the mixture was added 0.5ml of 0.2mM-Na2HPO4–citric acid buffer, pH 6.0. The suspensions were stirred for 30min and centrifuged at 10000g for 15h. The histones were recovered in the supernatant fraction. Non-histone (acidic) proteins were removed from the sedimented histone-
deficient chromatin by repeating the dissociation in 2M-NaCl–5m-urea, but in the presence of 10mM-Tris–HCl buffer, pH8.3, containing 5mM-NaHSO₃, followed by centrifugation at 100000g for 15h. The final residue remaining after this latter procedure contained 9–10% of the total protein relative to DNA. Protein-free prostatic DNA was prepared by the phenol-extraction procedure of Colter et al. (1962). All samples were finally resuspended in water to a final concentration of 20–50μg of DNA/100μl. Chromatin preparations were used within 24h of being suspended in water.

**Labelling of receptor proteins with 17β-hydroxy-5x-[1α,2α-3H₂]androstane-3-one**

Cytoplasmic receptors. The method employed was based upon that of Mainwaring & Peterken (1971). Ventral prostate tissue (six glands) (2.1–2.7 g wet wt.) was finely minced with scissors and homogenized in 5ml of 50mM-Tris–HCl buffer, pH7.4, containing 0.1mM-EDTA and 0.25mM-dithiothreitol (medium A). The supernatant from a preliminary centrifugation at 800g was re-centrifuged at 105000g for 1h. The resulting soluble supernatant (cytosol) fraction was made 4m with respect to 17β-hydroxy-5x-[1α,2α-3H₂]androstane-3-one (3H-labelled steroid), added in glycerol (50μl/ml of cytosol), and retained for 1h in an ice-bath. Saturated (NH₄)₂SO₄ (0.5vol.), adjusted to pH7.4 withaq. NH₃ soln., was gradually added, and the resulting precipitate collected by centrifugation at 10000g for 10min before re-dissolving in 5ml of medium A. Dialysis of this solution against 2.0 litres of medium A for 2–3h removed all traces of (NH₄)₂SO₄ and yielded a solution containing approx 1.5×10⁶ c.p.m./μg of protein per ml. Ammonium sulphate and free steroid could also be removed from the re-dissolved precipitate and from the 100000g supernatant (3.9×10⁶ c.p.m./μg of protein per ml) by passage through a 2cm×14cm column of Sephadex G-25 equilibrated with medium A.

Nuclear receptor. Purified nuclei were resuspended and washed several times 0.25 m-sucrose in medium A. Equal volumes of nuclear suspension (100–150μg of DNA) in medium A and labelled cytosol (adjusted with medium A so that the final concentration of 3H-labelled steroid in the mixture was 0.4nm) were incubated together in a shaking water bath at 37°C for 30min. At the end of this period, the mixture was chilled in ice and centrifuged at 800g for 20min. The nuclear pellet was washed several times in medium A, sedimented at 800g and was finally resuspended in 5ml of medium A containing 0.4m-KCl. The suspension was stirred in an ice bath for 30min and the viscous extract centrifuged at 100000g for 30min. This resulted in a solution containing 2.6×10⁴ c.p.m./0.18 mg of protein per ml.

No radioactivity was obtained in the nuclear extract when mixtures were incubated at 0°C. For use in studies with the solubilized RNA polymerase enzyme system, theionic strength of the nuclear extract was lowered by passage through a 2cm×14cm column of Sephadex G-25 equilibrated with medium A as outlined by Mainwaring & Peterken (1971).

Unlabelled receptor proteins for control systems were prepared by using cytosol and nuclear fractions from ventral prostate tissue of 48h castrated rats with the procedures described above, but without adding the 3H-labelled steroid. The non-radioactive preparations contained the same concentration of protein as did the 3H-labelled steroid–receptor complexes.

**Sucrose-density-gradient centrifugation.** Linear 5 ml (5–20%, v/v) sucrose density gradients in a uniform concentration of medium A were prepared at least 6h before use by using a Lucite block gradient-former (Martin & Ames, 1961). Some gradients also contained a uniform concentration of 0.5m-KCl. Samples for analysis (0.4ml) were layered over the gradients and centrifuged at 3–4°C for 18h at 100000g. One gradient in each set was layered with bovine serum albumin (s₂₀,₆ 4.6S) as sedimentation marker. After centrifugation, the base of each tube was pierced and two-drop fractions were collected. Radioactivity in fractions was measured in a mixture of 5ml of Aquasol and 0.5ml of water in a Nuclear–Chicago mark I liquid-scintillation spectrometer at a counting efficiency for tritium of 40%. To fractions from gradients containing bovine serum albumin, 1ml of water was added and the concentration of protein in each tube estimated spectrophotometrically.

**Solubilization of RNA polymerase activity.** This was accomplished by the methods of Roeder & Rutter (1970) and Mainwaring et al. (1971). Prostatic nuclei or nucleoli were suspended in 4ml of medium B (10mM-Tris–HCl buffer, pH7.9, containing 1.0m sucrose, 5mM-MgCl₂ and 5mM-dithiothreitol), and a necessary volume of 3.0m-(NH₄)₂SO₄ (adjusted to pH7.9 withaq. NH₃ soln.) to bring the final concentration to 0.3m. Samples of nucleoplasm were adjusted to the same concentrations. The viscous suspensions were sonicated for four periods of 10s at 20kHz in the MSE 150W ultrasonic disintegrator (high speed, setting 4; amplitude 13–16μm) with 20s periods for cooling. After rapid dilution with 2vol. of medium C (50mM-Tris–HCl buffer, pH7.9, containing 1mM-MgCl₂, 0.1mM-EDTA, 0.5mM-dithiothreitol and 25% (v/v) glycerol) the samples were centrifuged at 100000g for 1h. To clear supernatants was added solid (NH₄)₂SO₄ to give 0.42g/ml and, after standing for 30min, the precipitates were sedimented by centrifugation at 100000g for 30min.

These were then resuspended in medium C to a protein concentration of 0.6–0.8mg/ml and dialysed for 2h against this medium. Any precipitate formed
during dialysis was removed by brief centrifugation at 100000g and the supernatant, containing the RNA polymerase activity, was taken for further analysis. The final preparation, from prostatic nuclei from six 48h-castrated animals, contained 2-3mg of protein.

RNA polymerase prepared from whole nuclei was eluted from columns of DEAE-cellulose with a linear gradient of 0-0.8m-KCl as two discrete peaks of activity. The more rapidly eluted peak (0.3m-KCl) corresponded to activity from the nucleolus and the second peak (0.5-0.6m-KCl) to activity of extranucleolar origin. This was confirmed by the elution of enzyme prepared from nucleoli and nucleoplasm from similar columns. Both peaks contained Mg$^{2+}$- and Mn$^{2+}$-dependent activity, and preliminary experiments showed the optimum concentrations of the activating cations to be 5mm and 3mm for Mg$^{2+}$ and Mn$^{2+}$ respectively. More Mn$^{2+}$-dependent activity was observed in the nucleoplasmic peak. These results are in agreement with those of Mainwaring et al. (1971). The enzyme preparations during the latter stages are DNA-free, and show an absolute requirement for exogenous template. The activity is also dependent upon the presence of all four nucleoside triphosphates, and the enzyme reaction yields a ribonuclease-sensitive product.

**Measurement of RNA polymerase activity.** RNA polymerase activity was measured in a medium containing 60μmol of Tris–HCl buffer, pH8.1, 2.5μmol of MgCl$_2$, or 1.5μmol of MnCl$_2$, 15μmol of KCl, 200nmol of dithiothreitol, 300nmol of NaF, 300nmol each of ATP, GTP and CTP, 125pmol of $[^{14}C]$UTP and 20nmol of carrier UTP in a final volume of 500μl. In assays containing MnCl$_2$, the mixture also contained (NH$_4$)$_2$SO$_4$ at a final concentration of 0.4m. DNA template (10-25μg of DNA) and enzyme preparations were added in volumes of 50μl and 200μl respectively. In some experiments denatured calf-thymus DNA was used as template. Denaturation was carried out by heating DNA at 95°C for 10min, followed by rapid cooling in ice. In experiments on intact nuclei and nucleoli, DNA template and RNA polymerase were replaced by 250μl of nuclear (50-100μg of DNA) or nucleolar (20-50μg of DNA) suspension in 0.25m-sucrose–1mm-MgCl$_2$. In experiments involving E. coli RNA polymerase, 200μl of suspension (30μg of protein) was added to the assay. To investigate their effects on prostatic RNA polymerase activity, 17β-hydroxy-5α-androstan-3-one–protein receptor complexes were added to the assay system so that the final concentration of bound steroid, as determined by radioactivity measurement, was 0.25pmol/ml. Control fractions were added to the assay system in amounts, based on protein concentration, equal to the steroid–receptor complexes. Neither addition of these control fractions nor of free steroid (0.25pmol/ml) caused changes in enzymic activity. Enzyme reactions (15min at 37°C) were terminated by the addition of 2ml of 10% (w/v) trichloroacetic acid containing 1mm-Na$_2$P$_2$O$_5$. Mixtures were chilled in an ice bath and carrier yeast RNA (250μg in 250μl) was added. Washing of precipitates and preparation of acid-insoluble material for assessment of incorporated radioactivity have been previously described (Davies et al., 1972). Samples were counted in a Nuclear–Chicago mark I liquid-scintillation spectrometer at 55-60% counting efficiency for $^{14}C$ and zero counting efficiency for $^3$H.

After correction for controls in the absence of DNA the incorporation of $[^{14}C]$UMP into RNA was determined in terms of d.p.m/15min per mg of DNA. For nuclear and nucleolar suspensions RNA polymerase activity was also calculated as pmol of $[^{14}C]$UMP incorporated/mg of DNA and for purified enzyme the activity was also calculated as pmol of $[^{14}C]$UMP incorporated/100μg of enzyme protein in the presence of a constant quantity of DNA template.

**Chemical analyses**

DNA was determined by the diphenylamine procedure of Burton (1956) as modified by Giles & Myers (1965). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

**Results and Discussion**

**Characterization of 17β-hydroxy-5α-[1α,2α-3H$_2$]androstan-3-one–protein receptor complexes by sucrose-density-gradient centrifugation**

Centrifugation of labelled cytosol on linear 5–20% (w/v) sucrose density gradients showed two peaks of radioactivity (Fig. 1a). The faster-sedimenting fraction corresponded to a steroid–receptor complex of sedimentation coefficient approx. 8S, and the slower-sedimenting fraction to a complex of sedimentation coefficient approx. 3S. The 8S form was the first receptor to be identified by labelling whole tissue in vivo (Mainwaring & Peterken, 1971). Preliminary experiments indicated non-specific binding in the 3S region in that other steroids, such as $[\text{H}]$cortisol, $[\text{H}]$androstenedione, $[\text{H}]$estradiol and $[\text{H}]$testosterone, were retained by those proteins as well as 17β-hydroxy-5α-[1α,2α-3H$_2$]androstan-3-one, which was the only steroid bound in the 8S region. The high capacity of the 3S region was indicated by labelling cytosol in the presence of a 10000-fold excess of non-radioactive 17β-hydroxy-5α-androstan-3-one. Whereas the low-capacity 8S peak was abolished by this treatment, the radioactivity in the 3S peak was not significantly affected (Fig. 1a). Ammonium sulphate selectively precipitated the 8S peak, which was recovered by dissolution.
**Fig. 1. Analysis of labelled prostatic supernatant fractions by sucrose-density-gradient centrifugation**

Cell supernatant fractions (0.4 ml) were layered over linear 5–20% (w/v) sucrose density gradients prepared in uniform concentration of medium A and centrifuged at 100000 g for 18 h at 3–4°C. The radioactivity of two-drop fractions was determined. The direction of centrifugation was from right to left. Sedimentation marker (arrows) was bovine serum albumin (4.6S). (a) Supernatant labelled in vitro, 1 h at 0°C; ○, 17β-hydroxy-5α-[1α,2α-3H2]-androstan-3-one alone; ●, the 3H-labelled steroid plus a 10000-fold excess of the non-radioactive steroid. (b) ○, Receptor precipitated by 33% satd. (NH4)2SO4; •, supernatant after removal of 33% satd. (NH4)2SO4 precipitate by centrifugation at 10000g.

**Fig. 2. Analysis of nuclear 17β-hydroxy-5α-[1α,2α-3H2]-androstan-3-one–receptor complexes by sucrose-density-gradient centrifugation**

Samples (0.4 ml) were layered over linear 5–20% (w/v) sucrose density gradients prepared in medium A, and centrifuged at 100000 g for 18 h at 3–4°C. Some gradients contained a uniform concentration of 0.5M-KCl. Sedimentation marker (arrow) was bovine serum albumin (4.6S). Nuclear 3H-labelled steroid–receptor complex was formed in vitro by incubation of nuclei with labelled cytosol and extracted with 0.4M-KCl. ○, 0.4M-KCl-extracted receptor complex centrifuged through gradient containing 0.5M-KCl; ●, 0.4M-KCl-extracted complex after passage through Sephadex G-25, on a KCl-free gradient.

**Fig. 3. Comparison of labelling of cytosol preparations from different tissues with 17β-hydroxy-5α-[1α,2α-3H2]-androstan-3-one**

High-speed supernatant fractions were prepared from ventral prostate gland, spleen and liver of castrated rats and were made 4M with respect to 17β-[1α,2α-3H2]-hydroxy-5α-androstan-3-one. Samples (0.4 ml) of labelled cytosol were layered over linear 5–20% (w/v) sucrose density gradients and centrifuged at 100000 g for 18 h at 3–4°C. Centrifugation was from right to left. Sedimentation marker (arrow) was bovine serum albumin (4.6S). ○, Ventral prostate cytosol; ●, spleen cytosol; Δ, liver cytosol.
in medium A after re-centrifugation (Fig. 1b). Centrifugation of the nuclear extract on sucrose density gradients yielded one peak of radioactivity in the presence and absence of 0.5M-KCl, corresponding to a steroid–receptor complex of s20,w 4.5–5S (Fig. 2).

When cytosol fractions prepared from liver and spleen and labelled with the 3H-labelled steroid (4nm) were analysed by sucrose-density-gradient centrifugation, only one peak of radioactivity was observed (Fig. 3). This corresponded to a steroid–receptor complex of sedimentation coefficient of approx 3S. No 8S region was apparent. It is evident that the low-capacity 8S region of steroid–receptor proteins is restricted to androgen target organs. Incubation of prostatic, liver or spleen nuclei with labelled cytosol from liver or spleen resulted in no incorporation of radioactivity into nuclear fractions. The 3S steroid–protein regions can be precipitated by 70% saturation with (NH₄)₂SO₄.

These patterns of 3H-labelled steroid binding by cytoplasmic and nuclear receptor proteins are essentially similar to those reported by Mainwaring & Peterken (1971).

Transcription of chromatin by prostatic RNA polymerases

It is clear that one of the limiting factors in the development of a cell-free system which aims to reflect the effects of hormone–receptor complexes on RNA polymerase activity \textit{in vivo} would be the rate of transcription of native chromatin that can be achieved \textit{in vitro} by solubilized enzyme preparations. Usually, chromatin template activity has been studied by using bacterial DNA-dependent RNA polymerases with native chromatin or selectively deproteinized chromatin. Butterworth \textit{et al.} (1971) have shown that bacterial enzymes transcribe mammalian chromatin with differing degrees of efficiency, and bind to and transcribe from sites on the chromatin DNA different from those reported for the mammalian enzyme. In view of these authors’ observations on transcription of liver chromatin by liver RNA polymerases, it was considered necessary to investigate the transcriptional ability of the solubilized prostate enzyme fractions before studying the effects on the systems of the steroid–receptor complexes (Table 1). It can be seen that the nuclear enzyme transcribed prostatic nuclear chromatin with approx. 40–50% of the efficiency with which it transcribed DNA. Nucleolar RNA polymerase transcribed prostatic nuclear and nucleolar chromatin with only 15% and 19% respectively of the efficiency with which it transcribed DNA. The nucleoplasmic enzyme, however, transcribed nuclear chromatin much more efficiently, to a level of 42% of that observed with DNA, but did not transcribe nucleolar chromatin to the same extent. Transcription of chromatin by nucleolar enzyme was

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Template</th>
<th>[¹⁴C]UMP incorporated (pmol/100μg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5mm-MgCl₂–0.03m-KCl</td>
<td>3mm-MnCl₂–0.4m-(NH₄)₂SO₄</td>
</tr>
<tr>
<td>Whole nuclei</td>
<td>Calf thymus DNA</td>
<td>13.21</td>
</tr>
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<td></td>
<td>Nuclear chromatin</td>
<td>5.69 (43)</td>
</tr>
<tr>
<td></td>
<td>Nucleolar chromatin</td>
<td>6.77 (52)</td>
</tr>
<tr>
<td>Nucleoli</td>
<td>Calf thymus DNA</td>
<td>8.86</td>
</tr>
<tr>
<td></td>
<td>Nuclear chromatin</td>
<td>1.39 (16)</td>
</tr>
<tr>
<td></td>
<td>Nucleolar chromatin</td>
<td>1.71 (19)</td>
</tr>
<tr>
<td>Nucleoplasm</td>
<td>Calf thymus DNA</td>
<td>8.29</td>
</tr>
<tr>
<td></td>
<td>Nuclear chromatin</td>
<td>3.56 (43)</td>
</tr>
<tr>
<td></td>
<td>Nucleolar chromatin</td>
<td>0.86 (10)</td>
</tr>
<tr>
<td>E. coli</td>
<td>Calf thymus DNA</td>
<td>36.52</td>
</tr>
<tr>
<td></td>
<td>Nuclear chromatin</td>
<td>4.83 (13)</td>
</tr>
<tr>
<td></td>
<td>Nucleolar chromatin</td>
<td>6.72 (18)</td>
</tr>
</tbody>
</table>

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higher in a Mg\(^{2+}\)-0.03M-KCl environment, whereas
the nucleoplasmic enzyme was more effective in
Mn\(^{2+}\)-0.4M-(NH\(_4\)\(_2\))SO\(_4\), but was also high in the
Mg\(^{2+}\)-low salt environment. Presumably this indi-
cates that much of the DNA is available to the
nucleolar enzyme, probably that portion restricted
to the nucleolar chromatin, whereas the nucleoplasmic
enzyme has access to a greater number of initiation
sites. It could be worthwhile to compare transcription
rates of chromatin by the two enzyme forms under
various degrees of androgen-deprivation.

E. coli RNA polymerase transcribed approx. 13% of
prostatic nuclear chromatin and approx. 18% of
prostatic nucleolar chromatin as compared with that
achieved with purified calf thymus DNA. This shows
that at least one form of the prostatic enzyme is more
efficient than the bacterial enzyme at transcribing
prostate chromatin and is more able to gain access to
those large areas of the genome now reported to be
free of restricting chromosomal proteins (Clark &

Stimulation of nuclear and nucleolar RNA polymerase
activities

Incubation of intact nuclei and nucleoli in the
standard assay system for RNA polymerase to which
had been added cytoplasmic 8S or 3S steroid–receptor
complexes resulted in an increased incorporation of
[\(^{14}\)C]UMP into acid-insoluble material (Table 2). It is
noteworthy that the so-called non-specific binding
proteins in complex with 17β-hydroxy-5α-androstane-
3-one stimulated enzymic activity as well as the specific
binding proteins. This could suggest structural
similarities between the 3S and 8S proteins, or that the
8S is an aggregated form of certain 3S subunits
(Liao & Fang, 1970; Liao et al., 1972). The nuclear
steroid–receptor complex also produced an increase in
incorporation of [\(^{14}\)C]UMP when incubated with
fresh nuclei or nucleoli from 48h-castrated rats.

Stimulation of solubilized RNA polymerase activities

The various steroid–receptor complexes also
increased the activity of RNA polymerase solubilized
from prostatic nuclei (Table 3). Both cytoplasmic
complexes and the nuclear complex stimulated the
activity of the enzyme. In the presence of calf thymus
DNA as template, only a slight increase in the enzyme
activity was observed. The enhancement of activity
was much greater, however, when calf thymus DNA
was replaced by either purified prostatic nuclear or
nucleolar chromatin. The stimulatory effect was not
so marked with the nuclear steroid–receptor complex,
and it is of interest that with enzymes from endo-
metrium nuclei other workers have observed stimula-
tion only with the nuclear complex (Beziat et al.,
1970; Hough et al., 1970; Arnaud et al., 1971).

<table>
<thead>
<tr>
<th>Complex present</th>
<th>3S</th>
<th>Control</th>
<th>Complex</th>
<th>8S</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td>52.41±4.88</td>
<td>111.86±12.46</td>
<td>52.75±2.9</td>
<td>51.88±3.3</td>
<td>76.67±4.5</td>
</tr>
<tr>
<td>Nucleoli</td>
<td>26.14±2.02</td>
<td>39.19±3.33</td>
<td>26.32±1.90</td>
<td>46.61±1.78</td>
<td>26.34±1.92</td>
</tr>
</tbody>
</table>

Table 2. Effect of cytoplasmic and nuclear 17β-hydroxy-5α-androstane-3-one protein receptor complexes on RNA polymerase activity in nuclei and nucleoli.
Table 3. Effect of cytoplasmic and nuclear 17β-hydroxy-5α-androstan-3-one-protein receptor complexes on RNA polymerase solubilized from prostatic nuclei

RNA polymerase solubilized from prostatic nuclei was incubated in the standard assay system containing the steroid-receptor complexes prepared from cytoplasm or nuclei at a final steroid concentration of 0.25 pmol/ml based on radioactivity or in the presence of an equal quantity of 'steroid-free' protein. Incorporation of [3H]UMP is expressed as pmol/100 μg of protein. Values are the means of the results of at least four experiments ± S.D. Percentage increases are the average of the percentage increases observed in each determination ± S.D. The stimulation is measured in the presence of various templates (25 μg of DNA). Full details of the preparation of the various components are given in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Complex present</th>
<th>DNA template</th>
<th>[3H]UMP incorporated (pmol)</th>
<th>Increase in RNA polymerase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8S</td>
<td>3S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Complex</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td></td>
<td>12.25 ± 0.58</td>
<td>14.03 ± 0.84</td>
</tr>
<tr>
<td>Prostatic nuclear chromatin</td>
<td></td>
<td>4.69 ± 0.57</td>
<td>12.33 ± 2.07</td>
</tr>
<tr>
<td>Prostatic nucleolar chromatin</td>
<td></td>
<td>6.43 ± 0.74</td>
<td>14.19 ± 1.92</td>
</tr>
<tr>
<td>Liver chromatin</td>
<td></td>
<td>3.31 ± 0.36</td>
<td>3.48 ± 0.42</td>
</tr>
</tbody>
</table>

The requirement for prostate chromatin to promote maximum stimulation by the steroid-receptor complex was determined by the low stimulation observed with the 8S region of the RNA polymerase from both of these tissues. Labelled steroid-receptor complexes were also studied for their effect on the RNA polymerase activity. These results indicate that the specific binding of the steroid-receptor complex to the RNA polymerase from both of these tissues is 

To emphasize the specific binding of the steroid-receptor complex to the RNA polymerase from both of these tissues. Labelled steroid-receptor complexes were also studied for their effect on the RNA polymerase activity. These results indicate that the specific binding of the steroid-receptor complex to the RNA polymerase from both of these tissues is...
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Table 4. Comparison of the effects of prostatic and splanchnic 17β-hydroxy-5α-androstan-3-one-protein receptor complexes on solidified RNA polymerase activities.

<table>
<thead>
<tr>
<th>Spindle fractions</th>
<th>Prostatic complexes</th>
<th>Splanchnic complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.38 ± 1.35</td>
<td>8.90 ± 0.75</td>
</tr>
<tr>
<td>45S</td>
<td>7.78 ± 0.51</td>
<td>6.41 ± 0.25</td>
</tr>
<tr>
<td>8S</td>
<td>10.04 ± 1.51</td>
<td>10.92 ± 0.85</td>
</tr>
<tr>
<td>4S</td>
<td>3.14 ± 0.52</td>
<td>1.48 ± 0.40</td>
</tr>
</tbody>
</table>

The RNA polymerase was solubilized from both prostatic and splanchnic nuclei. Enzymes were incubated with 250 μg of calf thymus DNA, or prostatic chromatin or spleen chromatin. Some incubations contained 2.5% of either prostatic chromatin or spleen chromatin. Some incubations contained 2.5% of either prostatic chromatin or spleen chromatin. Some incubations contained 2.5% of either prostatic chromatin or spleen chromatin.

a DNA-like RNA respectively (Widnell & Tata, 1966; Hamilton et al., 1968). Both Mg2+ and Mn2+ dependent activities are present in both the nuclear and nucleoplasmic RNA polymerase purified from rat prostate (Table 5).

It has been observed that nucleoplasmic Mn2+ dependent RNA polymerase displays higher activity with denatured rather than native DNA as template. However, inclusion of denatured DNA as template in the assay mixture did not result in any significant stimulation of polymerase activity by steroid-receptor complexes.

Stimulation of nucleolar RNA polymerase by steroid-receptor complexes occurs preferentially in the presence of prostatic chromatin and 5mM-MgCl2, although some stimulation does occur in the presence of 3mM-MnCl2, particularly by the nuclear receptor complex. Although the nucleoplasmic enzyme transcribes prostatic chromatin more efficiently than the nucleolar enzyme, very little stimulation of this enzyme form occurred in the presence of either cation.

In fact, the cytoplasmic 8S complex caused no increase in enzyme activity when Mn2+ was present. It would appear that the stimulation of total nuclear enzyme by these complexes is a reflection of the nucleolar species it contains. However, it is noteworthy that the nuclear complex, with prostatic chromatin as template, stimulated the nucleoplasmic enzyme to some extent in the presence of Mn2+, suggesting that stimulation of DNA-like RNA synthesis can occur in vivo.

Other authors have shown that hormone-stimulated RNA synthesis is of a ribosomal type, which is synthesized in the nucleolus (Liao & Lin, 1967; Jacob et al., 1969; Raynaud-Jammet et al., 1971; Mainwaring et al., 1971). The results of our study have now shown an increase of RNA polymerase activity brought about by a steroid-receptor complex in intact nucleoli and also specifically in the purified nucleolar form. Stimulation also occurs in the presence of nucleolar chromatin. It is noteworthy that the enzyme form that transcribes native chromatin with the least efficiency is preferentially stimulated.

It is tempting to relate this to a situation in vivo. The absence of stimulation in high-salt conditions, observed by us and others (Baulieu et al., 1972), could be due to removal of some important protein factor from chromatin at high ionic strength. The stimulation caused by the nuclear complex under these conditions might suggest that the complex contains this factor. There are therefore many possibilities, which require further clarification.

Stimulation of RNA polymerase activity in the presence of altered templates

Further insight into the tissue specificity of this system in vitro was provided by the effects of the
Table 5. Effect of cytoplasmic (8 S) and nuclear (4.5 S) 17β-hydroxy-5α-androstan-3-one-protein receptor complexes on prostatic nucleolar and nucleoplasmic RNA polymerases.

Nucleolar and nucleoplasmic RNA polymerases solubilized from the respective subnuclear fractions were incubated with various templates and the steroid-receptor complexes (0.25 pmol of 17β-hydroxy-5α-androstan-3-one/ml) in assay mixtures containing MgCl₂ or MnCl₂ in high or low ionic strength, respectively. Values are the means of four or more experiments ± s.d. Percentage increases are the average of those increases observed in each determination ± s.d.

<table>
<thead>
<tr>
<th>Conditions of assay</th>
<th>Complex present</th>
<th>Template</th>
<th>Prostate nuclear chromatin</th>
<th>Liver chromatin</th>
<th>Prostate nuclear chromatin</th>
<th>Liver chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺-low salt</td>
<td>Control</td>
<td>Calf thymus DNA</td>
<td>10.89 ± 0.38</td>
<td>9.48 ± 0.46</td>
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<td>3.57 ± 0.31</td>
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<tr>
<td></td>
<td>Complex</td>
<td>Prostate nuclear chromatin</td>
<td>13.18 ± 0.63</td>
<td>12.16 ± 0.78</td>
<td>4.23 ± 0.79</td>
<td>1.76 ± 0.15</td>
</tr>
<tr>
<td>Mg²⁺-high salt</td>
<td>Control</td>
<td>Calf thymus DNA</td>
<td>10.66 ± 0.45</td>
<td>9.49 ± 0.49</td>
<td>3.70 ± 0.34</td>
<td>3.48 ± 0.64</td>
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<td></td>
<td>Complex</td>
<td>Prostate nuclear chromatin</td>
<td>13.48 ± 0.63</td>
<td>12.16 ± 0.78</td>
<td>4.23 ± 0.79</td>
<td>1.76 ± 0.15</td>
</tr>
<tr>
<td>Mn²⁺-low salt</td>
<td>Control</td>
<td>Calf thymus DNA</td>
<td>7.67 ± 0.46</td>
<td>7.82 ± 0.52</td>
<td>3.48 ± 0.64</td>
<td>3.57 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>Complex</td>
<td>Prostate nuclear chromatin</td>
<td>10.44 ± 1.35</td>
<td>9.49 ± 0.49</td>
<td>3.70 ± 0.34</td>
<td>3.48 ± 0.64</td>
</tr>
<tr>
<td>Mn²⁺-high salt</td>
<td>Control</td>
<td>Calf thymus DNA</td>
<td>7.67 ± 0.46</td>
<td>7.82 ± 0.52</td>
<td>3.48 ± 0.64</td>
<td>3.57 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>Complex</td>
<td>Prostate nuclear chromatin</td>
<td>10.44 ± 1.35</td>
<td>9.49 ± 0.49</td>
<td>3.70 ± 0.34</td>
<td>3.48 ± 0.64</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>[14C]UMP incorporated (pmol) Increase in activity (%)</th>
<th>Complex present</th>
<th>Template</th>
<th>Prostate nuclear chromatin</th>
<th>Liver chromatin</th>
<th>Prostate nuclear chromatin</th>
<th>Liver chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Calf thymus DNA</td>
<td>2.16 ± 0.42</td>
<td>1.76 ± 0.15</td>
<td>3.70 ± 0.34</td>
<td>3.48 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>Complex</td>
<td>Prostate nuclear chromatin</td>
<td>1.94 ± 0.15</td>
<td>1.71 ± 0.17</td>
<td>2.65 ± 0.46</td>
<td>2.65 ± 0.46</td>
</tr>
<tr>
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<td>Control</td>
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<td>2.41 ± 0.42</td>
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<td>3.48 ± 0.64</td>
</tr>
</tbody>
</table>
RNA polymerase activity in vitro

Table 6. Effect of cytoplasmic and nuclear 17β-hydroxy-5α-androstan-3-one–protein receptor complexes on nuclear RNA polymerase in the presence of selectively altered templates

RNA polymerase solubilized from prostatic nuclei was incubated in an assay medium containing steroid–receptor complexes (0.25 pmol of 17β-[1α,2α-3H]hydroxy-5α-androstan-3-one/ml). The template for RNA synthesis varied in composition: calf-thymus DNA, liver chromatin, prostatic chromatin intact, deficient of histones, deficient of histone and acidic proteins, and prostatic DNA. Full details are given in the Materials and Methods section. The enzyme activity in the presence of the complexes or an equal quantity of steroid-deficient receptor is expressed as pmol of [14C]UMP incorporated/μg of template DNA and the percentage increase in activity in the presence of steroid–receptor complex is specified in each case.

<table>
<thead>
<tr>
<th>[14C]UMP incorporated (pmol)</th>
<th>Control fractions</th>
<th>Increase in incorporation of [14C]UMP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex present</td>
<td>8 S</td>
<td>3 S</td>
</tr>
<tr>
<td>Template</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>1.12</td>
<td>1.22</td>
</tr>
<tr>
<td>Liver chromatin</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>Prostatic nuclear chromatin</td>
<td>0.46</td>
<td>0.56</td>
</tr>
<tr>
<td>Histone-deficient prostatic chromatin</td>
<td>0.63</td>
<td>0.72</td>
</tr>
<tr>
<td>Histone-deficient and non-histone-deficient prostatic chromatin</td>
<td>0.71</td>
<td>0.76</td>
</tr>
<tr>
<td>Prostatic DNA</td>
<td>0.89</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Cytoplasmic and nuclear steroid–receptor complexes on the nuclear enzyme in the presence of selectively altered templates (Table 6). The usual slight stimulatory effect with calf thymus DNA was noted but, as previously shown, the presence of prostatic nuclear chromatin as template produced the larger increase in enzymic activity. Removal of histone proteins from prostatic chromatin produced an increase of 17% in the rate of transcription, but did not alter the stimulatory effect. Removal of a large proportion of non-histone protein increased the rate of transcription by a further 25%, but again did not significantly alter the extent of stimulation. At this stage, 9–10% of the chromatin-associated protein remained. When protein-free, phenol-isolated prostatic DNA was introduced as template into this system in vitro, the template activity was increased but the percentage stimulation caused by the complexes fell to those low levels achieved with either calf thymus DNA or liver chromatin used as template.

The role of the chromatin-associated proteins is obviously of considerable interest. Steroid–receptor complexes are bound to chromatin DNA, under the control of these non-histone proteins (Mainwaring & Peterken, 1971), and they would now appear to be essential for stimulation of RNA polymerase by steroid–receptor complexes. Histones affect the template capacity, but not the stimulation of RNA polymerase activity, possibly by retaining a supercoiled structure as suggested by Shih & Bonner (1970), since they fail to produce an organ specificity for chromatin (Paul & Gilmour, 1968). This property is a feature of the non-histone proteins (Gilmour & Paul, 1970; Spelsberg & Hnilica, 1970), although the major portion of the non-histone fraction has little tissue specificity (Elgin & Bonner, 1970; MacGillivray et al., 1971). These properties are limited to certain proteins which are tightly bound to DNA (Teng et al., 1970) and which probably remain after chromatin dissociation in NaCl–urea at pH 8.3 (Mainwaring & Peterken, 1971). This would explain the lack of effect on the receptor complex stimulation of RNA polymerase, of removing most of the non-histone proteins from chromatin, and the major effect of the subsequent removal of the remaining small portion.

The exact mechanism of action of the steroid hormone, however, remains a problem of which chromatin-specificity is a part. It is evident that the ionic conditions for enzyme assay and the source of enzyme impose limits upon the extent of RNA polymerase stimulation, and probably further controlling factors which exist in vivo should be introduced into the system.

The authors are grateful to the Tenovus Organisation for their generous financial support.
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1975
HORMONAL EFFECTS IN VITRO ON RIBONUCLEIC ACID POLYMERASE IN NUCLEI ISOLATED FROM HUMAN PROSTATIC TISSUE

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(Received 14 May 1973)

Various metabolites of testosterone, compounds such as 5α-dihydrotestosterone and the 5α-androstanediols, appear to have specific roles in eliciting certain of the androgenic responses (Baulieu, Lasnitzki & Robel, 1968a, b). Furthermore, some of these compounds can stimulate in vitro the activity of DNA-dependent RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) of nuclei prepared from rat and dog prostatic tissue (Davies, Fahmy, Pierrepoint & Griffiths, 1972). To investigate this matter further, the effects of a series of testosterone metabolites on the RNA polymerase activity isolated from specimens of tissue from patients with benign prostatic hypertrophy (bph) were studied, and the inhibitory effects of various stilboestrol derivatives were also investigated.

Human bph specimens, obtained immediately after prostatectomy, were homogenized using a Latapie press in combination with a Potter–Elvehjem homogenizer. Nuclei were purified from homogenates by centrifugation through discontinuous gradients of hypertonic sucrose (0.8–1.8–2.1 mol/l) and were judged free of cytoplasmic fragments by biochemical and microscopic criteria (Widnell & Tata, 1964). The RNA polymerase activity of intact nuclei was measured in a medium essentially similar to that previously described (Davies et al. 1972), except [U-14C]UTP (sp. act. 514 mCi/mm) was used as labelled precursor. Steroids or stilboestrol analogues were added to the incubation mixture in cytosol (105,000 g supernatant) to give a final concentration of 4 μmol/l. Cytosol alone was added to control incubations. Incubations (15 min) were terminated by the addition of 10% trichloroacetic acid in 1 mm-Na₂HPO₄ and acid-insoluble precipitates were prepared for measurement of incorporated radioactivity as described by Davies et al. (1972).

The effects of the various compounds on the activity of RNA polymerase are shown in Table 1. The greatest degree of stimulation was produced by 5α-dihydrotestosterone. Significant stimulation was produced by 5α-androstane-3β,17β-diol and, to a lesser degree, by 5α-androstane-3α,17β-diol and by testosterone. These effects, taken in consideration with other observations in prostatic tissue from differing sources (Baulieu et al. 1968a, b; Farnsworth, 1970; Harper, Fahmy, Pierrepoint & Griffiths, 1970; Davies et al. 1972), infer a principal androgenic role for 5α-dihydrotestosterone within a pattern of metabolites, the maintenance of which is necessary for a controlled androgenic response.
The inhibitory effects of the stilboestrol derivatives are also noteworthy. Diethylstilboestrol and meso-dibutyldihydrostilboestrol effectively inhibited RNA polymerase activity, while (±)-dibutyldihydrostilboestrol showed negligible inhibition. Preliminary studies on the properties of I.C.I. 85966, a nitrogen-mustard derivative of stilboestrol (3,4-di[p-[(N-his-2-chloroethyl)carbamoyl]phenyl]hex-3-ene) showed varying degrees of inhibition of the human bph RNA polymerase.

Table 1. Effect of incubation of purified nuclei isolated from human benign prostatic hypertrophy tissue with various steroid and stilboestrol derivatives on the RNA polymerase activity

<table>
<thead>
<tr>
<th>Compound (μmol/l) added in cytosol to assay system</th>
<th>Activation or inhibition of RNA polymerase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>(a) (b) (c) (d) (e) (f) (g) (h)</td>
</tr>
<tr>
<td>0-3</td>
<td>3-7 13-2 6-8 63 9-2 7-7 9-2 4-0 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>3a-Hydroxy-5a-androstan-1-4-androst-4-ene-3,17-dione</td>
<td>75-3 33-3 41-6 27-5 29-8 29-1 24-1 28-5 ( P &lt; 0.001 )</td>
</tr>
<tr>
<td>5a-Androstane-3a,17a-diol</td>
<td>1-9 3-0 0-9* 2-0 1-7 0-2* 24 0-4* ( P &gt; 0.10 )</td>
</tr>
<tr>
<td>5a-Androstane-3a,17β-diol</td>
<td>2-9 6-6 2-6 3-4 5-5 2-3 1-3* 1-7 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>5a-Androstane-3β,17-dione</td>
<td>3-1* 0-2* 0-8* 0-8 0-6 0-4* 0-6* 0-6* ( P &gt; 0.10 )</td>
</tr>
<tr>
<td>5a-Androstane-3a,17β-diol</td>
<td>4-1 21-7 31-7 16-0 16-0 16-0 14-4 8-6 ( P &lt; 0.001 )</td>
</tr>
<tr>
<td>Androst-5-ene-3a,17β-diol</td>
<td>0-2 0-8* 4-8* 0-9 34 1-6* 1-6* 1-9* ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Androst-4-ene-3,17-dione</td>
<td>1-4* 0-3 0-9* 0-5* 1-9* 0-7* 0-2* 1-1 ( P &gt; 0.10 )</td>
</tr>
<tr>
<td>3β-Hydroxy-5α-androstane-17-one</td>
<td>0-3* 5* 2-4* 0 0 1-2 1-4 10 2-2* ( P &gt; 0.10 )</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0 0-4* 1-3* 0-7 0-5 1-5* 0-2 0-1 ( P &gt; 0.10 )</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0-8* 2-6* 0-2* 0-8 0-6 0-6* 0-4* 1-0 ( P &gt; 0.10 )</td>
</tr>
<tr>
<td>5α-Dihydroepiandrosterone</td>
<td>0 0-3 0-9* 2-3* 0 0-6 1-6* 1-3 ( P &gt; 0.01 )</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>37-1* 7-0* 14-0* 5-4* 58 11-7* 16-4* 7-8* ( P &gt; 0.01 )</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>45-2* 22-0* 29-0* 25-1* 23-1* 28-1* 23-8* 17-2* ( P &lt; 0.001 )</td>
</tr>
<tr>
<td>(±)-Dibutyldihydrostilboestrol</td>
<td>0-6 2-0 1-2* 0-7* 0-3 2-3* 0-2* 1-2* ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>meso-Dibutyldihydrostilboestrol</td>
<td>58-8* 25-2* 32-3* 24-5* 30-6* 30-4* 30-6* 19-6* ( P &lt; 0.001 )</td>
</tr>
<tr>
<td>I.C.I. 85966</td>
<td>43-8* 22-1* 20-7* 23-9* 25-8* 41-3* 20-7* 22-4* ( P &lt; 0.001 )</td>
</tr>
</tbody>
</table>

Nuclear preparations (50-100 μg DNA) were incubated for 15 min at 37°C in assay medium containing various substances (added in 50 μl cytosol) at a final concentration of 4 μmol/l. Duplicate assays were carried out. The incorporation of [14C]UMP into acid-insoluble material was determined, expressed as d.p.m./15 min/mg DNA, and calculated as pmol [14C]UMP incorporated/15 min/mg DNA (range of incorporation in eight preparations was 340±9±1 (s.e.m.) pmol/mg DNA). Increases and decreases in incorporation are expressed as % of the value obtained in the absence of added compounds. A two-way analysis of variance with replication (due to measurement of RNA polymerase per nuclear preparation) was performed on the control with each test group. The variance ratio and degrees of freedom were calculated for test groupings, prostates and interaction (Armitage, 1971). P values were calculated for each test substance v. controls. Letters refer to separate enzyme preparations.

* Inhibition; no asterisk, stimulation.

The authors are grateful to the Tenovus Organisation for their generous financial support, and would also like to thank Dr A. L. Walpole and Dr D. N. Richardson, I.C.I., Alderley Edge, for their co-operation in preparing the dibutyldihydrostilboestrol derivatives and also I.C.I. 85966.

REFERENCES


Effects of α-Amanitin on the Stimulation of Prostatic Ribonucleic Acid Polymerase by Prostatic Steroid–Protein Receptor Complexes

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(Received 22 March 1974)

Stimulation of prostatic RNA polymerase in vitro by prostatic 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone)–receptor complexes has been previously reported. By use of the selective inhibitor, α-amanitin, we have shown that both nucleolar and extranucleolar RNA polymerase activities may be stimulated, but stimulation is abolished at high ionic strength.

The stimulation of prostatic DNA-dependent RNA polymerase (nucleoside triphosphate–RNA nucleotidyltransferase, EC 2.7.7.6) by androgens has been well documented (Liao et al., 1965; Bashirelahi et al., 1969; Davies et al., 1972). The retention and relocation in hormone-responsive tissues, not only of androgens but of other steroid hormones, has been shown to depend on their binding to cytoplasmic protein receptor molecules (Mainwaring, 1970; Fang & Liao, 1971), and the transfer of the complex to the nucleus (Fang & Liao, 1971; O’Malley et al., 1972). A direct stimulation of prostatic RNA polymerase by prostatic dihydrotestosterone (17β-hydroxy-5α-androstan-3-one)–receptor complexes has been shown, that is dependent on the presence of a specific prostatic chromatin template (Davies & Griffiths, 1973a,b). The requirement for a specific template correlates with the association of steroid–receptor complexes specifically with chromatin from target tissues (Mainwaring & Peterken, 1971; Steggles et al., 1971). Further studies were done on the stimulation of prostatic RNA polymerase by prostatic steroid–receptor complexes, to elucidate the exact nature of the stimulated enzyme and of the type of RNA produced as a result of the stimulation. This involved the evaluation of the effects of ionic strength on the system and the use of the toxin α-amanitin. This substance has no effect on nucleolar RNA polymerase in vitro, but inhibits the activity of nucleoplasmic RNA polymerase (Stirpe & Fiume, 1967; Novello & Stirpe, 1969; Jacob et al., 1970a,b; Kedinguer et al., 1970; Lindell et al., 1970). Such studies are of value in elucidating the role of hormonal steroids in the modification of gene expression, and of the mechanism by which steroids instigate the chain of biochemical events in cells of hormone-responsive tissues.

Materials and methods

All preparative procedures were done on ventral prostatic tissue excised from 8–12-week-old male Sprague–Dawley rats 48 h after bilateral castration. Methods of purification of all necessary subcellular components and procedures for measuring the incorporation of labelled precursor into RNA have been described by Davies & Griffiths (1973a,b). Modifications and details of concentrations of (NH₄)₂SO₄ for individual experiments are given in the text. These solutions were adjusted to pH 8.1 with aq. NH₃ solution before addition to enzyme assays. When added, α-amanitin (Calbiochem Ltd., London W.1, U.K.) was in 120mM-Tris–HCl buffer, pH 8.1. Incubation mixtures were adjusted to constant volume with this buffer. The [³H]5α-dihydrotestosterone–receptor complexes were added to assay mixtures at a final radioactive 5α-dihydrotestosterone concentration of 250nmol/ml. Additional control assays containing 5α-dihydrotestosterone-free receptor proteins (Davies & Griffiths, 1973a,b) did not show an appreciably different [¹⁴C]UMP incorporation from the basic enzyme systems.

DNA content of subcellular preparations was determined by the method of Burton (1956) with calf thymus DNA as standard. Protein was measured by the method of Lowry et al. (1951) with crystalline bovine serum albumin (Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.) as standard.

Results and discussion

Preliminary investigations showed that maximum inhibition of soluble whole nuclear RNA polymerase was achieved with 60–80ng of α-amanitin in assays containing up to 50μg of enzyme protein. Dose response was similar in assays with both Mg²⁺–low-salt medium (25–35% inhibition) and Mn²⁺–high-salt medium (70–90% inhibition). Similarly, maximum inhibition of RNA polymerase in intact nuclei and in nucleoplasmic fragments was obtained with the use of those amounts of toxin/25–30μg of DNA.
Table 1. Effect of ionic strength and α-amanitin on stimulation of nuclear RNA polymerase by [3H]5α-dihydrotestosterone–receptor complexes

The activity of RNA polymerase in intact rat ventral-prostatic nuclei or in preparations solubilized from such nuclei prepared from rats castrated 48 h previously was measured in standard assay mixtures with and without various substances. Cytoplasmic (8S) and nuclear (4.5S) [3H]5α-dihydrotestosterone–receptor complexes were added to certain assays at a final [3H]steroid concentration of 0.25 pmol/ml. α-Amanitin was added to some assays in quantities of 60 ng assay. RNA polymerase activity of nuclei is expressed as pmol of [3H]UMP incorporated/100 µg of DNA and that of soluble preparations as pmol of [3H]UMP incorporated/100 µg of enzyme protein. Values are the means ± s.d. of at least quadruplicate determinations. P values for stimulation of activity by [3H]steroid–receptor complexes versus controls: * P < 0.001; † P > 0.1. P values for stimulation of α-amanitin-sensitive activity, as calculated from relative inhibition of activity by α-amanitin versus inhibition of controls: ‡ P > 0.1; § P < 0.1; || P < 0.01.

<table>
<thead>
<tr>
<th>Conditions of assay</th>
<th>Control</th>
<th>Control + α-amanitin</th>
<th>8S complex</th>
<th>8S complex + α-amanitin</th>
<th>4.5S complex</th>
<th>4.5S complex + α-amanitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Intact nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺ (5 mmol/litre), no (NH₄)₂SO₄</td>
<td>3.60 ± 0.34</td>
<td>2.47 ± 0.28</td>
<td>*7.22 ± 0.62</td>
<td>†5.78 ± 0.76</td>
<td>*5.18 ± 0.74</td>
<td>‡4.54 ± 0.58</td>
</tr>
<tr>
<td>Mn²⁺ (3 mmol/litre), (NH₄)₂SO₄ (0.4 mol/litre)</td>
<td>16.72 ± 0.92</td>
<td>1.63 ± 0.92</td>
<td>†15.78 ± 3.15</td>
<td>1.35 ± 0.47</td>
<td>†18.27 ± 1.42</td>
<td>1.60 ± 0.14</td>
</tr>
<tr>
<td>Mg²⁺ (5 mmol/litre), Mn²⁺ (3 mmol/litre), (NH₄)₂SO₄ (0.04 mol/litre)</td>
<td>9.23 ± 0.09</td>
<td>4.45 ± 0.63</td>
<td>†15.09 ± 0.94</td>
<td>9.80 ± 0.27</td>
<td>*16.37 ± 0.38</td>
<td>§9.98 ± 1.12</td>
</tr>
<tr>
<td>(2) Solubilized nuclear RNA polymerase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺ (5 mmol/litre), Mn²⁺ (3 mmol/litre), (NH₄)₂SO₄ (0.04 mol/litre)</td>
<td>1.43 ± 0.21</td>
<td>0.40 ± 0.08</td>
<td>*4.66 ± 0.21</td>
<td>0.80 ± 0.21</td>
<td>*4.70 ± 0.34</td>
<td>‡0.65 ± 0.12</td>
</tr>
</tbody>
</table>

Table 2. Effects of α-amanitin and 5α-dihydrotestosterone–receptor complexes on nucleoplasmic RNA polymerase activity

Nucleoplasm was prepared by sonication from ventral-prostatic nuclei of rats castrated 48 h previously. RNA polymerase activity of nucleoplasmic material and preparations solubilized from such material was measured in assay media containing Mn²⁺ (3 mmol/litre) and (NH₄)₂SO₄ (0.04 mol/litre). Cytoplasmic (8S) and nuclear (4.5S) [3H]5α-dihydrotestosterone–receptor complexes were added to certain assays at a final [3H]steroid concentration of 0.25 pmol/ml. α-Amanitin was added in quantities of 60 ng assay. RNA polymerase activity of nucleoplasmic material was expressed as pmol of [3H]UMP incorporated/100 µg of DNA and that of solubilized nucleoplasmic enzyme as pmol of [3H]UMP incorporated/100 µg of protein. Values are the means ± s.d. of at least quadruplicate determinations. P values for stimulation of activity by [3H]steroid–receptor complexes versus controls: * P < 0.001. P values for stimulation of α-amanitin-sensitive activity, as calculated from relative inhibition of activity by α-amanitin versus inhibition of controls: † P < 0.001; ‡ P < 0.01.

<table>
<thead>
<tr>
<th>RNA polymerase activity (pmol of [14C]UMP incorporated)</th>
<th>Control</th>
<th>Control + α-amanitin</th>
<th>8S complex</th>
<th>8S complex + α-amanitin</th>
<th>4.5S complex</th>
<th>4.5S complex + α-amanitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoplasmic fragments</td>
<td>5.81 ± 0.09</td>
<td>1.16 ± 0.16</td>
<td>*10.57 ± 0.62</td>
<td>†12.87 ± 0.50</td>
<td>*10.56 ± 0.58</td>
<td>‡4.15 ± 1.19</td>
</tr>
<tr>
<td>Solubilized nucleoplasm RNA polymerase</td>
<td>2.92 ± 0.10</td>
<td>1.00 ± 0.56</td>
<td>*7.40 ± 0.24</td>
<td>†2.75 ± 0.41</td>
<td>*7.29 ± 0.63</td>
<td>‡1.65 ± 0.49</td>
</tr>
</tbody>
</table>
Stimulation of RNA polymerase in intact nuclei occurred when cytoplasmic (8S) or nuclear (4.5S) 5α-dihydrotestosterone–receptor complexes were included in a low-salt medium containing Mg²⁺ (5mmol) (Table 1). This apparently was stimulation of nucleolar RNA polymerase, since it was not sensitive to α-amanitin. In a high-salt [(NH₄)₂SO₄; 400mmol/litre] medium containing Mn²⁺ (3mmol/litre), a considerable increase in α-amanitin-sensitive activity was observed. This probably represented salt-stimulation of nucleoplasmic RNA polymerase, but inclusion of receptor complexes in the assay caused no further stimulation. However, when nuclei were incubated in a medium containing (NH₄)₂SO₄ (40mmol/litre), a condition promoting Mn²⁺-dependent activity but not inhibiting Mg²⁺-dependent activity, enzyme stimulation by 5α-dihydrotestosterone–receptor complexes was observed. The stimulated activity appeared to be partly α-amanitin-sensitive and partly insensitive. Further studies were carried out with solubilized nuclear enzyme (Davies & Griffiths, 1973b) in an attempt to resolve the nature of the stimulated enzyme. Incubations were carried out in a medium containing (NH₄)₂SO₄ (40mmol/litre), with chromatin as template. The enzyme was stimulated to a great extent by 5α-dihydrotestosterone–receptor complexes, and most of the activity was α-amanitin-sensitive. This suggests that, under favourable ionic conditions, steroid–receptor complexes stimulate both nucleolar and extranucleolar RNA polymerase activity. To demonstrate this further, nucleoplasmic fragments and solubilized nucleoplasmic enzyme were incubated with steroid–receptor complexes in a medium containing (NH₄)₂SO₄ (40mmol/litre), with chromatin from prostatic nuclei as template. Again part of the stimulated RNA polymerase activity was α-amanitin-sensitive (Table 2). Apparently, stimulation of RNA polymerase activity by 5α-dihydrotestosterone–receptor complexes is inhibited under conditions of high ionic strength. This cannot be due entirely to inhibition of nucleolar enzyme (Raynaud-Jammet et al., 1971), since the activity of nucleoplasmic RNA polymerase, which was increased by high salt, was not stimulated by addition of the complexes. Any such stimulation caused by increased template activity would be countered, however, by the removal of chromatin-associated proteins at high salt concentrations. Further, binding of more polymerase molecules to initiation sites could be inhibited at high ionic strength or alternatively the high salt concentration may interfere with the essential interaction between the steroid–protein complex and chromatin.

We are grateful to the Tenovus Organization for their generous financial support.

FURTHER STUDIES ON THE STIMULATION OF PROSTATIC RIBONUCLEIC ACID POLYMERASE BY 5α-DIHYDROTESTOSTERONE–RECEPTOR COMPLEXES

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(Received 10 January 1974)

SUMMARY

The stimulation in vitro of prostatic RNA polymerase activity by prostatic 5α-dihydrotestosterone–receptor complexes has been previously reported. Further investigations into the nature of the stimulation have now been carried out. By use of the selective inhibitor, α-amanitin, and by varying the concentration of ammonium sulphate in the assay media, both the nucleolar and extranucleolar forms of RNA polymerase could be stimulated, depending upon the ionic conditions employed. High ionic strength inhibited stimulation, either by interference with the association between steroid-receptor complexes and chromatin components, or by blocking the conversion of cytoplasmic complexes to a more ‘active’ form of the complex. 5α-Dihydrotestosterone–receptor complexes appeared to affect the template availability of prostatic chromatin, possibly in a way similar to that of the chromatin-associated proteins.

INTRODUCTION

An essential primary step in the expression of the androgenic stimulus in the ventral prostate gland of the rat is the formation of complexes between 5α-dihydrotestosterone (5α-DHT; 17β-hydroxy-5α-androstan-3-one), a metabolite of testosterone, and receptor proteins with a selective high affinity for this steroid (Mainwaring, 1969a, b; Fang, Anderson & Liao, 1969). The 5α-dihydrotestosterone–protein receptor complex thus formed undergoes translocation to the nucleus in a specific manner (Fang & Liao, 1971) and associates with prostatic chromatin (Mainwaring & Peterken, 1971). The close association of a steroid–receptor complex with chromosomal sites in the nuclei of hormone-responsive tissues also is a feature in tissues other than those which respond to androgens (Spelsberg, Steggle & O’Malley, 1971; Steggle, Spelsberg & O’Malley, 1971; O’Malley, Toft & Sherman, 1971; O’Malley, Spelsberg, Schrader, Chytil & Steggle, 1972; Higgins, Rousseau, Baxter & Tomkins, 1973), the specificity of the association residing in the tissue chromatin (Steggle, Spelsberg, Glasser & O’Malley, 1971). This association, together with the early response of RNA synthesis to hormonal stimulation, would suggest that the steroid–receptor complex plays an integral role in the regulation of genome transcription in the target cell.
Preliminary studies (Davies, Fahmy, Pierrepoint & Griffiths, 1972) showed that certain androgenic steroids could stimulate, in vitro, the activity of DNA-dependent RNA polymerase (nucleosidetriphosphate: RNA nucleotidyltransferase: EC 2.7.7.6) in purified prostatic nuclei of rats. More recent work using a reconstituted system in vitro (Davies & Griffiths, 1973a, b), has shown that the stimulation of prostatic solubilized RNA polymerase by 5α-dihydrotestosterone–receptor complexes is dependent upon the presence of an intact native prostatic chromatin template. Further investigations have now been carried out on the nature of this increase in RNA polymerase activity and the 5α-DHT–receptor complexes involved.

MATERIALS AND METHODS

Materials

Animals

Male Sprague–Dawley rats (8–12 weeks old) were obtained from Charles River Ltd, Margate, Kent, U.K. Animals were castrated by the scrotal route under ether anaesthesia. Ventral prostatic tissue was removed from normal intact animals, or from rats 24 or 48 h after bilateral orchidectomy, as described in the text.

Chemicals

[1α,2α-3H]5α-Dihydrotestosterone (specific radioactivity, 47 Ci/mmol) and [U-14C]-UTP (specific radioactivity, 475–514 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. Dithiothreitol and 5α-DHT were purchased from Koch-Light Laboratories, Colnbrook, Bucks. Bovine serum albumin was a product of Sigma (London) Chemical Co. Ltd, Kingston-upon-Thames, Surrey. Tris base (Aristar grade), yeast RNA, calf thymus DNA and calf thymus histone (Type II-A) were obtained from B.D.H. Ltd, Poole, Dorset. α-Amanitin was purchased from Calbiochem (U.K.), London. Sephadex G-25 was supplied by Pharmacia (U.K.) Ltd, London.

Methods

Preparation of subcellular fractions

All preparative procedures, unless otherwise indicated, were carried out at 0–4 °C.

Nuclei, nucleoli and ‘nucleoplasm’

The preparation of nuclei and subnuclear fractions from rat ventral prostate glands was carried out as previously described (Davies & Griffiths, 1973b). The term ‘nucleoplasm’ refers to nuclear material remaining after removal of the nucleoli. Approximately 65% of homogenate DNA was recovered in the form of purified nuclei. Nucleoli and nucleoplasm contained 12–17 and 70–78% of nuclear DNA respectively. The mean protein:DNA ratios of nuclei, nucleoli and nucleoplasm from normal animals were 7·2 ± 0·3, 10·7 ± 0·5 and 5·6 ± 0·6, whereas from 48 h castrated rats the values were 5·6 ± 0·7, 8·2 ± 1·3 and 4·4 ± 0·4.

Chromatin

The preparation of chromatin from rat prostatic nuclei and nucleoli by means of various saline extractions has been described (Davies & Griffiths, 1973b). Extraction
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of chromatin with NaCl solution (0-35 mol/l) effectively removed all endogenous RNA polymerase activity. Preliminary characterization of chromatin preparations as regards DNA, RNA, histone and non-histone protein content was carried out by the methods of Marushige & Bonner (1966) and details of the chemical composition of chromatin are included in the Results section. Selective removal of chromatin-associated proteins was carried out as previously described (Davies & Griffiths, 1973b). Selective reconstitution of chromatin was achieved by the stepwise dialysis procedure described by Gilmour & Paul (1969).

Prostatic DNA was prepared by the phenol-deoxycholate method of Colter, Brown & Ellem (1962).

Labelling in vitro of receptor proteins with [3H]5α-dihydrotestosterone

The preparation of cytosol fractions (105 000 g supernatant) in Tris–HCl buffer (50 mmol/l), pH 7-4, containing EDTA (0-1 mmol/l) and dithiothreitol (0-25 mmol/l) (medium A) and labelling of these fractions with [3H]5α-DHT has been described (Davies & Griffiths, 1973b). Partial purification of receptor complexes was achieved by selective precipitation by the dropwise addition of 0-5 vol. saturated ammonium sulphate solution. Labelled nuclear fractions were prepared by the incubation of purified nuclei (100–150 µg DNA in 0-25 M-sucrose–1 mM-MgCl₂ solution) with equal volumes of labelled cytosol fractions and extraction of the sedimented nuclei with KCl (0-4 mol/l). Desalting of 5α-DHT–receptor complexes and removal of unbound 3H-labelled steroid was achieved by passage through small columns of Sephadex G-25 equilibrated with medium A.

Full details of these methods and of the preliminary analysis of labelled fractions on sucrose density gradients have been given (Davies & Griffiths, 1973b).

Incorporation of prostatic [3H]5α-dihydrotestosterone–receptor complexes into chromatin and DNA

Formation of complexes between [3H]5α-DHT receptors and chromatin or purified DNA preparations was performed according to the method of Mainwaring & Peterken (1971). Incorporation occurred at 0 °C but was accelerated at 37 °C.

Solubilization of RNA polymerase activity

Details of this method have been previously described (Davies & Griffiths, 1973b). Preparations solubilized from whole prostatic nuclei contained both Mg²⁺- and Mn²⁺-dependent activities. The enzyme showed an absolute requirement for exogenous DNA template, and the activity was dependent upon the presence of all four nucleoside triphosphates and yielded a ribonuclease-sensitive product.

Measurement of RNA polymerase activity

RNA polymerase activities were measured in Mg²⁺-low salt and Mn²⁺-high salt media, measurements being based on incorporation of [14C]UMP into acid-insoluble material (Davies & Griffiths, 1973a, b). Modifications of these media and the concentration of ammonium sulphate solutions to fit the requirements of individual experiments are described in detail in the text. Ammonium sulphate solutions were adjusted to pH 8-1 with aqueous ammonia before addition to assay media. When added, α-amanitin was in Tris–HCl buffer (120 mmol/l), pH 8-1. Incubation mixtures
were adjusted to constant volume with this buffer. Nuclear and nucleoplasmic fractions were usually added to assay media in quantities of 50–100 µg DNA, and the solubilized enzyme, unless otherwise stated, in quantities of about 60 µg protein. The \[^{3}H\]5α-DHT–protein complexes were added to assay mixtures at a final radioactive 5α-DHT concentration of 250 pmol/l. Additional control assays containing equal concentrations of free \(^{3}H\)-labelled steroid or equal quantities of '5α-DHT-free' receptor proteins did not show an appreciably different \(^{14}C\)UMP incorporation into polynucleotide linkage to that observed in the basic enzyme systems.

**Estimation of template availability**

The availability of template to prostatic RNA polymerase was determined in terms of the amounts of enzyme required to achieve maximum velocity of the enzyme reaction in the presence of a standard amount of template DNA. Using the usual enzyme assay medium containing MgCl₂ (5 mmol/l) and MnCl₂ (3 mmol/l) with ammonium sulphate (0.04 mol/l), varying amounts of nuclear RNA polymerase (10–60 µg enzyme-protein) were incubated with the different template preparations under study at a standard concentration of 25 µg DNA per assay. All other facets of the procedure were as for the standard enzyme activity estimation.

**Chemical analyses**

DNA content of preparations was determined by the diphenylamine procedure of Burton (1956) as modified by Giles & Myers (1965), using calf thymus DNA as standard. RNA content was determined by the orcinol method as described by Schneider (1957) using yeast RNA as standard. Protein estimations, including determination of chromatin-associated non-histone proteins, were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine serum albumin as standard. Histone proteins were also determined by the method of Lowry et al. (1951) using calf thymus histone as standard.

**RESULTS**

**Characterization of \[^{3}H\]5α-dihydrotestosterone–protein receptor complexes by sucrose density gradient centrifugation**

Profiles of radioactivity corresponding to \[^{3}H\]5α-DHT–receptor complexes, which are distributed through sucrose density gradients after highspeed centrifugation, have been previously described (Davies & Griffiths, 1973b). Representative profiles are shown in Fig. 1. Cytoplasmic extracts yielded two peaks of radioactivity (Fig. 1a). The higher molecular weight component was consistently localized in the region corresponding to sedimentation coefficients in the range 7.7–8.5 S, and the lower molecular weight complex had a sedimentation coefficient in the range 3–3.5 S. Higher levels of incorporation of \[^{3}H\]5α-DHT into complexes were observed in preparations from 24 h castrated rats than from 48 h castrated animals (Fig. 1a), probably due to a decrease in androgen receptor protein with time (Baulieu & Jung, 1970; Mainwaring, 1970). However, when determining the effects of \[^{3}H\]5α-DHT–receptor complexes upon RNA polymerase activity, preparations from 48 h castrated animals were used. This was to minimize the amount of endogenous unlabelled 5α-
DHT complexed to chromatin and to establish reproducible experimental procedures as regards constant amounts of 5α-DHT added to enzyme assay systems in the form of steroid–protein complexes.

Fig. 1. Analysis of labelled prostatic subcellular fractions by sucrose density gradient centrifugation. Samples (0.4 ml) of subcellular fractions labelled with [3H]5α-dihydrotestosterone (5α-DHT) were layered over linear 5–20% (w/v) sucrose density gradients and centrifuged at 100,000 g for 18 h at 3–4 °C. Some gradients also contained a uniform concentration of KCl (0.5 mol/l). Sedimentation marker (arrows) was bovine serum albumin (4–6 S). Direction of centrifugation was from left to right. The radioactivity of three-drop fractions was determined.

(a) Cell supernatant fractions from rat ventral prostate, labelled in vitro, 1 h at 0 °C; ○, rats castrated 24 h previously; ●, rats castrated 48 h previously. (b) [3H]5α-DHT–receptor complex precipitated from cell supernatant fractions; ●, precipitated by 33% saturation with ammonium sulphate; ○, precipitated by 75% saturation with ammonium sulphate. (c) Nuclear [3H]-labelled steroid–receptor complex formed in vitro by incubation of nuclei with labelled cytosol and extraction of the nuclei with KCl (0.4 mol/l); ○, extract from nuclei prepared from rats castrated 24 h previously and incubated with labelled cytosol from similar rats; ●, extract from nuclei prepared from rats castrated 48 h previously and incubated with labelled cytosol from similar rats. Gradients contained KCl (0.5 mol/l).

The ‘3 S’ and ‘8 S’ cytoplasmic complexes could be separated from each other by selective ammonium sulphate fractionation (Fig. 1b). The higher-molecular-weight complex was precipitated by 25–33% ammonium sulphate saturation and the low-molecular-weight complex by 70–75% saturation. [3H]5α-Dihydrotestosterone–receptor complexes were extracted from ventral prostatic nuclear fractions in a form of sedimentation coefficient 4.5–5.0 S (Fig. 1c). Throughout this report, the cytoplasmic high- and low-molecular-weight complexes and the nuclear complex are referred to by their sedimentation coefficient, i.e. ‘8 S’, ‘3 S’ and ‘4.5 S’, respectively.

Effects of [3H]5α-dihydrotestosterone–protein receptor complexes on RNA polymerase activity

These studies were concerned with the influence of ionic strength on the stimulation in vitro of prostatic RNA polymerase activity by 5α-DHT–receptor complexes and the effect of incubating the system in the presence of α-amanitin.

The effects of α-amanitin on the Mg2+-low salt- and Mn2+-high salt-stimulated
RNA polymerase activities of rat ventral prostate are shown in Fig. 2. Maximum inhibition of RNA polymerase in intact nuclei (Fig. 2a) was achieved with 60–80 ng α-amanitin/25–50 μg DNA and, similarly, maximum inhibition of soluble whole nuclear RNA polymerase was achieved using these quantities of toxin in assays containing up to 50 μg enzyme protein (Fig. 2b). Dose response was similar in both the Mg²⁺-low salt assays (25–35% inhibition) and the Mn²⁺-high salt assays (70–90% inhibition).

Fig. 2. Effect of α-amanitin on prostatic RNA polymerase activity. (a) Intact nuclei; each assay contained 50 μg of DNA. (b) Enzyme solubilized from whole nuclei; each assay contained 50 μg of enzyme protein. ●, RNA polymerase activity measured under conditions of low salt concentration in the presence of Mg²⁺; ■, RNA polymerase activity measured under conditions of high salt concentration in the presence of Mn²⁺.

![Graph](image1)

Fig. 3. Effect of ionic strength and α-amanitin on stimulation of nuclear RNA polymerase by [³H]5α-dihydrotestosterone (5α-DHT)-receptor complexes. The activity of RNA polymerase in rat ventral prostatic nuclei prepared from rats castrated 48 h previously was measured in standard assay mixtures with and without various added substances. [³H]5α-DHT-receptor complexes were added to certain assays at a final ³H-labelled steroid concentration of 0.25 pmol/ml. Values are the average of at least quadruplicate determinations ± s.d.

(a) Whole nuclei; medium containing Mg²⁺ (5 mmol/l), ammonium sulphate absent. (b) Whole nuclei; medium containing Mn²⁺ (3 mmol/l) and ammonium sulphate (0.4 mol/l). (c) Whole nuclei; medium containing Mg²⁺ (5 mmol/l), Mn²⁺ (3 mmol/l) and ammonium sulphate (0.04 mol/l). (d) Enzyme solubilized from whole nuclei; medium containing Mg²⁺ (5 mmol/l), Mn²⁺ (3 mmol/l) and ammonium sulphate (0.04 mol/l).

C = control assays; αn = assays containing α-amanitin (60 ng/assay); 8S = assays containing cytoplasmic 8S 5α-DHT-receptor complex; 4.5S = assays containing nuclear 4.5S 5α-DHT-receptor complex.

RNA polymerase activity was estimated by measuring the incorporation of [¹⁴C]uridine 5' monophosphate (UMP) into acid-insoluble material.

Stimulation of RNA polymerase activity in intact nuclei occurred when cytoplasmic (8 S) or nuclear (4.5 S) 5α-DHT–receptor complexes were included in a low-salt medium containing Mg²⁺ (5 mmol/l) (Fig. 3a). This was probably stimulation of
nucleolar RNA polymerase, since it was not sensitive to \( \alpha \)-amanitin. In a high-salt medium \( [(NH_4)_2SO_4 (400 \text{ mmol/l})] \) containing \( \text{Mn}^{2+} (3 \text{ mmol/l}) \), a considerable increase in the \( \alpha \)-amanitin-sensitive activity was observed. This probably represented salt stimulation of nucleoplasmic RNA polymerase, but inclusion of receptor complexes in the assay caused no further stimulation (Fig. 3b). It is interesting to compare these results with those of Raynaud-Jammet, Bieri & Baulieu (1971), who made similar observations using a rat uterine system. When nuclei were incubated in a medium containing \( (NH_4)_2SO_4 (40 \text{ mmol/l}) \), a condition which promotes \( \text{Mn}^{2+} \)-dependent activity, but does not inhibit \( \text{Mg}^{2+} \)-dependent activity, stimulation of RNA polymerase activity by 5z-DHT-receptor complexes was observed (Fig. 3c). The stimulated activity appeared to be partly sensitive and partly insensitive to \( \alpha \)-amanitin. A clearer picture was obtained when the enzyme solubilized from whole nuclei was incubated in a similar medium using native chromatin as template (Fig. 3d). The enzyme was greatly stimulated by 5z-DHT-receptor complexes, and most of the stimulated activity was \( \alpha \)-amanitin-sensitive. This suggests that in vitro, under favourable ionic conditions, 5z-DHT-receptor complexes can stimulate both nucleolar and extranucleolar RNA polymerase activity.

In order to demonstrate this further, nucleoplasmic fragments and solubilized nucleoplasmic enzyme were incubated with receptor complexes in a medium containing \( (NH_4)_2SO_4 (40 \text{ mmol/l}) \). Chromatin from prostatic nuclei was again used as template for the solubilized enzyme, since a native template is required for stimulation to occur under these conditions (Davies & Griffiths, 1973a, b). Once again, part of the stimulated RNA polymerase activity was \( \alpha \)-amanitin-sensitive (Figs. 4a, b). It would seem therefore that stimulation of RNA polymerase is inhibited under conditions of high ionic strength.

**Interrelationship of \([^3H]5\alpha\text{-dihydrotestosterone} – \text{receptor complexes and their affinity for prostatic chromatin}**

Salt-dependent interconversion of the cytoplasmic 8 S and 3 S 5z-DHT-receptor complexes (Baulieu & Jung, 1970; Mainwaring, 1970; Mainwaring & Mangan, 1973) and the temperature-dependent interrelationship of the 8 S and nuclear 4-5 S complex (Mainwaring & Irving, 1973; Mainwaring & Mangan, 1973) have been reported. Obviously, such interconversion could take place in the medium used for RNA polymerase assay.

Incubation of the cytoplasmic \( '8 \text{ S}' \)–receptor complex in the enzyme high-salt assay medium at 37 °C resulted in the formation of a 3 S complex (Fig. 5a). However, if the \( '8 \text{ S}' \) complex was incubated in the low-salt medium at 37 °C, a 4-4-5 S complex was formed (Fig. 5b), similar to the complex extractable from prostatic nuclei (Fig. 1c) and from prostatic chromatin which had been incubated with the \( '8 \text{ S}' \) complex (Fig. 5c). Although little conversion occurred at 0 °C, this was increased after a longer incubation period in the presence of chromatin. This suggests that a temperature-dependent conversion of the cytoplasmic 8 S complex is a prerequisite for the maximum formation of nuclear 5z-DHT-receptor complexes and also that, under certain conditions in vitro, the conversion may be influenced by chromatin components. Such a conversion may also be necessary for stimulation of RNA polymerase activity.
An essential step in the action of 5α-DHT-receptor complexes is their association with prostatic chromatin. The ability of the various complexes to form a further association with chromatin is shown in Fig. 6. The ‘8 S’ complex preferentially combined with prostatic chromatin at 37 °C although complexes were also formed at 0 °C (Fig. 6a). The association between the ‘8 S’ and calf thymus DNA and between free [3H]5α-DHT and prostatic chromatin were negligible when compared with the combination of [3H]5α-DHT-receptor complexes and native chromatin. The 8 S and

4.5 S complexes, however, combined with prostatic chromatin far more readily than did the 3 S complex (Fig. 6b). It is worthy of note that to achieve saturation levels (58–63 fmol of [3H]5α-DHT/50 μg DNA) only 65 fmol of [3H]5α-DHT in the form of 8 S or 4.5 S complexes had to be added, but to achieve similar levels, 536 fmol of [3H]-labelled steroid as 3 S complex was required. This was probably due to the presence of a large amount of non-specific steroid-binding proteins in the 3 S region containing [3H]-labelled steroid. To allow incorporation of the small proportion of specific 3 S [3H]-labelled steroid complex (possibly 8 S subunits) into prostatic
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chromatin, a large excess of 3 S complexes was required most of which did not associate with the chromatin.

The incorporation of \([^3H]5\alpha\)-DHT-receptor complexes into chromatin was abolished by high salt concentration. Whereas the amounts of \([^3H]5\alpha\)-DHT incorporated into prostatic chromatin after 20 min at 37 °C in the form of cytoplasmic 8 S steroid–receptor complex or nuclear 4-5 S steroid–receptor complex under low

salt conditions were 1116 fmol and 1175 fmol/mg DNA respectively, under high salt conditions these values fell to 169 fmol and 184 fmol/mg DNA respectively. The high \((NH_4)_2SO_4\) concentration may provide unfavourable ionic conditions for the formation of such complexes. Alternatively the conditions may result in the removal of tissue-specific proteins necessary for complex formation and enzyme stimulation, especially at pH values near 8-0 at which the enzyme activity was assayed.

Prostatic chromatin: chemical composition and template availability

In Table 1 are listed certain components of prostatic chromatin. Whole nuclear chromatin from castrated rats showed a lower protein:DNA ratio than that from
intact rats, the decrease in protein being mainly due to a decrease in the content of non-histone (acidic) proteins. Since these proteins are claimed to be responsible for the control of gene transcription and template availability such a decrease would suggest decreased available template. No significant differences in the content of histone proteins and chromosomal RNA between normal and castrated rats were observed.

![Graph](image)

**Fig. 6.** Association of \([^{3}H]5\alpha\)-dihydrotestosterone (5\alpha\-DHT)-receptor complexes with chromatin. The incorporation of \([^{3}H]5\alpha\)-DHT-receptor complexes prepared from subcellular fractions of ventral prostate glands of rats castrated 48 h previously into chromatin prepared from similar glands or into DNA was carried out according to the method of Mainwaring & Peterken (1971). \(^{3}H\)-Labelled steroid-receptor complexes were incubated with chromatin or DNA samples for varying periods and the radioactivity associated with sedimented chromatin or associated with DNA measured and expressed as incorporated \([^{3}H]5\alpha\)-DHT.

(a) Incorporation of cytoplasmic 8 S 5\alpha\-DHT-receptor complex into chromatin at 37 °C (●); and at 0 °C (○); incorporation of cytoplasmic 8 S complex into calf thymus DNA as comparison (■); incorporation of free \([^{3}H]5\alpha\)-DHT into prostatic chromatin as comparison (□). (b) Incorporation into prostatic chromatin of cytoplasmic 8 S \([^{3}H]5\alpha\)-DHT–receptor complex (●), curve from (a) for comparison; incorporation of nuclear 4-5 S \([^{3}H]5\alpha\)-DHT–receptor complex (■), and of cytoplasmic 3 S \([^{3}H]5\alpha\)-DHT–receptor complex (○).

### Table 1. Chemical composition of chromatins

<table>
<thead>
<tr>
<th></th>
<th>DNA (mg)</th>
<th>RNA (mg/mg DNA)</th>
<th>Protein (mg/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-histone</td>
</tr>
<tr>
<td>Whole nuclear chromatins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) From normal animals</td>
<td>1</td>
<td>0.038 ± 0.017</td>
<td>1.31 ± 0.19</td>
</tr>
<tr>
<td>(b) From castrated animals</td>
<td>1</td>
<td>0.046 ± 0.029</td>
<td>0.72 ± 0.17</td>
</tr>
<tr>
<td>Nucleolar chromatins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) From normal animals</td>
<td>1</td>
<td>0.150</td>
<td>2.36 ± 0.17</td>
</tr>
<tr>
<td>(b) From castrated animals</td>
<td>1</td>
<td>0.245</td>
<td>2.19 ± 0.12</td>
</tr>
</tbody>
</table>

Values are means ± s.d. (s.d. omitted when less than 3 values available).

Nucleolar chromatin from rat ventral prostate generally contained a high content of non-histone protein and of chromosomal RNA. Strangely, the histone protein content of castrated rat ventral prostatic nucleolar chromatin was less than that from normal animals, whereas the relative concentration of non-histone proteins and of chromosomal RNA did not alter.

The template availability of total nuclear chromatin was also investigated (Fig. 7).
In these experiments, a constant amount of DNA (25 μg) was used in each assay. A much greater amount of RNA polymerase was required to achieve maximum activity in the presence of calf thymus DNA than was required in the case of prostatic chromatin (Fig. 7a). However, the template availability of chromatin from castrated rats did not differ quantitatively from that of chromatin from normal rats. When prostatic chromatin, previously incubated with excess nuclear ‘4·5 S’ 5α-DHT-receptor complex, was used as template (Fig. 7b), more enzyme was required to achieve maximum activity. The template availability, however, did not reach the levels observed in the presence of unrestricted prostatic DNA. Although the observed increases in RNA polymerase activity (Figs 3, 4) in these reconstituted systems may not be fully accounted for by an increase in template capacity, such an increase could be responsible for a large proportion of stimulated RNA polymerase activity.

It is interesting to compare the effects of added [3H]5α-DHT–receptor complexes on template availability with the effects of homologous chromatin–associated proteins. Addition of histone proteins to previously de-repressed prostatic chromatin resulted in a decrease in template activity, the decrease being greater with increased histone concentration (Fig. 8a). Addition of non-histone (acidic) proteins to restricted chromatin, however, resulted in an increase in template activity, relative to the amount of added non-histone (Fig. 8b). It is possible that the effect of non-histone proteins and 5α-DHT–receptor complexes is similar. Addition of receptor complexes to chromatin from normal rats did not cause such an increase in template activity, probably because binding sites on the chromatin were already occupied by endogenous steroid.
The rates of transcription of prostatic nucleolar chromatin by homologous enzyme are of a very low level (Davies & Griffiths, 1973b) and, as yet, it has not been possible to detect changes in template capacity of prostatic nucleolar chromatin. Moreover, it has not been possible to demonstrate an association between [3H]5α-DHT–receptor complexes and nucleolar chromatin. Furthermore, although androgen–receptor complexes increased the nucleolar RNA polymerase activity in vitro (Davies & Griffiths, 1973a, b) and despite the isolation of an oestradiol–receptor complex from the nucleolus of uterine tissue (Arnaud, Beziat, Guilleux & Mousseron-Canet, 1971), we have been unable to isolate a [3H]5α-DHT–receptor complex from the nucleoli of rat ventral prostate cells.

**DISCUSSION**

The androgenic stimulation of RNA polymerase activity in rat ventral prostate has been well documented (Liao, Leininger, Sagher & Barton, 1965; Liao, Barton & Lin, 1966; Bashirelahi, Chader & Villee, 1969; Davies et al. 1972). In a reconstituted system in vitro it has been shown that stimulation of the solubilized enzyme by 5α-DHT–receptor complexes can occur if prostatic chromatin is provided as a template (Davies & Griffiths, 1973a, b). Stimulation was observed in a medium of low ionic strength but was abolished by high salt concentration. Depending upon whether RNA polymerase was assayed in the presence of Mg2+, or Mn2+ and ammonium sulphate, the product of the reaction was either a ribosomal-type RNA synthesized by nucleolar enzyme or a DNA-like RNA synthesized by nucleoplasmic enzyme.
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respectively (Widnell & Tata, 1966; Hamilton, Widnell & Tata, 1968). Earlier results from this laboratory (Davies & Griffiths, 1973a, b) suggested therefore a preferential stimulation of ribosomal RNA synthesis. However, since the optimal conditions for the assay of DNA-like (messenger) RNA synthesis can be regarded as somewhat hyperphysiological, it is possible that such conditions are unfavourable for the action of 5α-DHT–receptor complexes.

The results now reported clearly indicate that high salt concentrations interfered with the essential interaction between 5α-DHT–protein receptor complexes and prostatic chromatin and, possibly, in this enzyme assay medium in the absence of nuclear material, with the temperature-dependent conversion of the cytoplasmic ‘8 S’ complex into a ‘4·5 S’ complex. It may be necessary for the originally formed cytoplasmic steroid–protein complex to be converted to an ‘active’ form, in a manner similar to the reported conversion of the uterine cytoplasmic oestradiol–receptor complex (Mohla, DeSombre & Jensen, 1972). Conversion in the absence of nuclear components supports the concept of a ‘neo-nuclear’ receptor (Jung & Baulieu, 1971).

The studies on the effects of salt concentrations on the RNA polymerase assay are particularly interesting. By use of conditions of lower ionic strength, together with α-amanitin, which selectively inhibits nucleoplasmic RNA polymerase (Jacob, Sajdel & Munro, 1970; Jacob, Sajdel, Muecke & Munro, 1970; Kedinger, Gniazdowski, Mandel, Gissinger & Chambon, 1970; Lindell, Weinberg, Morris, Roeder & Rutter, 1970), stimulation of the synthesis of DNA-like RNA (as expressed by the activity of extra-nucleolar RNA polymerase) has been demonstrated. It can be argued that if the effect of the prostatic 5α-DHT–receptor complexes upon the prostatic cell is to promote tissue growth and initiate functional activity, the initial increase in RNA synthetic activity should be of an ‘informational’ nature, i.e. messenger RNA species. Synthesis of ribosomal RNA may be secondary to, and dependent upon, a prior increase in messenger RNA species (Schmid & Sekeris, 1973). However, increases in nucleolar RNA polymerase activities in androgen-dependent (Davies & Griffiths, 1973a, b) and oestrogen-dependent (Arnaud et al. 1971) tissues have been observed. Preliminary experiments with the reconstituted system now described indicate that under both low- and high-salt conditions, 5α-DHT–receptor complex induces an increase in heterogeneous nuclear RNA, with some minor increase in the small molecular weight RNA’s. Current work is aimed towards the clarification of the nature and time-sequence of RNA production during hormonal stimulation.

Current theories on the action of steroid–receptor complexes imply an alteration in the nature of transcription which is controlled by the steroid–receptor complex. The molecular mechanism by which this is achieved is still uncertain. It was hoped to obtain more information on the nature of the transcriptional response by measuring the amount of DNA in prostatic chromatin which is available, under differing conditions, to the RNA polymerase. Less enzyme was required to achieve maximum activity when prostatic chromatin was used as template than when prostatic DNA was employed. This indicated that much less DNA was available in the restricted chromatin template. Addition of saturating amounts of nuclear 5α-DHT–receptor complex apparently caused more template to become available, but no quantitative differences were observed between the chromatin templates from normal and castrated rats.
This would suggest that the final difference in chromatin template activity between intact and castrated states may be more qualitative than quantitative. However, the non-histone (acidic) protein content of normal prostatic chromatin was somewhat higher than that of prostatic chromatin from the castrated animal, which would normally suggest an increased template activity. Increases in the prostatic nuclear content of acidic proteins and in template activity of prostate chromatin in castrated rats after androgen administration have been reported (Couch & Anderson, 1973a, b). Obviously, chromatin changes in relation to hormone action must be monitored in greater detail.

The role of acidic chromosomal proteins in the control of available template sites is being established (see review by Spelsberg, Wilhelm & Hnilica, 1972). Addition of non-histone proteins to chromatin preparations increased template activity (Kamiyama & Wang, 1971) and homologous proteins caused greater increases (Kostraba & Wang, 1972). We have shown this to be the case in the rat ventral prostate. Receptor proteins are acidic in nature (Bruchovsky & Wilson, 1968; Mainwaring, 1969a, b) and can be regarded as tissue specific. Acidic proteins interact with histone proteins and DNA, and counteract histone inhibition of DNA and RNA polymerase activities in vitro. It is possible therefore that 5α-DHT–protein complexes could fulfil such a role in the prostatic cells. Although evidence exists for (Barker & Warren, 1967; Stackhouse, Chetsanga & Tan, 1968; Beato, Seifart & Sekeris, 1970) and against (Dahmus & Bonner, 1965; Barker & Warren, 1966) the direct stimulation in vitro of template activity by steroid–receptor complexes, under our experimental conditions at least, an increase in prostatic template availability occurred in the presence of an excess of 5α-DHT–protein complexes.

The importance of using homologous enzyme in these template activity studies must be stressed since the differences in transcription characteristics between bacterial and mammalian enzymes (Butterworth, Cox & Chesterton, 1971) could mean that such heterologous enzymes may not recognize regulatory mechanisms such as those imposed by steroid–receptor complexes on mammalian chromatin. Moreover, the possibility that the transcript induced by steroid–receptor complexes could provide for chromosomal RNA, which is involved in selective restriction of chromatin (Bekhor, Kung & Bonner, 1969; Huang & Huang, 1969) cannot be ignored. Systems are now being developed which will allow the comparison of transcripts of chromatin under different physiological conditions and which will assist investigations into the monitoring of the various genetic responses to hormonal stimuli.

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SIMILARITIES BETWEEN 5α-DIHYDROTESTOSTERONE-RECEPTOR COMPLEXES FROM HUMAN AND RAT PROSTATIC TISSUE: EFFECTS ON RNA POLYMERASE ACTIVITY

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Protein macromolecules specifically binding [3H]5α-dihydrotestosterone ([3H]DHT) have been identified in cytosol and in nuclei prepared from human benign hypertrophic prostate. These macromolecules have similar properties to receptor proteins from other androgen-dependent tissues, as regards sedimentation coefficients on sucrose gradients and steroid specificity. Cytosol preparations from androgen-dependent tissues were able to transfer [3H]DHT in a recoverable protein-bound form to nuclei of other androgen-dependent tissues but not to nuclei of androgen-independent tissues. No transfer of radioactive steroid from cytosol of these latter tissues to any nuclei could be achieved. Labelled cytosol preparations from androgen-dependent tissues could stimulate the RNA polymerase activity of nuclei from androgen-dependent tissues but not that of nuclei from androgen-independent tissues. Cytosol preparations from these latter tissues could not affect RNA polymerase activity. Under suitable ionic conditions, human cytosol preparations containing DHT could stimulate both α-amanitin-sensitive and -insensitive RNA polymerase activities of human prostatic nuclei. However, rat ventral prostatic DHT-cytosol protein complexes were equally as efficient in performing this function, suggesting the possible involvement of specific DHT-receptor complexes in this process. It is therefore suggested that receptor molecules from androgen-dependent tissues may not be species specific but may share properties which would facilitate research into the understanding and aetiology of pathological conditions.

Keywords: receptor; 5α-dihydrotestosterone; rat ventral prostate; human hypertrophic prostate; RNA polymerase.

The frequency with which benign enlargement of the prostate has been found to occur in older men has promoted considerable interest in the aetiology of the disease. Androgens appear to be implicated in this aetiology since the development of the condition seems to be dependent upon the presence of functional testes (Huggins, 1947). The human hypertrophic prostate gland has
been shown to possess an active 5α-reductase enzyme system (Shimazaki et al., 1965a; Chamberlain et al., 1966), responsible for the conversion of testosterone to 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one). This metabolite of testosterone is now considered to be the ‘active androgen’ in many tissues (Bruchovsky and Wilson, 1968), and has been shown to be the principal steroid formed from testosterone by the human hypertrophic prostate in vivo (Pike et al., 1970; Becker et al., 1972) and in vitro (Shimazaki et al., 1965a, b; Siiteri and Wilson, 1970; Giorgi et al., 1971). Moreover, the concentration of 5α-dihydrotestosterone (DHT) is higher in hypertrophic than in normal prostatic tissue (Siiteri and Wilson, 1970; Farnsworth, 1971).

These results direct attention to the possible androgenic role of DHT in the development of prostatic hypertrophy, especially in relation to the ability of this steroid to stimulate, in vitro, the RNA polymerase activity of nuclei isolated from specimens of benign hypertrophic prostate in the presence of cytosol from the same source (Davies and Griffiths, 1973a). This observation was of particular interest, especially considering the known specific retention of DHT by proteins of selective high affinity, found in both cytoplasmic and nuclear preparations from hypertrophic prostate (Hansson and Tveter, 1971; Hansson et al., 1971, 1972; Mainwaring and Milroy, 1973), and in view of the postulated role of the DHT–protein receptor complexes in controlling genetic transcription in the rat ventral prostate gland (Davies and Griffiths, 1973b, c; 1974a, b).

If this ‘active androgen’ is concerned in the pathogenesis of prostatic disease, then the formation and function of steroid–receptor complexes and the effects of such complexes on the genome must be intimately related to aetiology. This study was undertaken to investigate the role of such complexes in regulating RNA synthesis in the hypertrophic prostate. Furthermore, since certain similarities appear to exist among steroid–receptor complexes from various androgen-dependent tissues, the possibility that other basic biochemical properties may also be common to such complexes was investigated by a study (a) of the ability, when labelled with [3H]DHT, of the complexes to transfer radioactivity into nuclear preparations and (b) the stimulation of RNA polymerase activity of isolated nuclei.

MATERIALS AND METHODS

Animals and tissues

Male Sprague–Dawley rats were bred in the Institute Animal Unit and used when 8–12 weeks old. Animals were castrated by the scrotal route under ether
anaesthesia. Tissues were removed from animals 24 h after bilateral orchidooepididymectomy.

Human prostate tissue was obtained at retropubic prostatectomy for benign prostatic hypertrophy, with the cooperation of the surgical staff at local hospitals. All tissues were immediately placed in the ice-cold buffer appropriate for planned experimentation in vessels surrounded with crushed ice. The preparation of tissue extracts was begun immediately upon the arrival of the specimens in the laboratory. The age range of patients undergoing prostatectomy was 58–83 years with a mean of 69 years. Histological examination of all prostatic specimens confirmed the presence of benign hypertrophy only, without any evidence of malignancy. One specimen of non-adenomatous prostate gland, showing no indication of hyperplasia or other disease, was obtained, being removed from a cadaver within 12 h of death.

Chemicals

[1, 2, 4, 5, 6, 7 (α)-3H]5α-Dihydrotestosterone (spec. act. 175 Ci/mmol) and [U-14C]UTP (spec. act. 475–514 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. The disodium salt of ATP and the trisodium salts of CTP, GTP and UTP were purchased from the Boehringer Corporation (London) Ltd., Ealing, London. α-Amanitin was provided by Calbiochem (U.K.) Limited, London. Crystalline bovine serum albumin, calf thymus DNA, dithiothreitol, Tris-base (99% pure), Triton X-100 and 5α-dihydrotestosterone were bought from the British Drug Houses Ltd., Poole, Dorset, U.K. Sephadex G-25 was a product of Pharmacia (U.K.) Ltd. Ammonium sulphate was recrystallized twice from EDTA (5mM) before use. All other substances were of 'Analar' grade and were dissolved in water distilled from all-glass apparatus.

Centrifuges and rotors

All high-speed centrifugation procedures described in this paper were carried out in the Beckman L2-65B ultracentrifuge using the SW50.1 (6 × 5ml) swinging-bucket rotor ($r_{av}$ 8.35 cm).

Preparation of subcellular fractions

All procedures were carried out at 0–4 °C unless otherwise indicated.

(a) Preparation of nuclei from human tissue

Specimens of prostatic tissue were dissected free of necrotic areas and adherent connective tissue, weighed, washed in 50 mM Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM MgCl₂ (medium A), chopped finely
with a sharp scalpel blade and minced with scissors. The mince was passed through a Latapie press (pore size 1 mm) and the resulting suspension homogenised in medium A in a Potter–Elvehjem ground-glass homogenizer using a teflon pestle with a clearance of 0.15–0.23 mm. The pestle was driven by a motor (TriR Instruments, Jamaica, New York) at 3000–3500 rev./min. Crude nuclear pellets were prepared by centrifugation of homogenates at 800 g for 10 min, and washed twice by resuspension in and resedimentation from medium A at 800 g for 10 min. The pellet was resuspended in a maximum of 1.6 ml of 50 mM Tris–HCl buffer, pH 7.4, containing 0.88 M sucrose, 1 mM MgCl₂, per g of original tissue. Aliquots (1.6 ml) of nuclear suspension were layered on discontinuous gradients of 1.8 M sucrose over 2.1 M sucrose (1.7 ml of each both containing 1 mM MgCl₂ and 50 mM Tris–HCl buffer, pH 7.4), and centrifuged at 106 000 g<sub>av</sub> for 1 h. Purified nuclei formed as a pellet at the base of the tube. Routine assessment of purity of nuclear fractions was carried out by light microscopic examination at ×750 magnification. Nuclei were found to be free of cytoplasmic contamination and, based on DNA determinations, 45–65% of tissue nuclei was recovered in a purified form. The protein:DNA ratio of nuclei thus prepared from specimens of benign hypertrophic prostate was 8.5–9.5:1.

(b) Preparation of nuclei from rat tissues

Nuclei were prepared from rat tissues as previously described (Davies and Griffiths, 1973c). When preparing extracts from seminal vesicles, it was essential that all glandular secretions were expelled before homogenization. Accordingly, these glands were opened longitudinally and rinsed in several changes of 0.9% (w/v) NaCl and then deionized water before homogenization. When it was necessary to purify nuclei from many batches of tissue, crude nuclear pellets were washed three times in medium A containing 0.1% (v/v) Triton X-100, and finally resuspended in medium A. Satisfactory purity was obtained by this method as determined by light-microscopic assessment.

**Labelling of subcellular fractions with [³H]DHT**

Procedures for the labelling of receptor proteins in cytosol (100 000 g soluble supernatant) fractions and their transfer to nuclear preparations have been described (Davies and Griffiths, 1973c; 1974b). During the course of this investigation, cytosol fractions from a number of tissues were examined. In studies on the transfer of [³H]steroid from cytosol to nuclear fractions (tables 1 and 2) and on the effects of [³H]DHT–cytosol mixtures on RNA polymerase activities of isolated nuclei (table 3), cytosol preparations were diluted to a constant protein concentration of 5 mg/ml. Serum was also diluted to a similar
concentration. Cytosol was prepared and diluted in 50 mM Tris–HCl buffer, pH 7.4, containing 0.1 mM EDTA and 0.25 mM dithiothreitol (medium B). For some experiments, as detailed in the text, partial purification of cytoplasmic [3H]DHT–receptor complexes was achieved by 33% saturation of cytosol with (NH₄)₂SO₄ (Davies and Griffiths, 1973c). Precipitates were sedimented at 10 000 g, redissolved in medium B and desalted by passage through small (10 × 1 cm) columns of Sephadex G-25 packed and equilibrated in medium B. Unlabelled receptor proteins for control systems were prepared by using cytosol and nuclear fractions without addition of ³H-labelled steroid. Preliminary characterization of [³H]steroid–receptor complexes was carried out by sucrose density gradient analysis (Davies and Griffiths, 1973c).

Estimation of RNA polymerase activity

RNA polymerase activity was measured in a medium containing 60 μmol of Tris–HCl buffer, pH 8.1, 3 μmol of MgCl₂ or 1.5 μmol of MnCl₂, 15 μmol of KCl, 200 nmol of dithiothreitol, 300 nmol of NaF, 300 nmol each of ATP, GTP and CTP, 125 pmol of [¹⁴C]UTP and 20 nmol of carrier UTP, and 10% (v/v) glycerol in a final volume of 500 μl. Some assays contained (NH₄)₂SO₄, at either 0.4 M or 0.04 M as specified in the text. Solutions of (NH₄)₂SO₄ were adjusted to the pH of the assay with aqueous NH₃ solution before addition to the mixtures. α-Amanitin was added to assays as specified in 120 mM Tris–HCl buffer, pH 8.1. Assays were adjusted to constant volume with this buffer. Nuclear suspension (20–50 μg of DNA) was added to 100 μl volumes. To investigate their effects on RNA polymerase activity, [³H]DHT–receptor complexes, partially purified by ammonium sulphate fractionation, were added to the assay system so that the final concentration of bound [³H]steroid was 250 fmol/ml. Cytosol and serum samples, previously diluted to 5 mg/ml as regards protein concentration prior to labelling with [³H]steroid, were added in aliquots of 100 μl. ‘Control’ receptor fractions were added to assay systems in amounts, based on protein concentration, equal to DHT–receptor complexes, or DHT–cytosol mixtures. At the termination of assays (15 min at 37 °C, collection and processing of acid-insoluble material for estimation of incorporation of labelled UMP was performed as previously described (Davies et al., 1972). Incorporation of [¹⁴C]UMP was abolished by the inclusion of exogenous ribonuclease (10 μg) or NaOH (100 μl of a 10 M solution) in the assay mixture. Results were corrected for zero-time controls and activity was calculated as fmol [¹⁴C]UMP incorporation per 100 μg DNA/15 min.
Chemical analyses

DNA content of preparations was determined by the diphenylamine procedure of Burton (1956) as modified by Giles and Myers (1965), using calf thymus DNA as standard. RNA content was determined by the orcinol method described by Schneider (1975) using yeast RNA as standard. Protein determinations were carried out using the method of Lowry et al., (1951), using crystalline bovine serum albumin as standard.

RESULTS

Characterization of [3H]DHT-receptor complexes

Analysis by sucrose density gradient centrifugation of human benign hypertrophic prostatic cytosol preparations labelled with [3H]DHT (4 pmol/ml; 1 h at 0 °C) indicated two peaks of radioactivity corresponding to [3H]DHT-receptor complexes (fig. 1a). The higher molecular weight component sedimented in the region corresponding to a sedimentation coefficient in the range 7.5-8.5 S, and the lower molecular weight component was of sedimentation coefficient 3-4 S. This latter area of binding contained some non-specific binding in the sense that other [3H]steroids, such as [3H]testosterone, [3H]-androstenedione and [3H]cortisol could be bound in this region, and was also of high capacity, since the radioactivity in this region could not be decreased by incubation in the presence of a 100-fold excess of non-labelled samples of any of the steroids named above (fig. 1b). The 7.5-8.5-S (hereafter referred to as 8-S) proteins were specific in that only [3H]DHT was bound in this region.

Fig. 1. Analysis of cytoplasmic binding of [3H]DHT by sucrose density gradient centrifugation. Samples of cytosol (100,000 g supernatant) prepared from various specimens of human hypertrophic prostate glands were made 4 nM with respect to [3H]steroids and retained at 0 °C for 1 h. Aliquots (400 μl) of 3H-labelled cytosols were layered onto linear sucrose gradients [5 ml; 5-20% (w/v)] in medium B and centrifuged at 100,000 g, for 18 h at 3-4 °C. Direction of centrifugation was from left to right. Sedimentation marker (arrows) was in all cases bovine serum albumin (S20w 4.6 S). (a) Cytosol labelled with [3H]DHT in the absence (○) and presence (●) of 400 nM unlabelled DHT; (b) cytosol labelled with [3H]DHT (○), curve from (a) as comparison, or labelled separately with [3H]testosterone, [3H]cortisol or [3H]-androstenedione (●). Data in (a) and (b) represent studies on cytosol (9.8 mg protein per ml) prepared from one specimen of hypertrophied prostate gland. Studies on a number of specimens show varying levels of binding of [3H]DHT in the 8-S region. A cytosol (8.4 mg protein per ml) not displaying specific 8-S binding, labelled with [3H]DHT in the presence (●) and absence (○) of 400 nM unlabelled DHT is shown in (c).
and of low capacity in as much as a 100-fold excess of unlabelled DHT abolished the retention of radioactivity in this area (fig. 1a). No other unlabelled steroid could bring about this effect. It should be mentioned that the two-peak pattern of binding was observed in only 30% of the specimens of hypertrophic prostate examined; the remainder showed only the 3-4-S peak of protein-bound radioactivity which displayed the characteristics of non-specificity and high capacity described above (fig. 1c). In those specimens which did show specific binding, the size of the 8-S peak reflecting the amount of [3H]steroid bound varied considerably. This may be attributed to varying levels of endogenous DHT in the prostate gland of androgenically potent patients, and therefore to variations in the number of unoccupied binding sites for DHT. The two-peak specific and non-specific low-capacity and high-capacity pattern is similar to that observed in androgen-dependent tissues, such as rat ventral prostate and rat seminal vesicle (fig. 2a), and also in non-adenomatous human prostate gland (fig. 2b), and the single-peak non-specific high-capacity pattern is shared with cytosol preparations from such androgen-independent tissues as rat liver, spleen and rat serum (fig. 2a).

The 8-S [3H]DHT-receptor complex could be precipitated from hypertrophic prostatic cytosol by 33% saturation with (NH₄)₂SO₄, and after redissolution and salting on Sephadex G-25, retained the same sedimentation coefficient (fig. 2c). However, after the preparation had been incubated at 30 °C for 15 min, or 37 °C for 10 min, a [3H]DHT-receptor complex of sedimentation coefficient 4-5 S was observed (fig. 2c). (At greater periods of time, loss of

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Fig. 2. Analysis of cytoplasmic binding of [3H]DHT by sucrose density gradient centrifugation. Samples of cytosol (100,000 g supernatant) prepared from various tissues were made 4 nM with respect to [3H]DHT and retained for 1 h at 0 °C. Aliquots (400 μl) of [3H]-labelled cytosol were layered on to linear sucrose gradients, [5 ml, 5-20% (w/v)] in medium B and centrifuged at 100,000 gav for 18 h at 3-4 °C. Direction of centrifugation was from left to right. Sedimentation marker (arrows) was in all cases bovine serum albumin (S₂₀,w 4.6 S). (a) Cytosols prepared from tissues of rats castrated 24 h previously were diluted with medium B to a constant protein concentration of 5 mg/ml prior to labelling with [3H]DHT. [3H]-Labelled cytosols were analysed from ventral prostate (○), seminal vesicle (●), and liver, spleen, skeletal muscle or serum (▲); (b) cytosol (7.3 mg protein per ml) prepared from nonadenomatous human prostate gland labelled in the absence (○) and presence (●) of 400 nM unlabelled DHT. (c) Human benign hypertrophic prostate (8.2 mg protein per ml) was incubated with [3H]DHT (4 nM) for 1 h at 0 °C, and made 33% with (NH₄)₂SO₄. The precipitate was collected by centrifugation at 10,000 g suspended in medium B and desalted by passage through Sephadex G 25, at 0-4 °C. Aliquots of the eluate (1.8 mg of protein per ml) were analysed by sucrose density gradient centrifugation after incubation at 0 °C (●) or at 37 °C for 10 min (○).
Androgen receptors in human prostate
radioactivity from the complex was observed. However, in the presence of nuclei, the incubation could be continued at time periods up to 30 min at 30 °C, or 15 min at 37 °C, to allow for maximum incorporation of [3H]steroid into nuclei, or for optimum activity of RNA polymerase. A temperature-dependent conversion of the 8-S [3H]DHT-receptor complex of the rat ventral prostate to a 4–5-S form has been observed, and has been postulated to be important in the mechanism of action of androgens (Mainwaring and Irving, 1973; Davies and Griffiths 1974b).

The ability of cytosol preparations from human benign hypertrophic prostate, and from rat ventral prostate, seminal vesicle, liver and spleen, and rat serum, previously labelled with [3H]DHT (4 pmol/ml; 1 h at 0 °C), to transfer radioactivity into homologous and heterologous nuclear fractions was investigated (table 1). It can be seen that transfer of radioactivity among rat ventral prostatic, seminal vesicular and hypertrophic prostatic fractions was consistently higher than among those from androgen-independent tissues. Uptake of radioactivity into nuclei of rat liver and spleen, or transfer by cytosol fractions from these tissues or rat serum, was only rarely significantly higher than that achieved with free [3H]steroid. There was, however, a significant cytosol-dependent transfer of [3H]steroid from cytosol of androgen-dependent tissues into homologous and heterologous nuclear fractions from such tissues. It is obvious, however, for reasons outlined above, that the transfer of radioactivity from human hypertrophic prostatic cytosol and the uptake of radioactivity by human hypertrophic prostatic nuclei will vary from specimen to specimen due to differing concentrations of endogenous DHT. The data in table 1 represent results obtained using the same pools of labelled cytosol and the same preparations of nuclei. In individual experiments performed when only smaller pools were available, the uptake by human prostatic nuclei and the transfer of [3H]steroid by human prostatic cytosol was observed to vary from negligible levels to values above those shown in table 1. A consistent fact, however, was that when a 8-S peak could be demonstrated by sucrose density gradient centrifugation, transfer of radioactivity from [3H]-labelled human hypertrophic cytosol to nuclei of androgen-dependent tissues could also be achieved.

To obviate the possibility that such uptake of radioactivity represented only non-specific nuclear adsorption in some manner, nuclei similarly labelled were extracted with KCl (0.4 M) and extracts were analyzed by sucrose density gradient centrifugation (fig. 3). These results do not represent studies using the same pools of cytosol and nuclei. It is clear that only nuclei from androgen-dependent tissues (hypertrophic prostate, rat ventral prostate and seminal vesicle) previously incubated with labelled cytosol from such tissues, either
Table 1

Uptake of [3H]DHT by nuclear fractions. Samples of cytosol from human hypertrophied prostate glands and from various tissues of rats castrated 24 h previously, as well as serum from similar rats, were diluted to a protein concentration of 5 mg/ml and incubated with [3H]DHT (4 nM) for 1 h at 0 °C. Aliquots of ³H-labelled cytosols and serum (500 µl) were incubated with equivalent volumes of homologous and heterologous nuclear fractions (50-100 µg of DNA) at 37 °C for 15 min. After incubation, mixtures were chilled, sedimented at 800 g, washed several times in ice-cold 0.25 M sucrose in medium B, and finally extracted in 1 ml of 0.4 M KCl in medium B. Extracts were centrifuged at 100,000 g for 10 min, and aliquots of supernatant counted for ³H. Values are the means ± S.D. for at least six determinations carried out using the same pools of nuclei and cytosol.

<table>
<thead>
<tr>
<th>Source of [³H]DHT (Labelled cytosols)</th>
<th>Uptake of [³H]DHT (dpm/mg nuclear DNA)</th>
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<tbody>
<tr>
<td></td>
<td>Benign hypertrophic prostate</td>
</tr>
<tr>
<td>Benign hypertrophic prostate</td>
<td>153846 ± 4662</td>
</tr>
<tr>
<td>Rat ventral prostate</td>
<td>129759 ± 5439</td>
</tr>
<tr>
<td>Rat seminal vesicle</td>
<td>270396 ± 103341</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>68376 ± 9324</td>
</tr>
<tr>
<td>Rat liver</td>
<td>60606 ± 24476</td>
</tr>
<tr>
<td>Labelled serum</td>
<td>72650 ± 7770</td>
</tr>
<tr>
<td>Free steroid</td>
<td>64103 ± 10878</td>
</tr>
</tbody>
</table>
homologous or heterologous, yielded a definite peak of protein-bound radioactivity, with sedimentation coefficient 4-5 S. Any other combination of cytosol and nuclear fractions yielded indeterminate profiles corresponding to unbound steroid. This also held true for cytosol and nuclear preparations from hypertrophic prostate specimens which displayed no 8-S peak. Apparently, therefore, an interrelationship exists between the macromolecules specifically binding DHT in these androgen-dependent tissues and the ability of nuclei to accept these complexes. Both cytoplasmic and nuclear complexes were found to be proteinaceous in nature, being sensitive to trypsin but resistant to nuclease.

The amount of radioactivity transferred from benign hypertrophic prostatic \(^3\)H-labelled cytosol could be markedly decreased if nuclei were pre-incubated with human prostatic cytosol or rat ventral prostatic cytosol containing unlabelled DHT. Pre-incubation of nuclei with liver cytosol together with DHT or with DHT alone had no such effect. A similar picture emerged using rat ventral prostatic nuclei. Nuclear uptake of \(^3\)H steroid was decreased by pre-incubation with homologous cytosol together with DHT and with human prostatic cytosol together with DHT, but not with liver preparations or free steroid. This suggests that DHT–receptor complexes from human prostatic and rat prostatic cytosols compete for the same limited number of sites in either nuclear preparations, and that the transfer of \(^3\)H steroid from cytosol to heterologous nuclei is not to spurious or artificial non-specific sites. In separate experiments it was shown that incubations such as these shown in table 2

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Fig. 3. Analysis of nuclear retention of \(^3\)H DHT by sucrose density gradient centrifugation. Samples of nuclei (25-50 μg of DNA in 500 μl) from various tissues were incubated with equivalent volumes of \(^3\)H DHT-labelled cytosol preparations (5 mg of protein per ml) for 30 min at 30 °C. Nuclear preparations were sedimented, washed and extracted with 1 ml of 0.4 M KCl in medium B and aliquots of extracts (400 μl) were layered over linear sucrose gradients [5ml: 5-20% (w/v)] in medium B containing an uniform concentration of KCl (0.5 M). (a) Extracts of benign hypertrophic prostatic nuclei previously incubated with \(^3\)H-labelled cytosol from benign hypertrophic prostate (○), rat ventral prostate (●), or with \(^3\)H-labelled rat serum (▲); (b) extracts of benign hypertrophic prostatic nuclei previously incubated with \(^3\)H-labelled cytosol from rat seminal vesicle (○) or from rat spleen, liver or skeletal muscle (▲), and extracts of rat seminal vesicular nuclei previously incubated with \(^3\)H-labelled cytosol from benign hypertrophic prostate (●); (c) extracts of rat ventral prostatic nuclei previously incubated with \(^3\)H-labelled cytosol from hypertrophic prostate gland (○), rat ventral prostate (●), or ventral prostate nuclei previously incubated with rat seminal vesicular \(^3\)H-labelled cytosol, or vice versa (▲). All rat tissues were from animals castrated 24 h before sacrifice. Direction of centrifugation was from left to right. Sedimentation markers (arrows) were in all cases bovine serum albumin (S\(_{20,w}\) 4.6 S).
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Table 2

Competition for $[^3H]DHT$ binding sites in nuclei. Samples of cytosol from human hypertrophied prostate glands and from various tissues of rats castrated 24 h previously were diluted to a protein concentration of 5 mg/ml and aliquots were incubated with either 4 nM $[^3H]DHT$ or 4 nM unlabelled DHT for 1 h at 0 °C. Nuclei were prepared from human hypertrophied prostate gland or ventral prostate glands of rats castrated 24 h previously and aliquots of nuclear preparation (500 μl containing 50–100 μg of DNA) were incubated at 37 °C for 15 min with equivalent volumes of medium B, medium B containing 4 nM unlabelled DHT, or of bovine serum albumin (5 mg/ml) or cytosols from human hypertrophied prostate gland, rat ventral prostate gland or rat liver, all previously incubated with unlabelled steroid. After incubation, nuclei were chilled, sedimented at 800 g, washed several times in ice-cold 0.25 M sucrose in medium B, resuspended in 500 μl of this medium and reincubated at 37 °C for 15 min with equivalent volumes of either $[^3H]$-labelled human prostatic cytosol or $[^3H]$-labelled rat ventral prostatic cytosol. After incubation, nuclei were sedimented, washed, and extracted in 1 ml of 0.4 M KCl in medium B. Extracts were sedimented at 100,000 gav and aliquots of supernatant counted for $^3$H. Values in the table (dpm per mg of DNA) are the average of duplicate determinations in one experiment using the same pools of cytosol and nuclei. Similar observations have been made in subsequent experiments.

<table>
<thead>
<tr>
<th>Source of $[^3H]$steroid</th>
<th>Uptake of $[^3H]$DHT (dpm per mg of DNA)</th>
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<tr>
<td></td>
<td>Human benign hypertrophic prostatic nuclei</td>
</tr>
<tr>
<td>(a) $[^3H]$-labelled benign hypertrophic prostatic cytosol</td>
<td></td>
</tr>
<tr>
<td>Nuclei preincubated with:</td>
<td></td>
</tr>
<tr>
<td>buffer (medium B)</td>
<td>181587</td>
</tr>
<tr>
<td>free steroid</td>
<td>178254</td>
</tr>
<tr>
<td>bovine serum albumin with DHT</td>
<td>176746</td>
</tr>
<tr>
<td>human prostatic cytosol with DHT</td>
<td>26349</td>
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<tr>
<td>rat ventral prostatic cytosol with DHT</td>
<td>31270</td>
</tr>
<tr>
<td>rat liver cytosol with DHT</td>
<td>178810</td>
</tr>
<tr>
<td>(b) $[^3H]$-labelled rat ventral prostatic cytosol</td>
<td></td>
</tr>
<tr>
<td>Nuclei preincubated with:</td>
<td></td>
</tr>
<tr>
<td>buffer (medium B)</td>
<td>156111</td>
</tr>
<tr>
<td>free steroid</td>
<td>156349</td>
</tr>
<tr>
<td>bovine serum albumin with DHT</td>
<td>153571</td>
</tr>
<tr>
<td>human prostatic cytosol with DHT</td>
<td>27460</td>
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<tr>
<td>rat ventral prostatic cytosol with DHT</td>
<td>23968</td>
</tr>
<tr>
<td>rat liver cytosol with DHT</td>
<td>154921</td>
</tr>
</tbody>
</table>
could cause a depression of peaks of radioactivity upon analysis of labelled nuclear fractions by sucrose density gradient centrifugation.

Effects of DHT-receptor complexes on RNA polymerase activity

In view of the effects described above, it was considered important to discover whether the various cytosol preparations in combination with DHT could elicit a biological response in the nuclei of androgen-dependent tissues. Firstly, the abilities of the various cytosol DHT mixtures to stimulate the RNA polymerase activities of the various nuclear preparations in the combinations shown in table 1 were investigated. It was shown (table 3) that only those combinations which showed uptake of \(^{3}\text{H}\)DHT into nuclei caused an increase in RNA polymerase activity. ‘Control’ cytosols i.e. cytosols prepared from ventral prostate gland of castrated rats but without addition of DHT did not cause an increase in the activity of RNA polymerase, whereas such cytosols previously incubated with \(^{3}\text{H}\)DHT caused increases in \(^{1}\text{C}\)UMP incorporation in nuclei from androgen-dependent tissues. It should be noted, however, that ‘control’ unlabelled cytosol from human hypertrophic prostate gland was able to bring about an increase in the RNA polymerase activity of nuclei from androgen-dependent tissues. This may be attributed to the presence of a high concentration of specifically-bound endogenous steroid, although a further increase does occur in most cases in the presence of exogenous steroid, probably due to a complete saturation of the system. No increase in the RNA polymerase activity of rat liver nuclei could be brought about by combination with any labelled cytosol, nor could rat liver cytosol and rat serum stimulate nuclear RNA polymerase activity under any conditions. Also, if nuclei from one of the androgen-dependent tissues was incubated sequentially with DHT-cytosols from two tissues no greater stimulation of the RNA polymerase activity could be observed, even if both cytosols were from androgen-dependent tissues. This may again suggest a limited number of nuclear sites, occupation of which by DHT-receptor complexes can result in an increase in genetic transcription, and also that heterologous complexes are occupying specific sites and not artificial ones.

The stimulation of RNA polymerase activity was investigated further as to the nature of the RNA synthesized and the role of the DHT-receptor complex in the process. It would have been preferable to use the nuclear steroid-receptor complex for this purpose, since stimulation of rat ventral prostatic RNA polymerase by the homologous nuclear complex has been observed (Davies and Griffiths, 1973b, c). Unfortunately, extracts from hypertrophic prostatic nuclei were contaminated with RNA polymerase activity and nuclear RNA which could interfere with enzyme assays. In the assays described below,
Table 3

Effects of cytosol and DHT on nuclear RNA polymerase activities. Samples of cytosol from human hypertrophied prostate gland and from various tissues of rats castrated 24 h previously, as well as serum from similar rats, were diluted to a protein concentration of 5 mg/ml and incubated with DHT (4 nM) at 0 °C. Nuclei were prepared from human prostate gland and from rat ventral prostate and rat liver and aliquots of nuclear preparation (50–100 µg of DNA in 500 µl) were incubated at 37 °C for 15 min in the RNA polymerase assay mixture with 100 µl volumes of buffer, buffer containing DHT (4 nM), the various labelled cytosol fractions, or control cytosol fractions (not incubated with DHT). In some instances (marked with an asterisk *) duplicate samples of nuclei were reincubated with a second cytosol–DHT fraction, as specified in the table. The RNA polymerase activities (fmol [¹⁴C]UMP incorporated per 100 µg of DNA/15 min) were determined. Values in the table are results of duplicate determinations obtained from one experiment using the same pools of cytosol and nuclei. Observations were verified in similar experiments.

<table>
<thead>
<tr>
<th>Additions to assay mixtures</th>
<th>RNA polymerase activities (fmol[¹⁴C]UMP incorp./100 µg DNA/15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human benign hypertrophic prostatic nuclei</td>
</tr>
<tr>
<td>Buffer</td>
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<tr>
<td>Buffer with DHT</td>
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<tr>
<td>Benign hypertrophic prostatic cytosol</td>
<td>3939</td>
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<tr>
<td>Benign hypertrophic prostatic cytosol labelled with DHT</td>
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</tr>
<tr>
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<tr>
<td>Rat ventral prostatic cytosol labelled with DHT</td>
<td>4299</td>
</tr>
<tr>
<td>*Rat ventral prostatic cytosol labelled with DHT, benign hypertrophic prostatic cytosol labelled with DHT</td>
<td>4193</td>
</tr>
<tr>
<td>Rat seminal vesicle cytosol</td>
<td>3516</td>
</tr>
<tr>
<td>Rat seminal vesicle cytosol labelled with DHT</td>
<td>4182</td>
</tr>
<tr>
<td>*Rat seminal vesicle cytosol labelled with DHT, rat ventral prostatic cytosol labelled with DHT</td>
<td>–</td>
</tr>
<tr>
<td>Rat liver cytosol</td>
<td>3451</td>
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<td>Rat liver cytosol labelled with DHT</td>
<td>3054</td>
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</table>

therefore, rat and human cytoplasmic 8-S steroid–receptor complexes were utilized after partial purification by ammonium sulphate fractionation and desalting on Sephadex G-25. The rat ventral prostatic cytoplasmic 8-S complex
Androgen receptors in human prostate

has been shown to stimulate the RNA polymerase activity of rat ventral prostatic nuclei (Davies and Griffiths, 1973b, c), presumably due to its conversion to an ‘active’ form under the conditions used for assay of RNA polymerase (Davies and Griffiths, 1974b). Similar characteristics of temperature-dependent conversion have been shown for the cytoplasmic 8-S steroid–receptor complex of the human hypertrophic prostate (fig. 2c).

DHT–receptor complexes were incubated with hypertrophic prostatic nuclei and RNA polymerase activity assayed under three separate sets of conditions (table 4). Stimulation of RNA polymerase activity of intact nuclei occurred when steroid–receptor complexes were included in a medium containing Mg²⁺ ions in the absence of (NH₄)₂SO₄. Under these ionic conditions, the RNA synthesized should be mainly of a ribosomal type, synthesized by nucleolar RNA polymerase. The activity is, however, decreased by 50% by α-amanitin, and this can be related to the fact that this stimulated activity is insensitive to α-amanitin. It should be noted however that ‘control’ unlabelled 8-S proteins from hypertrophic prostatic cytosol also bring about stimulation of RNA polymerase activity, to varying degrees, and that addition of DHT caused a further small but significant increase in activity. This is a situation similar to that described above, for stimulation by cytosol. Under conditions of high ionic strength (0.4 M (NH₄)₂SO₄) in the presence of Mn²⁺ ions, RNA polymerase activity is greatly increased, probably due to hyperphysiological disruption of the nuclear matrix, as well as stimulation of enzyme activity, and this activity is mainly α-amanitin-sensitive, suggesting activity of nucleoplasmic RNA polymerase B activity, promoting messenger RNA synthesis. However, this activity is not stimulated by DHT–receptor complexes. Possibly, if this great stimulation by salt is due to some extent to derestricion of the template, then the biochemical role of the steroid–receptor complex in affecting transcriptions of chromatin would be obliterated. At lower concentrations of (NH₄)₂SO₄ (0.04 M), in the presence of Mg²⁺ and Mn²⁺ ions, stimulation of α-amanitin-sensitive RNA polymerase activity did occur, suggesting that, under suitable conditions, DHT–receptor complexes can stimulate the RNA polymerase enzymes catalyzing both ribosomal and messenger RNA synthesis in hypertrophic prostatic nuclei. This is a picture similar to that obtained in a study on rat ventral prostatic nuclei (Davies and Griffiths, 1974a, b), and it is extremely interesting to note that [³H]DHT–receptor complexes from rat ventral prostate stimulate the RNA polymerase activity of hypertrophic prostatic nuclei in a similar manner to the homologous complexes. In this case, the control fractions did not stimulate the enzyme activity, emphasizing the role of the steroid–receptor complex in the stimulatory effect and also the interrelationship of the complexes from separate androgen-dependent tissues,
Stimulation of nuclear RNA polymerase activity by [\(^3\)H]DHT–receptor complexes. The activity of RNA polymerase in intact nuclei prepared from human hypertrophic prostate gland was measured in the absence and in the presence of cytoplasmic 8-S [\(^3\)H]DHT–receptor complexes prepared from rat ventral prostate gland and human hypertrophic prostate gland. Activities are expressed as fmol [\(^{14}\)C]UMP incorporated /100 pg DNA/ 15 min. Cytoplasmic 8-S complexes were prepared by addition of 0.5 volume of saturated (NH₄)₂SO₄ solution to rat and human prostate cytosol preparations previously labelled with [\(^3\)H]DHT (4 nM) at 0 °C. Precipitates were sedimented at 10,000 g, resuspended in medium B and desalted by passage through columns of Sephadex G25. ‘Control’ cytosol fractions were prepared by treatment of rat ventral prostate fractions or human hypertrophic prostatic fractions without prior labelling with [\(^3\)H]steroid. Labelled fractions were added to assays at a radioactive steroid concentration of 250 fmol/ml. Assays were carried out at various ionic strengths and in the presence and absence of a-amanitin. (60 ng/assay). RNA polymerase activity of nuclei is expressed as fmol [\(^{14}\)C]UMP incorporated per 100 pg of DNA/15 min. Values are the means ± S.D. of at least ten determinations using the same pools of bulked cytosol and nuclear fractions at each ionic strength. P values for stimulation of activity by control cytosol fractions versus buffer controls: \(^a\)P<0.001, \(^b\) non-significant; P values for stimulation of activity by \(^3\)H-labelled fractions versus control cytosol fractions: \(^c\)P<0.001, \(^d\) non-significant.

<table>
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<tr>
<th>Control</th>
<th>Mg(^{2+}), NO(NH₄)₂SO₄</th>
<th>Mn(^{2+}), (NH₄)₂SO₄ (0.4 M)</th>
<th>Mg(^{2+}), Mn(^{2+}), (NH₄)₂SO₄ (0.4 M)</th>
</tr>
</thead>
<tbody>
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<td>—a-amanitin</td>
<td>2231 ± 123</td>
<td>5051 ± 447</td>
<td>3639 ± 299</td>
</tr>
<tr>
<td>+a-amanitin</td>
<td>1190 ± 231</td>
<td>1018 ± 60</td>
<td>1133 ± 93</td>
</tr>
<tr>
<td>Unlabelled human receptor</td>
<td>—a-amanitin</td>
<td>2717 ± 119(^a)</td>
<td>5808 ± 574(^a)</td>
</tr>
<tr>
<td></td>
<td>+a-amanitin</td>
<td>1789 ± 248(^a)</td>
<td>1016 ± 38(^b)</td>
</tr>
<tr>
<td>Labelled human receptor</td>
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<td>5293 ± 308(^b)</td>
<td>5697 ± 729(^d)</td>
</tr>
<tr>
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<td>+a-amanitin</td>
<td>5460 ± 337(^c)</td>
<td>1017 ± 83(^d)</td>
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<td>Unlabelled rat receptor</td>
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<td>5654 ± 908(^b)</td>
</tr>
<tr>
<td></td>
<td>+a-amanitin</td>
<td>1746 ± 65(^a)</td>
<td>1087 ± 142(^b)</td>
</tr>
<tr>
<td>Labelled rat receptor</td>
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<td>5874 ± 686(^d)</td>
</tr>
<tr>
<td></td>
<td>+a-amanitin</td>
<td>4240 ± 903(^c)</td>
<td>1061 ± 109(^d)</td>
</tr>
</tbody>
</table>
Androgen receptors in human prostate

since steroid-protein mixtures from androgen-insensitive tissues do not affect RNA polymerase activity (Davies and Griffiths, 1973c and table 3).

DISCUSSION

The result presented in this paper indicate the presence of receptors specific for DHT in human hypertrophic prostate gland. Our observations complement those of Hansson et al. (1971, 1972) and Mainwaring and Milroy (1973). The steroid-receptor complexes found in the human hypertrophic prostate exhibit similar characteristics to those observed in androgen-dependent tissues of the rat, and were shown to be intertransferable as regards cytosol and nuclear preparations from heterologous tissues.

The transfer of radioactivity from cytosol to nuclei could be depressed by the pre-incubation of nuclei with homologous cytosol preparations containing DHT, and also with similar preparations from heterologous androgen-dependent tissues, but not with such preparations from androgen-independent tissues, or with free steroid. Moreover, in these combinations when [3H]-steroid could be shown to transfer from cytosol to nuclei, increases in the RNA polymerase activity of the nuclei were observed. Again, heterologous cytosol was as efficient as homologous cytosol in this respect, and the specificity of the process was suggested in that addition of homologous cytosol did not supplement the increases in RNA polymerase activity produced by heterologous cytosols. These two sets of results indicate a limited number of nuclear acceptor sites for steroid-receptor complexes, and that heterologous complexes associate with the same areas of chromatin as homologous complexes. The comparatively low levels of RNA polymerase in nuclei of androgen-dependent tissues of castrated animals suggested a deficiency in gene transcription which could be rectified by androgen-receptor complexes.

As previously pointed out by Mainwaring and Milroy (1973), there are two possible reasons for the inability to detect androgen receptors in all specimens of the hypertrophic glands studied. The first possibility concerns the loss or damage of receptor proteins during homogenization, but improved expertise and extreme caution did not result in an increased percentage success. Much more likely is the presence of high concentrations of endogenous androgen in certain samples of hypertrophied tissue, possibly in the peri-urethral area (Siiteri and Wilson, 1970). Since it was not possible, due to shortage of experimental material, to correlate the presence of receptors with histologically selected areas of tissue, it is reasonable to suppose that certain experiments were carried out on tissue from areas or nodules taken from regions containing such a high concen-
tration of steroid. Future studies will concentrate on selected rather than random samples, thus relating the degree of androgen binding with histological appearance.

The interrelationship apparent between the androgen receptors of the human hypertrophied gland and the ventral prostate gland and seminal vesicles of the rat may have an important bearing on future investigations on the diseased prostate. In a research environment directed towards the estimation of tissue receptor proteins in relation to therapy, it is becoming increasingly obvious that the development of immunological methods to measure receptor protein itself rather than unoccupied and possibly spurious receptor sites is desirable. It is equally obvious that the raising of antibodies to receptor proteins from experimental animals may well be easier than from human prostate tissue especially if structural as well as functional similarities could be demonstrated. The development of the elegant purification techniques for such receptor proteins (Mainwaring and Irving, 1973) could be well applied to such an investigation. The results reported in this paper regarding the ability of human prostatic cytosol steroid–receptor complex to enter rat prostatic nuclei as a complex of increased affinity, and vice versa, suggests that the overall mechanism of action is 'specific' to androgen-dependent tissues but not to species, and that such studies as those outlined above may well be worthwhile.

Such a concept is supported by the preliminary studies on the effects of steroid–receptor complexes on hypertrophic prostatic nuclear RNA polymerase activity. Steroid–receptor complexes from both hypertrophic prostate and rat ventral prostate were apparently able to stimulate α-amanitin-sensitive and -insensitive RNA polymerase activities, in a manner similar to that observed with rat ventral prostatic nuclei (Davies and Griffiths, 1974a, b). This effect probably represents an inter-dependent stimulation of precursor RNA synthesis, necessary to promote tissue growth and functional activity. Furthermore, the stimulation of the RNA polymerase activity by the cytoplasmic steroid–receptor complex from hypertrophic prostate under conditions of temperature which cause it to undergo a molecular transformation suggests an 'activation' similar to that observed in the rat ventral prostate (Davies and Griffiths, 1974b) and reported for the uterine oestradiol–receptor complex (Mohla et al., 1972). It is implied that the steroid–receptor complex controls and regulates the nature of transcription in a target organ. Moreover it could be argued, that since increased concentrations of DHT have been reported in the hypertrophied prostate gland of dog (Gloyna et al., 1970) and man (Siiteri and Wilson, 1970) and that this steroid has been shown, in vivo, to promote abnormal prostatic growth in dog (Gloyna et al., 1970) and cellular hyperplasia in
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vitro in explants of prostate tissue in culture (Baulieu et al., 1968), then it may be possible that in the peri-urethral region where hyperplasia arises, the concentration of DHT is increased in such a way as to saturate available receptor. In such a system, this may override the regulatory mechanisms and provide the stimulus for extensive cell division.

It cannot be suggested at this stage that the rat ventral prostatic steroid-receptor complexes stimulate the synthesis of the same messenger RNA species in hypertrophic prostatic tissue as do the homologous complexes. Studies are now in progress into the initiation of RNA polymerase on isolated chromatin from hypertrophied prostatic tissue and are directed towards studying the effect of steroid-receptor complexes on the transcription of the genome. However, the results of the present report indicating structural similarities of receptor proteins from different species, and suggesting the presence of similar nuclear acceptor sites for gene modification may be of considerable value in developing further studies into the etiology of prostatic cancer.

ACKNOWLEDGEMENTS

The authors are extremely grateful to Mr. D. G. Morris and Mr. R. W. Rees, University Hospital of Wales, Heath Park, Cardiff, and Mr. W. B. Peeling, St. Woolo’s Hospital, Newport, Gwent, for their kind co-operation in providing specimens of human hypertrophic prostatic tissue; and to the Tenovus Organization for their generous financial support.

REFERENCES


Chapter 18

HORMONAL EFFECTS IN VITRO ON PROSTATIC RNA POLYMERASE AND DNA POLYMERASE ACTIVITY*

P. DAVIES, M. E. HARPER, AND K. GRIFFITHS

During the past few years, reports from a number of research centers have clearly indicated that protein receptor molecules have an essential role in transmitting the effect of steroid hormones within the cells of responsive tissues. Steroid hormones combine with cytoplasmic receptor proteins to form a complex that is transferred to the nucleus where another complex having certain chromosomal sites composed of DNA and nuclear acidic protein is formed. However, the function of such steroid-protein complexes within the nucleus and the nature of the effects that the various components of this system may have upon gene transcription is still uncertain.

The present study has been concerned with the effects of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one, 5α-DHT) upon the activity of DNA-dependent RNA polymerase (nucleoside triphosphate-RNA nucleotidyl transferase, EC 2.7.7.6) in androgen-dependent prostatic tissue. The aim of the investigation was to reconstruct from various purified cellular components an in vitro system that would enable the steroid-receptor complexes formed in the prostate to bring about an increase in the rate of RNA synthesis. Preliminary investigations had indicated that such experiments might well increase our understanding of the mechanism of steroid action in the prostate, since it was

*The authors are grateful to the Tenovus organization (Cardiff) for their generous financial support.
shown that specific steroids were capable of stimulating in vitro the activity of RNA polymerase in purified nuclear preparations from rat and canine prostatic tissue (Table 18–1). It has been recently shown, by use of various conditions of assay for RNA polymerase activity, and by use of the selective inhibitor, α-amanitin, that 5α-DHT-receptor complexes can stimulate the activity of both nucleolar and nucleoplasmic RNA polymerase, indicating stimulation of both ribosomal RNA and messenger RNA synthesis. These studies have since been extended to nuclear preparations isolated from human tissue specimens exhibiting benign prostatic hypertrophy (Table 18–11). The results indicated that out of a series of testosterone metabolites, only 5α-DHT and 5α-androstane-3β, 17β-diol consistently stimulated RNA polymerase to any significant extent in the human nuclear preparation. Both compounds had similar effects on the RNA

<table>
<thead>
<tr>
<th>Compound Added To Assay</th>
<th>% Activation of RNA Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat Prostate</td>
</tr>
<tr>
<td></td>
<td>Nuclear Preparation</td>
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<tr>
<td>Testosterone</td>
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</tr>
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</tr>
<tr>
<td>5α-androstane-3α,17α-diol</td>
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</tr>
<tr>
<td>5α-androstane-3α,17β-diol</td>
<td>27.0</td>
</tr>
<tr>
<td>5α-androstane-3β,17α-diol</td>
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</tr>
<tr>
<td>5α-androstane-3β,17β-diol</td>
<td>31.0</td>
</tr>
</tbody>
</table>

* Nuclei were purified from tissue homogenates by centrifugation through hypertonic sucrose. RNA polymerase activity was assayed in a medium containing various steroids at a final concentration of 40 μM. Enzyme reactions were initiated by the addition of 250 μl of nuclear suspension (50–100 μg of DNA) in 0.25 M of sucrose plus 1 mM MgCl₂. Incubations were at 37°C for 15 min. Incorporation of [1⁴C]UMP into acid-insoluble material was determined as d.p.m./15 min/100 μg DNA. Activity achieved in the presence of a steroid is expressed as a percentage of increase over that value in the absence of any steroid. A similar pattern of stimulation was observed in the presence of 4 μM concentrations of these steroids, but at such concentrations the system showed a requirement for the presence of cytosol (soluble supernatant fraction).
TABLE 18-11
EFFECT OF VARIOUS STEROIDS ON NUCLEAR RNA POLYMERASE
ACTIVITY IN HUMAN TISSUE SPECIMENS EXHIBITING BENIGN
PROSTATIC HYPERTROPHY

<table>
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<tr>
<th>Compound Added</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>6.8</td>
<td>6.3</td>
<td>9.2</td>
<td>7.7</td>
<td>9.2</td>
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</tr>
<tr>
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<td>2.6</td>
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<td>5α-dihydroepitestrone</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

* Tissue was homogenized in 0.25 M sucrose containing 1 mM MgCl₂ using a Latapie press in combination with a Potter homogenizer with a motor-driven Teflon pestle. Nuclei were purified by centrifugation through discontinuous gradients of hypertonic sucrose. RNA polymerase activity was assayed in the medium described in the text to which was added 50 μl of cytosol (105,000 x g supernatant) containing steroid such that the final concentration of the medium was 4 μM. Steroids were added to cytosol in glycerol (50 μl/ml of cytosol). The enzyme reaction was initiated by the addition of 250 μl of nuclear suspension (50-100 μg of DNA) in 0.25 M sucrose and 1 mM MgCl₂. Incubations were for 15 min at 37° C. Incorporation of [¹⁴C]UMP into acid-insoluble material was determined as d.p.m./15 min/100 μg DNA. Activity achieved in the presence of steroids is expressed as a percentage of increase over the activity observed when cytosol containing only glycerol was added to the assay medium.

polymers activities in rat and canine prostatic nuclear preparations, although 5α-androstane-3α, 17α-diol also stimulated the enzyme in the canine preparation. Such studies indicate the need to investigate further the role of various testosterone metabolites within the nucleus. The following experiments were therefore undertaken as a preliminary to a more detailed investigation of steroid action in the prostate.

All preparative procedures were carried out on ventral prostatic tissue taken from rats that had been castrated 48 hours previously. Purification of nuclei was performed as described by Mainwaring, except that 1 mM MgCl₂ was used instead of CaCl₂ in all preparative media, and 2.2 M
Hormonal Effects In Vitro on Prostatic Activity

Sucrose was preferred as the medium for the final centrifugation of the preparation. Satisfactory purity was assessed by both biochemical and microscopic criteria. Nucleoli were prepared from nuclei by sonication and centrifugation through 0.88 M sucrose. Percentage disintegration of nuclei was assessed by staining with Azure C. Solubilization and purification of nuclear and nucleolar RNA polymerase activities was achieved by the method of Mainwaring, et al. Nucleoplasm and nucleoplasmic RNA polymerase were prepared by a method based on that of Roeder and Rutter. RNA polymerase activity was measured in a medium containing 60 μmol tris-HCl buffer (pH 8.1); 2.5 μmol MgCl₂ or 1.5 μmol MnCl₂; 15 μmol KCl; 200 nmol dithiothreitol; 300 nmol NaF; 300 nmol each of ATP, GTP, and CTP; 125 pmol [³⁵C] UTP (514 mCi/mmol); and 20 nmol carrier UTP in a final volume of 500 μl. In assays utilizing MnCl₂, the mixture also contained (NH₄)₂SO₄ at a final concentration of 0.4 M. DNA template (10–25 μg) was added in 50 μl of water, and the purified enzyme (50–80 μg) was added in 200 μl of buffer. In experiments with intact nuclei and nucleoli, DNA and the enzyme were replaced by either a nuclear or nucleolar suspension (50–100 μg and 20–50 μg of DNA respectively) in 250 μl of 0.25 M sucrose with 1 mM MgCl₂. The incubations were conducted for 15 min at 37°C and were terminated by the addition of 2 ml of 10% (w/v) trichloroacetic acid containing 1 mM Na₄P₂O₇. Washing of precipitates and preparation of acid-insoluble material for counting of radioactivity has been described previously. Solubilized nuclear RNA polymerase was eluted from columns of DEAE-cellulose as two peaks corresponding to nucleolar and nucleoplasmic forms of the enzyme (Fig. 18-1). Chromatin was prepared from nuclei and nucleoli using the method described by Mainwaring, et al., and preliminary chemical analysis was performed using the methods of Marushige and Bonner. Chromatin was devoid of RNA polymerase activity.

Cytoplasmic 5α-DHT-receptor complexes were prepared according to Mainwaring and Peterken. Cytosol (105,000 g
Figure 18-1. Elution profile of RNA polymerase solubilized from rat prostatic nuclei from columns of DEAE-cellulose. RNA polymerase was solubilized from purified nuclei by sonication at high-salt concentration. Enzyme preparations in a minimum volume of buffer were applied to 2 x 14 cm columns of DEAE-cellulose and were eluted from the columns using a linear gradient of 0 to 0.8 M KCl at a flow rate of 6 ml/hr. Fractions (2 ml) were collected and assayed in the presence of MgCl₂. Two peaks of RNA polymerase activity were eluted from the columns: form I corresponding to activity of nucleolar origin and form II corresponding to activity of extra-nucleolar origin.

supernatant) was made 4 nM with respect to [1α,2α-³H]5α-DHT; (47 Ci/mmol) added in glycerol, (50 μl/ml of cytosol). Preliminary characterization of the cytosol steroid-binding proteins on linear 5 to 20% sucrose-density gradients showed two distinct peaks of radioactivity (Fig. 18-2). The faster sedimenting fraction corresponded to a 5α-DHT-receptor complex with a sedimentation coefficient of approximately 8S, and the slower sedimenting fraction corresponded to a complex with a sedimentation coefficient of approximately 3S. Nonspecific steroid binding in the 3S-region was indicated by labelling with [³H]5α-DHT in the presence of a
Figure 18-2. Characterization of cytoplasmic [3H]5α-DHT-receptor complexes by sucrose-density-gradient centrifugation. Ventral prostate glands from 48-hour castrated rats were minced finely with scissors and homogenized in 50 mM tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA and 0.25 mM dithiothreitol. The supernatant from a preliminary centrifugation at 800 g was recentrifuged at 105,000 g for 1 hour, the "cytosol" fraction made 4 nM with respect to [1α,2α-3H]5α-DHT (47 Ci/mmol) and retained at 0°C for 1 hour. Linear 5 ml 5 to 20% (w/v) sucrose-density gradients in uniform concentration of 50 mM tris-HCl, 0.1 mM EDTA, and 0.25 mM dithiothreitol were prepared at least 6 hours before use. Samples for analysis (0.4 ml) were layered over the gradients and centrifuged at 100,000 x g for 18 hours at 3°C. One gradient in each set was layered with BSA (S20,w 4.6S) as sedimentation marker. Direction of centrifugation was from right to left. At the completion of centrifugation, two-drop fractions were collected from the bottom of the tube, and radioactivity was measured. Cytosol labelled with [3H]5α-DHT yielded two peaks of radioactivity corresponding to steroid-protein receptor complexes corresponding to sedimentation coefficients of 8S and 3S (o-o-o). Cytosol labelled with [3H]5α-DHT in the presence of a 10,000-fold excess of nonradioactive 5α-DHT yielded only one peak, in the 3S region (○-○-○).

10,000-fold excess of nonradioactive 5α-DHT. This treatment abolished the 8S peak, but radioactivity was retained in the 3S region (Fig. 18-2). The 8S receptor complex was selectively precipitated from cytosol by 33 percent saturation with
ammonium sulphate, while the 3S was retained in the supernatant. The 8S complex was collected by centrifugation, resuspended in buffer, and dialysed to remove traces of ammonium sulphate without changing the sedimentation coefficient or bound radioactivity (Fig. 18–3).

Figure 18–3. Sucrose-density-gradient centrifugation of cytoplasmic [3H]5α-DHT-protein receptor complexes. Cytosol was labelled with 4 nM [1α,2α-3H]5α-DHT (47 Ci/mm) for 1 hour at 0°C. Labelled receptor was precipitated by the gradual addition of 0.5 vol of saturated (NH₄)₂SO₄ adjusted to pH 7.4 with aqueous NH₃ and was collected by centrifugation at 10,000 x g for 15 minutes. The precipitate was redissolved in 50 mM of tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA and 0.25 mM dithiothreitol. Residual traces of (NH₄)₂SO₄ were removed by dialysis. Samples of redissolved precipitate and supernatant (0.4 ml) were layered over 5 ml 5 to 20% (w/v) sucrose-density gradients with a uniform concentration of 50 mM tris-HCl, 0.1 mM EDTA, and 0.25 mM dithiothreitol and centrifuged at 100,000 x g for 18 hours at 3°C. Direction of centrifugation was from right to left. One in each set of gradients was layered with BSA (S₂₀,w 4.6S) as sedimentation marker. After centrifugation, two-drop fractions were collected from the bottom of the tube and counted for radioactivity. The redissolved 33% (NH₄)₂SO₄ precipitate yielded one peak of radioactivity corresponding to a sedimentation coefficient of 8S (—–—–). The supernatant yielded one peak of radioactivity in the 3S region (• – • – • – •).
The nuclear steroid-receptor complex was prepared by incubating equal volumes of labelled cytosol and nuclear suspension (100–150 µg of DNA) at 37°C for 30 min. Nuclear 5α-DHT-receptor complex was extracted with 0.4M KCl and contained less than 20 percent of the original radioactivity. Sucrose-density-gradient centrifugation yielded one peak of radioactivity in the presence or absence of KCl; this peak corresponded to a steroid-receptor complex with a sedimentation coefficient of 4.5–5S (Fig. 18–4). The ionic strength of the nuclear complex was lowered for use in the reconstituted system by passage through a Sephadex G-25 column.

In order to investigate RNA polymerase activity, the 5α-DHT-receptor complexes were added to the assay system so that the final concentration of bound 5α-DHT was in all cases 0.25 pmol/1 ml, as indicated by the bound radioactivity. Control systems were prepared from cytosol and nuclear fractions that were isolated from 48-hour castrated rats but were not exposed to radioactive 5α-DHT. Control fractions were added to the assay system in amounts that were equivalent in protein concentration to the 5α-DHT-receptor complexes. Addition of these control fractions to the assay systems produced no alteration in RNA polymerase activity.

Incubation of intact nuclei and nucleoli in an RNA polymerase assay system containing cytoplasmic 8S or 3S 5α-DHT-receptor complexes resulted in an increased incorporation of [14C]UMP into acid-insoluble material (Table 18–III). It was interesting to note that the so-called “non-specific” binding proteins in complex with 5α-DHT, as well as the “specific” binding proteins, stimulated enzyme activity. This could suggest that there are structural similarities between the 3S and the 8S proteins or that the 8S complex is an aggregated form of certain 3S subunits.16 The nuclear 5α-DHT-receptor complex also increased the incorporation of [14C]UMP when incubated with either fresh nuclei or nucleoli.

The various complexes increased the activity of solubilized nuclear RNA polymerase when assayed in a purified system (Table 18–IV). Once again, both cytoplasmic complexes and
Figure 18-4. Sucrose-density-gradient centrifugation of nuclear $[^3$H]$5\alpha$-DHT-protein receptor complexes. Cytosol (105,000 x g supernatant) was made 4 nM with $[1\alpha,2\alpha[^3$H]$5\alpha$-DHT (47 Ci/mmol) and retained at 0°C for 1 hour. After this period, equal volumes of labelled cytosol and nuclear suspension (100-150 μg of DNA) were incubated together at 37°C for 30 minutes. The mixtures were then chilled in ice, and nuclei were collected by centrifugation at 800 x g and washed several times in 50 mM tris-HCl (pH 7.4) containing 0.1 mM EDTA and 0.25 mM dithiothreitol. The nuclei were extracted at 0°C with 0.4 M KCl, and the resulting viscous extract centrifuged at 100,000 x g for 30 minutes. KCl could be removed from the supernatant by passage through a Sephadex G-25 column. Samples of the supernatant with and without KCl were layered on 5 ml 5 to 20% (w/v) sucrose-density gradients in a uniform concentration of 50 mM tris-KCl, 0.1 mM EDTA, and 0.25 mM dithiothreitol. Some gradients contained uniform concentrations of 0.5 M KCl. One gradient in each set was layered with bovine serum albumin $(S_{20,\text{w}} 4.6S)$ as sedimentation marker. Gradients were centrifuged at 100,000 x g for 18 hours at 3°C. Direction of centrifugation was from right to left. After centrifugation, two-drop fractions were collected from the bottom of the tubes and their radioactivity measured. In the presence (o-o-o) and absence (●-●-●) of KCl, one peak of radioactivity was found, corresponding to a sedimentation coefficient of approximately 4.5 to 5S.
TABLE 18-III

EFFECT OF CYTOPLASMIC AND NUCLEAR 5α-DHT-PROTEIN-RECEPTOR COMPLEXES ON RNA POLYMERASE ACTIVITY IN NUCLEI AND NUCLEOLI

<table>
<thead>
<tr>
<th></th>
<th>Incorporation in Presence of</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytoplasmic Receptors</td>
<td>Nuclear Receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;8S&quot;</td>
<td>&quot;3S&quot;</td>
<td>&quot;4.5S&quot;</td>
<td></td>
</tr>
<tr>
<td>Nuclei</td>
<td>107.0</td>
<td>115.2</td>
<td>53.5</td>
<td></td>
</tr>
<tr>
<td>Nucleoli</td>
<td>50.5</td>
<td>77.5</td>
<td>72.1</td>
<td></td>
</tr>
</tbody>
</table>

*Nuclei were purified by centrifugation through 2.2 M sucrose containing 1 mM MgCl₂. Nucleoli were prepared by sonication of intact nuclei and centrifugation through 0.88 M sucrose. Cytoplasmic and nuclear 5α-DHT-protein-receptor complexes were added to the assay mixture so that the final concentration of 5α-DHT, based on bound radioactivity, was 0.25 pmol/ml. Enzyme reactions were initiated by addition of 250 µl of nuclear suspension (50-100 µg of DNA) or nucleolar suspension (20-50 µg of DNA). Incorporation of [³⁴C]UMP into acid-insoluble material was calculated as d.p.m./15 min/100 µg of DNA. Activities in the presence of 5α-DHT-receptor complexes are expressed as percentage increases above those observed in the presence of an equivalent amount (based on protein concentration) of protein receptor that was free of 5α-DHT. Addition of this receptor protein not equilibrated with radioactive 5α-DHT caused no increase in nuclear or nucleolar RNA polymerase activities.

The nuclear complex stimulated the activity of the enzyme. In the presence of calf-thymus DNA (25 µg) as template, only a slight increase in the enzymic activity was observed. The enhancement of activity was much greater, however, when calf-thymus DNA was replaced by either purified prostatic nuclear or nucleolar chromatin (10-25 µg DNA), but the increases in activity were not so marked with the nuclear 5α-DHT-receptor complex. Other workers, using enzymes from endometrial nuclei, have observed stimulation only with the nuclear complex.¹⁴¹³

The relationship of prostatic chromatin to the tissue-specificity of the action of 5α-DHT-receptor complexes are
Normal and Abnormal Growth of the Prostate

### TABLE 18-IV

EFFECT OF 5α-DHT-PROTEIN-RECEPTOR COMPLEXES ON RNA POLYMERASE SOLUBILIZED FROM PROSTATIC NUCLEI*

<table>
<thead>
<tr>
<th>Template</th>
<th>Cytoplasmic Complexes</th>
<th>Nuclear Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;8S&quot;</td>
<td>&quot;3S&quot;</td>
</tr>
<tr>
<td>Calf-Thymus DNA</td>
<td>14.4</td>
<td>10.1</td>
</tr>
<tr>
<td>Prostatic Nuclear Chromatin</td>
<td>158.2</td>
<td>91.9</td>
</tr>
<tr>
<td>Prostatic Nucleolar Chromatin</td>
<td>116.3</td>
<td>85.8</td>
</tr>
<tr>
<td>Liver Chromatin</td>
<td>6.2</td>
<td>8.1</td>
</tr>
</tbody>
</table>

*Nuclear RNA polymerase was solubilized from purified nuclei by sonication in 0.3 M (NH₄)₂SO₄. Incubations (15 min at 37°C) were initiated by the addition of 200 µl of enzyme solution. 5α-DHT-receptor complexes were added to the assay medium so that the final concentration of 5α-DHT, based on radioactivity, was 0.25 pmol/ml. Enzyme activity was measured in the presence of either calf-thymus DNA (25 µg), prostatic nuclear or nucleolar chromatin (10-25 µg of DNA), or liver chromatin (10-25 µg of DNA) as template. Chromatin was prepared by saline extraction of the respective nuclear or subnuclear fractions. Incorporation of [¹⁴C]UMP was calculated as pmol [¹⁴C]UMP/µg of template DNA. Activities are expressed as percentage increases above those observed in the presence of an equivalent amount (based on protein measurement) of 5α-DHT-free receptor.

Demonstrated by the low level of stimulation of enzymic activity observed when chromatin prepared from liver nuclei was used in the system (Table 18-IV). Furthermore, if the prostatic enzyme was replaced in the system by *E. coli* RNA polymerase (Sigma Ltd., Surrey, U.K.), an increase in the [¹⁴C]UMP incorporation was also produced by the addition of the 5α-DHT-receptor complexes, again supporting the view that specificity of tissue-binding of steroid-receptor complexes resides in tissue chromatin.

An interesting but complex aspect of the action of 5α-DHT-receptor complexes is their differential effect on the RNA polymerase activities purified from subnuclear fractions (Table 18-V). It appears that the degree of stimulation brought about by the complexes is dependent not only upon the DNA template provided but also upon the intranuclear source of the enzyme and the ionic conditions employed. The product of the reaction is a ribosomal-type RNA or a
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TABLE 18-V

EFFECTS OF CYTOPLASMIC (8S) AND NUCLEAR (4.5S) 5α-DHT-PROTEIN-RECEPTOR COMPLEXES ON PROSTATIC NUCLEOLAR AND NUCLEOPLASMIC RNA POLYMERASES*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Template</th>
<th>% Increase in Enzyme Activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>&quot;8S&quot;</td>
</tr>
<tr>
<td>Nucleolar (Form I)</td>
<td>Calf-Thymus DNA</td>
<td>11.8</td>
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<tr>
<td></td>
<td>Prostatic Nuclear Chromatin</td>
<td>96.6</td>
</tr>
<tr>
<td>Mg⁺⁺/Low Salt</td>
<td>Prostatic Nuclear Chromatin</td>
<td>143.5</td>
</tr>
<tr>
<td></td>
<td>Liver Chromatin</td>
<td>4.5</td>
</tr>
<tr>
<td>Mn⁺⁺/High Salt</td>
<td>Calf-Thymus DNA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Prostatic Nuclear Chromatin</td>
<td>5.7</td>
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<td>Nucleoplasmic (Form II)</td>
<td>Calf-Thymus DNA</td>
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<td>Prostatic Nuclear Chromatin</td>
<td>12.8</td>
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<td>Mg⁺⁺/Low Salt</td>
<td>Liver Chromatin</td>
<td>8.0</td>
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<tr>
<td>Mn⁺⁺/High Salt</td>
<td>Calf-Thymus DNA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Prostatic Nuclear Chromatin</td>
<td>0</td>
</tr>
</tbody>
</table>

* Nucleolar and nucleoplasmic RNA polymerase were solubilized from the respective subnuclear fractions by sonication in 0.3 M (NH₄)₂SO₄. Both forms were assayed in media containing Mg⁺⁺ (5 mM) and low salt (0.3 M KCl) or Mn⁺⁺ (3 mM) and high salt (0.4 M (NH₄)₂SO₄). DNA templates used were calf-thymus DNA (25 µg), prostatic nuclear or nucleolar chromatin (10-25 µg of DNA), or liver chromatin (10-25 µg of DNA). Chromatin was prepared by saline extraction of the respective nuclear or subnuclear fractions. The 5α-DHT-receptor complexes were again added to give a final concentration of 0.25 pmol 5α-DHT/ml assay mixture. Incorporation of [³⁵S]UMP was calculated as pmol [³⁵S]UMP/µg template DNA. Activities are expressed as percentage increases above those observed in the presence of an equivalent amount of 5α-DHT-free receptor protein, which caused no stimulation of enzyme activity.

DNA-like RNA, depending upon whether the enzyme is assayed in the presence of Mg⁺⁺ or of Mn⁺⁺ and (NH₄)₂SO₄.¹⁰,²⁹ Both Mg⁺⁺- and Mn⁺⁺/(NH₄)₂SO₄-dependent activities have been shown to be present in the nucleolar and the nucleoplasmic RNA polymerases purified from rat prostate.²¹ Our findings confirm this.

Stimulation of nucleolar RNA polymerase by 5α-DHT-receptor complexes occurs preferentially in the presence of prostatic chromatin and 5 mM MgCl₂, although some stimu-
lation does occur in the presence of 3 mM MnCl₂, particularly by the nuclear complex. Even though the nucleoplasmic enzyme transcribes prostatic chromatin more efficiently than the nucleolar enzyme, especially in the presence of Mn⁺⁺ and (NH₄)₂SO₄ (0.4 M), very little stimulation of this form of the enzyme occurred in the presence of either cation. In fact, the cytoplasmic 8S complex did not stimulate the nucleoplasmic enzyme when Mn⁺⁺ was present. It would appear from these results that the stimulation of total nuclear enzyme by these complexes is principally a reflection of the stimulation of the nucleolar species it contains. It is interesting to note, however, that the nuclear complex, with prostatic chromatin as template, stimulated the nucleoplasmic enzyme to some extent in the presence of Mn⁺⁺, suggesting that stimulation of DNA-like RNA synthesis may well occur in an in vivo situation.

The results showing preferential stimulation of nucleolar RNA synthesis as measured in intact nucleoli or with the purified RNA polymerase and those showing stimulation of the nuclear enzyme in the presence of nucleolar chromatin assume greater interest if considered in light of reports from other centers. The RNA synthesized in response to hormonal stimulation is mainly of the ribosomal type,¹⁴-¹⁷ which is synthesized in the nucleolus.²⁹,³¹ Estradiol-induced stimulation of RNA polymerase activity in intact nuclei occurs in the presence of α-amanitin, which is a specific inhibitor of the nucleoplasmic enzyme,³⁰ and a lack of hormonal effect has been reported in the presence of high-salt buffer.³ Androgen effects in the prostate are mainly directed towards Mg⁺⁺-dependent activity, especially that of the nucleolus.²¹ It would appear, therefore, that our results contribute to the large body of evidence indicating that one of the primary effects of steroid hormones in target tissue is to increase the rate of ribosomal RNA synthesis.

Further insight into the tissue specificity of the system is provided by the results shown in Table 18-VI. The effects of the cytoplasmic 5α-DHT-receptor complexes on the nuclear enzyme in the presence of altered templates were
**TABLE 18-VI**

**EFFECT OF CYTOPLASMIC 5α-DHT-PROTEIN-RECEPTOR COMPLEXES ON NUCLEAR RNA POLYMERASE IN THE PRESENCE OF VARIOUSLY TREATED TEMPLATES**

<table>
<thead>
<tr>
<th>Template</th>
<th>% Increase in [14C]UMP Incorporation With</th>
<th>pmol [14C]UMP Incorporation/μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;8S&quot;</td>
<td>&quot;3S&quot;</td>
</tr>
<tr>
<td>Calf-Thymus DNA</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Total Nuclear Prostatic Chromatin</td>
<td>154</td>
<td>66</td>
</tr>
<tr>
<td>Liver Chromatin</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Histone-Deficient Prostatic Chromatin</td>
<td>144</td>
<td>50</td>
</tr>
<tr>
<td>Histone &amp; Nonhistone Protein Deficient Prostatic Chromatin (NaCl Urea)</td>
<td>128</td>
<td>49</td>
</tr>
<tr>
<td>Prostatic DNA (Phenol Treated)</td>
<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>

* RNA polymerase was solubilized from prostatic nuclei by sonication in 0.3 M (NH₄)₂SO₄; Chromatin was prepared by saline extraction from prostate or liver nuclei. Histones were dissociated from prostatic chromatin in a mixture of 2 M NaCl plus 5 M urea in 0.2 M Na₂HPO₄ and citric acid buffer (pH 6.0). Histone-deficient chromatin was sedimented by centrifugation at 100,000 x g for 15 hr. The major portion of the nonhistone proteins was dissociated from histone-deficient chromatin in 2 M NaCl plus 5 M urea in 10 mM tris-HCl buffer (pH 8.3) containing 5 mM NaHSO₃. Chromatin was resedimented as described above. Prostatic DNA (protein free) was prepared by extraction of chromatin in 0.5% Na deoxycholate and water-saturated phenol. All templates were added to the assay in quantities of 10–25 μg of DNA. Incorporation of [14C]UMP was calculated as pmol [14C]UMP/μg DNA and was also expressed as the percentage of increase above that measured in the presence of 5α-DHT-free receptor proteins.

investigated. The usual slight stimulatory effect in the presence of calf-thymus DNA was noted; however, much larger increases in enzymic activity were observed with prostatic nuclear chromatin as template. Histone proteins were dissociated from chromatin in a mixture of 2 M NaCl and 5 M urea in 0.2 M Na₂HPO₄-citric acid buffer (pH 6.0), and the histone-deficient chromatin was collected by centrifugation at 100,000 g for 15 hours. This treatment produced an increase in the rate of transcription by 17 percent but did not
cause any alteration in the degree of enzymic stimulation. Nonhistone proteins were then removed from the histone-deficient chromatin by dissociation in 2 M of NaCl plus 5 M urea in 10 mM of tris-HCl buffer (pH 8.3) containing 5 mM NaHSO₃. The histone- and nonhistone-deficient chromatin was collected by centrifugation as previously described. This procedure increased the rate of transcription by an additional 25 percent without altering the extent of stimulation resulting from incubation with the complexes. At this stage, 9 to 10 percent of the protein, relative to DNA, remained. When the prostatic chromatin was completely stripped of protein by extraction with phenol and deoxycholate, the template activity was further increased, but the percentage of stimulation caused by the cytoplasmic complexes fell to the same low levels that are achieved using either calf-thymus DNA or liver chromatin as template.

The role of the chromatin-associated proteins is obviously worthy of considerable study. Steroid-receptor complexes

### Table 18-VII

<table>
<thead>
<tr>
<th>Tissue</th>
<th>T</th>
<th>epiT</th>
<th>5αT</th>
<th>An</th>
<th>3α,17α</th>
<th>3α,17β</th>
<th>3β,17α</th>
<th>3β,17β</th>
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</table>

DNA polymerase activity was measured as cpm [³H]TMP incorporated into DNA after 2 hours of incubation. Incubation conditions were described previously (M. E. Harper, et al., "The Effect of Prostatic Metabolites of Testosterone and Other Substances on the Isolated Deoxyribonucleic Acid Polymérase of the Canine Prostate," Biochemical Journal, 119:785, 1970). Four replicat assays were performed. Steroids were added to give a 40 μM final concentration. T, testosterone; epiT, epitestosterone; 5αT, 5α-dihydotestosterone; An, androsterone; 3α,17α-diol, 5α-androstane-3α,17α-diol; 3α,17β-diol, 5α-androstane-3α,17β-diol; 3β,17α-diol, 5α-androstane-3β,17α-diol; 3β,17β-diol, 5α-androstane-3β,17β-diol; A, androstenedione.
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are bound to the DNA of chromatin, and the number of available sites is controlled by the nonhistone chromatin-associated proteins. Furthermore, our results show that steroid-receptor stimulation of solubilized RNA polymerase is also dependent upon the presence of nonhistone proteins if the effect is to be maximal (Table 18-VI). Histones affect the template capacity, but not the stimulation of RNA polymerase activity, possibly by retaining a supercoiled structure, since they fail to produce an organ-specificity for chromatin. This property is, however, a feature of the nonhistone proteins, although the major portion of the nonhistone fraction has little tissue specificity. It would seem that these properties are limited to certain proteins that are tightly bound to DNA and that probably remain after chromatin dissociation in NaCl-urea (pH 8.3). This would explain (1) the lack of effect on the 5α-DHT-receptor-complex stimulation of RNA polymerase caused by removing most of the nonhistone proteins from chromatin and (2) the major effect

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Specific Activity (nmol TTP incorporated/mg protein/hr)</th>
<th>% Inhibition</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5 Acid PPT. Fraction</td>
<td>0.06</td>
<td>24.1</td>
<td>15.2</td>
</tr>
<tr>
<td>DEAE Cellulose Chromatography</td>
<td>0.50</td>
<td>15.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Phosphocellulose Chromatography</td>
<td>0.72</td>
<td>18.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Hydroxyapatite Chromatography</td>
<td>1.04</td>
<td>27.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Various purification stages are shown and have been described elsewhere (M. E. Harper, et al., "Effects of Steroids and Stilboestrol Analogues in vitro on Purified Prostatic DNA Polymerase," submitted for publication). DNA polymerase activity was measured as cpm [3H]TMP incorporated into DNA after 2 hours of incubation under standard conditions. Steroids were added in ethanol (5 μl) to give a final concentration of 40 μM. DES, diethylstilboestrol; T, testosterone; 5αT, 5α-dihydrotestosterone; αα-diol, 5α-androstane-3α,17α-diol.
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Figure 18-5. Effect of cytoplasmic receptor protein complexes on the isolated DNA polymerase from rat prostate. The DNA polymerase was prepared from the ventral prostate of 12-week-old Sprague-Dawley rats. Cytosol preparations of ventral prostate (105,000 x g) were obtained from 24-hour castrated rats. One portion was incubated with 4 nM [1α,2α-3H] 5α-DHT for 1 hour at 4°C before sucrose-density-gradient (5-20%; w/v) centrifugation. Samples collected for radioactivity measurement (top diagram) showed the receptor proteins. A second portion of unlabelled prostatic cytosol was also run on a similar gradient, and the samples of the fractions collected were added to tubes set up for DNA polymerase assay with and without the addition of 1 μM 5α-DHT. Assays were in quadruplicate.
produced by the removal of the final small protein fraction. It is clearly evident that chromatin specificity is not the whole problem because the conditions for enzyme assay and the source of enzyme also impose limits upon the extent of stimulation. Any results obtained from in vitro systems must be assessed with some caution, and it is still possible that further controlling factors, which exist in vivo, should be introduced into the in vitro assay.

Some of these problems have recently been researched by our group. Two years ago Harper, et al. described the stimulation by testosterone and 5α-androstane-3α,17α-diol of the semi-purified F₂-fraction DNA polymerase enzyme system (EC 2.7.7.7.) in the canine prostate (Table 18-VII). Subsequent assay of a twentyfold purified enzyme preparation showed that although diethylstilboestrol consistently inhibited the activity of the enzyme, the testosterone and 5α-androstane-3α,17α-diol failed to induce any degree of stimulation (Table 18-VIII). Currently, studies are in progress to investigate the effect of steroid-protein receptor complexes on the activity of purified canine DNA polymerase; however, it is interesting that whereas none of the steroids investigated have been shown to stimulate semi-purified DNA polymerase enzyme from the rat prostate, the activity of this enzyme preparation was stimulated by 5α-DHT in the presence of the 8S protein receptor complex (Fig. 18-5). It is obvious that a considerable amount of experimental work will be necessary to elucidate the mechanism of steroid action within the nucleus of the cell.

REFERENCES

Normal and Abnormal Growth of the Prostate


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INFLUENCE OF STEROID-RECEPTOR COMPLEXES ON TRANSCRIPTION BY HUMAN HYPERTROPHIED PROSTATIC RNA POLYMERASES

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The effects of 5α-dihydrotestosterone–receptor complexes on transcription in human hypertrophied prostate tissue were studied in a cell-free system reconstituted from the various subcellular fractions prepared from specimens of the diseased gland. Two major RNA polymerase species were isolated from human hypertrophied prostate. These were designated A and B and were distinguishable by their preference for divalent cations and their sensitivity to salt and α-amanitin. Moreover, RNA polymerase B, but not RNA polymerase A, could effectively transcribe a prostate chromatin template. Any enzyme activity endogenous to some chromatin preparations was shown to be characteristic of RNA polymerase B. 5α-Dihydrotestosterone–receptor complexes were transferred into prostatic chromatin both steroid- and tissue-specifically. The association of steroid–receptor complexes with chromatin produced changes in template activity and increased the transcription of the chromatin by exogenous and endogenous RNA polymerase B. With a number of specimens, however, there was considerable variation in accessible cytoplasmic receptor sites, uptake of steroid–receptor complexes by chromatin preparations, the template activity of the chromatin and its response to steroid–receptor stimulation. Nevertheless, the transcription characteristics of human hypertrophied prostatic chromatin appear to be influenced by steroid–receptor complexes, and the extent of the response to added complexes would undoubtedly be governed by pre-existing complexes having had an earlier effect.

Keywords: prostate; RNA polymerase; 5α-dihydrotestosterone; androgen receptor.

The overwhelming evidence that the ventral prostate gland of the rat is dependent upon androgenic steroids for the maintenance of its growth (see reviews by King and Mainwaring, 1974; Minguell and Sierralta, 1975) has led to the belief that a similar mechanism of androgenic regulation may be inherent in the human hypertrophied prostate, particularly since this condition will not arise in the absence of functioning testes (Huggins, 1947). This concept is supported to some extent by the observation that the principal metabolite of testosterone in the human hypertrophied prostate in vivo (Pike et al., 1970; Becker et al., 1972), in vitro (Farnsworth and Brown, 1963; Shamazaki et al., 1965a,b; Siiteri and Wilson, 1970; Giorgi
et al., 1971) and in organ culture (McMahon et al., 1974) has been shown to be 
5α-dihydrotestosterone (5α-DHT; 17β-hydroxy-5α-androstane-3-one), now estab-
lished, at least in the rat ventral prostate as the most active androgen (Bruchovsky
and Wilson, 1968). Although studies from these laboratories have clearly indicated
the importance of other metabolites of testosterone, in particular 5α-androstan-3α,
17α-diol in the androgen-dependent glands of the dog, a species also susceptible to
the development of prostatic hypertrophy (Evans and Pierrepoint, 1975), the
elevated concentration of 5α-DHT in the human hypertrophied prostate (Siiteri and
Wilson, 1970; Farnsworth, 1971; Millington et al., 1975), particularly in the peri-
urethral area (Siiteri and Wilson, 1970), and the ability of this steroid to promote
proliferation of human hypertrophied prostatic tissue in culture (Ghanadian et al.,
1975) would suggest a mechanism analogous to that shown to exist in the rat ven-
tral prostate.

A major difficulty in the development of a study of human hypertrophied pros-
tate as an androgen-dependent gland has been an inconsistency in the recognition of
protein components specifically binding androgens in the cytoplasm of these
glands. Since the uptake of steroid by such receptor proteins has been established
as a necessary prerequisite to its subsequent effect at nuclear or genomic sites, it
would seem necessary to demonstrate 5α-DHT—protein interaction in human hy-
pertrophied prostatic cytoplasmic extracts. Certain proteins present in human pros-
tatic extracts have, however, been described as ‘storage proteins’ (Grant and Giorgi,
1972), whereas other groups (Becker et al., 1975; Mobbs et al., 1975; Cowan et al.,
1975) have been unable to distinguish prostatic ‘intracellular’ proteins and serum
steroid-hormone-binding globulin (SHBG), although uptake of [3H]5α-DHT by
prostatic extracts was in excess of that attainable by plasma contamination (Steins
et al., 1974). Specific receptor proteins for androgens in cytoplasmic and nuclear
extracts, however, have been identified as being distinct from plasma components
(Hanson et al., 1971, 1972; Mainwaring and Milroy, 1973).

In view of these observations, it would seem reasonable to classify steroid-bind-
ing proteins according to their ability to perform a well-defined function within the
cell. Transfer of protein-bound steroid to nuclei (Mainwaring and Milroy, 1973;
Davies and Griffiths, 1975), together with the similarity of 5α-DHT—receptor com-
plexes from both rat ventral prostate and human hypertrophied prostate, in in-
voking a stimulation of activity of RNA polymerase (EC 2.7.7.6 ribonucleoside tri-
phosphate: RNA nucleotidyltransferase) in isolated nuclei (Davies and Griffiths,
1975; Davies, 1975), certainly suggest a regulatory role of steroid—protein com-
plexes in the human prostate gland.

The purpose of the work described in this paper was to develop preliminary
studies (Davies and Griffiths, 1973a, 1975; Davies, 1975), in order to investigate
the association of 5α-DHT—protein complexes with various templates and their ef-
fect on transcription by native RNA polymerases. This necessitated the isolation
and characterization of various enzyme species and a study of their transcrip-
tive properties directed towards human prostatic chromatin. The investigation provided
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further evidence of the involvement of androgen—receptor complexes in controlling genetic transcription in a purified system in vitro.

MATERIALS AND METHODS

Tissues

With the co-operation of the surgical staff at local hospitals, human prostatic tissue was obtained at retropubic prostatectomy for benign prostatic hyperplasia. All tissues were placed in ice-cold media immediately after excision. Preparation of tissue extracts was begun immediately the specimens arrived in the laboratory. The age range of patients undergoing prostatectomy was 51—69 years old, with a mean of 63 years. Histological examination of all prostatic specimens confirmed the presence of benign hyperplasia only, with no evidence of malignancy.

Tissues were also obtained from male Sprague-Dawley rats (aged 8—12 weeks) bred in the Institute Animal Unit. These animals had either undergone no surgery or had been castrated at various times prior to sacrifice, as indicated in the text.

Chemicals

5α-Dihydro-[1,2,4,5,6,7(n)-3H]testosterone (spec. radioact. 107—130 Ci/mmol), [2,4,6,7(n)-3H]oestradiol (spec. radioact. 85—110 Ci/mmol), [monoethyl-3H]diethylstilboestrol (spec. radioact. 50—100 Ci/mmol) and [5-3H]UTP (spec. radioact. 10—14 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks., UK. The disodium salt of ATP and the trisodium salts of CTP, GTP and UTP were purchased from the Boehringer Corporation (London) Ltd., Ealing, London who also provided RNAase, DNAase, actinomycin D and α-amanitin. Rifamycin AF/013 was the generous gift of Dr. R. Cricchio and Professor L.G. Silvestri of Gruppo Lepetit, Milan, Italy. The British Drug Houses Ltd., Poole, Dorset, UK provided crystalline bovine serum albumin, calf-thymus DNA, calf-thymus histone (Type II-A), dithiothreitol, Tris base, Triton X-100, DEAE—cellulose (Whatman DE 23) and phosphocellulose (P11). Sephadex G-25 was a product of Pharmacia (UK) Ltd., London and Macherey-Nagel cellulose, type 2200ff, was obtained from Camlab Ltd., Cambridge, UK. Human serum albumin was a gift from the Lister Institute, Elstree, Herts. Ammonium sulphate was recrystallised twice from EDTA (5 mM) before use. All other substances were of ‘Analar’ grade and were dissolved in water distilled from all-glass apparatus.

Centrifuges and rotors

High-speed centrifugation procedures described in this paper were carried out in a Beckman L2-65B or L5-65B ultracentrifuge using a SW50.1 (6 X 5 ml) swinging-bucket rotor ($r_{av}$ 8.35 cm), or a SW60 (6 X 4 ml) swinging-bucket rotor ($r_{av}$ 9.17 cm).
Preparation of subcellular fractions from rat tissues
These methods have been described in detail in previous publications (Davies and Griffiths, 1973b, 1974). Any slight modifications necessitated by experimental procedure will be specified as required in the text.

Preparation of subcellular fractions from human hypertrophied prostate tissue
All procedures were carried out at 0—4°C unless otherwise indicated.

Nuclei Purified nuclei were prepared from specimens of human hypertrophied prostate nuclei as previously described (Davies and Griffiths, 1975).

Chromatin Dependent on the method of preparation, chromatin may be obtained with differing template characteristics (de Pomerai et al., 1974). The procedure generally employed for the experiments described in this paper provided chromatin devoid of endogenous RNA polymerase activity at concentrations of KCl up to 0.6 M (Mainwaring et al., 1971) and was therefore suitable for studies using exogenous purified RNA polymerase. To provide chromatin with significant endogenous RNA polymerase activity, the method of Butterworth et al. (1971) was used. Preliminary chemical analysis of chromatin preparations was carried out as described by Marushige and Bonner (1966). The average protein: DNA ratio for chromatin devoid of RNA polymerase activity was 1.96:1 and for chromatin with associated RNA polymerase activity, the ratio was 2.20:1. Details of histone:nonhistone:DNA ratios of individual chromatin preparations are given where necessary in the text. Chromatin was usually used within 36 h of preparation, after which time deleterious changes were observed despite storage at −20°C. Over 80% of nuclear DNA was recovered in chromatin prepared by either procedure.

DNA DNA was prepared relatively rapidly, by ‘deproteinization’ of human hypertrophied prostate chromatin. Chromatin was mixed with CsCl (60%, w/v) and the mixture centrifuged at 170 000 g for 20 h. The resulting pellets (E260/E280 > 2.0) were gently dissolved in 10 mM Tris—HCl, pH 7.4, containing EDTA (0.5 mM), to a concentration of 50—100 μg of DNA/ml and dialyzed against 1000 vol. of this medium for 16 h.

Labelling in vitro of subcellular fractions with [3H]5α-DHT
Procedures for the labelling of receptor proteins in cytosol (100 000 g supernatant) fractions prepared from human hypertrophied prostate and their transfer to nuclear preparations have been previously described (Davies and Griffiths, 1975). Published methods were also used for the partial purification of [3H]5α-DHT—protein complexes by selective precipitation with ammonium sulphate (Davies and Griffiths, 1973b) and by adsorption to immobilized DNA (Mainwaring and Irving, 1973; Irving and Mainwaring, 1974). The incorporation of cytoplasmic [3H]5α-DHT—receptor complexes into chromatin was according to Mainwaring and Peterken (1971) with variations in times of incubation, temperature and concentration of
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[3H]steroid–protein complex as described in the text. Details of protein and DNA concentrations of various fractions and of preparations included in RNA polymerase assays are given in the Results section.

Estimation of accessible receptor sites for 5α-DHT in human hypertrophied prostate cytosol

The presence of sites available for occupation by endogenous 5α-DHT was measured by sedimentation analysis on sucrose density gradients (Davies and Griffiths, 1975) or by saturation analysis and removal of free steroid by charcoal adsorption. In this latter method, samples of cytosol or fractionated cytosol (200 μl) were incubated (2 h at 0–4°C) with aliquots (200 μl) of solutions of [3H]5α-DHT to give final steroid concentrations between 50 and 1000 pg/0.2 ml (0.86–17.24 nM). After incubation, 400 μl of charcoal suspension (0.2% (w/v) Norit A; 0.02% (w/v) dextran T50) was added and the mixtures shaken for 15 min at 0–4°C. Charcoal was sedimented at 800 g and duplicate aliquots (200 μl) of supernatant assessed for [3H]steroid by liquid scintillation counting. Bound and free [3H]steroid were calculated with reference to concurrent standards and controls and values plotted according to Scatchard (1949). Non-specific binding was assessed in similar assays also containing a 100-fold higher concentration of unlabelled 5α-DHT. Where possible, similar incubations were carried out with [3H]oestradiol and [3H]diethylstilboestrol to assess binding due to serum proteins or to specific oestrogen-binding proteins. Preliminary assays using both sedimentation and saturation analysis gave similar results concerning specific binding, but throughout the investigation, the nature and the number of assays performed was dependent upon the amount of available tissue.

Solubilization of RNA polymerase activity

The preparation of RNA polymerase from human prostate tissue was by a procedure based on described methods (Roeder and Rutter, 1970; Mainwaring et al., 1971; Kedinger et al., 1972), involving sonication at high ionic strength, selective precipitation of contaminating nucleic acid with protamine sulphate and fractionation with ammonium sulphate. Selective extraction of nucleolar (form A) RNA polymerase was achieved by the method of Chesterton and Butterworth (1971).

Chromatographic separations DEAE–cellulose and phosphocellulose were pre-washed, packed in columns (1 X 14 cm), equilibrated and used for separation of RNA polymerase species essentially as described by Chesterton and Butterworth (1971). Active fractions from peaks, excluding fractions where enzyme activities overlapped, were bulked together and could be stored at –20°C for 6–8 weeks without loss of activity. For use in assay systems, enzyme fractions were concentrated to 50–100 μg/ml by dialysis against polyethylene glycol 6000 [30% (w/v)] in the appropriate buffer.
Estimation of RNA polymerase activity

RNA polymerase activity was measured in a medium containing 60 μmol Tris-HCl buffer, pH 7.9, 3 μmol MgCl₂ or 1.5 μmol MnCl₂, 15 μmol KCl, 200 nmol dithiothreitol 300 nmol NaF, 300 nmol each of ATP, GTP and CTP, 1 μCi [³H]UTP, 20 nmol carrier UTP and 10% (v/v) glycerol in a final volume of 500 μl. Certain assays contained 100 μg of bovine serum albumin, and some (NH₄)₂SO₄, usually 40 mM, as specified in the text. Solutions of (NH₄)₂SO₄ were adjusted to the pH of the assay with aq. NH₃ solution before addition to the mixtures. DNA template and enzyme preparations were added separately in volumes of 50 μl. Substances not standard to the assay (α-amanitin, actinomycin D) were added in 120 mM Tris-HCl buffer, pH 7.9. Rifamycin AF/013 was dissolved in a minimal volume of dimethyl sulphoxide and made up to the required concentrations with Tris-buffer, which was also used to adjust assays to constant volume.

Assays (15 min at 37°C) were terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid containing 1 mM Na₂P₂O₇, and collection and processing of acid-insoluble material for estimation of incorporation of labelled UMP into polynucleotide linkage was performed as previously described (Davies et al., 1972). Results were corrected for zero-time controls and for assay in the absence of DNA, when applicable.

Chemical analyses DNA content of preparations was determined by the diphenylamine procedure of Burton (1956) as modified by Giles and Myers (1965) using calf-thymus DNA as standard. Protein determinations, including determinations of chromatin-associated nonhistone proteins, were carried out by the method of Lowry et al. (1951), using human serum albumin as standard. Histone proteins were also estimated by the method of Lowry et al. (1951) using calf-thymus histone as standard.

RESULTS

Characterization of RNA polymerase activity isolated from human hypertrophied prostate tissue

The enzyme solubilized from prostatic nuclei by sonication at high ionic strength was eluted from columns of DEAE-cellulose as two major peaks of activity (fig. 1a). The first peak, designated form A, was eluted at 0.18–0.22 M KCl and the second designated form B, was eluted at 0.29–0.32 M KCl. The peaks contained both Mg²⁺ and Mn²⁺-dependent activity, the Mn²⁺/Mg²⁺ activity ratios being in the ranges 1.1–1.4 for enzyme A and 1.8–2.2 for enzyme B, for a number of preparations. The salt concentrations required for elution of the enzyme activities are similar to those necessary to separate the enzymes from a number of other tissues (Jacob, 1973), but are lower than those necessary to elute rat prostatic enzymes (Mainwaring et al., 1971). A species difference may therefore exist since our purifi-
Fig. 1. Chromatography of RNA polymerases on DEAE—cellulose. (a) RNA polymerase was released from human prostatic nuclei by sonication at high ionic strength. Dialyzed enzyme (200—500 µg of protein) was applied to columns of DEAE—cellulose in medium A (50 mM Tris—HCl buffer, pH 7.9, containing 1 mM MgCl₂, 0.5 mM dithiothreitol, and 25% (w/v) glycerol), and eluted with a linear gradient developed from 60 ml of medium A containing 0.05 M KCl and 60 ml of medium A containing 0.5 M KCl. Fractions (30 drops, approx. 1.9 ml) were assayed for RNA polymerase activity in the presence of Mg²⁺ ions (●) or Mn²⁺ ions (○). (b) RNA polymerase was extracted from nuclei by the method of Chesterton and Butterworth (1971). Dialyzed enzyme (200—500 µg of protein) was applied to columns of DEAE—cellulose, washed with medium A + 0.05 M KCl and medium A + 0.1 M KCl until no more protein was eluted, and the enzyme was eluted with a linear gradient developed from 60 ml of medium A + 0.1 M KCl and 60 ml of medium A + 0.5 M KCl. Fractions were assayed for RNA polymerase activity in the presence of Mg²⁺ ions.

cation studies on rat prostate RNA polymerases (Davies et al., 1975) show similar elution patterns to those reported by Mainwaring et al. (1971). The enzyme prepared by the selective extraction procedure was predominantly form A (fig. 1b), being eluted from columns of DEAE—cellulose at 0.20—0.25 M salt.

Rechromatography of the enzyme form A prepared from both procedures, on
Fig. 2. Chromatography of RNA polymerases on phosphocellulose. Enzyme preparations eluted from columns of DEAE-cellulose (fig. 1) were adjusted to 50% (v/v) with glycerol and 0.2 M with KCl and applied to columns of phosphocellulose. Columns were washed with medium B (10 mM Tris–HCl, pH 8.0, containing 0.1 mM EDTA, 1 mM dithiothreitol and 50% (v/v) glycerol), containing 0.2 M KCl, until no more protein was eluted. A linear gradient developed from 60 ml of medium B + 0.2 M KCl and medium B + 0.8 M KCl was then applied. Fractions (30 drop, approx. 1.9 ml) were collected and aliquots (50 μl) assayed for RNA polymerase activity in the presence of Mg<sup>2+</sup> ions. Enzymes rechromatographed on phosphocellulose were (a) RNA polymerase A (sonication); (b) RNA polymerase A (procedure of Chesterton and Butterworth, 1971); and (c) RNA polymerase B (sonication).

Columns of phosphocellulose (fig. 2) showed that the enzyme from the selective extraction procedure could be resolved into two peaks of activity, AI and ALL (fig. 2b), eluted at 0.30–0.34 M KCl and 0.36–0.40 M KCl, respectively, while
Table 1
Effect of various assay conditions on human hypertrophied prostate RNA polymerase activities.
Apart from the factors under test, RNA polymerase assays were standard incubations (see Methods). Enzymes AI and AII were obtained by phosphocellulose chromatography of parent enzyme A, prepared according to Chesterton and Butterworth (1971). Enzyme B was obtained by sonication at high ionic strength. Enzyme AI acquired by this latter procedure demonstrated similar properties to that obtained by selective extraction. In order to study the properties of chromatin-associated RNA polymerase, the template was prepared in such a way as to retain appreciable endogenous enzyme activity (see Methods). DNAase (10 μg) was pre-incubated for 10 min at 37°C in the reaction mixtures before addition of exogenous enzyme. RNAase (10 μg) or NaOH (100 μl of a 10 M solution) were added after 5 min and the reaction allowed to continue for a further 10 min. Values are the means of quadruplicate assays ± S.D. Values in parentheses are average percentage values of that obtained in the standard incubation (100%). Incubations containing RNAase or NaOH are compared with a standard incubation to which 100 μl of water was added after 5 min. All assays containing exogenous enzyme transcribing calf-thymus DNA were in the absence of ammonium sulphate; assays of chromatin-associated RNA polymerase contained 0.1 M ammonium sulphate.

<table>
<thead>
<tr>
<th>Conditions of assay</th>
<th>RNA polymerase activities</th>
<th>Chromatin-associated RNA polymerase (fmol [3H]UMP incorp/100 μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme A</td>
<td>Enzyme AI</td>
</tr>
<tr>
<td>Standard assay</td>
<td>931 ± 87.5 (100)</td>
<td>763 ± 32.7 (100)</td>
</tr>
<tr>
<td>Without DNA</td>
<td>17 ± 4.4 (1.9)</td>
<td>14 ± 1.4 (1.9)</td>
</tr>
<tr>
<td>Without ATP</td>
<td>235 ± 156.6 (31.7)</td>
<td>161 ± 6.6 (21.1)</td>
</tr>
<tr>
<td>Without GTP</td>
<td>171 ± 15.2 (18.3)</td>
<td>132 ± 16.9 (17.3)</td>
</tr>
<tr>
<td>Without CTP</td>
<td>300 ± 90.0 (32.2)</td>
<td>142 ± 17.0 (18.6)</td>
</tr>
<tr>
<td>Without cation</td>
<td>142 ± 48.4 (15.2)</td>
<td>78 ± 10.9 (10.2)</td>
</tr>
<tr>
<td>Plus water (100 μl)</td>
<td>1029 ± 66.8 (100)</td>
<td>746 ± 34.2 (100)</td>
</tr>
<tr>
<td>Plus alkali (100 μl 1.0 M NaOH)</td>
<td>330 ± 111.9 (32.1)</td>
<td>200 ± 37.4 (26.9)</td>
</tr>
<tr>
<td>Plus DNAse (10 μg)</td>
<td>275 ± 164.0 (26.7)</td>
<td>111 ± 38.4 (14.9)</td>
</tr>
<tr>
<td>Plus DNAse (10 μg)</td>
<td>336 ± 45.4 (36.1)</td>
<td>205 ± 13.6 (26.8)</td>
</tr>
<tr>
<td>Plus Actinomycin D (10 μg)</td>
<td>382 ± 57.5 (41.1)</td>
<td>175 ± 36.2 (23)</td>
</tr>
<tr>
<td>Plus α-amanitin (40 ng)</td>
<td>583 ± 63.7 (62.6)</td>
<td>507 ± 53.8 (66.5)</td>
</tr>
<tr>
<td>Plus α-amanitin (20 μg)</td>
<td>672 ± 46.4 (72.2)</td>
<td>631 ± 61.6 (82.7)</td>
</tr>
<tr>
<td>Plus rifamycin AF/013 (100 μg)</td>
<td>46 ± 4.9 (4.9)</td>
<td></td>
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</tbody>
</table>
that prepared by the sonication procedure was predominantly form AI (fig. 2a) as judged by the salt concentration required for its elution and the appearance of a vestigial form AII. Rechromatography of form B on phosphocellulose was not successful in separating the enzyme into possible subunits (Kedinger et al., 1971; Kedinger and Chambon, 1972; Jacob, 1973). One peak of activity was recovered eluted at a concentration of 0.60–0.70 M KCl (fig. 2c). The activities of the fractions eluted from phosphocellulose were dependent upon the addition of carrier protein, either bovine serum albumin or inactive protein eluted at 0.2 M KCl. No differing effects were observed from the use of either bovine serum albumin or inactive protein from the phosphocellulose eluate.

Separate experiments showed that all enzyme forms had pH optima in the range 7.8–8.2 and that the optimal concentrations for activating cations were 5.75–6.25 mM for Mg$^{2+}$ and 3.0–3.6 mM for Mn$^{2+}$. The enzyme reaction was dependent upon the presence of all four nucleoside triphosphates and showed an absolute requirement for either Mg$^{2+}$ or Mn$^{2+}$ ions (table 1). The reaction was dependent upon a DNA template, as shown by the loss in activity after treatment of the template with excess DNase. The product of the reaction is sensitive to treatment with alkali and RNAase. Form A enzyme is more sensitive to inhibition by actinomycin D than the form B enzyme, but more refractory to inhibition by α-amanitin. However, there is not complete insensitivity of form A to the toxin. This is similar to the pattern shown by the rat ventral prostate RNA polymerases (Mainwaring et al., 1971;
Davies and Griffiths, 1974). The slight inhibition of activity of parent enzyme A could not be explained by different levels of inhibition of AI and AII which have similar properties to the parent enzyme in all respects (table 1), nor could increased inhibition of any form be achieved with 20 μg of α-amanitin. The transcription of DNA by RNA polymerases A and B was effectively inhibited by 100 μg of rifampycin AF/013.

The properties of chromatin-associated RNA polymerase are also shown in table 1. This endogenous RNA polymerase activity showed similar properties to form B polymerase in its requirements for nucleoside triphosphates and cations and in its sensitivity to RNAase and α-amanitin. It was, however, insensitive to rifampycin.

The transcription of prostatic DNA and prostatic chromatin by human prostatic RNA polymerases AI and B is shown in fig. 3. Transcription of prostatic DNA by polymerase B, but not AI, was significantly stimulated by salt. Transcription of chromatin by the enzymes appears to be quite complex. No transcription of chromatin by form A was discernible in the absence of endogenous RNA polymerase activity. Since these studies were initially performed with enzyme AI from the sonication procedure, it was thought that the lack of AII might account for the absence of transcription, but neither AI or AII alone, nor the parent enzyme (prepared as such by selective extraction, or by remixing AI and AII) could transcribe a chromatin template. RNA polymerase B, however, transcribed chromatin efficiently and augmented any existing endogenous activity. This transcription displayed a biphasic response to salt concentration (fig. 3b), the first stimulation probably due to an effect on the enzyme (Widnell and Tata, 1966), the second to a dissociation of proteins from the template (Butterworth et al., 1971). Endogenous activity was apparent at low and high ionic strength (fig. 3c), but was higher under conditions when dissociation of proteins from the chromatin would have occurred (Butterworth et al., 1971).

Components specifically binding $[^3H]5α$-DHT, their association with chromatin and effects on transcription

Centrifugation through gradients of sucrose is probably the best possible procedure to detect cytoplasmic components which specifically bind steroids. Unfortunately, the procedure is also relatively time-consuming, and the biochemical integrity of other subcellular fractions derived from the same tissue may not be retained if stored until receptor analysis has been carried out. This problem need not occur when using ventral prostate tissue from a number of rats which can be adjusted to equivalent hormonal status. In this case, cytoplasmic fractions and subnuclear fractions prepared from glands of different animals may feasibly be mixed. Specimens of human hypertrophied prostate, however, vary considerably in receptor content (Davies and Griffiths, 1975) and this may influence chromatin structure and hence the activities of the enzymes associated with the genome. To make definitive observations on the interactions of the various subcellular components, therefore, it is desirable that they are all prepared from the same specimen, thus
necessitating swift demonstration of the presence of receptor molecules.

The rapid identification of specific 5α-DHT-binding proteins was carried out by the incubation of either radioactive 5α-DHT, oestradiol or diethylstilboestrol with aliquots of cytosol preparations, with and without a 100-fold higher concentration of the respective unlabelled compound, followed by removal of free steroid with dextran-coated charcoal. A decrease in bound radioactivity in the presence of cold competitor indicated saturable binding of the steroid. Fractionation of labelled cytosol using 33% ammonium sulphate and investigation of saturable binding in the

![Diagram](image-url)

Fig. 4. Uptake of [3H]5α-DHT–receptor complexes into chromatin. The retention of [3H]-5α-DHT by various templates was studied using the basic method of Mainwaring and Peterken (1971). [3H]-Labelled steroid–receptor complexes were incubated with templates for varying periods and the radioactivity retained expressed as incorporated [3H]5α-DHT. (a) Incorporation of human prostate [3H]5α-DHT–receptor complex into human prostate chromatin at 37°C (○) and at 0°C (●); (b) incorporation into human prostate chromatin of [3H]5α-DHT derived from human prostate cytosol (●), rat ventral prostate cytosol (○), rat spleen cytosol (●), human serum (○) or free [3H]steroid (○); (c) incorporation of [3H]5α-DHT derived from human prostate cytosol into human prostate chromatin (●), rat liver chromatin (○), rat spleen chromatin (○) and rat kidney chromatin (●); (d) incorporation into human prostate chromatin of [3H]steroid derived from human prostate cytosol labelled with [3H]5α-DHT (●), [3H]cortisol (○), [3H]oestradiol (○) or [3H]progesterone (●). Tissues were excised from rats castrated 24 h previously.
Transcription of human RNA polymerase

Table 2

Uptake of $[^3H]5\alpha$-DHT by human prostate chromatin in relation to template activity.

Chromatin was prepared from a number of specimens of human hypertrophied prostate so as to be devoid of RNA polymerase activity. Preparations were separated into four portions and used as follows: (a) for determinations of histone protein, nonhistone protein and DNA content; (b) for uptake of $[^3H]5\alpha$-DHT from samples of labelled cytosol, partially purified by ammonium sulphate fractionation and DNA-cellulose chromatography; (c) for determination of template activity by titration against RNA polymerase as described in legend to fig. 6: chromatin preparations were incubated with cytosols as in (b) but labelled with cold $5\alpha$-DHT at equal concentrations (4 nM) to $[^3H]$steroid; (d) for determination of template activity of chromatin without incubation with cytosol.

Template activity is expressed as the amount of chromatin DNA required to saturate 28.6 ng of RNA polymerase B relative to the necessary amount of naked DNA as 1; all chromatin preparations were incubated with the cytosol from the same gland, except specimen 1. This cytosol had no accessible specific binding sites for $[^3H]5\alpha$-DHT; chromatin was therefore incubated with $[^3H]$steroid—protein and steroid—protein from cytosol of specimen 2.

<table>
<thead>
<tr>
<th>Chromatin preparation</th>
<th>Histone DNA</th>
<th>Nonhistone DNA</th>
<th>$[^3H]5\alpha$-DHT uptake (dpm/mg DNA)</th>
<th>Template activity without steroid</th>
<th>Template activity with steroid</th>
<th>Percentage increase</th>
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</tbody>
</table>

resuspended precipitate yielded more satisfactory and less equivocal information concerning the presence of $[^3H]$steroid—receptor complexes than experiments with whole cytosol. In the studies reported here, saturable binding of $[^3H]$diethylstilbestrol or $[^3H]$oestradiol was never observed in resuspended precipitates from fractionated cytosol, whereas saturable binding of $[^3H]$oestradiol was frequently observed with preparations of whole cytosol concomitant with saturable binding of $[^3H]5\alpha$-DHT, indicating contamination with serum components. All those specimens showing saturable binding of $[^3H]5\alpha$-DHT in the resuspended precipitate from salt-fractionated cytosol also showed an 8 S peak of protein bound $[^3H]$steroid on gradient analysis. Such profiles have received adequate exposure previously (Davies and Griffiths, 1975). Fractionation of cytosol with ammonium sulphate prior to addition of $[^3H]$steroid invariably reduced the amount of binding and this procedure was not amenable to Scatchard analysis for similar reasons. Saturation analyses carried out on whole cytosol yielded apparent dissociation constants for $[^3H]5\alpha$-DHT—protein complexes in the range 1.4—2.8 nM and accessible binding site concentrations between 28 and 92 fmol/mg cytosol protein. Values for $[^3H]5\alpha$-DHT-binding by normal human male serum were 1.02—1.13 nM and 258—336 fmol/mg protein, respectively.
Table 3
Effects of 5α-DHT–receptor complexes on RNA polymerase activity associated with human hypertrophied prostate chromatin.
Chromatin was prepared from human hypertrophied prostate gland and rat spleen so as to contain appreciable endogenous RNA polymerase activity. Cytosol was prepared from human prostate gland and separate aliquots incubated with and without 5α-DHT (4 nM) and partially purified by salt fractionation and adsorption to immobilised DNA. Rat spleen cytosol was treated similarly. Normal human male serum was diluted to 20 mg protein/ml and then steroid was added, as necessary. Aliquots of cytosol (100 µl) were added to standard RNA polymerase assays containing approx. 10 µg of chromatin DNA. Free 5α-DHT (100 µl of a 4 nM solution) was added in 120 mM Tris buffer, pH 7.9, which was also added to standard assays to attain constant volume. Spleens were removed from rats 24 h after castration.

<table>
<thead>
<tr>
<th>Source of chromatin</th>
<th>Additions to assay</th>
<th>RNA polymerase activity (fmol [³H]UMP incorp./100 µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human hypertrophied prostate</td>
<td>None</td>
<td>911 ± 30</td>
</tr>
<tr>
<td></td>
<td>5α-DHT</td>
<td>942 ± 32</td>
</tr>
<tr>
<td></td>
<td>Human prostate-cytosol</td>
<td>1040 ± 20</td>
</tr>
<tr>
<td></td>
<td>Human prostate cytosol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 5α-DHT</td>
<td>1190 ± 38</td>
</tr>
<tr>
<td></td>
<td>Rat spleen cytosol</td>
<td>868 ± 42</td>
</tr>
<tr>
<td></td>
<td>Rat spleen cytosol + 5α-DHT</td>
<td>887 ± 22</td>
</tr>
<tr>
<td></td>
<td>Human serum</td>
<td>895 ± 33</td>
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<td></td>
<td>Human serum + 5α-DHT</td>
<td>965 ± 53</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>None</td>
<td>984 ± 30</td>
</tr>
<tr>
<td></td>
<td>5α-DHT</td>
<td>980 ± 67</td>
</tr>
<tr>
<td></td>
<td>Rat spleen cytosol + 5α-DHT</td>
<td>955 ± 70</td>
</tr>
<tr>
<td></td>
<td>Human prostate cytosol</td>
<td>960 ± 36</td>
</tr>
<tr>
<td></td>
<td>Human prostate cytosol + 5α-DHT</td>
<td>1012 ± 55</td>
</tr>
</tbody>
</table>

Human prostatic cytoplasmic [³H]5α-DHT–receptor complexes can enter nuclei of normal (Mainwaring and Milroy, 1973) and hypertrophied (Davies and Griffiths, 1975) prostate. The uptake of [³H]5α-DHT–receptor complexes by chromatin preparations from human hypertrophied prostate was more rapid at 37°C than at 0°C (fig. 4a), although dissociation also appeared quicker at the higher temperature. The process was steroid-specific (fig. 4d) and essentially tissue-specific for cytosol (fig. 4b) and chromatin (fig. 4c). It should be stressed that although the uptake of [³H]steroid by a particular chromatin preparation was naturally dependent upon the amount of [³H]steroid–receptor added in the form of a given preparation, it was not possible to predict the amount of steroid which could be bound by another
Fig. 5. Template activity of prostate chromatin. Template activity of human prostate chromatin and human prostate DNA was determined by titrating increasing quantities of template (1–250 μg of DNA/assay) against a fixed quantity of RNA polymerase (26.8 μg of protein/assay) and assessing incorporation of [3H]UMP in the usual manner (see Methods). (a) Template activity of human prostate DNA ('deproteinised' chromatin) (○), and of two representative human prostate chromatin preparations, one of high template activity (●) and one of relatively low template activity (■). (b) Human prostate chromatin was incubated with an excess of 5α-DHT–protein complexes, resedimented and used for determination of template activity. Studies were performed before (●) and after (○) incubation with steroid–protein complexes. All chromatin preparations were devoid of endogenous RNA polymerase activity.

chromatin preparation from these data. There was no reproducibility between one cytosol–chromatin system and another.

There is abundant evidence to indicate that association of 5α-DHT–receptor complexes with rat prostatic chromatins results in increased transcription of these chromatins by RNA polymerase (Davies and Griffiths, 1973b, 1974; Mainwaring and Jones, 1975). Preliminary studies have also suggested that this occurs in the human hypertrophied prostate (Davies, 1975).

Incubation of partially purified steroid–receptor complexes with preparations of chromatin from human hypertrophied prostate brought about an increase in the template activity of the chromatins when transcribed by RNA polymerase B. The amount of chromatin DNA required to saturate a given quantity of enzyme (26.8 μg protein) compared to deproteinized prostatic DNA ranged from 10 to 35 (fig. 5a). In most cases, the association of steroid–receptor complexes with chromatin resulted in a reduction in the amount of chromatin DNA required to saturate the same amount of enzyme (fig. 5b). This reduction varied between 20 and 40%. Samples of human hypertrophied prostate which contained no accessible specific binding sites for 5α-DHT, invariably had higher template activities than those which had such sites and which exhibited cytoplasmic to nuclear transfer of [3H]-steroid. The template activity of chromatin varied directly with the nonhistone content of the preparation and inversely with the histone content (table 2). With a number of specimens, however, there was no correlation between the amount of steroid–receptor complex which was taken up by the chromatins and their
Fig. 6. Influence of association of 5α-DHT with chromatin on its transcription by RNA polymerase. Chromatin was prepared from human prostate glands in such a way as to be devoid of RNA polymerase activity (a) or to have significant endogenous RNA polymerase (b). Samples of chromatin were used to measure uptake of partially purified [3H]5α-DHT–receptor complexes, added in various amounts. Concurrently, samples of chromatin were incubated with samples of the same cytosol but including nonlabelled 5α-DHT. These latter chromatin preparations were resedimented and their transcription by excess exogenous RNA polymerase B (a) or by endogenous RNA polymerase (b) was measured. In (a) and (b), uptake of [3H]5α-DHT (●) and transcription (incorporation of [3H]UMP; ○) are shown.

change in template activity. This may suggest that not all the complexes were involved in the modification of transcription characteristics.

The influence of steroid–protein complexes on transcription by endogenous RNA polymerases was also investigated. It can be seen (table 3) that the activity of chromatin-associated, α-amanitin-sensitive, RNA polymerase was increased after incubation with a saturating concentration of steroid–receptor complexes. The process was highly specific, being dependent upon human prostatic preparations and could not be reproduced using either human serum preparations or chromatin from rat spleen. In individual specimens, the stimulation of RNA polymerase activity closely followed the uptake of steroid–receptor into chromatin and similar dose–response curves could be obtained using chromatin transcribed by endogenous or exogenous form B RNA polymerase (fig. 6).

DISCUSSION

These studies indicate the presence of two major forms of DNA-dependent RNA polymerase in human hypertrophied prostate tissue. Although such criteria as metal ion dependence and ionic strength optima cannot be considered as invariant charac-
teristics of RNA polymerase species (Gissinger et al., 1974), these properties of the two forms under controlled conditions, as well as their relative sensitivities to α-amanitin, resemble those of other mammalian RNA polymerases (Jacob, 1973). RNA polymerase A, being α-amanitin-insensitive, corresponded to activity from the nucleolus, catalyzing ribosomal RNA synthesis, and RNA polymerase B, being sensitive to the toxin, corresponded to activity from the extranucleolar regions, synthesizing DNA-like RNA (Blatti et al., 1970). The fact that these enzymes did not react completely in one way or another to α-amanitin is difficult to explain, but cannot be attributable to the presence of a third RNA polymerase entity with intermediate sensitivity, since further inhibition could not be brought about by much higher concentrations of α-amanitin (Weil and Blatti, 1975). Both polymerases synthesized RNA using a naked DNA template, but only RNA polymerase B could effectively use a chromatin template. Transcription of chromatin by RNA polymerase A in vivo may be a more complex procedure than for RNA polymerase B, or may be dependent upon factors lost from either the chromatin or the enzyme during the purification procedure. Certainly the quality of transcription in vitro by RNA polymerases appears to be dependent, to a considerable extent, upon the method of preparation of the template (Flint et al., 1974; de Pomerai et al., 1974). Moreover, observations that under certain conditions, nucleolar RNA synthesis may be influenced by prior extranucleolar synthesis (Jacob et al., 1970; Schmid and Sekeris, 1973; Borthwick and Smellie, 1975), suggest the necessity of nucleoplasmic transcription simultaneously with nucleolar transcription.

According to Gissinger et al. (1974), salt stimulation of RNA polymerase A is confined to the initiation step, while both initiation and elongation of RNA chains by RNA polymerase B may be affected by salt. Thus, at low salt concentrations in which initiation can occur, both enzymes were stimulated slightly, whereas at higher salt concentrations which prevent initiation (Cedar and Felsenfeld, 1973), RNA polymerase A was inhibited, while RNA polymerase B activity was greatly increased, especially under conditions of ionic strength which would cause dissociation of chromosomal proteins. Chromatin-associated RNA polymerase activity was enhanced greatly by high salt concentrations, and was sensitive to α-amanitin but insensitive to rifamycin AF/013, thus identifying it as pre-initiated RNA polymerase B activity.

The presence of proteins specifically binding 5α-DHT in cytoplasmic fractions prepared from human hypertrophied prostate was shown by both sedimentation analysis and the demonstration of saturable high-affinity binding of the steroid in both whole cytosol and cytosol fractionated by 33% saturation with ammonium sulphate. Although contamination of such specimens by serum proteins can undoubtedly occur and is difficult to preclude, receptor proteins can be demonstrated by the techniques described. Problems concerned with nonspecific binding may be eliminated by the development of synthetic steroids specifically associating with receptors (Bonne and Raynaud, 1975). However, there will always remain a major disadvantage of studying tissues with high levels of endogenous steroid and contain-
ing receptor proteins not amenable to experimentation at temperatures conducive to steroid-exchange techniques. An inability to achieve reproducibility between different specimens of human prostate will restrict investigations to subcellular components derived from one specimen.

In this study, increasing concentrations of steroid—receptor complexes stimulated transcription of one particular chromatin preparation. The same concentration of steroid—receptor complexes, however, did not produce the same quantitative response in transcription of a different prostate chromatin preparation. It is envisaged that a difference in accessible cytoplasmic receptor sites may be reflected in variations in accessible nuclear sites, template activity and the response of templates to stimulation by steroid—receptor complexes. This is supported by the results show in table 2 and fig. 5. The range of response to template activity to varying uptake of steroid—receptor complexes by chromatin preparations also indicates the presence of non-specific binding sites in the template or that superfluous steroid—receptor complexes may bind to chromatin without causing any response.

The transfer of 5α-DHT into human prostate chromatin in cell-free systems is, however, extremely specific as regards steroid and cytosol. Only 5α-DHT, of the steroids tested, was transferred into chromatin. Cytosol fractions from human or rat prostate are required, and chromatin from non-target tissues did not readily accept the steroid. The association of steroid—receptor complex with chromatin appeared to modify its structure in such a way as to increase its template activity. Several possibilities exist as to how this is brought about (Mainwaring and Jones, 1974), such as displacement of chromatin-associated proteins, unwinding of DNA or facilitated movement of RNA polymerase along transcribable regions of DNA. Preliminary results suggest that the effects are biased towards increased elongation of RNA chains, in a similar manner to that observed in rat ventral prostate (Mainwaring and Jones, 1974; Davies, 1975) and this is supported by the parallel effects on preinitiated chromatin-associated RNA polymerase. In view of the effects observed in other steroid-hormone-dependent systems (Schwartz et al., 1975), it is by no means impossible that a combination of effects is involved. However, these problems may not be clarified until a greater understanding of initiation and elongation of RNA synthesis and of the association of steroid—receptor complexes with the regions of the genome is achieved.

ACKNOWLEDGEMENTS

The authors are extremely grateful to Mr. D.G. Morris and Mr. R.W. Rees, University Hospital of Wales, Heath Park, Cardiff, and Mr. W.B. Peeling, St. Woolo’s Hospital, Newport, Gwent, for their kind co-operation in providing specimens of human hypertrophic prostatic tissue, and to Professor L.G. Silvestri and Dr. R. Cricchio of Gruppo Lepetit, Milano, Italy for their gift of rifamycin AF/013. They would also like to thank the Tenovus Organization for their generous financial support.
REFERENCES

THE INFLUENCE OF STEROID-RECEPTOR COMPLEXES ON THE STAGES OF TRANSCRIPTION OF TARGET-TISSUE CHROMATIN

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Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, CF4 4XX, Wales

SUMMARY

Earlier investigations established that complexes formed between 5α-dihydrotestosterone and receptor macromolecules derived from rat ventral prostate subcellular fractions cause changes in the nature and extent of transcription of prostate chromatin by homologous RNA polymerases. The present report describes an investigation into relationships between concentrations of cellular receptor sites and initiation and elongation of RNA chains on prostatic chromatin from rats after castration or after androgen administration to castrated animals. Occupied receptor sites were measured by precipitation with progesterone sulphate and replacement of endogenously bound steroid with exogenous [3H]-steroid. Initiation of RNA chains was measured by incorporation of [γ-32P]-purine nucleotides and chain elongation by incorporation of [3H]-UTP under conditions which precluded reinitiation i.e. in the presence of rifamycin AF/013 or under high salt conditions. Castration resulted in a marked depletion of cellular receptors, particularly nuclear sites, and a decrease in initiation sites on chromatin. Testosterone propionate, depending on the time of administration, partially or completely maintained the nuclear content of receptor, but not cytoplasmic receptor. Androgen increased initiation sites above levels observed on prostatic chromatin of castrated rats. RNA chain length appeared dependent on androgenic status, but other reasons for this effect are discussed.

INTRODUCTION

The ventral prostate gland of the rat is a steroid-hormone target organ, as shown by its dependence upon an adequate supply of androgens for maintenance of its growth and function [see reviews 1, 2]. The intracellular localisation of the hormonal steroid and fulfillment of its postulated function within the cell are apparently dependent upon the preliminary formation of a cytoplasmic complex between the steroid and receptor proteins [1-3]. Studies in this laboratory [4-7] and elsewhere [8, 9] indicate that complexes formed in rat ventral prostate subcellular fractions between 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and proteins of selective high affinity profoundly influence the transcripive processes of the cell. Since changes in template activity can be due to alterations in any one of the many events involved in production of RNA chains [10], it is now necessary to separate the stages of transcription and observe steroid-receptor-induced effects on each stage individually. Moreover, if the steroid hormone–receptor complex is intimately or directly involved at the level of transcription, as previous studies suggest, the number or concentration of receptor sites within the cell should be in some way related to the amount or quality of transcription. This present communication serves as an introduction to a more detailed study of the interrelationship of receptors and transcription. Information is presented concerning the number of cytoplasmic and nuclear 5α-dihydrotestosterone (DHT)-receptor complexes and the initiation and elongation of RNA chains on rat ventral prostate chromatin at various times after castration and administration of androgen to castrated rats.

EXPERIMENTAL

Chemicals

1,2,4,5,6,7(α)-[3H]-5α-Dihydrotestosterone (specific radioactivity 107–130 Ci/mmol), [5-3H]-UTP (specific radioactivity 10.1 Ci/mmol), [γ-32P]-ATP (reference date specific radioactivity 15–17 Ci/mmol) and [γ-32P]-GTP (reference date specific radioactivity 13–16 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks, U.K. The disodium salt of ATP and the trisodium salts of CTP, GTP and UTP were purchased from the Boehringer Corporation (London) Ltd., Ealing, London, U.K. Protamine sulphate (from salmon roe) was obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Crystalline BSA, calf-thymus DNA, dithiothreitol, Tris-base (99% pure) and 5α-dihydrotestosterone were bought from the British Drug Houses, Poole, Dorset, U.K. Other unlabelled steroids and diethylstilbestrol were purchased from either Koch-Light Laboratories, or Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Rifamycin AF/013 was generously provided by Dr. R. Cricchio and Professor L. G. Silvestri of Gruppo Lepetit, Milan, Italy. All other substances were of ‘Analar’ grade and were dissolved in water distilled from all-glass apparatus.
Animals and tissues

Male Sprague–Dawley rats were bred in the Institute Animal Unit and used when 8–12 weeks old. Animals were eutropated or castrated (400 µg/100 g body weight, subcutaneously, beginning immediately, 24 h or 48 h after operation. Ventrall prostate tissue was carefully dissected from animals killed when required (see ‘Results’), placed in vessels surrounded with crushed ice and used immediately for the preparation of subcellular fractions (see below).

Centrifuges and rotors

All high-speed centrifugation procedures were carried out in a Beckman L2-65B or L5-65B ultracentrifuge using either the SW50.1 (6 × 5 ml) swinging-bucket rotor (r, 8.35 cm) or the SW60 (6 × 4 ml) swinging-bucket rotor (r, 9.17 cm).

Preparation of subcellular fractions

Methods for the preparation of soluble supernatants, nuclei and chromatin devoid of endogenous RNA polymerase activity have been previously described [4].

Measurement of receptor sites

(a) Cytoplasmic sites. Those receptor sites present in soluble supernatant (cytosol) fractions which were unoccupied by endogenous steroid were estimated by incubation (2 h at 0–4°C) of aliquots (100 µl) of cytosol (7–12 mg protein/ml) with equal vol. of [3H]-DHT (2–25 nmol/l) with and without a 100-fold higher concentration of unlabelled DHT. Free and some nonspecifically bound steroid were removed by mixing with 400 µl of charcoal suspension (2% (w/v) Norit A, 0.2% (w/v) Dextran T-70) for 15 min. Charcoal was sedimented at 800 g for 10 min, and radioactivity present in the supernatant was assessed by liquid scintillation counting. Total (occupied and unoccupied) sites were assessed by a method dependent upon prior precipitation of receptor sites by protamine sulphate. Protamine sulphate (100 µl of 1 mg/ml solution) was added to equal vol. of cytosol and precipitates were washed 5 times with 1 ml of buffer. Precipitates were then dispersed in [3H]-steroid solution (see above) at various temperatures (usually 15°C, see ‘Results’) for various periods of time (usually 16 h, see ‘Results’). After incubation, precipitates were re-sedimented, washed 6 times in ice-cold buffer, extracted twice with ethanol (1 ml), the combined extracts evaporated in scintillation vials, and radioactivity assessed as above.

(b) Nuclear sites. The number of receptor sites present in purified nuclei [4, 7] was estimated using an exchange assay. Protamine sulphate (100 µl of 1 mg/ml solution) was added to equal vol. of nuclear suspension (30–100 µg of DNA). Resulting precipitates were washed, incubated with [3H]-steroid, washed and extracted as described above for total cytoplasmic sites.

Purification of rat ventral prostate RNA polymerase B

RNA polymerase B was solubilised from rat ventral prostate nuclei and purified in a similar manner to published procedures [11–14], involving sonication at high ionic strength, selective precipitation of contaminating nuclear acid with protamine sulphate, fractionation with ammonium sulphate, and chromatography on columns of DEAE-cellulose and phosphocellulose.

Estimation of RNA polymerase activity

Routine assessment of RNA polymerase B activity was carried out in a reaction mixture (500 µl) containing Tris–HCl buffer (120 mmol/l), pH 7.9, KCl (30 mmol/l), dithiothreitol (400 µmol/l), NaF (600 mmol/l), MnCl2 (3 mmol/l), ATP, GTP and CTP (all 600 mmol/l), [3H]-UTP (0.5 µCi), UTP (40 µmol/l) and glycerol (10% (v/v)). DNA template (10–25 µg) and RNA polymerase (4–20 µg protein) were added in 100 µl and 50 µl respectively. Certain assays contained (NH4)2SO4 (40 mmol/l) and aminopterin (40 ng/assay). Incubations (15 min at 37°C) were terminated and acid-insoluble material processed and assessed for incorporated radioactivity as described previously [4, 6].

Certain modifications were necessary to study separately RNA chain initiation and elongation. This was done in either of two ways. (a) By use of rifampicin AF/013. The final concentrations of reactants was as described above. RNA polymerase was preincubated (15 min at 37°C with template in the absence of nucleoside triphosphates, which were then added together with rifampicin AF/013 (50 µg/assay). As well as [3H]-UTP, this mixture contained either [γ-32P]-ATP or [γ-32P]-GTP (0.5 µCi in each case). Incubation was continued for a further 15 min, then terminated and material processed as above. (b) By initiation in low salt followed by elongation in high salt. RNA polymerase was preincubated (15 min at 37°C) in a mixture (270 µl) containing the same concentrations of substances as in the routine assay, including labelled nucleoside triphosphates, but with MnCl2 at 1 mmol/l and excluding CTP. The initiation reaction was stopped and reinitiation prevented by addition of 85 µl of (NH4)2SO4 (1.6 mol/l) and chain propagation begun by addition of MnCl2 (final concentration 3 mmol/l) and CTP. Incubation and processing was carried out as for the routine assay. All assays performed under any of these regimes were corrected for zero-time controls, controls in the absence of DNA, and incorporation of radioactivity in the absence of enzymes.

Chemical analyses

Standard procedures were employed for the determination of DNA [15], using calf-thymus DNA as standard, and protein [16] using crystalline BSA as standard.
RESULTS

Estimation of receptor sites in cytoplasmic and nuclear preparations

Difficulties have been experienced in attempting to demonstrate rat ventral prostate DHT-receptor binding by conventional Dextran-coated charcoal techniques [see 17], but certain success has been reported [18-21]. Using the protocol described above (see 'Experimental'), it was possible to show specific accessible binding sites for exogenous \(^{3}H\)-DHT, including a high-affinity saturable component with a dissociation constant \((K_d)\) of 1-5 nmol/l. Full information will be published elsewhere. However, measurement of \(^{3}H\)-DHT replacement of endogenous DHT occupying receptor sites requires elevated temperature and prolonged incubation time. Thermolability of receptor sites precludes such an assay being performed with receptor free in solution. Precipitation of receptor by protamine sulphate [17] conferred greater stability on the receptor and allowed an exchange assay to be performed.

As shown in Fig. 1a, negligible exchange occurred at 0-4°C, while at higher temperatures (15°C and 25°C) the exchange process was more rapid. At 15°C, a plateau was reached after 12 h extending to 24 h of incubation. Increased temperature (25°C) increased the exchange rate, reaching a maximum after 4 h. Further elevation of the temperature to 30°C or 37°C (data not shown) resulted in rapid degradation of receptor making these temperatures unsuitable for the assay. For convenience and because of better reproducibility the assay was routinely employed at 15°C for 16 h. These conditions combined maximum replacement with \(^{3}H\)-steroid with minimal loss of binding capacity. Specific binding of \(^{3}H\)-DHT under these conditions, determined by bound radioactivity in the absence and presence of a 100-fold higher concentration of unlabelled DHT, was saturable, and in the cytosol preparations used in these studies, had an apparent \(K_d\) of 2-10 nmol/l.

The number of receptor sites in prostate nuclei was measured by a similar method. Originally, \(^{3}H\)-steroid was exchanged directly with endogenous steroid in purified nuclear preparations. This method was successful in that salt-extraction of nuclei previously incubated with \(^{3}H\)-steroid yielded a \(^{3}H\)-DHT-receptor complex of sedimentation coefficient 3.5-4.5 S when analysed on sucrose-density gradients (Fig. 2). The peak of radioactivity was depressed if incubations also contained unlabelled DHT, indicating low capacity for steroid and was characteristic

![Fig. 1. Effect of time and temperature on the \(^{3}H\)-DHT exchange assay for rat ventral prostate receptor. Aliquots (100 \(\mu\)l) of (a) cytosol (7-12 mg protein/ml) or (b) nuclei (30-100 \(\mu\)g DNA) were mixed with equal vol. of protamine sulphate solution (1 mg/ml), and resulting precipitates were sedimented and washed. Precipitates were incubated with \(^{3}H\)-DHT (20 nmol/l) alone, or with \(^{3}H\)-DHT plus 100-fold excess of unlabelled DHT at various temperatures. At indicated times, incubations were stopped and the amount of specifically bound \(^{3}H\)-DHT determined as described in 'Experimental'. Each value represents the mean of three determinations.](image_url)

![Fig. 2. Sedimentation analysis of nuclear \(^{3}H\)-DHT-receptor complex. Nuclei were prepared from the ventral prostate glands of normal intact rats and aliquots (25-50 \(\mu\)g DNA) were incubated under exchange conditions (without protamine sulphate precipitation) with \(^{3}H\)-DHT (20 nmol/l) (O) or with \(^{3}H\)-DHT plus 100-fold excess of unlabelled DHT (●). Nuclei were sedimented and washed 5 times with ice-cold buffer, and extracted with KCl (0.4 mol/l) for 30 min at 0-4°C with continual stirring. Debris was sedimented at 100,000 \(g_{av}\) for 30 min, and samples (400 \(\mu\)l) of extract were layered over linear 5 ml sucrose gradients (5-20\% w/v) containing an uniform concentration of KCl (0.5 mol/l). Gradients were centrifuged and fractionated as described previously [4, 5] and the sedimentation coefficient of nuclear receptor protein based on the position of radioactivity peaks calculated with reference to sedimentation markers.](image_url)
of specific binding [4, 5]. However, as with cytoplasmic sites, nuclear receptor was extremely susceptible to thermal degradation, resulting in rapid loss of binding. At the lowest temperature used (15°C) it was not possible to observe receptor levels similar to those obtained in vivo using animals of similar age and hormonal status, or reproducibility regarding optimal time and temperature for the assay. Therefore, precipitation of nuclear receptor prior to incubation was necessary. Under these conditions (Fig. 1b), exchange was minimal at 0–4°C, rapid but to an inconsistent level at 25°C and higher temperatures, but amenable to assay at 15°C. Saturation analysis of DHT binding by nuclei showed that the amount of [3H]-steroid specifically retained was a saturable function (Fig. 3a), representing a single class of binding sites with an apparent Kd in the region 4–5 nmol/l (Fig. 3b). These results show the presence of low-capacity, high-affinity binding sites for DHT in rat ventral prostate nuclei.

Nuclear binding sites in rat ventral prostate show stereospecificity in that oestradiol, diethylstilboesterol, progesterone and cortisol did not compete with [3H]-DHT for these sites, whereas unlabelled DHT significantly depressed the amount of bound radioactivity (Table 1). As expected if DHT-binding sites are due to specific receptor, they occur only in androgen target tissues (Table 2). Ventral prostate and seminal vesicle nuclei contain significant numbers of binding sites while rat liver, spleen and skeletal muscle do not.

Effects of androgen deprivation and restoration on cellular receptor sites

The concentration of intracellular DHT-binding sites in rat ventral prostate was studied in the intact rat, at 24 h, 48 h, and 72 h after castration, and in the glands of castrated animals which were left untreated for 72 h, or received daily doses of testosterone propionate beginning immediately or 24 h or 48 h after orchidectomy.

Not unexpectedly, level of cytoplasmic and nuclear binding sites decreased markedly after castration (Table 3). Total numbers of cytoplasmic receptors had fallen by 50%, 24 h after orchidectomy, by 48 h had decreased to 30% of initial levels, and 72 h after castration the level was 15% of that in prostate cells of intact animals. During this period, accessible cytoplasmic sites increased in number, and then declined. When expressed as a percentage of total sites, those unoccupied by endogenous steroid were increased with longer post-castration times. The number of nuclear receptor sites had decreased by 70%, 24 h after castration and by over 90%, 72 h after castration.

Administration of testosterone propionate to orchidectomised rats maintained levels of nuclear receptor if given immediately or 24 h after castration in daily doses (Table 4). A single dose of androgen given 48 h after castration raised the level of nuclear receptor to 200% of that observed in the castrated control. Androgen did not affect cytoplasmic sites so dramatically. Over 72 h of treatment, cytoplasmic receptor increased to 300% of control (castrated) levels and the percentage of sites accessible decreased to below 10%, but levels did not attain those observed in non-castrated animals. This lower reaction of cytoplasmic receptor to androgen was obviously reflected in total cellular receptor sites which in 3 day-treated castrated rats were lower than those levels seen in prostates of non-castrated animals.

Table 1. Steroid specificity of nuclear DHT-receptor complex. Rat ventral prostate nuclei from intact rats were incubated for 16 h at 15°C with [3H]-DHT (20 nmol/l) alone or in the presence of a 100-fold excess of indicated unlabelled steroids and the total amount of bound radioactivity determined at the end of incubation

<table>
<thead>
<tr>
<th>Competitor</th>
<th>d.p.m./100 μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18141 ± 664</td>
</tr>
<tr>
<td>DHT</td>
<td>5194 ± 327</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>17699 ± 412</td>
</tr>
<tr>
<td>Diethylstilboesterol</td>
<td>18997 ± 771</td>
</tr>
<tr>
<td>Progesterone</td>
<td>17401 ± 964</td>
</tr>
<tr>
<td>Cortisol</td>
<td>19532 ± 1385</td>
</tr>
</tbody>
</table>

Table 2. Tissue specificity of nuclear DHT-receptor complex. Nuclei were prepared from the indicated tissues of normal intact rats [6] and were incubated under optimal conditions of exchange with [3H]-DHT (20 nmol/l) and total amount of bound radioactivity was determined.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>d.p.m./100 μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>20390 ± 894</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>16085 ± 1592</td>
</tr>
<tr>
<td>Liver</td>
<td>2295 ± 270</td>
</tr>
<tr>
<td>Spleen</td>
<td>3206 ± 331</td>
</tr>
<tr>
<td>Muscle</td>
<td>1404 ± 406</td>
</tr>
</tbody>
</table>
Table 3. Concentrations of receptors after castration. Subcellular fractions were prepared from the ventral prostate glands of intact rats or from glands of rats castrated 24 h, 48 h and 72 h prior to experimentation. Total and accessible cytoplasmic DHT-binding sites and nuclear DHT-binding sites were determined as described in 'Experimental'.

<table>
<thead>
<tr>
<th>Days after castration</th>
<th>Total receptor sites</th>
<th>Cytoplasmic Receptor sites</th>
<th>Nuclear receptor sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Accessible</td>
<td>Total</td>
</tr>
<tr>
<td>0</td>
<td>31771</td>
<td>18445</td>
<td>3282(18)*</td>
</tr>
<tr>
<td>1</td>
<td>13538</td>
<td>9803</td>
<td>6600(67)</td>
</tr>
<tr>
<td>2</td>
<td>7544</td>
<td>6215</td>
<td>5014(81)</td>
</tr>
<tr>
<td>3</td>
<td>3960</td>
<td>3140</td>
<td>2769(88)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate accessible receptor as a percentage of total receptor.

**Effects of castration and androgen administration on RNA chain initiation and elongation on rat ventral prostate chromatin**

Conventional analysis of chromatin template activity was carried out using saturating concentrations of ventral prostate RNA polymerase B and nucleotides, and showed that incorporation of precursors into RNA was proportional to the amount of template using a fixed amount of polymerase until a titration point or plateau is reached. Homologous RNA polymerase B was used since previously it was observed that bacterial (E. coli) RNA polymerase transcribed prostate chromatin with different characteristics to prostate enzyme B [4] and may not respond to hormonal stimuli [8]. Preliminary results (Thomas and Davies, unpublished) showed that ventral prostate chromatin from young mature intact rats contained one binding site for homologous RNA polymerase B per 6000 nucleotide pairs and one initiation site per 120,000 nucleotide pairs. This suggests that 20 RNA polymerase molecules may be bound to chromatin for each RNA chain initiated.

Initiation of RNA chains on rat ventral prostate chromatin by homologous RNA polymerase B was measured by monitoring incorporation of [3H]-precursors under conditions precluding reinitiation during chain propagation. Elongation of RNA chains was measured by incorporation of [3H]-UMP derived from [3H]-UTP. Under the conditions of assay employed (see 'Experimental'), the preincubation times was sufficient to ensure initiation at all specific sites on chromatin, and incubation in the presence of rifampicin or high-salt was sufficiently long to allow completion of chain propagation.

The number of initiation sites on ventral prostate chromatin decreased after castration (Fig. 4), falling from over 10,000 sites per pg DNA in prostate of intact animals to 6000 sites per pg DNA in prostate of rats castrated 3 days previously. Administration of androgen to castrated rats did not completely maintain the level of initiation sites, although the number was increased over longer treatment time (Fig. 4). Interestingly, androgenic status apparently influenced prostate RNA chain length, since longer chains were synthesised from prostate chromatin from intact animals, or animals receiving an extended supply of androgen, than from prostate chromatin of animals totally deprived of or receiving a more limited dosage of androgen (Fig. 5). In this respect, it is noteworthy that preliminary results showed that the initial rate of elongation of RNA chains off intact-rat ventral prostate chromatin was 10 nucleotides/s, while that off prostate chromatin of 3-day castrates was 7 nucleotides/s. However, under the conditions of assay, RNA chain elongation had ceased in all cases.

Table 4. Concentrations of receptor after androgen administration to castrated rats. Groups of 6 or 7 rats were castrated and left untreated for 72 h, or received daily doses of testosterone propionate (see 'Experimental') beginning immediately or 24 h or 48 h after operation. Animals not receiving the androgen were injected with vehicle alone. Subcellular fractions were prepared from ventral prostate glands of those rats, and the numbers of receptor sites present in these factors were assessed as described in 'Experimental'.

<table>
<thead>
<tr>
<th>Days of androgen administration</th>
<th>Total receptor sites</th>
<th>Cytoplasmic Receptor sites</th>
<th>Nuclear receptor sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Accessible</td>
<td>Total</td>
</tr>
<tr>
<td>0</td>
<td>4306</td>
<td>2317</td>
<td>2129(92)*</td>
</tr>
<tr>
<td>1</td>
<td>7626</td>
<td>3366</td>
<td>1717(51)</td>
</tr>
<tr>
<td>2</td>
<td>18823</td>
<td>4734</td>
<td>3010(64)</td>
</tr>
<tr>
<td>3</td>
<td>21029</td>
<td>7096</td>
<td>583(8)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate accessible receptor as a percentage of total receptor.
Fig. 4. Concentration of nuclear receptors and RNA initiation sites on chromatin after castration and androgen administration to castrated rats. Experiments were carried out on 8 groups of 6 or 7 rats, which were (a) sham-castrated or castrated 24 h, 48 h or 72 h before use; and (b) castrated and left untreated for 72 h, or receiving androgen (see 'Experimental') in daily doses commencing immediately, or 24 h, or 48 h after operation. Animals not receiving androgen were injected with vehicle alone. The concentration of nuclear receptor sites was determined using the $[^3H]$-DHT exchange assay (see 'Experimental') and initiation sites on chromatin for RNA synthesis were estimated by incorporation of $[^3H]$-purine nucleotides.

**DISCUSSION**

This report describes preliminary investigations into possible relationships between cellular receptor levels and transcriptional ability of the rat ventral prostate cell under differing conditions of androgenic status. This involved the development of methods to measure receptor sites, based on the experiences of others.

Certain considerations complicate the quantitation of prostate specific DHT-binding [17]. Dextran-coated charcoal techniques have been developed [18-21], but studies have been directed towards the separation of specific from nonspecific components by preferential precipitation, either by ammonium sulphate [19,22] or protamine sulphate [17]. The latter method has been used to separate specifically bound DHT for assessment by radioimmunoassay [17], and has now been used to measure occupied receptor by exchange assay. The method is suitable for the estimation of receptor in cytoplasm and nuclei, and apparently measures steroid- and tissue-specific sites, although improvements for the complete elimination of nonspecific binding [17] could be incorporated.

Depletion of nuclear [17] and cytoplasmic [23-25] receptor at various times after castration has been reported and confirmed using the exchange assay. This depletion has been attributed to increased proteolytic activity [24], although decreases have been observed before the onset of proteolysis [17]. Restoration or maintenance of varying levels of receptor by differing dosages of androgen [25, and above] may therefore be accounted for by inhibition of proteolytic activity or by de novo synthesis of receptor under the influence of the hormonal steroid. Increase of receptors in the absence of protein synthesis [17] has led to conjecture concerning microsomal [26] or soluble [17] precursors.

Preferential accumulation of nuclear receptor after testosterone treatment (Table 4) may be due to rapid translocation of cytoplasmic receptor, activated by DHT [5,8], to the nucleus. If this occurs, then although total receptor is diminished, concentration of receptor within the nucleus might be sufficient to stimulate cellular processes during continued replenishment of cytoplasmic receptor. However, certain observations [27,28] that nuclear receptor can arise without intervention of cytoplasmic receptor suggest

Fig. 5. Concentration of nuclear receptors and elongation of RNA chains after castration or androgen administration to castrates. Groups of rats were used as described in the legend to Fig. 4. During the experiment, the number-average length of polynucleotide chains synthesised by previously initiated RNA polymerase under conditions precluding reinitiation was calculated from incorporation of $[^3H]$-UMP from $[^3H]$-UTP assuming that UMP comprises 28% of RNA chains.
that androgen dependent maintenance of cytoplasmic and nuclear receptor may be independent of each other. Nevertheless, there are indications that cytoplasmic receptor has physical properties suggestive of a precursor relationship to nuclear receptor [5], and comparatively low values of cytoplasmic receptor in androgen-treated animals may be the result of destruction of $^3$H]-DHT binding by residual proteolytic activity [27].

The major purpose of this investigation was to determine any existing link between receptor concentration and RNA initiation and elongation. DHT-receptor complexes influence the relative concentration of protein constituents and template availability of prostate chromatin [5]. Any modification of prostate chromatin by changing hormonal environment does not alter the numbers of binding sites for RNA polymerase B (Thomas and Davies, unpublished observations), so alterations in transcribable ability [5] should be due to an increased ability of the enzyme to initiate or propagate RNA synthesis.

Studies on the effect of oestradiol on gene expression in the chick oviduct [29, 30] report that the major influence of steroid is exerted towards the number of initiated RNA chains with no significant effects on the rate or extent of chain elongation, with a temporal correlation between nuclear-bound oestriadiol receptors and initiation sites for RNA synthesis. However, studies on the effects in vitro of DHT-receptor complexes on the transcription of prostate chromatin by homologous RNA polymerase have been shown to be directed toward chain elongation [7, 8]. The present investigation, however, after castration or androgen administration in vivo, show effects on chain initiation and elongation, indicating possible diverse actions of the steroid–receptor complexes. Obviously, from the maximum number-average chain lengths shown in Figs. 4 and 5, elongation in vitro is severely impaired. This is not due to nuclease action, but possibly to the loss of elongating factors during chromatin isolation [31]. It is difficult to envisage an increasing loss of elongating factors reducing chain length or rate of chain propagation with increased postcastration time unless these factors themselves are androgen-dependent, as described for prostate proteins with high affinity for DNA [32] including a DNA-unwinding protein [32, 33]. The existence of possible factors causing premature termination of RNA chains must also be taken into consideration.

To summarise, castration caused a decrease in ventral prostate receptor sites, chromatin sites for RNA chain initiation, and also the length of RNA chains. Androgen administration maintained all these parameters to some extent. While no stoichiometric relationship could be ascertained to exist between nuclear-bound receptor and RNA chain initiation or elongation, it is by no means unlikely that the association of receptor with chromatin produces fundamental effects at the transcriptional level. Whether the DHT-receptor complex acts by displacing chromatin-associated proteins so as to increase the number of available initiation sites, by increasing template activity by increasing unwinding of DNA, by inactivating spurious termination factors, or by a combination of all these processes, awaits a more detailed appraisal of the many interactions essentially involved in these events.

Acknowledgements—We wish to express our gratitude to the Tenovus Organisation for their generous financial support.

REFERENCES


DISCUSSION

Jensen. When you speak of the number of nuclear sites decreasing after castration and being restored, I take it you do not mean what we call acceptor sites or the capacity to bind transformed complex but rather the actual number of androgen-receptor complexes that are translocated to the nucleus.

Davies. Yes, this is what I mean, that's what I was referring to.

Spelsberg. I just want to throw out a word of caution. Even though one has a ribonuclease inhibitor, you can't be sure that you are not cleaving any RNA. One or two cleavages can reduce the size of molecular weight tremendously. Secondly, did you analyze your chromatin after these incubations. For instance, histone integrity, etc. With long incubations at high temperatures, nuclease/protease action can be significant.

Davies. We only made preliminary observations on this, but under the conditions of assay we don't lose histones to any great extent.

Hamilton. I have two questions to ask you of a methodological nature, but I think you can forgive me for asking this because we are trying to do similar things with chromatin of uterus from the ovariectomized rat. I'd like to know how much DNA in the form of chromatin you add to each reaction vial in your measurement of initiation sites and then what is the percent of variation in replicate experiments?

Davies. We add about 10 micrograms. We try to get 10 pg exactly but it varies with chromatin DNA. We have an interassay variation about 10-12%.

McGuire. Your comments about the receptors disappearing rapidly after castration possibly partly being due to polymerase activity, have you taken any 0 and 3 day extract from cytoplasm and nuclei and mixed them and assayed? Have you done a classical mixing experiment?

Davies. No, we have not done that.
Initiation and Elongation of Polyrribonucleotide Chains on Rat Ventral-Prostate Chromatin Transcribed by Homologous Ribonucleic Acid Polymerase B

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The characteristics of initiation of RNA synthesis and the elongation of RNA chains on rat ventral-prostate chromatin by RNA polymerase B were investigated by two methods. 1. Initiation was carried out under low-salt conditions with three ribonucleoside triphosphates, and elongation was begun in the absence of reinitiation by the addition of the fourth ribonucleoside triphosphate and increasing the salt concentration. 2. Stable initiation complexes were formed by preincubation of enzyme with template at 37°C, elongation was started by the addition of all four ribonucleoside triphosphates and reinitiation or spurious RNA synthesis was prevented by rifamycin AF/013. The latter method gave more reliable results. The dependence of those parameters on the androgenic status of the animal was studied. During the first 24h after castration, elongation was mainly affected, whereas after 72h a smaller number of initiation sites for RNA polymerase B on chromatin was evident. Considerable diurnal variations in the various parameters were observed. Changes in the relative concentrations of the chromatin-associated proteins were also observed after castration. In the rat ventral-prostate gland androgenic steroids may not only influence one stage of the transcriptional process, but may affect many factors involved in the control of gene expression.

The regulation of macromolecular synthesis in the rat ventral prostate depends on the binding of androgens to intracellular proteins (King & Mainwaring, 1974; Liao & Liang, 1974; Minguell & Sierralta, 1975). Complexes formed between 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and a specific cytoplasmic macromolecule are removed to the nucleus, where they are retained for a finite period of time, probably by selective components (Mainwaring et al., 1976). Studies performed in vitro show a direct effect of androgen-receptor complexes on the transcription of prostate chromatin by RNA polymerase (EC 2.7.7.6; nucleoside triphosphate–RNA nucleotidyltransferase) (Davies & Griffiths, 1973a,b, 1974a,b; Davies, 1975; Mainwaring & Jones, 1975; Hu et al., 1975). Although these studies establish the general effects, certain drawbacks are evident (Mainwaring, 1975). Extensive purification of the androgen receptor and complete understanding of hormonal effects on the several stages of the transcriptional process are essential. Measurement of template activity reflects the amount of DNA in chromatin available to RNA polymerase but, as Tsai et al. (1975) emphasized, the complexity of the process renders precise understanding of the mechanism of hormone action difficult. Template activity may be modified by (a) the binding to chromatin of RNA polymerase, (b) its dissociation and reassociation until formation of stable initiation complexes with DNA, (c) the initiation of RNA synthesis, (d) the rate of elongation of RNA chains and (e) termination and reinitiation of RNA synthesis. Each process should be studied without interference from the others.

The object of this work was to establish experimental conditions for the measurement of initiation sites for RNA polymerase on rat ventral-prostate chromatin and for the determination of the initial rates of elongation, and absolute lengths, of RNA chains. The application of these techniques to a study of androgen effects on transcription is described.

Methods and Materials

Experimental animals

Male Sprague–Dawley rats were bred in the Animal Unit of the Tenovus Institute, and used when 8–12 weeks old. Animals were castrated via a median scrotal incision under ether anaesthesia at various times before death. Ventral-prostate tissue was carefully dissected from animals killed when required (see the Results section), placed in vessels surrounded
with crushed ice, and used immediately for the preparation of subcellular fractions.

**Chemicals**

- [5-3H]UTP (specific radioactivity 10–30Ci/mm), [5-32P]ATP (specific radioactivity 23Ci/mm), [γ-32P]GTP (specific radioactivity 11-16Ci/mm) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The disodium salt of ATP, the trisodium salts of CTP, GTP and UTP, and α-amanitin were purchased from Boehringer Corp. (London) Ltd., London, W.5. U.K. Crystalline bovine serum albumin, calf-thymus DNA, calf-thymus histone (type II-A), dithiothreitol, Triton X-100 and Tris base (99% pure) were from the BDH, Poole, Dorset, U.K. Rifamycin AF/013 was generously provided by Dr. R. Cricchio and Professor L. G. Silvestri of Gruppo Lepetit, Milan, Italy. All other substances were of ‘AnalaR’ grade (BDH) and were dissolved in water distilled from all-glass apparatus.

**Centrifuges and rotors**

High-speed centrifugation procedures were carried out in a Beckman L2-65B or L5-65B ultracentrifuge by using either the SW.50.1 (6 x 5ml) swinging-bucket rotor (rav. 8.35cm) or the SW.60 (6 x 4ml) swinging-bucket rotor (rav. 9.17cm).

**Preparation of subcellular fractions**

Nuclei were purified from homogenates of rat ventral-prostate tissue as previously described (Davies & Griffiths, 1973b). Chromatin devoid of endogenous RNA polymerase activity was prepared by the method of Mainwaring & Peterken (1971). Chromatin with associated enzyme activity was prepared as described by Butterworth et al. (1971). Preliminary chemical analysis of chromatin was performed as described by Marushige & Bonner (1966). In these studies prostate chromatin from normal animals had a protein/DNA ratio (± s.d.) of 2.4 ± 0.55:1 (histone/non-histone protein/DNA, 1.77 ± 0.49:0.54 ± 0.22:1).

**Purification of rat ventral-prostate RNA polymerase B**

RNA polymerase B was solubilized from rat ventral-prostate nuclei and purified by a method derived from a combination of published procedures (Roeder & Rutter, 1970; Mainwaring et al., 1971; Kedinger et al., 1972; Davies et al., 1975). This involved sonication at high ionic strength, selective precipitation of contaminating nucleic acid with protamine sulphate, fractionation with (NH₄)₂SO₄, and chromatography on columns of DEAE-cellulose and phosphocellulose.

**Determination of RNA polymerase activity**

(a) Routine assessment. RNA polymerase activity was assessed as a routine during purification procedures or to determine the specific activity of samples at various periods after preparation. The reaction mixture (500μl) contained Tris/HCl buffer (120mm), pH 7.9, KCl (30mm), dithiothreitol (400μm), NaF (600μm), MnCl₂ (3mm), ATP, GTP and CTP (all 600μm), [γ-32P]UTP (0.5μCi), UTP (40μm) and glycerol (10%, w/v). DNA template (5–60μg) and RNA polymerase (4–50μg of protein) were added in 100 and 50μl respectively. Certain assays contained (NH₄)₂SO₄ (40mm) and α-amanitin (40ng/assay). Incubations (20 min at 37°C) were terminated and acid-insoluble material was processed and assessed for incorporated radioactivity as described previously (Davies & Griffiths, 1973b, 1975).

(b) Determination of RNA synthesis from rifamycin-resistant sites. The method used for this study was based on that of Chesterton et al. (1974). RNA polymerase (4–50μg of protein in 100μl) was pre-incubated (15 min at 37°C) with template (5–60μg of DNA in 100μl), in a reaction mixture (250μl) containing Tris/HCl buffer (120mm), pH 7.9, KCl (60mm), dithiothreitol (800μm), NaF (1.2mm), MnCl₂ (6mm) and glycerol (10%, w/v). At the end of this period, a mixture (250μl) containing ATP or GTP, CTP (1.2mm), [γ-32P]UTP (0.5μCi, UTP (80μm), Tris/HCl buffer (120mm), pH 7.9, and rifamycin AF/013 (60μg) was added. In each tube, the respective unlabelled purine nucleoside triphosphate was replaced with [γ-32P]ATP or [γ-32P]GTP (0.5μCi in each case). Rifamycin AF/013 was dissolved in a minimal volume of dimethyl sulphoxide before being taken up in the correct volume of Tris/HCl buffer to give the required concentration. The incubation was allowed to proceed for a further 20 min at 37°C, then terminated and acid-insoluble material processed as described above.

(c) Measurement of initiation in low-salt followed by elongation in high-salt conditions. A method similar to that described by Cedar (1975) was used. The final concentrations of the components of the reaction mixture were as in method (b). RNA polymerase was preincubated (15 min at 37°C) in a mixture (270μl) containing the same reactants as in method (a), but with [γ-32P]ATP or [γ-32P]GTP as well as [γ-32P]UTP, and MnCl₂ (1mm) and without CTP. The initiation reaction was stopped by the addition of 85μl of (NH₄)₂SO₄ (at various concentrations; see the Results section), and chain propagation begun by the addition of MnCl₂ (final concentration 3mm) and CTP (final concentration 300μm). Incubation and processing was as for method (b) above.

All assays performed by any of these methods were corrected for zero-time controls, controls in the
absence of DNA and absence of enzymes. The product of the reaction was sensitive to ribonuclease digestion and to alkali (0.2M-NaOH), and the reaction was dependent on the presence of a DNA template and a bivalent cation (Mn²⁺), and was inhibited by α-amanitin. Variations in the above assay were introduced to check certain aspects (see the Results section). The initial rate of elongation was measured by termination of the reaction at various periods up to 80s of incubation. The number of initiation sites was calculated from [γ-³²P]ATP and [γ-³²P]GTP incorporated. The rate of elongation and RNA chain length were calculated from the amount of [³H]-UMP incorporated, assuming that UMP represents 25% of total ribonucleoside monophosphate present in RNA.

Measurement of template availability

The amount of DNA in a template that was available to RNA polymerase was estimated by a method similar to that described for routine assessment of RNA polymerase activity, except that the amount of DNA added to the assay was varied, but the quantity of enzyme remained constant.

Chemical analyses

DNA content of fractions was measured by the diphenylamine procedure of Burton (1956) with modifications described by Giles & Myers (1965), with calf-thymus DNA as standard. Protein content, including non-histone protein, was measured by the procedure of Lowry et al. (1951), with crystalline bovine serum albumin as standard. Histone was measured as described by Lowry et al. (1951), with calf thymus histone as standard.

Results

Inhibition of prostate RNA polymerase B by α-amanitin and rifamycin AF/013

RNA polymerase B of rat ventral prostate (Mainwaring et al., 1971; Davies & Griffiths, 1974a,b) and other tissues (Jacob, 1973) is sensitive to α-amanitin. The maximum degree of inhibition of the rat prostate enzyme and the human prostate enzyme (Davies & Griffiths, 1976) was about 70–90%, but after further purification inhibition of prostate RNA polymerase B was above 95%.

RNA polymerase B from rat liver (Butterworth et al., 1971) and calf thymus (Meihac & Chambon, 1973) are sensitive to rifamycin AF/013 under certain conditions, but the enzyme is resistant to the inhibitor after the formation of the first internucleotide linkage. The activity of exogenous prostate RNA polymerase B was inhibited completely by rifamycin AF/013 at a concentration of 50μg per assay (Fig. 1a), for the enzyme concentrations used in this study. When the addition of the antibiotic was delayed, the extent of RNA synthesis was dependent on the period of delay (Fig. 1b). Endo-

![Graphs showing the effects of rifamycin AF/013 on the activity of rat ventral-prostate RNA polymerase B](image)

**Fig. 1. Effects of rifamycin AF/013 on the activity of rat ventral-prostate RNA polymerase B**

The transcription of ventral-prostate chromatin from normal rats by homologous RNA polymerase B was measured in the presence of various concentrations of rifamycin AF/013: 12μg of chromatin DNA and 34μg of enzyme protein were used in (a) and (b). The activity of RNA polymerase was measured in the presence of concentrations of rifamycin AF/013 between 0 and 100μg per assay (a), and in (b) when rifamycin AF/013 (50μg) was added 0min (●), 1min (○), 3min (▲), 6min (●) or not added (□) after the start of the assay. The effect of rifamycin AF/013 on the activity of chromatin-associated RNA polymerase (three different samples ○, ●, ▲, ○) was also investigated. In all cases RNA polymerase B activity was monitored by the incorporation of [³H]-UMP; all activity was sensitive to α-amanitin (60ng/assay).
genous RNA polymerase activity in chromatin was not inhibited by rifamycin AF/013, and could be stimulated (Fig. 1c), as previously observed (Butterworth et al., 1971). Apparently uninitiated and reinitiated, but not preinitiated, polymerase was sensitive to the toxin. The formation of rifamycin-resistant enzyme-template complexes was temperature-dependent, since all RNA synthesis was sensitive to the antibiotic if preincubation of RNA polymerase and template took place at 0-4°C.

Tritration of rat ventral-prostate RNA polymerase with template

The amounts of available DNA in various templates may be assessed by the titration of the template against a fixed amount of RNA polymerase (Cedar, 1975). The amount of template required to achieve maximum enzyme activity indicates the number of initiation, or binding, sites available on the template. As the amount of template is increased, the number of RNA chains synthesized is correspondingly increased until a plateau is reached. In the experiment shown (Fig. 2a), the titration point for a naked DNA template was 3 µg and that for the ventral-prostate chromatin template was approx. 40 µg. There are therefore 12-14 times as many sites on DNA as on ventral-prostate chromatin. By assuming maximum specific activity of RNA polymerase B and a mol. wt. 500000 (Kedinger & Chambon, 1972), this would suggest 3.4 x 10⁶ molecules of enzyme per pg of naked DNA or 0.24 x 10⁶-0.28 x 10⁶ molecules per pg of chromatin DNA. These values are probably an overestimate of actual initiation sites.

The titration curves of RNA polymerase with DNA and chromatin did not achieve the same plateau value (Fig. 2a) owing to a difference in the size of the product synthesized from each template. Whereas the RNA synthesized from DNA had a number-average size of 810 ± 26 (± s.d., n = 12) nucleotides, that synthesized from intact-rat prostate chromatin had 528 ± 75 (± s.d., n = 8) nucleotides. This discrepancy was more obvious when titration curves of prostate chromatin from normal rats and from animals that had been castrated 24 h earlier were compared (Fig. 2b). Although chromatin appeared to bind RNA polymerase to the extent of approx. 0.3 x 10⁶ molecules per pg of DNA, the number-average size of RNA synthesized from chromatin of castrated rats was 396 ± 25 (± s.d., n = 8) nucleotides (see below). Apparent Kₘ values for the interaction between RNA polymerase and chromatin of normal and castrated rats were 115 and 112 pm respectively.

Initiation in low-salt conditions followed by chain propagation in high-salt conditions

This procedure was a modification of that of Cedar (1975). RNA polymerase B was allowed to initiate RNA synthesis in low salt with only ATP, GTP and [³H]UTP present. After the preincubation period, the mixture was adjusted to 0.4 M with (NH₄)₂SO₄ to prevent further initiation and CTP was added to permit elongation to proceed. The preincubation time of 15 min was sufficient to allow subsequent synthesis from all initiation sites; in fact, initiation was complete after 8 min preincubation (Fig. 3). Initiation sites were measured by inclusion of [γ-³²P]ATP or [γ-³²P]GTP in the preincubation mixture. These experiments were carried out under conditions of enzyme excess. Increasing the RNA polymerase concentration had no effect on the incorporation of [³H]UMP, but doubling the amount of template doubled RNA synthesis.

Fig. 2. Tritration of RNA polymerase B with DNA and chromatin

Increasing amounts of DNA (○), chromatin from ventral prostates of normal rats (●) or chromatin from ventral prostates of rats castrated 24 h previously (△) were incubated under standard assay conditions of 8.56 µg of RNA polymerase B. Enzyme activity was monitored by incorporation of [³H]UMP into acid-precipitable material. (a) Comparison of DNA (○) and chromatin from prostates of normal rats (●); (b) comparison of prostate chromatin from normal (●) and castrated (△) rats.
Measurement of RNA chain initiation and elongation is complicated by the contamination of commercial nucleoside triphosphates with small amounts of the other nucleoside triphosphates, resulting in the synthesis of oligonucleotide chains during the low-salt preincubation procedures. Considerable incorporation of \(^{[3]H}\)UMP occurred before propagation of RNA chains in high-salt media (Fig. 4). In a similar experiment, using 10\(\mu\)g of chromatin DNA and 23\(\mu\)g of RNA polymerase, low-salt incorporation of \(^{[3]H}\)UMP was 146.8 pmol/100\(\mu\)g of protein and, in parallel experiments, 15.44 pmol of \(^{[3]H}\)CMP was incorporated per 100\(\mu\)g of protein. The degree of contamination can vary from preparation to preparation, and from one nucleoside triphosphate to another. This oligonucleotide formation may not affect the absolute length of RNA chains, but will influence the rates of elongation measured in the first minute of propagation.

Comparison of RNA chain initiation and elongation as measured by low-salt/high-salt technique and by rifamycin AF/013 challenge

The initiation and elongation of RNA chains synthesized from normal rat ventral-prostate chromatin was studied by these two methods. The number of initiated sites per pg of DNA was 10057 \(\pm\) 866 (\(\pm\) S.D., \(n = 8\)) measured by the low-salt/high-salt technique and 11 429 \(\pm\) 1089 (\(\pm\) S.D., \(n = 8\)) measured by the rifamycin-challenge assay. The number-
average chain length of synthesized RNA was 546 ± 60 and 515 ± 165 (±s.d., n = 8) nucleotides respectively. The ratio [\( ^{32} P \)ATP]/[\( ^{32} P \)GTP incorporated was in the range 2.11 ± 0.65 (±s.d., n = 16) for both methods. The methods therefore give reasonable agreement for chain length and for the number of initiation sites per pg of DNA, although this number is considerably less than the estimated number of molecules of RNA polymerase that may associate with the template. However, when the initial rate of elongation was measured over the first 80s of propagation time (Fig. 5), a discrepancy was observed. In this particular experiment, the initial rate of elongation in the rifamycin assay was 8.23 nucleotides/s, but using a low-salt/high-salt procedure, the apparent rate was 27.1 nucleotides/s, a high value which may be attributed to the extent of preincubation incorporation of [\( ^{3} H \)UTP. Moreover, ...
elaboration was completed more rapidly in high-salt incubation.

Preincubation of RNA polymerase with template in the absence of all nucleotides allows the formation of rifamycin-resistant complexes at specific initiation sites (Chesterton et al., 1974; Fig. 1). Similar preincubation does not render complexes resistant to salt. Initiation was inhibited by 0.1M-(NH₄)₂SO₄ (Fig. 6). As reinitiation will be prevented by this concentration, it would be more effective in the measurement of elongation in vitro, as a concentration of 0.15M is necessary for dissociation of chromatin-associated proteins (Butterworth et al., 1971).

Both methods described could be used to determine the number of specific initiation sites on chromatin and the number-average nucleotide length of RNA chains. If, however, the rate of elongation is to be considered, then the rifamycin-propagation method, which precludes propagation until after the preincubation period, should be the method of preference.

Effect of castration on the number of initiation sites on rat ventral-prostate chromatin, the rate of elongation of RNA chains, and the number-average chain length

This study used rifamycin AF/013 to determine specific initiation sites and to prevent reinitiation, for reasons stated above. Two periods of castration were investigated: (a) a 'short' term, to study effects produced in the first 24h of castration, and (b) a 'long' term, to observe changes over a 3-day post-castration period. Since diurnal variations in RNA polymerase B activity, reported for this enzyme in other tissues (Glasser & Spelsberg, 1972; Spelsberg & Cox, 1976), could interfere with the short-term study, the number of initiation sites, the rate of elongation and the chain length were measured by using prostate chromatin in intact rats killed at various times over a 24h period (Fig. 7). A considerable variation in all three parameters occurs. Values for initiation sites and chain length were lowest at 16:00h and highest at 12:00h (Fig. 7a), but the elongation rate was lowest at 24:00h, but again highest at 12:00h (Fig. 7b). The diurnal variations in these parameters were sufficiently significant (±8.4% for initiation sites, ±27% for chain length and ±18% for elongation rate) to warrant rigorous control systems during the first 24h after castration, so that results for animals castrated at various times during the day could be compared with average normal values. When animals were castrated at daily intervals, 12:00h was taken as the time-point for experimentation.

Over the first 24h after castration (Table 1), the number of chromatin initiation sites and the rate of elongation were slightly decreased. The length of the synthesized RNA chains was most affected, but elongation was more severely impaired than is usual in
suggest castration, the various these limits are sites of RNA
transcription and that appears involved with the latter ribonucleotide chain various stages of steroid-receptor complex with the

discussion

Gross effects of steroids on the activity of RNA polymerases (Mainwaring et al., 1971; Davies & Griffiths, 1973a,b; 1974a,b) only give a slight indication of the processes concerned with gene activation. Independent assessment of the interaction of the steroid–receptor complex with the genome and the various stages involved in the production of the polyribonucleotide chain by RNA polymerase associated with the template is necessary. Our laboratory has concerned itself first with the latter problem. Homologous RNA polymerase B was used in these studies as differences in transcription characteristics of eukaryotic and bacterial RNA polymerases have been observed (Butterworth et al., 1971; Reeder, 1973; Cedar, 1975), and differing responses of Escherichia coli RNA polymerase to androgen–receptor complexes have been reported (Davies & Griffiths, 1973a; Mainwaring & Jones, 1975). Although the bacterial enzyme has been used successfully in other steroid-hormone-dependent systems (Schwartz et al., 1975; Tsai et al., 1976), it may have an impaired or decreased ability to recognize ‘activated’ sites on the prostate template.

Preliminary experiments (Fig. 2) showed that more sites were available for RNA polymerase on naked DNA than on a native chromatin template. The calculated numbers of polymerase molecules bound per unit of DNA vastly exceeded the actual number of initiation sites. A number of regions on the template may retain RNA polymerase but do not provide the correct conditions for the formation of a specific initiation complex. Similar amounts of RNA polymerase associate with the prostate chromatin from both normal and castrated rats, although shorter RNA chains are synthesized in the latter case.

There are two principal means by which the measurement of initiation and elongation can be separated. These involve either rifamycin AF/013 inhibition of reinitiation and initiation at nonspecific sites or alteration of salt concentration and nucleotide availability thereby preferentially controlling conditions to allow initiation or elongation. Rifamycin AF/013 was an effective inhibitor of non-preincubated prostate RNA polymerase B (Fig. 1). Delayed addition of rifamycin allowed an increased synthesis of RNA, probably because only reinitiation was being inhibited and the number of reinitiations became less as time progressed. As illustrated in Fig. 1(c) preinitiated RNA polymerase B could not be inhibited. This may be due to the insensitivity of the enzyme–DNA complex to the drug or the rapid rate of initiation by the complex (Chamberlin, 1974). No reinitiation in the presence of rifamycin occurred during the incubation period.

The rifamycin–challenge assay has distinct advantages over the low-salt/high-salt technique which can produce incorrect calculations of chain length, possibly owing to dissociation of chromatin-associated proteins as well as oligonucleotide formation in low-salt conditions. The introduction of high salt is essential to inhibit reinitiation, but dissociation of

Table 2. Changes in template characteristics of rat ventral-prostate chromatin after castration
Chromatin was prepared from the ventral prostate glands of unoperated rats and rats castrated 24, 48 or 72h previously. The various parameters were measured as described in the legend to Table 1.

<table>
<thead>
<tr>
<th>Time after castration (days)</th>
<th>Initiation sites (no./pg of DNA)</th>
<th>Number-average chain length (nucleotides)</th>
<th>Elongation rate (nucleotides/s)</th>
<th>Protein/DNA</th>
<th>Histone/non-histone protein/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10640</td>
<td>520</td>
<td>11</td>
<td>2.90</td>
<td>2.06:0.7:1</td>
</tr>
<tr>
<td>1</td>
<td>10066</td>
<td>418</td>
<td>7</td>
<td>2.94</td>
<td>1.84:0.98:1</td>
</tr>
<tr>
<td>2</td>
<td>7707</td>
<td>379</td>
<td>*</td>
<td>1.53</td>
<td>1.10:0.31:1</td>
</tr>
<tr>
<td>3</td>
<td>6214</td>
<td>349</td>
<td>*</td>
<td>1.56</td>
<td>1.08:0.46:1</td>
</tr>
</tbody>
</table>

* Could not be determined accurately.
chromatin-associated proteins could be decreased by using a final salt concentration of 0.1 M. Moreover, similar chain lengths were observed using either the rifamycin-challenge assay or the low-salt/high-salt technique. Oligonucleotide formation is dependent on the extent of contamination of nucleotide preparations and could probably be overcome only by stringent purification. As the rifamycin assay allows addition of all nucleotides after preincubation, spurious elongation rates caused by nucleotide contamination or salt would not be observed (Fig. 5).

Transcription of chromatin was impaired in vitro in relation to transcription of DNA. This could be attributed to the chromatin-associated proteins, but, since equally short chain lengths were observed by using rifamycin and 0.4 M salt, which might be expected to promote elongation, other factors might possibly be involved. These problems should be taken into consideration, together with castration-induced effects.

The observed diurnal variations (Fig. 7) in the number of initiation sites, number-average chain length and elongation rate necessitated the use of controls over the first 24 h post-castration period. During this time, the major effects of castration were on chain length. This agrees with previous observations (Mainwaring & Jones, 1975; Davies, 1975) that initiation sites were not significantly decreased 24 h after castration. However, 48 and 72 h after castration the numbers of initiation sites were decreased, with further decreases in the number-average nucleotide size of RNA chains.

The decreased length of RNA chains transcribed from chromatin in vitro can be attributed to the loss of elongation factors from the template during preparation (Cox, 1976). The results from castration studies suggest that androgen withdrawal may further decrease the number or efficiency of such factors. These substances may themselves be dependent on androgens for their maintenance or synthesis.

The absolute length of RNA chains, as well as the rate of elongation, may be controlled by certain chromatin-associated proteins (Solage & Cedar, 1976). Alterations in the proportion of non-histone and histone proteins are evident during post-castration periods (Table 2) and may be related to variations in transcription (Couch & Anderson, 1973a,b; Davies & Griffiths, 1974a; Nyberg & Wang, 1976).

An important feature of the transcription of chromatin in vitro is the involvement of ribonucleases. Inhibitors of these enzymes vary in efficiency (Jones, 1976). The decrease in chain length may be independent of nuclelease action (Davies et al., 1976) but the production of RNA is susceptible to numerous degradative enzymes.

The androgenic status of the animal influences more than one aspect of the transcription of prostate chromatin by homologous RNA polymerase B. The present data provide a basis for a more detailed appraisal of the many interactions involved in these events. A relationship exists between the concentration of nuclear receptors and transcription (Davies et al., 1976), but it is uncertain whether the androgen-receptor complex acts by displacement of chromatin-associated proteins to reveal initiation sites, by assisting in the unwinding of DNA, by promoting elongation factors or by inactivating termination factors.

We are grateful to the Tenovus Organization for their generous financial support.

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MEASUREMENT OF FREE AND OCCUPIED CYTOPLASMIC AND NUCLEAR ANDROGEN RECEPTOR SITES IN RAT VENTRAL PROSTATE GLAND

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SUMMARY

A method has been developed which allows the estimation of occupied and unoccupied androgen receptor sites in both cytoplasmic and nuclear fractions of rat ventral prostate. The procedure involves precipitation of receptor proteins and incubation of precipitates with labelled 5α-dihydrotestosterone. Uptake of 3H-labelled steroid at 0–4 °C gives an indication of free receptor, whereas binding at a raised temperature (15 °C) allows estimation of occupied receptor. Non-specific binding was measured in the presence of a 100-fold excess of unlabelled 5α-dihydrotestosterone. The exchange method was specific for androgens, and specific binding was detected only in fractions of androgen-dependent tissues. The method can be applied to cytosol, whole nuclei, chromatin and salt-extractable and salt-resistant protein preparations from nuclear fractions, and gives a reliable estimate of total receptor sites when occupied as compared with control measurements of unoccupied sites.

INTRODUCTION

The available information concerning the physico-chemical properties of macromolecules which selectively retain androgens in rat ventral prostate (Liao & Fang, 1969; King & Mainwaring, 1974; Liao & Liang, 1974; Minguell & Sierralta, 1975) has indicated a high affinity for 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one: 5α-DHT), shown to be the active metabolite of testosterone in this tissue (Bruchovsky & Wilson, 1968). The distribution of androgen receptors in prostatic cytoplasm and nucleus (King & Mainwaring, 1974; Liao & Liang, 1974; Bruchovsky, Lesser, Van Doorn & Craven, 1975a), and their postulated involvement in transcriptional (Davies & Griffiths, 1973, 1974; Davies, 1975; Hu, Loor & Wang, 1975; Mainwaring & Jones, 1975; Davies, Thomas & Griffiths, 1976) and post-transcriptional (Ichii, Izawa & Murakami, 1974; Liang & Liao, 1975) processes, has emphasized the desirability of a more complete quantitative assessment of ligand retention and ligand–receptor interaction. Such an approach might define the imprecise relationship of cytoplasmic and nuclear receptors (Bruchovsky, Rennie & Vanson, 1975b) and clarify the involvement of receptors in synthesis of macromolecules (Davies et al. 1976). This has, however, proved difficult (Blondeau, Corpechot, Le Goascogne, Baulieu & Robel, 1975).

Only partial success has been achieved using conventional or modified dextran-coated charcoal techniques (Boesel & Shain, 1974; Blondeau & Robel, 1975; Shain & Boesel, 1975; Shain, Boesel & Axelrod, 1975), and certain disadvantages are inherent in the gel exclusion and filtration techniques (Topert, Zabel & Ziegler, 1974; Bruchovsky & Craven, 1975). Possibly the best results which can be obtained involve precipitation of receptor with
ammonium sulphate (Verhoeven, Heyns & De Moor, 1975) or protamine sulphate (Blondeau et al. 1975). Steroid hormone receptors are acidic proteins which can be precipitated selectively by protamine sulphate (Mainwaring, 1969; Stegglels & King, 1970), and this method has been used to measure specifically bound androgen by 'radioimmunoassay' (Blondeau et al. 1975), cytoplasmic androgen- and progestin-binding in human breast cancer cells (Horwitz, Costlow & McGuire, 1975), and occupied nuclear oestrogen receptors by exchange (Zava, Harrington & McGuire, 1976; Nicholson, Davies & Griffiths, 1977). The application of the technique to the measurement of occupied and unoccupied cytoplasmic and nuclear receptor sites in rat ventral prostate tissue is now reported.

**MATERIALS AND METHODS**

**Chemicals**

[1,2,4,5,6,7-3H]5α-Dihydrotestosterone (specific activity 107–130 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks. [6,7-3H]17β-Hydroxy-17α-methyl-oestra-4,9,11-trien-3-one ([3H]R1881) and unlabelled R1881 were a gift from Dr J.-P. Raynaud, Centre de Recherches Roussel-Uclaf, Romainville, France. Crystalline bovine serum albumin, calf-thymus DNA, dithiothreitol, EDTA, Tris-base (99% pure) and 5α-DHT were purchased from the British Drug Houses, Poole, Dorset. Other unlabelled steroids and diethylstilboestrol were bought from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, or Koch-Light Laboratories, Colnbrook, Bucks., who also provided protamine sulphate (from salmon roe).

**Animals and tissues**

Male Sprague–Dawley rats were bred in the Institute Animal Unit and generally used when 8–12 weeks old. Some animals were castrated by the scrotal route under ether anaesthesia when 8 weeks old. Both intact and castrated rats received injections of [3H]5α-DHT (usually 50 μCi) or 5α-DHT (400 μg/100 g body weight) either subcutaneously, in a maximum volume of 200 μl sesame oil, or intravenously. Ventral prostate tissue was carefully dissected from rats killed when required, placed in vessels surrounded by crushed ice, and used immediately.

**Preparation of subcellular fractions**

Details of the preparation of soluble supernatant (cytosol) fractions, nuclei and chromatin have been published previously (Davies & Griffiths, 1973).

**Detection of subcellular components retaining 3H-labelled steroid**

The basic buffer system used in these studies comprised Tris–HCl buffer (10 mmol/l) at pH 7.4, containing EDTA (0.1 mmol/l) and dithiothreitol (0.25 mmol/l).

**Cytoplasmic sites**

Receptor sites occupied by endogenous steroid were measured in cytosol by a method based on that of Bonne & Raynaud (1976). Whereas these authors used the synthetic androgen R1881 in exchange methods in free solution, in this study 5α-DHT was incubated with receptor precipitated by protamine sulphate. It has been reported that the amount of specifically bound 5α-DHT which is precipitated, is dependent upon protamine sulphate concentration (with maximum precipitation between 0.6 and 1 mg/ml), is the same between 5 and 60 min of incubation, and is proportional to protein concentration below 15 mg/ml (Blondeau et al. 1975). The present method was influenced by these observations. Protamine sulphate (100 μl of 1 mg/ml solution) was added to an equal volume of cytosol (7–12 mg protein/ml) and the resulting precipitates were washed five times with 1 ml buffer. Precipi-
tates were then dispersed in $^3$H-labelled steroid solution (2–25 nmol/l), with and without a 100-fold higher concentration of radioinert steroid, at various temperatures and for differing periods of time. After incubation, precipitates were sedimented at 800 g for 10 min, washed six times in ice-cold buffer and extracted twice with ethanol (1 ml). The combined extracts were evaporated in scintillation vials and radioactivity was measured.

**Nuclear sites**

A similar method to that described above was employed to measure occupied receptor sites in nuclei and nuclear fractions. Protamine sulphate (100 μl of 1 mg/ml solution) was added to equal volumes of nuclear suspension (10–30 μg DNA). Resulting precipitates were washed, incubated with $^3$H-labelled steroid with and without unlabelled steroid, washed again and extracted as described above. The method was applied to whole nuclei, chromatin and the salt extract and salt residue of chromatin, with certain modifications as described in the text.

Whenever possible, the nature of the proteins binding $^3$H-labelled androgens was analysed by sedimentation through gradients of sucrose (Davies & Griffiths, 1973, 1974). In some instances, particularly in the case of nuclear fractions, free steroid was removed by adsorption on a charcoal pellet (derived from 1 ml of a solution of 2% (w/v) Norit A and 0-2% (w/v) Dextran T-70). Exposure to charcoal (10 min at 0 °C) was ended by centrifugation (800 g for 10 min) and samples of the supernatant were layered on gradients. In the case of salt-extracted $^3$H-labelled nuclear fractions, the $^3$H-labelled steroid refractory to this procedure was extracted from the residue with 1 ml ethanol, evaporated in scintillation vials and the radioactivity measured.

**Chemical analyses**

Protein content of fractions was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine serum albumin as standard. Protein was precipitated from preparations with 10% (w/v) trichloroacetic acid and washed twice with 5% (w/v) trichloroacetic acid before analysis to prevent interference by buffer components. DNA was measured by the method of Burton (1956) using calf-thymus DNA as standard.

**RESULTS**

**Measurement of occupied and unoccupied androgen receptor sites in rat ventral prostatic cytoplasmic fractions**

In standard steroid–receptor binding systems, it is accepted that the association of the ligand with accessible binding sites can take place at 0–4 °C, while replacement of endogenous steroid occupying receptor sites by exogenous $^3$H-labelled steroid requires a higher temperature and, possibly, prolonged incubation time. In the case of the rat ventral prostate, several factors preclude such an assay being carried out with 5z-DHT and receptors in free solution (Blondeau et al. 1975; Bonne & Raynaud, 1976). However, precipitation of the receptor with protamine sulphate confers greater stability on the receptor (Blondeau et al. 1975) and allows an exchange assay to be performed.

As shown in Fig. 1a, negligible exchange occurred at 0–4 °C, while at higher temperatures (15 and 25 °C) the exchange process was more rapid. At 15 °C, a plateau was reached after 2 h, extending to 24 h of incubation. Increased temperature (25 °C) increased the exchange rate, which reached a maximum after 4 h. Further increase of temperature to 30 or 37 °C resulted in rapid degradation of receptor making these temperatures unsuitable for the assay. For convenience and for better reproducibility due to favourable conditions of ability and dissociation (Figs 1b, c), the assay was routinely employed at 15 °C for 16 h.

Parallel assays carried out at 0–4 °C gave the accessible receptor concentration. Specific
Fig. 1. Effect of time and temperature on the cytoplasmic \([\text{H}]5\alpha\)-dihydrotestosterone-(\([\text{H}]5\alpha\)-DHT) receptor complex. Aliquots (200 µl) of cytosol (7–12 mg/ml) were mixed with an equal volume of protamine sulphate solution (1 mg/ml) and resulting precipitates were incubated for various periods at 4 °C (○), 15 °C (●), 25 °C (△) or 30 °C (□). For exchange assay (a), precipitates were incubated with \([\text{H}]5\alpha\)-DHT (20 nmol/l) with and without 5α-DHT (2 µmol/l), and specific binding was calculated from the difference in uptake of \(^3\text{H}\)-labelled steroid. For measurement of stability (b), cytosol was pre-incubated at 0 °C with \([\text{H}]5\alpha\)-DHT (20 nmol/l) in the presence and absence of radio-inert ligand (2 µmol/l) and aliquots were precipitated and incubated as shown. For the measurement of dissociation (c), radio-inert 5α-DHT (2 µmol/l) was added after precipitation of \(^3\text{H}\)-labelled cytosol.

Fig. 2. Saturation analysis of \([\text{H}]5\alpha\)-dihydrotestosterone (\([\text{H}]5\alpha\)-DHT) binding to cytoplasmic receptor. Aliquots (200 µl) of cytosols (8·5 mg/ml, ●; 9·4 mg/ml, ○) were mixed with equal volumes of protamine sulphate solution (1 mg/ml) and the resulting precipitates were incubated with various concentrations of \([\text{H}]5\alpha\)-DHT (2–25 nmol/l), with and without radio-inert 5α-DHT (200–2500 nmol/l), for 16 h at 15 °C. Specific binding was calculated from the difference in retained radioactivity in the absence and presence of unlabelled steroid and plotted according to Scatchard (1949).
Table 1. Measurement of cytosol androgen receptors in intact and castrated rats of various ages (values are means ± S.D. of four determinations)

(Each age group was sub-divided so that one set received injections of [3H]5α-dihydrotestosterone ([3H]5α-DHT) (100 μCi) before death and the cytosol fractions were prepared and [3H]5α-DHT receptors measured on sucrose gradients. The other sub-groups were killed, prostate cytosol fractions prepared, and [3H]5α-DHT receptors assessed by direct labelling at 4 °C with 3H-labelled steroid followed by gradient analysis, or by exchange assay. Average values for accessible sites are shown in parentheses.)

<table>
<thead>
<tr>
<th>Animals</th>
<th>Exchange assay</th>
<th>Labelling in vitro</th>
<th>Labelling in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 weeks old</td>
<td>265 ± 26 (0)</td>
<td>Not detectable</td>
<td>283 ± 9</td>
</tr>
<tr>
<td>12 weeks old</td>
<td>233 ± 14 (49)</td>
<td>53 ± 6*</td>
<td>246 ± 24</td>
</tr>
<tr>
<td>20 weeks old</td>
<td>217 ± 20 (38)</td>
<td>44 ± 4</td>
<td>229 ± 28</td>
</tr>
<tr>
<td>Castrated (8 weeks old):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>129 ± 13 (100)</td>
<td>119 ± 25</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>2 days</td>
<td>56 ± 23 (50)</td>
<td>63 ± 18</td>
<td>48†</td>
</tr>
<tr>
<td>3 days</td>
<td>38 ± 8 (38)</td>
<td>Not detectable</td>
<td>27†</td>
</tr>
<tr>
<td>7 days</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

* One aliquot showed no binding: value is for three determinations only.
† Values are for one determination only.

binding of [3H]5α-DHT under these conditions, determined by bound radioactivity in the absence and presence of unlabelled 5α-DHT, was saturable, and in the cytosol preparations used in these studies, had an apparent dissociation constant (Kd) of 2–10 nmol/l (Fig. 2). However, in routine studies it was deemed sufficient to use a one-point assay using 20 nmol [3H]5α-DHT/l with and without 2 μmol unlabelled 5α-DHT/l. In studies when such an assay could be compared with a known receptor concentration calculated from specific binding on parallel sucrose gradients, excellent agreement was achieved (Table 1).

Measurement of androgen receptor sites in rat ventral prostatic nuclei

In preliminary studies (Davies et al. 1976) a direct exchange of [3H]5α-DHT with endogenous steroid in nuclei was attempted, but met with only partial success. However, by employing previous precipitation with protamine sulphate, exchange methods could be applied to prostatic nuclei, the optimal temperature and time being 15 °C for 16 h (Fig. 3a). Saturation analysis of [3H]5α-DHT binding showed that the amount of 3H-labelled steroid specifically retained was a saturable function representing a single class of binding sites with an apparent Kd in the region of 5–10 nmol/l (Figs 3b, c). Similar dissociation constants and receptor concentrations were obtained whether the competitor used to determine non-specific binding was unlabelled DHT or R1881 (Figs 3b, c). Similar concentrations were obtained if [3H]-R1881 was used with either unlabelled steroid as competitor, with Kd values in the range 6–8 nmol/l. Levels of apparent non-specific binding were lower using [3H]R1881.

Nuclear receptor sites in rat ventral prostatic nuclei showed specificity for androgens in that only 5α-DHT, R1881, and to a lesser extent, testosterone and 5α-androstane-3β,17β-diol, could reduce the binding of [3H]5α-DHT (Table 2). 5α-Androstane-3β,17β-diol has been shown previously to have some effect on macromolecular synthesis in the rat ventral prostate (Davies, Fahmy, Pierrepoint & Griffiths, 1972). All other compounds tested were ineffective. Specific uptake of [3H]5α-DHT under exchange conditions has been shown to be restricted to androgen target tissues (Davies et al. 1976).
Fig. 3. Nuclear binding of [3H]5α-dihydrotestosterone ([3H]5α-DHT). Aliquots of nuclear suspension (200 µl) were mixed with equal volumes of protamine sulphate solution (1 mg/ml) and (a) precipitates were incubated with [3H]5α-DHT (20 nmol/l), with and without unlabelled 5α-DHT (2 µmol/l), for various periods at 4°C (O), 15°C (△) or 25°C (●). Specific binding was calculated as described in the Materials and Methods; precipitates were incubated with [3H]5α-DHT (2-25 nmol/l), with and without (b) 5α-DHT (200-2500 nmol/l) or (c) 17β-hydroxy-17α-methyl-oestra-4,9,11-trien-3-one (R1881) (200-2500 nmol/l) for 16 h at 15°C. Specific binding was calculated and plotted according to Scatchard (1949).

Table 2. Steroid specificity of nuclear androgen-receptor complex (values are means ± S.D. of four estimations)

<table>
<thead>
<tr>
<th>Competitor</th>
<th>d.p.m./100 µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13561 ± 469</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>3617 ± 442</td>
</tr>
<tr>
<td>Testosterone</td>
<td>8344 ± 831</td>
</tr>
<tr>
<td>R1881</td>
<td>3676 ± 590</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>12637 ± 367</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>10517 ± 979</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>13960 ± 259</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>13798 ± 609</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>13539 ± 249</td>
</tr>
<tr>
<td>Cortisol</td>
<td>12678 ± 269</td>
</tr>
<tr>
<td>Progesterone</td>
<td>13229 ± 525</td>
</tr>
<tr>
<td>R1881, 17β-Hydroxy-17α-methyl-oestra-4,9,11-trien-3-one</td>
<td>13539 ± 249</td>
</tr>
</tbody>
</table>

It has previously been reported that not all non-covalently bound steroid in the nuclei of androgen-sensitive cells can be extracted with salt (Mainwaring, 1969; Van Doorn, Craven & Bruchovsky, 1976). This was also the case in this study (Table 3). The material refractory to extraction with KCl (0-6 mol/l) comprised between 10 and 25% total nuclear radioactivity, but between 16 and 26% specifically retained 3H-labelled steroid. As expected, increasing doses of 3H-labelled steroid resulted in an increasing proportion of non-specifically retained material, which could cause discrepancies in the comparison of total nuclear radioactivity and uptake of 3H-labelled steroid in the exchange assay.

Maximum extraction of nuclear radioactivity was achieved with 0-6 mol KCl/l (Fig. 4a), in two extractions. For example, in one nuclear preparation 6316 d.p.m. were recovered on one extraction, 616 d.p.m. in the second, no radioactivity in three subsequent salt extractions and 713 d.p.m. were recovered from the residue in ethanol. Only the first extraction yielded
Exchange assay for androgen receptors

Table 3. Uptake of \([^{3}H]5\alpha\)-dihydrotestosterone (\([^{3}H]5\alpha\)-DHT) into rat prostatic nuclei

(Intact rats were injected with various doses of \([^{3}H]5\alpha\)-DHT (50–200 \(\mu\)Ci) and nuclei were prepared from ventral prostate glands 60 min later. Samples of nuclei were extracted with ethanol (total nuclear extract) or with KCl (0·6 mol/l). Samples of the latter extract were counted directly or layered on 5–20 % (w/v) sucrose gradients and the radioactivity in the receptor peak was calculated. Material resistant to salt extraction (KCl residue) was extracted with ethanol. *Samples of the same extract were counted directly or analysed on gradients and calculated back to total volume. †Gradient input was calculated similarly.)

<table>
<thead>
<tr>
<th>(\text{H})-labelled steroid injected ((\mu)Ci)</th>
<th>Total nuclear extract</th>
<th>KCl extract*</th>
<th>KCl residue</th>
<th>Protein-bound*</th>
<th>Gradient input†</th>
</tr>
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<tbody>
<tr>
<td>50</td>
<td>27129</td>
<td>22579</td>
<td>7245</td>
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<td>22334</td>
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<td>88891</td>
<td>8804</td>
<td>44657</td>
<td>87834</td>
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<tr>
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<td>89486</td>
<td>86959</td>
<td>9386</td>
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</tbody>
</table>

A peak on gradient centrifugation. Increasing salt concentrations resulted in larger peaks on sucrose gradients (Fig. 4b). For purposes of efficient precipitation of specifically bound material with protamine sulphate, however, the salt concentration had to be reduced to 0·1–0·2 mol/l (Fig. 4c).

A comparison of data obtained from injections of animals with \([^{3}H]5\alpha\)-DHT, or with 5\(\alpha\)-DHT followed by exchange with \([^{3}H]5\alpha\)-DHT gave very good agreement for total nuclear radioactivity, and salt-extractable or non-extractable radioactivity (Table 4). These experiments were carried out with the lowest dose of \(\text{H}\)-labelled steroid (see Table 3) to minimize ‘non-specific’ uptake into nuclei.

![Graphs showing effect of salt concentrations on recovery of \([^{3}H]5\alpha\)-dihydrotestosterone (\([^{3}H]5\alpha\)-DHT) from nuclei and precipitation of receptor-bound steroid with protamine sulphate.](image-url)
Table 4. Uptake of $[^{3}H]5\alpha$-dihydrotestosterone ($[^{3}H]5\alpha$-DHT) into prostatic nuclei from castrated rats (values are means ± S.D. of four determinations)

(Animals were castrated 24 h before injection with $[^{3}H]5\alpha$-DHT (50 µCi) or 5α-DHT (400 µg/100 g body wt) and nuclei were prepared from ventral prostate glands 60 min later. In the animals injected with $[^{3}H]5\alpha$-DHT, radioactive steroid was assessed directly and, in the animals injected with 5α-DHT, receptor sites were analysed by exchange.)

<table>
<thead>
<tr>
<th></th>
<th>Salt extract</th>
<th>Salt residue</th>
<th>Total nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals injected with $[^{3}H]5\alpha$-DHT</td>
<td>10091 ± 304</td>
<td>1237 ± 70</td>
<td>13130 ± 1414</td>
</tr>
<tr>
<td>Animals injected with 5α-DHT; nuclei used in exchange assay with $[^{3}H]5\alpha$-DHT</td>
<td>9436 ± 1372</td>
<td>1608 ± 383</td>
<td>11349 ± 1314</td>
</tr>
</tbody>
</table>

Application of exchange methodology to chromatin preparations

The value of the method in measuring the uptake of radioactivity into chromatin was assessed. In these experiments chromatin prepared from prostate glands of animals castrated 24 h previously was incubated with cytosol charged with $^{3}$H-labelled steroid (Davies & Griffiths, 1974). In some instances cytosol was incubated with 5α-DHT, and the number of binding sites was calculated using the exchange method. It can be seen that data obtained from the exchange method closely paralleled direct $[^{3}H]5\alpha$-DHT measurement of the time-course of $^{3}$H-labelled steroid incorporation into nuclei (Fig. 5), the effects of cytosol receptor concentration (Fig. 6) and the effect of chromatin DNA concentration (Fig. 7) on this incorporation. As would be expected, the uptake of cytosol steroid–receptor was dependent upon time, and was saturable under certain conditions of protein concentration, the number of steroid–receptor molecules bound being dependent upon the number of nuclear acceptor sites available. Results obtained by direct uptake of $^{3}$H-labelled steroid and by the exchange method are in accord with previous observations (Mainwaring & Peterken, 1971; Davies & Griffiths, 1974).

Fig. 5. Uptake of steroid into chromatin. Incorporation of 5α-dihydrotestosterone (5α-DHT) into chromatin was carried out according to Davies & Griffiths (1974). Nuclear suspension (16-40 µg DNA) was incubated with equal volumes of cytosol at 30 °C for various periods up to 60 min. In (a) cytosol was prelabelled with $[^{3}H]5\alpha$-DHT (20 nmol/l) and steroid incorporation into chromatin calculated by direct counting of radioactivity. In (b) cytosol was pre-incubated with radio-inert 5α-DHT (20 nmol/l), and steroid incorporation into chromatin measured by the exchange assay. Incorporation into material extractable by (>) and refractory to (●) salt (0-6 mol/l) was monitored. Fractions were prepared from prostate glands of rats castrated 24 h previously.
Fig. 6. Effect of cytosol receptor concentration on uptake of steroid into chromatin. Nuclear suspension (13-6 µg DNA) was incubated with increasing concentrations of cytosol 5α-dihydrotestosterone-(5α-DHT) receptor complex for 45 min at 30 °C. In some cases (○), cytosol protein concentration varied as diluted and in others (●) cytosol protein concentration was made constant by adjustment with heat-treated (75 °C for 15 min) cytosol. Cytosol was pre-incubated with [3H]5α-DHT (20 nmol/l) (a, b) or 5α-DHT (c, d) and incorporation measured as described in legend to Fig. 5. Incorporation of steroid into material extractable by (a, c) or refractory to (b, d) salt (0-6 mol/l) was monitored. Cytosol steroid–receptor added was checked by sucrose gradient centrifugation or by exchange assay.

Fig. 7. Effect of DNA concentration on incorporation of steroid into chromatin. Conditions of the experiment were as described in Figs 5 and 6. Varying amounts of chromatin, as shown, were incubated at 30 °C for 45 min, with constant amounts of cytosol, prelabelled with (a) [3H]5α-dihydrotestosterone ([H]5α-DHT) (20 nmol/l) or (b) 5α-DHT (20 nmol/l) followed by exchange assay. Incorporation of steroid into material extractable by (○) or refractory to (●) KCl (0-6 mol/l) was monitored. Input of cytosol receptor, as calculated from gradient analysis or exchange assay, was 33·8 fmol.
Translocation of receptor from cytoplasm to nucleus

The exchange assay was tested by monitoring a well-known phenomenon: the localization of \(^{3}\)H-labelled steroid in the nucleus and concomitant depletion of cytoplasmic \(^{3}\)H-labelled steroid–receptor complex. As shown in Fig. 8, after intravenous injection of either \(^{3}\)H-labelled steroid or unlabelled steroid, a similar pattern was observed. Due to the possibility of non-specific uptake of \(^{3}\)H-labelled steroid, the exchange method probably gave a more accurate assessment of specifically bound material.

**DISCUSSION**

Numerous difficulties have been experienced in the quantitation of androgen receptors in the rat ventral prostate (Blondeau et al. 1975). These stem mainly from the binding of \(^{3}\)H\(5\alpha\)-DHT to non-specific binding components and the extensive metabolism of this steroid in rat prostatic cytosol (Gore & Baron, 1965). Obviously, it is possible to separate specific receptors from non-specific binding components by virtue of their sedimentation characteristics (Mainwaring, 1969), but although this may allow measurement of the amount of steroid bound, no estimation of affinity can be achieved. The use of dextran-coated charcoal has proved inefficient in the separation of specific and non-specific components (Blondeau & Robel, 1975). Protamine sulphate precipitation of cytosol receptor is efficient, accurate and reliable and confers stability on the complex (Blondeau et al. 1975) and, unlike precipitation with ammonium sulphate (Bonne & Raynaud, 1975; Shain & Boesel, 1975 Shain et al. 1975), renders the steroid refractory to enzymic action (Blondeau et al. 1975).

Moreover, the application of this method to cytosol and nuclear preparations allows the determination of free and occupied receptor sites in either or both subcellular compartments.

It can be seen (Fig. 1) that protamine sulphate precipitation confers upon the cytoplasmic receptor–steroid complex characteristics of stability and dissociation amenable to an exchange assay. These characteristics are similar to those reported for the \(^{3}\)H\(18\)81–receptor complex (Bonne & Raynaud, 1976). The calculation of specific binding sites by subtraction of \(^{3}\)H-labelled steroid uptake in the presence of radioinert steroid from that in its absence correlated well with control sedimentation studies (Table 1). The added advantage of the assay is the calculation of the dissociation constant (Fig. 2). This also offers advantages over th
use of a synthetic steroid (Bonne & Raynaud, 1976) since the interaction of the receptor and the physiologically active steroid is of major interest.

The method also allows the determination of nuclear receptor sites by exchange with endogenous steroid. This is clearly of use in relating receptor sites with changes in metabolic processes. Changes in the incorporation of steroid to specific sites on chromatin can be carefully monitored. Moreover, the method can be used on whole nuclei, chromatin and salt-extracted or salt-resistant material. The exact role of the last is as yet undetermined (Mainwaring, Symes & Higgins, 1976), but may be of some importance (Clark & Peck, 1976). Whatever the case, the presence of limited, saturable sites cannot be dismissed lightly until their relationship, or lack of relationship, with hormone-mediated events can be established. A number of different androgen–receptor complexes have been postulated to exist in the cell (Rennie & Bruchovsky, 1972; Katsumata & Goldman, 1974; Bruchovsky et al. 1975b) and this presence may be reflected by different retention characteristics in nuclei.

Although the method described herein was designed mainly to link with transcriptional studies in the rat ventral prostate, it obviously has a more widespread potential use. The development of assays to detect receptor sites, usually occupied, in human benign hyper¬trophic and malignant prostate has always been fundamental to the understanding of prostatic dysfunction.

We would like to express our gratitude to the Tenovus Organization for their generous financial support.

REFERENCES


Androgenic Regulation of Elongation of Polyrribonucleotide Chains on Rat Ventral-Prostate Chromatin

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The kinetics of polynucleotide-chain elongation by rat ventral-prostate RNA polymerase B with homologous chromatin as a template were investigated. Chain elongation was measured under conditions wherein all initiation had occurred, no re-initiation took place and the reaction rate was constant. The kinetic behaviour of prostate RNA polymerase B was consistent with a mathematical model formulated for the multi-substrate enzyme. The addition of each nucleoside triphosphate was independent of the other three. The overall rate of chain elongation was lower when prostate chromatin from castrated rats was used than with prostate chromatin from normal rats. The inclusion of dihydrotestosterone-receptor complexes stimulated the rate of elongation. Androgenic effects did not appear to be directed towards the addition of individual nucleoside triphosphates, but probably towards one of the other major events in RNA-chain elongation, i.e. unwinding of DNA or movement of the enzyme along the template.

The specific transfer of dihydrotestosterone (17β-hydroxy-5α-androstan-3-one)-receptor complexes to defined sites (Mainwaring & Peterken, 1971; Mainwaring et al., 1976) within rat ventral-prostate chromatin results in elevated activity of RNA polymerase (EC 2.7.7.6; nucleoside triphosphate-RNA nucleotidyltransferase), reflected in an increased production of mRNA (Mainwaring et al., 1974a,b; Nyberg et al., 1976). Studies in vitro have clearly indicated that androgen–receptor complexes influence directly the transcription of prostate chromatin by homologous RNA polymerase B (Davies & Griffiths, 1974a,b; Mainwaring & Jones, 1975; Hu et al., 1975). The initial effects of the dihydrotestosterone–receptor complex involve alterations in RNA-chain elongation (Mainwaring & Jones, 1975; Davies, 1975), with changes in the number of accessible initiation sites occurring at a later stage (Davies et al., 1976; Thomas et al., 1977). Further attempts to elucidate the nature of the primary effects of the dihydrotestosterone–receptor complex on the elongation of polynucleotide chains by rat ventral prostate RNA polymerase B are now described.

Materials and Methods

Experimental animals

Details of experimental animals and operative procedures are the same as reported previously (Thomas et al., 1977). Tissue was processed by using similar rotors and centrifuges to those previously described (Thomas et al., 1977).

Chemicals

Except for those described below, the source and nature of the chemicals used have been listed by Thomas et al. (1977). 5α-Dihydro[1α,2α-3H2]testosterone (56Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Dihydrotestosterone was a product of Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Compound R1881 (methyltrienolone: 17β-hydroxy-17α-methyl oestra-4,9,11-trien-3-one) and [6,7-3H2]labelled compound R1881 (specific radioactivity 58.2 Ci/mmol) were gifts from Dr. J.-P. Raynaud, Centre de Recherches Roussel-Uclaf, Romainville, France. (NH4)2SO4, specially low in heavy metals (BDH, Poole, Dorset, U.K.), was adjusted to the required pH value (see below) with eq. 2m-NH3. Machery–Nagel cellulose (type 2200ff) was supplied by Camlab Ltd., Cambridge, U.K., and was used for the preparation of DNA–cellulose as described in detail by Mainwaring & Peterken (1971). On the basis of the release of deoxyribonucleotides by hot-acid digestion (Alberts et al., 1968), each 1g of washed DNA–cellulose contained 400–500µg of immobilized DNA. Cellulose (Whatman; medium fibre) was also supplied by BDH, and was cycled successively in 0.5m-NaOH, water, 0.5m-HCl and water before use. Sephadex G-25 (medium-grade) was purchased from Pharmacia (U.K.) Ltd., London W.5,
U.K. All other chemicals were of the highest available commercial grades, and glass-distilled water was used in making up all solutions.

Preparation of subcellular fractions

All procedures were carried out at 0-4°C, unless otherwise stated.

Partial purification of dihydrotestosterone–receptor complexes. Cytoplasmic dihydrotestosterone–receptor complexes were prepared from ventral-prostate glands of normal intact rats and of rats castrated 24h previously. In the former case unlabelled dihydrotestosterone was included in buffers when necessary, and in the latter case 3H-labelled steroid was used. All other aspects of the procedure were similar.

Cytosol (100,000g, supernatant) was prepared from a homogenate (0.4–0.6g of tissue/ml in medium A (50 mM-Tris/HCl buffer, pH 7.4, containing 0.25 mM-EDTA and 0.5 mM-dithiothreitol), and 0.1 vol. of glycerol was added. Cytosol was made 5 mM with dihydrotestosterone, and a 'receptor-enriched' fraction (Davies & Griffiths, 1973; Irving & Mainwaring, 1974) was prepared by the dropwise addition of 0.5 vol. of satd. (NH₄)₂SO₄ solution (pH 7.4) in medium B [medium A containing 10% (v/v) glycerol], also containing 1 mM-dihydrotestosterone. Precipitates were collected, resuspended in medium B and desalted on columns of Sephadex G-25 as previously described (Davies & Griffiths, 1973). Chromatography on columns of DNA–cellulose prepared from native (double-stranded) DNA from rat ventral-prostate gland or calf thymus was done as described by Mainwaring & Irving (1973). Columns were made from a mixture of 1.5 g (wet wt.) of DNA–cellulose and 0.5 g of Whatman cellulose, both previously equilibrated with medium B. Samples of cytoplasmic steroid–receptor complex were applied in medium B (2 ml; 0.5–1 mg of protein/ml). Adsorbed proteins were eluted with medium B containing 0.5 M-KCl, and samples were desalted before analysis or use in other systems by passage through columns of Sephadex G-25. This process also removed the excess of free steroid present during the preparative procedure to adjust for dissociation of endogenous steroid.

The preparations eluted from columns of DNA–cellulose were free of nucleic acid contaminants and were devoid of RNA polymerase, ribonuclease and deoxyribonuclease activities, as determined by the following criteria: [³H]UMP was not incorporated into an acid-insoluble product in the presence of a DNA template and the DNA–cellulose eluate but in the absence of RNA polymerase; the eluted material could cause no degradation of [³H]RNA synthesized by authentic RNA polymerase from rat ventral prostate, nor of [³H]DNA at either pH 5.0 or pH 8.0. The characterization of ³H-labelled steroid–protein complexes throughout the procedure was done by centrifugation through linear 5–20% (w/v) sucrose gradients (Davies & Griffiths, 1973, 1974b). Complexes formed between receptor proteins and unlabelled dihydrotestosterone were identified by exchange techniques based on precipitation with protamine sulphate and addition of exogenous dihydro[³H]testosterone (Davies et al., 1977) or on addition of ³H-labelled compound R1881 (Bonne & Raynaud, 1976). The latter conditions were used when subsequent gradient analysis of the ³H-labelled steroid–receptor complex was necessary.

Preparation of chromatin and DNA. Nuclei were purified from homogenates of rat ventral-prostate tissue as previously described (Davies & Griffiths, 1973). Chromatin devoid of endogenous RNA polymerase activity was prepared by the method of Mainwaring & Peterken (1971). Prostate DNA was prepared rapidly from chromatin as described by Davies & Griffiths (1975).

Purification of rat ventral-prostate RNA polymerase B

The scheme for purification of RNA polymerase B has been described (Thomas et al., 1977). Transcription of chromatin by this enzyme was sensitive to low concentrations of a-amanitin (Davies & Griffiths, 1974a,b; Thomas et al., 1977).

Interactions of dihydrotestosterone–receptor complexes and chromatin

The transfer of dihydrotestosterone–receptor complexes into chromatin was carried out under the conditions defined by Mainwaring & Peterken (1971). The specific uptake of ³H-labelled steroid was assessed by direct solubilization and measurement of radioactivity (Davies & Griffiths, 1973). The specific uptake of receptor bound to endogenous unlabelled steroid was monitored by the exchange procedure of Davies et al. (1977).

Determination of RNA polymerase activity

RNA polymerase activity was routinely assessed as described by Thomas et al. (1977). The rate of elongation of RNA chains was determined as previously described (Thomas et al., 1977), except that a final concentration of KCl of 0.1 M was used to prevent reinitiation by prostate RNA polymerase B. All other aspects of the reaction and characterization of the product were as reported (Thomas et al., 1977).

Counting of radioactivity

The ³H radioactivity in acid-insoluble precipitates and aqueous samples was counted in a Nuclear-Chicago (Mark I or II) scintillation spectrometer in 1978.
a phosphor comprising 5g of 2,5-diphenyloxazole in a mixture of 500ml of Triton X-100 and 1 litre of toluene at an efficiency of 20–40% determined by external standardization.

Chemical analyses

DNA and protein contents of fractions were measured as previously reported (Thomas et al., 1977).

Results

Partial purification of dihydrotestosterone-receptor complex

$^3$H-labelled cytosol (3.54×10$^4$d.p.m./mg of protein) was 33% saturated with (NH$_4$)$_2$SO$_4$ to yield a 'receptor-enriched' fraction (3.75×10$^4$d.p.m./mg of protein). Subsequent chromatography on DNA-cellulose provided a 100-fold purified dihydro-$^3$H-testosterone-receptor complex (4.02×10$^6$d.p.m./mg of protein after desalting), on the basis of specific radioactivities. As shown by Mainwaring & Irving (1973), free or non-specifically bound dihydro-$^3$H-testosterone was not retained by DNA-cellulose. The procedure provided a rapid means of separating the androgen–receptor complex from non-specific binding components in a suitable form for inclusion in reconstituted systems. Similar results were obtained with rat prostate DNA or calf thymus DNA.

At 1 day after castration, total cytoplasmic receptors are decreased by 50% (Davies et al., 1976). Moreover, $^3$H-labelled steroid–receptor complexes could not be added to a reaction mixture containing $[^3$H]UTP. For these reasons, prostate glands from normal rats were used in later experiments. The integrity of steroid–protein complexes was checked at each stage of purification by exchange with $^3$H-labelled compound R1881 and sedimentation analysis. A characteristic two-peak binding profile was obtained with samples of whole cytosol (Fig. 1a). Samples of material precipitated by 33% saturated (NH$_4$)$_2$SO$_4$, and resuspended, were resolved into a single specific 8S peak on gradients (Fig. 1b), whereas the supernatant after salt fractionation comprised only non-specific proteins (Fig. 1c). It is noteworthy that the 8S peak retained its form even after prolonged incubation of samples at 15°C during the exchange procedure. From the data in Fig. 1, it can be calculated that whole cytosol bound $^3$H-labelled compound R1881 to the extent of 241 fmol/mg of protein (Fig. 1a), whereas the salt-precipitated fraction (Fig. 1b) bound 2086 fmol/mg of protein. Similar

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**Fig. 1.** Sedimentation analysis of $^3$H-labelled compound R1881–receptor complexes

Cytosol was prepared from ventral-prostate glands of normal intact rats and adjusted to 33% satd. (NH$_4$)$_2$SO$_4$. The precipitate was sedimented at 10000g, for 30 min. Samples of the resuspended precipitate and of the supernatant were desalted through columns (10 cm × 1 cm) of Sephadex G-25. Samples of the various preparations were incubated with $^3$H-labelled compound R1881 (20 nM), alone (a) or with compound R1881 (2 μM) (c) under the conditions described by Bonne & Raynaud (1976), and, after removal of steroid by charcoal adsorption [final concentration 1% (w/v) Norit A, 0.1% (w/v) Dextran T-70], samples (400μl) were layered on linear sucrose density gradients [5–20% (w/v)] in a uniform concentration of medium A], and analysed as previously described (Davies & Griffiths, 1973, 1974b). (a) Unfractionated cytosol: protein input 5.57mg; specific binding equivalent to 26800 d.p.m. (1337 fmol); (b) resuspended salt precipitate: protein input 280μg; specific binding equivalent to 12073 d.p.m. (602 fmol); (c) supernatant after salt fractionation: protein input 2.36mg; no specific binding. In all cases, centrifugation was from left to right. Fractions of volume approx. 170μl were collected. Sedimentation marker (arrows) was bovine serum albumin (S20,w 4.6S).
values were obtained with dihydro[^H]testosterone by the protamine sulphate-exchange method. The degree of enrichment of the receptor fraction observed here is not identical with that obtained with ^3H-labelled cytosol (see above), but falls within the range achieved over a number of experiments using either method (8-12-fold). Further purification by adsorption on immobilized DNA (Fig. 2) yielded a fraction that specifically retained ^3H-labelled compound R1881 or dihydro[^H]testosterone to the extent of 21000fmol/mg of protein.

Uptake of androgen-receptor complexes into chromatin and effects on transcription by RNA polymerase B

The specific uptake of the partially purified dihydrotestosterone-receptor complex into prostate chromatin from rats castrated 1 day previously was measured by exchange with dihydro[^H]testosterone (Fig. 3a). The saturation value was 2250fmol of dihydrotestosterone/mg of DNA. Since this method measures all the potential nuclear binding sites in 24h-castrated animals, it can be calculated that approx. 11000 molecules of dihydrotestosterone are required to saturate all such sites in a single prostate cell. Such a number has been previously reported (Davies et al., 1977).

An input of steroid-receptor complex of approx. 100fmol of dihydrotestosterone (10000fmol/mg of DNA) was required to saturate all nuclear binding sites (Fig. 3a) and to produce maximum stimulation of transcription by RNA polymerase B (Fig. 3b). A preincubation period of 30min at 37°C was also necessary for maximum stimulation of transcription (Fig. 4). The optimum conditions thus determined for saturation of chromatin acceptor sites and for increased transcription were used in subsequent experiments.

Rate of elongation of RNA chains

In these studies it was essential to establish conditions in which the initial reaction was linear with time during the period of measurement. To measure the initial rate of RNA-chain elongation independently of the initiation reaction, initiation of RNA molecules was completed before extensive elongation was allowed to proceed (see the Materials and Methods section). Elongation, when measured in this way, was linear with time up to 80s (Fig. 5), when either the amount of template or the concentration of nucleoside triphosphate became rate-limiting.

Kinetics of nucleoside triphosphate addition

The process of polyribonucleotide-chain elongation probably involves several steps. The kinetics of nucleoside triphosphate addition can be investigated by examining the rate of the reaction as a function of the nucleoside triphosphate concentrations. The average velocity of incorporation (v) in terms of nucleotides per unit time per chain and the average time of incorporation per nucleotide (t) are given by:

\[ \frac{1}{v} = t = \sum_{x=\text{A},\text{U},\text{G},\text{C}} f_x t_x \]  

(1)

where \( f_x \) is the mole fraction of nucleoside triphosphate \( x \) in the product RNA chain, and \( t_x \) is the mean time for the addition of \( x \) to the chain, after the previous nucleoside triphosphate has been incorporated. The quantities \( t_x \) are calculated as follows: consider a polymerase-DNA-RNA complex (ES), which is at a site where \( x \) is to be incorporated. The site is free (E) or has the nucleoside triphosphate (S) bound in an enzyme-substrate complex (ES). The corresponding concentrations are [E], [E^S], [S], [ES]. The reactions for the reversible formation of the enzyme-substrate complex are:

\[ E^S + S \xrightleftharpoons[k_{-1}]{k_{1}} ES \]  

(2)

and

\[ [E^S] = [E] + [ES] \]  

(3)

1978
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Fig. 3. Incorporation of dihydrotestosterone-receptor complex into prostatic chromatin, and effects on transcription
(a) Chromatin from the prostate glands of rats castrated 1 day earlier was incubated with increasing concentrations of dihydrotestosterone–receptor complex (160 fmol of dihydrotestosterone/100 µl). The protein concentration was kept constant by addition of the necessary quantities of heat-denatured androgen–receptor complex. The amount of dihydrotestosterone specifically associated with chromatin was measured by an exchange technique (Davies et al., 1977).
(b) Increasing concentrations of dihydrotestosterone–receptor complex (as above) were incubated with chromatin and activity of RNA polymerase was determined with the chromatin as template. The activity is given as the increase in incorporation of [3H]UMP over that observed in the absence of dihydrotestosterone–receptor complex (47.2 fmol/min).

Fig. 4. Effect of preincubation of dihydrotestosterone–receptor complex with chromatin on transcription properties
Ventral-prostate chromatin from rats castrated 24 h previously was preincubated with dihydrotestosterone–receptor complex (160 fmol of dihydrotestosterone/10 µg of DNA) for various periods of time (up to 60 min) before addition of RNA polymerase. Activity is expressed as incorporation of [3H]UMP above that observed in the absence of androgen–receptor complex (58.1 fmol/min).

The overall velocity of reactions (2) and (4) is obtained by the standard Michaelis–Menten argument as:

\[
\frac{1}{[E_x]} \cdot \frac{d[P]}{dt} = \frac{k_{2a}[S_x]}{K_a + [S_x]}
\]  

(5)

where:

\[
K_a = \frac{k_{-1x} + k_{2a}}{k_{1x}}
\]  

(6)

Thus the velocity and propagation time for step \( x \) per chain are given by:

\[
\frac{1}{v_x} = \frac{K_a + [S_x]}{k_{2a}[S_x]} = \frac{K_a}{k_{2a}[S_x]} + \frac{1}{k_{2a}}
\]  

(7)

Then, from eqn. (1), the overall rate of incorporation per chain is given by:

\[
\frac{1}{v} = t = \sum_{x=A,G,C,U} \left[ \frac{f_x}{k_{2a}} + \frac{f_x K_a}{k_{2a}[S_x]} \right]
\]  

(8)

In a typical experiment, one nucleoside triphosphate \( (S_p) \) is adjusted to a lower concentration than the other three, and this concentration is varied. Eqn. (8) predicts that a plot of \( 1/v \) versus \( 1/[S_p] \) should be a straight line with a slope of \( f_p K_p/k_{2p} \). The intercept on the \( 1/v \) axis at \( 1/[S_p] = 0 \) is:

\[
1/v(1/[S_p] = 0) = \sum_{x=A,G,C,U} \frac{f_x}{k_{2a}} + \sum_{x=A,G,C,U} \frac{f_x K_a}{k_{2a}}
\]  

(9)

Data supporting the rate-law eqn. (8) have been presented for the bacterial enzyme (Bremer, 1967; Hyman & Davidson, 1970; Rhodes & Chamberlin, 1974; Solage & Cedar, 1976a).
Effect of dihydrotestosterone-receptor complexes

When the concentrations of three of the nucleoside triphosphates are kept constant while the concentrations of the fourth (β) is varied, a straight line is obtained from a plot of 1/ν against 1/[Sβ]. The slope of this line (fβKβ/K2β) should be independent of the concentrations of the other nucleoside triphosphates. These kinetic data hold true for the transcription of rat ventral-prostate chromatin by homologous RNA polymerase B (Fig. 6). The slope of the graph remains constant over a wide range of fixed concentrations of the other nucleoside triphosphates.

Effect of dihydrotestosterone–receptor complexes on the rate of elongation of polyribonucleotide chains

The effects of dihydrotestosterone–receptor complexes on the initial rate of elongation of RNA chains with prostate chromatin as template were investigated. In preliminary experiments the range of values (nucleotides/s, ±S.D.) under optimum conditions of nucleoside triphosphates were as follows: with chromatin from normal rats as template, 11 ± 1.9 (n = 6); with chromatin from normal rats plus dihydrotestosterone–receptor complexes, 14 ± 2.6 (n = 4); with chromatin from normal rats plus denatured complexes, 9 ± 1.8 (n = 4); with chromatin from castrated rats as template, 7 ± 0.7 (n = 10); with chromatin from castrated rats plus dihydrotestosterone–receptor complexes, 12 ± 2.2 (n = 7). The inclusion of mixtures of dihydrotestosterone and proteins from serum or liver cytosol at similar concentrations to dihydrotestosterone–receptor complexes had no significant effect on the initial rate of elongation of RNA chains with prostate chromatin from either normal or castrated rats as template. With the system outlined above, it was observed that, although the rate of elongation of RNA chains with chromatin from castrated rats as template is less than that with chromatin from normal rats as template (Fig. 7), no change is observed in the slope of the graph, i.e. nucleoside triphosphate addition. Also, whereas the inclusion of dihydrotestosterone–receptor complexes at saturating concentration increased the rate of elongation on both templates, particularly on chromatin from castrated rats, and the addition of heat-denatured steroid–receptor complexes appeared to inhibit chain elongation slightly, in no instance was a change in the slope of the graph evident.

Discussion

The development of exchange techniques (Bonne & Raynaud, 1976; Davies et al., 1977) enabled the purification of endogenous 5α-dihydrotestosterone–receptor complexes to be carried out free of the doubts attached to calculating endogenous contents from parallel labelled preparations. Such complexes were purified to similar extents to those obtained by Mainwaring & Irving (1973). It is noteworthy that under exchange conditions the 8S form of the receptor is maintained. This could be due to the ineffectiveness of the incubation temperature and time (15°C for 16h) to promote previously reported conformational changes (Mainwaring & Irving,
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In the present study, RNA-chain elongation has been measured under conditions designed to isolate propagation from the other steps in RNA synthesis. RNA synthesis is initiated in low salt in the presence of three nucleoside triphosphates producing RNA polymerase-DNA complexes with short oligonucleotide chains which cannot be elongated without the fourth nucleoside triphosphate. After the addition of the fourth nucleoside triphosphate, chain elongation is linear with time (Fig. 5) and during this period (80s) no new initiations can occur (Thomas et al., 1977). Thus RNA propagation can be studied under various conditions, although initiation occurs under constant conditions.

When three of the nucleoside triphosphates (α) were kept at a fixed concentration and the fourth (β) was varied, the kinetic parameters of β were independent of α. As shown in Fig. 6, the slope obtained by plotting 1/ν against 1/[Sb] was independent of the concentrations of other nucleoside triphosphates.

Apart from nucleoside triphosphate addition, RNA-chain elongation also involves DNA unwinding and translocation of RNA polymerase from one site on DNA to another (Solage & Cedar, 1976a). A comparison of RNA-chain elongation on different templates showed that prostate chromatin from castrated rats supported a lower rate of elongation than that on chromatin from normal rats, thereby confirming previous observations (Davies et al., 1976; Thomas et al., 1977). This may be due to altered relative concentrations of chromatin proteins (Couch & Anderson, 1973a,b; Davies & Griffiths, 1974a; Thomas et al., 1977) affecting propagation (Solage & Cedar, 1976b). Inclusion of dihydrotestosterone-receptor complexes alleviated the inhibition. Heat-denatured complexes inhibited propagation, possibly owing to non-specific association with templates. However, in these kinetic studies, no change in the slope of the graph was observed, suggesting that the action of androgen-receptor complexes is not directed towards the addition of nucleoside triphosphates.

These studies therefore emphasize the influence of androgen-receptor complexes on RNA-chain elongation by RNA polymerase B. They also eliminate the involvement of one of the steps in elongation and suggest that androgen-receptor complexes affect the rate of DNA unwinding or the movement of RNA polymerase from one nucleotide to the next.

We are grateful to the Tenovus Organisation for their generous financial support.

References


Fig. 7. Effect of dihydrotestosterone-receptor complexes on kinetics of elongation

Elongation of polynucleotide chains was measured at various concentrations ([Sb]) of (a) UTP or (b) GTP, by using RNA polymerase B transcribing chromatin from normal rats (○), chromatin from castrated (24h) rats (□), chromatin from castrated rats preincubated with androgen-receptor complexes (▲), chromatin from normal rats preincubated with androgen-receptor complexes (△) or denatured androgen-receptor complexes (●). Data are adjusted for equal amounts of enzyme-DNA complex.

1973; Davies & Griffiths, 1974a,b). Alternatively, compound R1881 may not bind with the necessary characteristics to produce an ‘activated’ receptor.

The use of reconstituted systems in vitro (Davies & Griffiths, 1974a,b; Mainwaring & Jones, 1975; Hu et al., 1975) has provided valuable information about the regulation of transcription by androgen-receptor complexes. Effects on RNA polymerase activity, however, do not necessarily specify the individual processes intimately involved. Earlier studies (Mainwaring & Jones, 1975; Davies, 1975; Davies et al., 1976; Thomas et al., 1977) indicated that chain elongation was primarily affected by androgen-receptor complexes. However, chain elongation can be separated into several steps. Kinetic studies have now been used in an attempt to clarify the initial site of action of dihydrotestosterone-receptor complexes.

10. Receptor–Genome Interaction

REGULATION OF TRANSCRIPTION OF THE PROSTATE GENOME BY ANDROGENS

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SUMMARY

The effect of androgen withdrawal on a number of parameters of transcription has been studied using reconstituted systems based on rat ventral prostate chromatin or nuclei. Nuclear receptors, total RNA polymerase activity, the numbers of RNA polymerase molecules active in transcription, RNA-chain lengths and the rates of their elongation are all significantly decreased 1 day after castration and continue to decrease, less dramatically, until 7 days after castration. Both a-amanatin-sensitive and a-insensitive RNA polymerase activities were affected. The changes in initiation sites on chromatin available to RNA polymerase B do not correlate with alterations in receptor levels and RNA synthesis in intact nuclei. Possible reasons for these discrepancies are discussed. Hybridization of cellular poly(A)-containing RNA from prostates of normal rats and rats castrated 3 days previously suggested that castration resulted in the reduced concentration of a small number of highly-abundant poly(A) RNA sequences.

INTRODUCTION

Cells of the rat ventral-prostate gland contain receptor proteins which selectively retain androgens, particularly 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) [1, 2]. The transference of complexes thus formed to, and their retention by, specific nuclear components [3–5] are characteristic features of androgen-responsive cells. The consequences of this intranuclear localization include stimulation of the enzymes responsible for the cytoplasmatic protein-synthesizing machinery [10, 11], the general enhancement of mRNA synthesis [12, 13], and regulation of the processes leading to replication [14, 15] and cell proliferation [16]. The possibility exists that relatively late events stem from the initial improvement of prostate chromatin as a template for RNA polymerase (EC 2.7.7.6; nucleoside triphosphate-RNA nucleotidyltransferase) B [17–20], instigating subsequent happenings through interdependent control processes thus set in motion, although the continued presence of the androgen-receptor complex appears necessary for complete response. This situation will probably be clarified by the study of steroid-inducible specific mRNAs [21, 22] and proteins [23, 24].

Although steroid-mediated effects upon mRNA availability are elicited to a major extent through transcrip[tive pathways, they may not be limited to a single mechanism. For instance, oestrogens have been reported to modulate mRNA turnover [25] and processing [26], and certain androstane derivatives appear to influence translational systems [27]. Depen-

dent upon metabolism and the target tissue, androgens stimulate the formation of a variety of products [27], and may even act dissimilarly in different sexual accessory glands of the rat [cf. 21 and 28]. These facts suggest that regulation at the genome operates at a level much more tissue-specific than that of steroid-receptor interaction. For this reason, the mechanism of action of androgens cannot be expressed generally beyond its most basic aspects. It is our contention [18–20] that the influence of androgens, and androgen-receptor complexes, on the prostate genome should be appraised critically with reference to the factors involved in intracellular regulation of RNA polymerase activity and selectivity [29] and of template activity [19, 30]. Undoubtedly, our understanding of androgenic regulation of transcription, particularly that of specific genes, will benefit from a fastidious scrutiny of the multiplicity of processes involved in the production of mRNA.

MATERIALS AND METHODS

Experimental animals. Details of experimental animals, operative procedures, and preliminary processing of tissues are given in previous publications [18–20].

Chemicals. The suppliers and grades of chemicals not included herein have been listed previously [18–20]. The Radiochemical Centre, Amersham, Bucks., U.K. provided [d8-3H]-ATP (specific radioactivity 10–30 Ci/mmol), [d5-3H]-CTP (specific radioactivity 10–30 Ci/mmol), [d8-3H]-GTP (specific radioactivity 10–30 Ci/mmol) and poly[5-3H]-uridylic acid (specific radioactivity 400–600 Ci/mmol of nucleoside residue). Unlabelled nucleosides and nucleotides, 5'-phospho-[2'-deoxy-thymidylyl-(3'-5')]12–18.
deoxy-thymidine [oligo(dT)], actinomycin D, RNA-dependent DNA polymerase (reverse transcriptase) from avian myoblastosis virus, DNase I (EC 3.1.4.5; deoxyribonucleate 3'-oligonucleotidohydrolase) from bovine pancreas (Grade II) and subtilisin (EC 3.4.21.14; subtilopeptidase A) from Bacillus subtilis were obtained from the Boehringer Corporation (London) Ltd., U.K. S1 Nuclease was purchased from Sigma (London) Chemical Co. Ltd., U.K. Polyethyleneimine (PEI)-cellulose coated sheets were obtained from Camlab Ltd., U.K.

Preparation of subcellular fractions. Methods for the preparation of soluble supernatants, nuclei and chromatin have been previously described [7].

Measurement of receptor sites. Cytoplasmic and nuclear androgen receptors were identified and quantitatively assessed by sedimentation analysis [7], gel exclusion chromatography [31] and/or exchange procedures after precipitation with protamine sulphate [32]. Cytoplasmic and nuclear volumes and intracellular concentrations of androgens were calculated according to Bruchovsky et al.[33]. Physical characteristics of receptor proteins were derived from established relationships [see 33].

**Figure 1.** A scheme for the quantitation of \(^{3}H\)-uridine and \(^{3}H\)-UMP in alkaline hydrolysates of RNA. The rationale for the scheme is outlined below. First, the total radioactivity in the alkaline hydrolysate is measured per mg nuclear DNA when both parameters can be conveniently quantitated. This radioactivity consists entirely of uridine and UMP, since all unincorporated precursor is removed by extensive washing. Values are corrected for changes in the specific activity of exogenous \(^{3}H\)-UTP due to nucleoside triphosphate pools in nuclei, and for hydrolysis of UMP to uridine. Incorporation of radioactivity into uridine and UMP per µg DNA was calculated from the initial ratio and the total radioactivity incorporated into RNA per unit DNA. Assays were carried out in the presence and absence of α-amanitin. Assuming that prostate nuclei contain 7.5 µg DNA per cell and knowing the specific activity of \(^{3}H\)-UTP in the assay, approximate values for the numbers of actively transcribing RNA polymerases sensitive and insensitive to α-amanitin can be calculated.
Synthesis of cDNA. The synthesis of [3H]-labelled DNA complementary to poly(A)RNA (cDNA_{poly(A)RNA}) was essentially as described for ovalbumin cDNA [40]. [3H]-Labelled dGTP, dATP and dCTP (100 μCi of each) were dried down and resuspended in a solution (1 ml) containing 50 mM-Tris-HCl, pH 8.3, 20 mM-dithiothreitol, 6 mM MgCl₂, 100 mM-KCl, 200 μM-dTTP, oligo(dT) at 5 μg/ml, actinomycin D at 36 μg/ml and poly(A)RNA at 25 μg/ml. The mixture was chilled on ice then reverse transcriptase was added to a final concentration of 60 units/ml, and the mixture briefly vortexed. Incubations (30 min at 46°C) were terminated by adjustment of the mixture to 0.5% (w/v) with sodium dodecylsulphate, 10 mM with respect to EDTA and 50 μg/ml with respect to heat-denatured, sonicated calf-thymus DNA. The fraction excluded from a column of Sephadex G50 equilibrated 100 mM-Tris-HCl, pH 7.6, containing 5 mM-EDTA, was precipitated in 2 vol. ethanol containing 200 mM-sodium acetate, pH 5.5. The precipitate was redissolved in 200-400 μl of 100 mM-NaOH, containing 10 mM-EDTA, heated at 60°C for 30 min, the pH adjusted to 5.5 with acetic buffer, and cDNA was reprecipitated with ethanol, dissolved in a minimal volume of water and stored at -20°C. [3H]-cDNA was analyzed on alkaline sucrose gradients [40].

Rat globin mRNA prepared from cultures of fetal rat-liver by a modification of the method of Ross [41] was used for the synthesis of cDNA_{globin}.

Hybridization of poly(A)RNA with cDNA. Hybridization [21] was carried out in 240 mM-sodium phosphate buffer (equimolar), containing 0.05% (w/v) sodium dodecylsulphate and 1 mM-EDTA, at 70°C, in vol. of 50 μl. Excess poly(A)RNA (for concentrations see Results) was mixed with sufficient cDNA to give 1000 c.p.m./sample, and overlaid with paraffin oil. Driver RNA concentration was calculated from optical density measurements assuming that 1 pg/mg unit/ml is equivalent to 40 pg RNA. The extent of hybrid formation was related to resistance to S₁ nuclease. Aliquots (40 μl) of samples were added to mixtures (800 μl) containing 0.4 M-sodium acetate, pH 4.5, 0.8 M-NaCl, 5 mM-ZnSO₄ and 25 μg of denatured DNA. This mixture was divided into duplicate samples. To one was added S₁ nuclease (100 units), and both were incubated at 37°C for 2 h. Assays were terminated by addition of 10% (w/v) trichloroacetic acid and bovine serum albumin to 1 mg/ml. After mixing and retention in ice-salt for 15 min, precipitates were washed, collected, dried and analyzed after treatment with reaction conditions).

Computer programs. Radioactivity was estimated as previously described [20]. Protein and DNA were measured by the usual methods [see 20].

RESULTS

Determination of cellular receptor levels

Androgen receptor sites were measured after labeling in vitro [7] or in vivo [32] by: (a) sedimentation analysis; (b) gel-exclusion chromatography; and (c) saturation analysis combined with exchange with exogenous [3H]-steroid. Techniques (a) and (b) proved practicable for reproducibility with tissue from castrated animals, whereas technique (c) was the most suitable for the measurement of receptor sites in tissue fractions from intact animals, and for the rapid determination of dissociation constants (Kₐ). Values given below are the means of several determinations ± S.D. The rat ventral prostate nuclear androgen-receptor complex detected by sedimentation analysis (Fig. 2a) had a sedimentation coefficient of 3.3 ± 0.14 S (n = 10), and, by gel-exclusion chromatography (Fig. 2b) was shown to have a Stokes radius of 2.6 ± 0.12 nm (n = 8). From these data, it was calculated that the complex has a mol. wt. of 35,500 and a frictional ratio of 1.21. No nuclear receptor was detected 7 days after castration during these experiments, so any alterations in the physical characteristics of the receptor [16] could not be confirmed.

Saturation analysis (e.g. Fig. 2c) revealed that nuclear binding was a saturable function representing a single class of binding sites with an apparent Kₐ in the range 5–10 nmol/l. Similar analyses of cytoplasmic receptors (not shown) yield (a: in high salt) a sedimentation coefficient of 4.4 S, a Stokes radius of 4.5 nm, a mol. wt. of 83,000 and a frictional ratio of 1.56 (b: in low salt) a sedimentation coefficient of 7.7 S, a Stokes radius of 7.75 nm, a mol. wt. >240,000 and a frictional ratio of 1.89, and Kₐ values in the range 2–10 nmol/l. A combination of the three techniques showed that a nuclear receptor concentration (molecules/nucleus in each case) of 14,000 ± 1800 (n = 12) decreased to 5500 ± 450 (n = 8) 24 h after castration, and was further reduced to 1500 ± 200 (n = 4) and 800 ± 150 (n = 4) 2 and 3 days, respectively, after castration. Nuclear receptor was virtually undetectable 7 days after castration. Cytoplasmic receptors (molecules/cell in each case) increased from 13,000 ± 250 (n = 4) in intact animals to 21,000 ± 650 (n = 5) 18–24 h after castration, and thereafter decreased to 10,000 ± 500, 6200 ± 100 and 3100 ± 150 (n = 5 in each case), 24–48 h, 48–60 h, and 72 h, respectively, after castration, and to a basal level of 2000 ± 300 (n = 5) 7 days after castration. Concomitantly, the nuclear [3H]-androgen concentration fell from a control level of >60 nmol/l to 23 ± 2 nmol/l (n = 8) 1 day after castration, 7 nmol/l 2 days after castration, approx. 4 nmol/l 3 days after castration, and <0.5 nmol/l 7 days after castration. Similarly, cytoplasmic androgen concentrations decreased from >16 nmol/l, to 9 nmol/l, to 6 nmol/l, to 2.5 nmol/l.

The effect of castration on parameters of transcription measured in vitro

A number of transcription parameters have been studied using either purified intact nuclei, or purified chromatin as a template. The use of nuclei made the study of both α-amantatin-sensitive and -insensitive
Fraction No. Elution Volume (ml) Bound Steroid (pmol/l)

Fig. 2. Identification and measurement of nuclear receptor sites. Nuclear receptor sites were measured by sedimentation analysis (a), gel-exclusion chromatography (b) and saturation analysis combined with exchange of endogenous steroid and exogenous [3H]-DHT. (a) Nuclei were prepared from the prostate glands of rats injected with 200 µCi of 1α,2α-[3H]-testosterone 1 h before death, extracted with 0.6 M NaCl and extracts were layered over linear 5 ml 5–20% (w/v) sucrose gradients containing an uniform concentration of 1 M NaCl and centrifuged at 100,000 g for 16 h at 4°C. The radioactivity in 3-drop (~0.17 ml) fractions was measured. Centrifugation was from left to right. The sedimentation marker (arrow) was bovine serum albumin (S_{20,w} 4.6 S). Radioactivity input was 17,250 d.p.m. (b) Nuclear extracts were prepared and processed on columns of Sephadex G-200 according to Bruchovsky et al.[31]. The arrow indicates the void volume of the column. Radioactivity (●) and protein content (broken line) were monitored. Under these conditions, 88% of macromolecule-bound radioactivity was released from excluded components and corresponded to androgen-receptor complex. (c) Nuclear receptor was quantitated according to Davies et al.[32]. Data shown are corrected for binding in the presence of a 100-fold excess of unlabelled competitor.

Fig. 3. Effect of castration on transcription characteristics of prostate chromatin and nuclei. Several parameters of transcription were measured using intact nuclei or chromatin prepared from the ventral prostate glands of normal intact rats or of rats castrated at various times before death. All parameters are expressed as percentages of the values per cell observed using subcellular fractions from intact animals. Transcription insensitive (a) and sensitive (b) to low concentrations (40 ng/assay) of α-amanitin was measured; parameters studied were total RNA synthesis (●), elongating RNA polymerase molecules (○), the rate of elongation (△) and the number-average chain length (□). The cellular complement of cytoplasmic (○) and nuclear (■) androgen-receptor complexes, total (α-amanitin-sensitive and -insensitive) RNA polymerase activity (●) and chromatin initiation sites (▲) are shown in (c). Actual values for transcription parameters are shown in Table 1; values for receptor concentration are included in the first section of Results.
RNA polymerase activities possible, whereas, with chromatin, it was feasible to monitor only α-amanitin-sensitive RNA polymerase (i.e. RNA polymerase B), as RNA polymerase A could not initiate efficiently on chromatin in vitro. The nuclear system was adapted to measure 3'-termini generated by the individual RNA polymerase molecules active in transcription, and, from the proportions of [3H]-uridine and [3H]-UMP present in alkaline-hydrolysates (see Fig. 1), the rates of chain elongation and the number-average lengths of polynucleotide chains were calculated. The limitations of these calculations are discussed later, and the various correction factors which must be employed are detailed in the legend to Fig. 3.

It was observed that approx. 62% of endogenous RNA synthesis was insensitive to low concentrations of α-amanitin, whilst 38% was sensitive to the drug. Both activities were dramatically reduced following castration (Fig. 3a, b), although α-amanitin-sensitive activity declined to lower levels (Fig. 3b), representing, on average, 25% of total activity after castration. The decrease in activity could be attributed in both cases to a reduction in the numbers of transcribing molecules of enzyme (actual numbers are detailed in Table 1), although significant and correlative diminutions in chain lengths and elongation rates may be observed (Fig. 3a, b), especially in the case of α-amanitin-sensitive activity (Fig. 3b). The reduction in the rate of RNA synthesis corresponds closely to the fall in nuclear androgen-receptor concentration (Fig. 3c), but the number of initiation sites on prostate chromatin accessible to endogenous RNA polymerase B, although showing a tendency to decrease after castration, do not exhibit so absolute a dependence on endocrine status (Fig. 3c).

If α-amanitin-sensitive activity in nuclei can be directly compared with the activity of RNA polymerase B, the data obtained using nuclei and chromatin show certain disparities (Table 1), which may arise from discrepancies inherent in the methodologies. An assessment of elongation rates, for instance, showed that those achieved by RNA polymerase B on chromatin are far in excess (>20-fold) of those using nuclei. The refinement of nuclei to chromatin may remove regulatory factors which restrict transcription. However, elongation in vitro is probably more rapid than that using either system in vitro, which could indicate the loss of elongation factors during the preparation of nuclei. These apparently contradictory aspects serve to emphasize the complexity of nuclear transcription and the hazards implicit in reconstruction experiments in vitro. Whatever the case, and whether or not elongation in vitro reflects the situation in vivo, androgen withdrawal affected chain elongation further. The number-average chain length, similar in both systems, was reduced after orchidectomy. However, although the number of transcribing RNA polymerase B molecules was reduced by more than 60%, 24 h following castration, the number of chromatin sites potentially available for initiation of RNA synthesis did not diminish during this period. Clearly, interpretation of those data obtained from studies in vitro must be critically reappraised if regulatory mechanisms probably allied to receptor systems still in control in intact nuclei can be overridden with sub-nuclear preparations using vast excesses of exogenous enzyme.

Further investigation of elongation of RNA polymerase B using chromatin as a template showed that the process follows the kinetics of a multisubstrate enzyme system in which the addition of each nucleotide is independent of the concentrations of the others (Fig. 4a). The kinetics of this reaction are evaluated in Ref. 20. Adaptation of this system to include androgen-receptor complexes indicated that, although these affect the gross rate of chain elongation, the influences were not directed towards nucleotide triphosphate addition (Fig. 4b).

Studies on the complexity of poly(A)RNA from rat prostate

An alternative approach to the measurement of transcription in reconstituted systems in vitro is the study of the complexity of poly(A)RNA present in the rat prostate. Analysis of the kinetics of hybridization of poly(A)-containing RNA with its complementary DNA can provide information on the numbers of different sequences in mammalian cells [42]. Application of this method to rat ventral prostate may indicate whether particular RNA species are under androgenic control. A great deal of similar work has been published [21, 22], therefore only the essentials are presented here.

Cellular poly(A)-containing RNA was isolated from the prostates of normal rats and of rats castrated 3 days earlier and hybridized to [3H]-DNA complementary to normal prostate poly(A)RNA. The cDNA used in these experiments sedimented in alkaline sucrose gradients at a position corresponding to a chain length of 300-350 nucleotides relative to standards. The number-average nucleotide chain length of poly(A) RNA, as determined by sucrose-gradient centrifugation followed by poly-[3H]-U hybridization of each fraction across the gradient, was in the range 1600-1650 for poly(A)RNA from both normal and castrated animals. Hybridization of cDNA with both types of poly(A)RNA is shown in Fig. 5 relative to the hybridization of globin mRNA with its complementary DNA. Globin mRNA hybridized over approx. two orders of magnitude of R_{d}, whereas homologous and heterologous hybridizations of prostate RNA took place over five orders of magnitude of R_{d}. The complexity of the various abundance classes of poly(A)RNA thus revealed to be present in prostate RNA was determined by comparing the R_{d/2} values of each class with that obtained for the globin mRNA, i.e. 5.4 × 10^{-4} mol/s. Globin mRNA coding for α and β chains has a complexity of 4 × 10^{5} daltons, and the prostate poly(A)RNA has a complex-
Table 1. Parameters of transcription of rat ventral prostate nuclei and chromatin at various times after castration

<table>
<thead>
<tr>
<th>Post-castration period (days)</th>
<th>Initiation sites (No./cell)</th>
<th>Molecules of RNA polymerase actively transcribing (No./cell)</th>
<th>Elongation rate (nucleotides/min)</th>
<th>Number-average chain length (nucleotides)</th>
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<tr>
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<td>x-Amanitin</td>
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<td></td>
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<td>Total</td>
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<td>Insensitive</td>
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<td>79,500</td>
<td>37,800</td>
<td>12,800</td>
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<td>75,500</td>
<td>17,000</td>
<td>4,200</td>
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<td>2</td>
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<td></td>
<td>46,600</td>
<td>15,000</td>
<td>5,600</td>
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<tr>
<td>7</td>
<td></td>
<td>14,600</td>
<td>5,100</td>
<td>9,500</td>
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</table>

The data in this Table were obtained using purified nuclei or chromatin from prostate glands. Transcription on chromatin was totally inhibited by x-amanitin, since chromatin was prepared in such a way as to be devoid of endogenous activity and absolutely dependent upon addition of RNA polymerase B. Initiation sites on purified chromatin were measured using rifamycin AF/013 or increased salt concentration to prevent reinitiation. Elongation rates on chromatin were estimated using the rifamycin challenge assay only. Details of the methodologies not available in this manuscript can be found in Refs. 19 and 20. The essential difference between assays on chromatin and nuclei is that initiation sites on chromatin are measured by incorporation of the first, or 5'-nucleotide, whilst the numbers of molecules active in transcription are calculated from the last, or 3'-nucleotide of a chain being synthesized. Numbers per cell are calculated assuming each prostate cell contains 7.5 pg of DNA. Dashes indicate that a particular determination has not been made.
Transcription in the prostate

Fig. 4. Kinetics of elongation of polyribonucleotide chains by RNA polymerase B. (a) Dependence of chain elongation on concentration of GTP. Elongation of polyribonucleotide chains was studied separately from initiation under conditions where the reaction rate was still linear [see Ref. 20]. The concentrations of ATP and CTP were saturating throughout (600 μM). The concentration of GTP was varied (see the abscissa) and the reaction rate was measured at 100 μM—(O), 150 μM—(△) and 200 μM—(●) UTP. (b) Effect of DHT-receptor complexes. Elongation was measured at various concentrations (see the abscissa) of GTP by using RNA polymerase B transcribing chromatin from normal rats (△), chromatin from castrated (24 h) rats (O), chromatin from castrated rats incubated with androgen-receptor complexes (100 fmol of DHT/10 μg chromatin DNA) (●), chromatin from normal rats incubated with heat-denatured androgen-receptor complexes (▲). Data are adjusted for equal amounts of enzyme-DNA complex.

Fig. 5. Hybridization of normal rat prostate cDNA with prostate poly(A)RNA from normal and castrated animals. Total cellular poly(A)-containing RNA prepared from the prostate glands of normal rats and rats castrated 3 days before the experiment was hybridized to DNA complementary to the poly(A)RNA from normal rats. The homologous reaction is represented by the open symbols and the heterologous reaction by the closed symbols. Concentrations of RNA between 2.4 and 2400 μg/ml were used. The hybridization of globin mRNA with its complementary DNA is shown (broken line) as reference.

As homologous and heterologous reactions proceed to almost the same level (Fig. 5), sequences in “normal” RNA are not absent but are still present as a lower abundance class in “castrate” RNA. The shift in the initial portion of the log curve of about 0.5 log units suggests that the more abundant sequences are present at a lower concentration, and are reduced in 3 day-castrated animals to about 60% of that in normal animals. Although a post-castration period of

ity of approx. 5.7 × 10⁵ daltons. The data for RNA from normal animals are consistent with the presence of three discrete abundance classes of poly(A)-containing RNA whereas those for castrated-animal RNA are consistent with the presence of two abundance classes. As shown in Table 2, prostate RNA from normal animals contains about five sequences in very high abundance, approx. 170 sequences of moderate abundance and about 6800 scarce sequences. RNA from castrated animals possessed a moderate abundance class of about 31 sequences and a low abundance class of approx. 7400 sequences. These data are essentially similar to those of Parker and Mainwaring [21] as absolute reliance cannot be placed on the estimates of numbers of sequences in each class [21, 42-44]. In this respect, the RNA used in these studies was assumed to comprise 75% poly(A)RNA.
3 days may be inadequate to compensate for sequences with long half-lives, these data show the androgen-dependence of certain highly-abundant sequences of poly(A)RNA, especially in the light of results [21] showing a further 10-fold decrease in the concentration of the abundant sequences at longer times after castration and restoration of hybridization at lower orders of magnitude of $R_{d1/2}$ by administration of testosterone.

**DISCUSSION**

A number of studies have indicated that the effects of testosterone on prostate RNA polymerase activities in vivo [6] may be reproduced in vitro using DHT-receptor complexes and prostate nuclei or chromatin [7-9, 17, 45]. Responses using chromatin appear limited to transcription by RNA polymerase B [17-20], and particularly to elongation of polyribonucleotide chains by this enzyme [17-20, 46]. Studies with reconstituted systems in vitro have been subject, justifiably, to criticism [27], due to use of crude cytoplasmic fractions containing receptor [7-9] possibly resulting in nonspecific effects. The use of bacterial RNA polymerase [45, 47] may also be criticised in view of differing characteristics of transcription by pro- and eukaryotic enzymes [7, 17]. For these reasons, the use of homologous [17-20] or, at least, mammalian [48] RNA polymerases and of partially-purified steroid-receptor complexes [17-20] is recommended. Discrepancies, however, remain evident.

Androgen withdrawal causes remarkable decreases in the numbers of $\alpha$-aminatin-sensitive and -insensitive RNA polymerase molecules active in transcription (Fig. 3, Table 1). If it is assumed that a large proportion of the activity insensitive to low concentrations of the drug corresponds to RNA polymerase A activity, its high level is consistent with the rapid turnover and renewal of endoplasmic reticulum elements observed in this secretory gland [10, 11]. Furthermore, although RNA polymerase B activity falls ultimately to a lower relative percentage (approx. 10%) of its own original activity, the drop in $\alpha$-aminatin-insensitive activity 1 day after castration (approx. 70%) represents a greater decrease overall in RNA synthesis, as previously observed [6]. As the androgen-receptor complex does not enter nuclei to any appreciable extent [49] its effects upon RNA polymerase A are probably indirect, and its direct effects upon chromatin may be more discernible via the activity of RNA polymerase B.

Precise measurement of 3'-termini is complicated [35] by ribonuclease and phosphatase activities and by the necessity of correction factors to allow for possibly irreproducible changes in uridine:UMP ratios during processing. From the results presented here, prostate cells contain approx. $1.3 \times 10^4$ RNA polymerase B molecules active in transcription per diploid genome. Assuming that the data in Table 2 reasonably estimate the number of mRNA encoded genes, then the ratio enzyme:gene is almost 2:1. As the number of different poly(A)RNA sequences is undoubtedly an underestimate of mRNA sequences, the actual ratio would be somewhat lower. The relative scarcity of sequences may be a reflection of preferential transcription, in which case a cellular concentration of $>1 \times 10^4$ copies of a single sequence [22] could be the result of higher levels of packing of RNA polymerase on a particular gene. This concept does not take into consideration variation in mRNA half-life, but emphasizes rates of initiation or accessibility of promoter sites, both being susceptible to regulation by chromosomal proteins [9, 47], and nuclear pools of free, nontranscribing RNA polymerases (currently under investigation).

Whatever the case, it is clear from Table 1 that the number of measured initiation sites is far in excess of either the numbers of transcribing molecules or the number of poly(A)RNA sequences. The latter could be explained by more than one DNA sequence coding for one poly(A)RNA sequence. The former suggests that: (a) regulation or repression indicated by concomitant decreases of nuclear receptor and elongating enzymes is not resistant to a barrage of exogenous RNA polymerase; (b) selectivity of transcription can be controlled by a balance between free and bound RNA polymerase; (c) even using homologous RNA polymerase and rifamycin AF/013 spurium initiation occurs at sites other than specific pro-
motor regions; (d) chromatin preparations have lost some structural or regulatory properties present in intact nuclei; (e) some RNA polymerase molecules form initiation complexes and maybe the first phosphodiesters bond but do not elongate further to produce 3'-termini measurable as uridine. The disappearance of nuclear receptor appears temporally linked to diminished elongation [18: Fig. 3], but the methods employed measure number-average chain lengths only and rates of elongation based on these. True relationships would be established by determination of these parameters for specific RNA chains.

These problems could be surmounted or circumvented in vitro by adequate analysis of changes in the spectrum of chain lengths of RNA populations in differing endocrine environments, the relationships of elongating RNA polymerases to actual RNA production rate in vitro, the relationship of free and bound RNA polymerases under differing androgenic status, and the elimination of variations in chromatin properties [50, 51] by use of preparations mirroring the situation in vivo [e.g. 52]. A new avenue of research is opened by the presence of specific androgen-dependent sequences of mRNA [21, 22; Fig. 5, Table 2]. A major effect of testosterone appears to be the regulation of the relative concentrations of RNA sequences [21]. This may be related to differential rates of production of mRNA, possibly through rates of elongation (see above), processing and turnover. Specific cDNA probes are potentially available, either from the DNA unannealed in heterologous relative to homologous hybridization reactions or, if this is not practical or if it is not the DNA complementatory to androgen-inducible sequences, by hybridization over a narrow range of low R0 values so that the only androgen-dependent RNA sequences form hybrids, followed by destruction of the RNA. Such a probe could be used to monitor the production and fate of mRNA in prostate cells. Hopefully, such information, coupled with data from improved reconstructed systems, will result in greater understanding of androgenic regulation of specific gene expression.

Acknowledgements—We are grateful to the Tenovus Organization for their generous financial support, and to the British Council for enabling Dr. Jariya Boonjawat to participate in these studies.

REFERENCES

chapter 17

Androgen Receptors in Human Normal, Hypertrophied, and Carcinomatous Prostates

Peter Davies and Keith Griffiths

The development of androgen receptor assays for use in assigning therapy in cases of prostatic disease has been impeded by a combination of three main factors. These are: (1) the occupation of the majority of receptor sites by endogenous steroid; (2) the instability of the androgen receptor; (3) the relatively high affinity for natural androgens shared by the receptor and by serum proteins. These problems hinder standardization and saturation analyses, and a variety of exchange procedures, as previously discussed. However, an understanding of the possible involvement of receptor complexes in, at least, some of the processes of diseased prostatic tissue requires a complete quantitative assessment of receptor retention and ligand-receptor interaction, and this has been achieved, above difficulties overcome, by two methods. The first involves the use of an androgen, R1881 (methyltrienolone: 1,17β-hydroxyoestra-4,9,11-trien-3-one), which binds only to receptor protein so doing confers greater stability to the receptor. The second method, used in our laboratories, involves the precipitation and stabilization of the receptor with protamine sulphate. This procedure has been shown to measure androgen receptor sites in rat ventral prostate tissue with accuracy, and has also been applied to human prostate tissue. Details of the method follow.

Samples of human prostate cytosol (7–12 mg of protein/ml) or nuclear suspension (10–30 μg of DNA/ml) were mixed with an equal volume (100 μl) of protamine sulphate solution (1 mg/ml in medium A, 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA and 0.25 mM dithiothreitol). The resulting precipitates were washed five times with 1 ml of medium A and then dispersed in 3H-labeled steroid solution (2–25 nmol/l), with and without a 100-fold higher concentration of radio-inert steroid. Incubations at 0–4°C for 16 hr were used for measurement of accessible sites, and incubations at 15°C for 16 hr were used to assess total (occupied and unoccupied) sites. In most samples, more than 90% of sites were occupied. After incubation, precipitates were washed six times with 1 ml of medium A, and extracted twice with ethanol (1 ml). The combined extracts were evaporated in scintillation vials, and radio-
activity was measured. In conventional fashion, specifically retained radioactivity was estimated by subtraction of steroid bound in the presence of competitor from that in the absence of competitor. Analysis of binding data according to Scatchard showed a single class of high-affinity binding sites with dissociation constants ($K_d$) in the range 0.1–1.0 nmol/l.

The protamine sulphate procedure, as expected, showed greater specificity than charcoal adsorption techniques. Whereas, using charcoal assays, [3H]dihydrotestosterone binding in hypertrophied prostate cytosol was displaced 83 ± (S.D.)6% by unlabeled dihydrotestosterone, 77 ± 14% by testosterone, 72 ± 13% by 5α-androstane-3α, 17β-diol, 76 ± 7% by 5α-androstane-3β, 17β-diol, 61 ± 8% by estradiol, and insignificantly by R1881 and diethylstilbestrol, using the protamine sulphate assay, binding of [3H]dihydrotestosterone was displaced 80 ± 7% by dihydrotestosterone, 37 ± 17% by testosterone, 79 ± 4% by R1881, 17 ± 6% by 5α-androstane-3α, 17β-diol, 24 ± 13% by 5α-androstane-3β, 17β-diol, 3 ± 2% by estradiol, and negligible displacement was achieved using diethylstilbestrol. Evidently, charcoal adsorption detects a mixture of specific and nonspecific components, while the protamine sulphate method measures only receptor.

The question remains regarding what value even the most accurate assay of androgen receptors has in treatment of prostate disease. Certainly, receptor proteins present in human hypertrophied prostate cytosol are translocated with dihydrotestosterone nuclear sites, where they exert an effect via transcriptional mechanisms. Functional aspects apart, does the presence of androgen receptor proteins in diseased prostate provide a basis for a particular therapy? In all specimens of prostate tissue so far studied, whether normal, hypertrophied, or cancer, receptor proteins specific for dihydrotestosterone have been found in both cytoplasmic nuclear fractions (see Table I). These data are limited, but may indicate some trends. The cellular content of androgen receptors (cytoplasmic plus nuclear) appears to be higher in hypertrophied and carcinomatous prostate than in the normal gland. Cytoplasmic nuclear ratios show a different distribution of receptor; this may suggest a less efficient translocation mechanism in diseased prostate tissue, but since, in these tissues, nuclear content of receptors is usually higher, this phenomenon may more likely indicate an overloading of the system. The apparent dissociation constants imply a higher affinity for ligand-receptor interaction in the carcinomatosus prostate. This achieves greater interest in the light of the fact that testosterone shows an increased ability to compete for [3H] dihydrotestosterone binding sites in prostate cancer tissue (Fig. 1). In combination with reports concerning decreased metabolism of testosterone to dihydrotestosterone in prostate cancer and observations on the binding of [3H] testosterone in this tissue, this fact suggests that the regulation of cell growth by a testosterone-receptor system may be an important facet of the development, maintenance, or detection of prostate cancer.

The value of androgen receptor assays in prostate disease will not be determined fully until a greater range of values for each state of the prostate is obtained. This, together with information concerning the reproducibility of such assays and their relation to the histological and pathological nature of specimens, will or will not establish such assays as a clinical adjunct. Other than this, the study of androgen receptors in diseased prostate tissue will yield essential information concerning the functionality of such a system during development of disease, and provide a basis for the testing of new antiandrogens.

**Acknowledgments**

The authors are grateful to the Tenovus Organization for its generous financial support.
Androgen Receptors in Prostate

Table I. Concentration of Cytoplasmic and Nuclear Receptors in Prostate Tissue*

<table>
<thead>
<tr>
<th>RECEPTOR CONCENTRATION</th>
<th>NORMAL</th>
<th>HOPEPTROPHY</th>
<th>CANCER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K_D</strong> (n mol/l)</td>
<td>0.98-1.29</td>
<td>1.56-2.81</td>
<td>0.69</td>
</tr>
<tr>
<td>CYTOPLASMIC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RECEPTOR CONCENTRATION</strong></td>
<td>44-57</td>
<td>38-92</td>
<td>63</td>
</tr>
<tr>
<td><strong>f mol/mg protein(DNA)</strong></td>
<td>1041-1354</td>
<td>2423-5461</td>
<td>3363</td>
</tr>
<tr>
<td>Molecules/cell</td>
<td>44-57</td>
<td>38-92</td>
<td>63</td>
</tr>
<tr>
<td>NUCLEAR</td>
<td>1.02-1.32</td>
<td>1.17-1.41</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>RECEPTOR CONCENTRATION</strong></td>
<td>197-248</td>
<td>412-668</td>
<td>546</td>
</tr>
<tr>
<td><strong>f mol/mg protein(DNA)</strong></td>
<td>758-955</td>
<td>1387-3922</td>
<td>1529</td>
</tr>
<tr>
<td>Molecules/cell</td>
<td>197-248</td>
<td>412-668</td>
<td>546</td>
</tr>
</tbody>
</table>

*The concentration of cytoplasmic receptors is expressed as fmol/mg protein and that of nuclear receptors as pmol/mg DNA.

![Graph](attachment:graph.png)

Total receptor sites for [³H] dihydrotestosterone in prostate cytosol were measured by the protamine sulphate method. [³H] Steroid was included at 20 nmol/liter and the competitors at various concentrations up to 2,500 nmol/liter.

References

1. and Raynaud, J. P.: Steroids 26, 227
2. and Raynaud, J. P.: Steroids 27, 497
I. INVESTIGATIONS RELATING TO CANCER OF THE PROSTATE.

B. STUDIES ON THE METABOLISM OF STEROIDS BY PROSTATIC TISSUE.
Testosterone Metabolism in vivo by Human Prostatic Tissue

By A. Pike, W. B. Peeling, M. E. Harper, C. G. Pierrepont and K. Griffiths

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(Received 24 August 1970)

Although 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) has been accepted as an active androgen in the rat for a number of years (Dorfman, 1956; Saunders, 1963), evidence has only recently been obtained to suggest that this compound, rather than testosterone, may well be the active hormone in the accessory sex organs of this species (Bruchovsky & Wilson, 1968a,b; Anderson & Liao, 1968; Mainwaring, 1970). The formation of 5α-dihydrotestosterone from testosterone by preparations of human benign hyper trophic prostatic tissue in vitro has also been demonstrated (Farnsworth & Brown, 1963; Ofner, Smakula, Wotiz, Lemon & Mescon, 1965; Chamberlain, Jagarice & Ofner, 1966), and the stimulatory effect this steroid has on cell division with the induction of epithelial hyperplasia in maintained explants of rat prostate (Baulieu, Lasnitzki & Robel, 1968a,b) is noteworthy in this respect.

More recently, Kowarski, Shalf & Migeon (1969) reported the presence of 5α-dihydrotestosterone in high concentration in the prostates of dogs after the intravenous infusion of [3H]testosterone. This communication now describes preliminary investigations to study the metabolites found in the prostate gland after infusion via the cephalic vein of [7α-3H]testosterone (10 μCi, specific radioactivity 1.5 Ci/mmol) into human subjects 20 min before a Millen retropubic prostatectomy was performed on men with benign prostatic hypertrophy. The enucleated tissue was transferred to the laboratory on ice, chopped into smaller pieces and homogenized in 5 vol. of acetic acid containing 500 μg each of the carrier non-radioactive steroids investigated. The filtrates and acetone washes of the mixtures were pooled and dried in vacuo. Steroids were fractionated as described by Fahmy, Griffiths, Turnbull & Symington (1968) and isolated on thin layers of silica gel HF254/366 and alumina HF254/366 and also on Welm alumina. Table 1 also shows the derivatives formed and indicates the t.l.c. systems adopted for their isolation. The following solvent systems were used: solvent I, chloroform-acetone (37:3, v/v); solvent II, chloroform-acetone (150:1, v/v); solvent III, cyclohexane-ethyl acetate (7:3, v/v); solvent IV, cyclohexane-ethyl acetate (9:11, v/v); solvent V, cyclohexane-ethyl acetate (4:1, v/v); solvent VI, ether-ethyl acetate (19:1, v/v); solvent VII, cyclohexane-ethyl acetate (1:1, v/v).

Portions of the neutral steroid fraction were applied as bands to thin layers of silica gel. After chromatography in solvent I, bands corresponding in mobility to 5α-androstan-3,17-dione (RF 0.75), androstenedione (androst-4-ene-3,17-dione) (RF 0.60), androsterone (3α-hydroxy-5α-androstan-17-one) and 5α-dihydrotestosterone (RF 0.46), testosterone and epitestosterone (17α-hydroxyandrost-4-en-3-one) (RF 0.30), 5α-androstan-3α,17β-diol, 5α-androstan-3β,17α-diol and 5α-androstan-3β,17β-diol (RF 0.22) and 5α-androstan-3α,17α-diol (RF 0.14) were eluted. Androsterone and 5α-dihydrotestosterone were acetylated and separated by re-running in solvent II (RF 0.45 and 0.58 respectively). After hydrolysis the free steroids were oxidized to 5α-androstan-3,17-dione and chromatographed in solvent III on silica gel (RF 0.42).

Testosterone and epitestosterone were separated by running on Welm alumina plates in solvent IV (RF 0.50 and 0.39 respectively). Further purification was obtained by acetylation and re-running again on alumina in solvent III. The 5α-androstanediols, 3α,17β, 3β,17α and 3β,17β, were separated as described elsewhere (Harper, Pierrepont, Fahmy & Griffiths, 1970a).

Derivatives were prepared as described previously (Griffiths, Grant & Whyte, 1963). Δ4-3-Oxo steroids were measured after elution by their selective absorption of light at 240 nm, and the remaining steroids and their derivatives were determined by g.l.c. [1% XE-60 on Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa., U.S.A.) at 200°C]. Radioactivity was measured by using a Nuclear-Chicago liquid-scintillation spectrometer. The specific radioactivities of the carrier steroids and their derivatives were used to calculate the total radioactivity associated with each steroid investigated. These results are expressed as percentages of the total radioactivity isolated in the prostate.

Table 1 shows the evidence for the identification of the radioactive carrier steroids isolated from the human benign hypertrophic prostatic tissue and their contribution, as a percentage, to the total radioactivity extracted from the tissue. In Expts.
Table 1. Evidence of the identification of testosterone metabolites isolated from human prostatic tissue

The dashes indicate that the compounds were lost during processing. S, Silica gel; Al, alumina.

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvents used for purifications</th>
<th>Sp. radioactivities (d.p.m./μmol)</th>
<th>% of total radioactivity in prostate tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I (S), I (Al)</td>
<td>Trace</td>
<td>2350</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), VII (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (Al), VII (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>I (S), VII (S)</td>
<td>11150</td>
<td>13250</td>
</tr>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>III (S), V (S), I (S)</td>
<td>11450</td>
<td>12490</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone acetate</td>
<td>II (S), III (S)</td>
<td>11010</td>
<td>13990</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>I (S), I (Al)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epitestosterone acetate</td>
<td>I (S), VII (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androstenedione</td>
<td>III (Al), VII (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), VII (S)</td>
<td></td>
<td>8360</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I (S), VII (S)</td>
<td></td>
<td>8600</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (S)</td>
<td></td>
<td>7930</td>
</tr>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>I (S), V (S)</td>
<td>1220</td>
<td>460</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>IV (S)</td>
<td>1850</td>
<td></td>
</tr>
<tr>
<td>5α-Androstane 3β,17β-diacetate</td>
<td>III (S)</td>
<td>1170</td>
<td>420</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>I (S), VI (S)</td>
<td></td>
<td>2760</td>
</tr>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>V (S), I (S)</td>
<td>0</td>
<td>2760</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>IV (S)</td>
<td>2850</td>
<td></td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>I (S), VI (S), VII (Al)</td>
<td>2800</td>
<td>3510</td>
</tr>
<tr>
<td>5α-Androstane-3β,17α-diol</td>
<td>V (S), I (S)</td>
<td>4500</td>
<td>0</td>
</tr>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>IV (S)</td>
<td>4800</td>
<td></td>
</tr>
</tbody>
</table>
1, 2 and 3, 0.20, 0.38 and 0.35% of the administered radioactivity were isolated respectively in the prostatic tissue extracts. Extensive metabolism of the tritiated testosterone by the tissue would appear to occur with the formation of radioactive metabolites similar to those described from studies with this tissue in vitro (Farnsworth & Brown, 1963; Farnsworth, 1970). Their studies showed that the hypertrophic tissue converted testosterone into a number of products, the major one being 5α-dihydrotestosterone, thus tending to support the recent concept (Bruchovsky & Wilson, 1968a,b) that this steroid is the active androgen in this target organ. Although it is often implied that the metabolic pattern of steroid formation from incubation studies is of little significance, it would seem that the investigations carried out in vitro by Farnsworth (1970) are closely related to the situation as it pertains in vivo, and demonstrated by the experiments now described. The amount of 5α-dihydrotestosterone formed in vivo would suggest that it may have a significant role as the principal androgen in the human prostate. It is noteworthy that 5α-dihydrotestosterone has been shown to stimulate DNA nucleotidyltransferase (EC 2.7.7.7) isolated from human benign hypertrophic prostatic tissue (Harper, Fahmy, Pierrepoint & Griffiths, 1970). The formation from testosterone of compounds with a 17α-hydroxy group, epitestosterone (17α-hydroxyandrost-4-en-3-one) and 5α-androstane-3α,17α-diol, may be important, possibly in relation to studies with the canine prostate (Harper, Pierrepoint, Fahmy & Griffiths, 1970b) indicating that 5α-androstane-3α,17α-diol may have some role in regulating DNA polymerase activity in this tissue. The significance of the presence of these diols must await further study, perhaps along the lines of Baulieu et al. (1968a,b), who have described a differential action of the androstanediols and 5α-dihydrotestosterone on rat prostate in culture. Such studies may indicate enzyme systems that are hyperactive in diseased prostatic tissue, thus causing a steroid imbalance and a hyperplastic condition.

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TESTOSTERONE METABOLISM IN THE DOG PROSTATE WITH REGARD TO ITS GROWTH AND FUNCTION
Keith Griffiths, M. E. Harper, M. A. Groom, A. W. Pike, A. R. Fahmy and C. G. Pierrepoint

The androgen dependent nature of human prostatic cancer is well known, and its treatment by anti-androgen therapy such as castration or diethylstilboestrol administration has become established following the pioneering studies of Huggins and his colleagues thirty years ago [1, 2]. Extensive studies by many groups of investigators have provided a considerable background of information regarding the relationship between testis and prostate, and the nature of the primary dysfunction related to the induction and development of prostatic hyperplasia or neoplasia. Now, the recent studies of Bruchovsky and Wilson [3, 4] and Anderson and Liao [5] have indicated 5a-dihydrotestosterone, rather than testosterone, as the androgenic hormone in the rat, opening up a new realm of investigation. Our own research programme has been directed towards a study of the canine prostate, with parallel investigations of rat and human tissues for comparative purposes.

It is well known that the prostate gland requires testicular ‘hormones’ for its normal function, and it is generally accepted that abnormalities in the total or relative hormonal content of the testicular secretion may be related to prostatic dysfunction. The prostate itself however, may induce its own diseased state by the transformation of steroids presented to it in the plasma to more potent androgens. In the dog, the prostate gland commonly undergoes hypertrophy and hyperplasia but rarely succumbs to neoplasia, thus providing a necessary model for the study of the relationship between the two organs in both normal and abnormal states.

Incubation studies of normal canine prostatic tissue with isotopically-labelled testosterone indicate (Table 1) that many of the metabolites, previously shown by Dr Farnworth and Dr. Ofner to be formed from this substrate in vitro by human hypertrophic prostate, are also

Table 1. INCUBATION OF [14C]TESTOSTERONE WITH MINCED CANINE PROSTATIC TISSUE

<table>
<thead>
<tr>
<th>Steroids Investigated</th>
<th>Untreated animals</th>
<th>Oestrogen treated animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>TESTOSTERONE (unmetabolised)</td>
<td>62.8</td>
<td>81.7</td>
</tr>
<tr>
<td>EPITESTOSTERONE</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>5a-DIHYDROTESTOSTERONE</td>
<td>3.1</td>
<td>5.3</td>
</tr>
<tr>
<td>ANDROSTENEDIONE</td>
<td>1.7</td>
<td>4.8</td>
</tr>
<tr>
<td>ANDROSTERONE</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>5α-HYDROXYANDROSTENEDIONE</td>
<td>0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>TESTOSTERONE SULPHATE</td>
<td>0</td>
<td>0.01</td>
</tr>
</tbody>
</table>

2 g. minced tissue were incubated in Krebs-Ringer bicarbonate glucose in the absence of added cofactors, at 38.5°C for 2 hr in O2/CO2, 95/5. After incubation the steroids were analysed as described [10]. The oestrogen-treated dog received 15 mg oestradiol implanted subcutaneously at 3 wks. and 5 wks. prior to incubation studies.
synthesized by this tissue. Consistent with the earlier human studies, was the finding that 5α-dihydrotestosterone was one of the major metabolites, although testosterone sulphate and epitestosterone were also formed. Prostatic tissue from dogs pretreated with oestradiol-17β showed an extensive metabolism of testosterone with a particularly high conversion to very polar, as yet unidentified, metabolites. Dr. Farnsworth has already emphasized the regulatory role oestrogens may have on steroid metabolism in the prostate cell.

**EFFECT OF DIETHYLPHTALOESTROL ON PROSTATIC DNA POLYMERASE**

<table>
<thead>
<tr>
<th>Neoplasia</th>
<th>Hyperplasia</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3)</td>
<td>(7)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Figure 1.
The effect of 10 μmol diethylstilboestrol on the DNA polymerase activity of three neoplastic and seven hyperplastic human prostates, and six canine prostates, each experiment completed in triplicate. The range for different preparations is shown.

Previous studies from this laboratory [6] have shown that certain natural oestrogens, oestradiol-17β, oestradiol and 16-epi-oestradiol, stimulated the DNA polymerase enzyme system (DNA nucleotidyltransferase, E.C. 2.7.7.7) isolated from calf thymus gland by the procedure of Shepherd & Keir [7]. More recently, Fahmy and Griffiths [8] described the inhibition of this enzyme system by diethylstilboestrol and some of its analogues, and it seemed that further studies concerned with the hormone responsiveness of the similar enzyme system from prostate tissue could be particularly germane to our understanding of the mechanism of action of these hormones in this tissue.

Diethylstilboestrol was found to inhibit the activity of the DNA polymerase from both hyperplastic and neoplastic human prostate tissue (Fig. 1) and also from the normal canine prostate gland. On average, there was a 40% inhibition of the enzyme activity in all tissues. Also observed in these investigations, was the stimulation of the enzyme derived from two hyperplastic prostate glands by testosterone and 5α-dihydrotestosterone. At a final concentration of 40 μM, testosterone stimulated the DNA polymerase by 14 and 38% and 5α-dihydrotestosterone by 32 and 30% respectively. Neither of these compounds had any effect on the enzyme from human neoplastic prostate tissue, nor on the calf thymus enzyme system.

The results obtained from enzyme preparations of normal dog prostates are shown in Table 2. It is noteworthy that the activity of the DNA polymerase was enhanced by only two steroids, testosterone and 5α-androstane-3α,17α-diol but not, notably by 5α-dihydrotestosterone. Significance is also attached to the inhibition of the canine enzyme system by the
TABLE 2

In vitro effects of various compounds on DNA polymerase activity isolated from canine prostatic tissue.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Stimulation</th>
<th>% Change in Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>35</td>
<td>%</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>5α-Dihydrotestosterone</td>
<td>%</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>5α-Dihydroepitestosterone</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>5α-Androstane-3α,17α-diol</td>
<td>13</td>
<td>%</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>5α-Androstane-3β,17α-diol</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>0</td>
<td>%</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Dihydrodibutylstilboestrol</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Hexoestrol</td>
<td></td>
<td>%</td>
</tr>
</tbody>
</table>

DNA polymerase activity was analysed as described [9]. The DNA polymerase preparations were obtained from prostates of young mature dogs.

The early studies [8] on calf thymus preparations had suggested an interesting relationship between the structure of this analogue of stilboestrol and the degree of inhibition of DNA polymerase. The alkyl substitution in the αα'-positions altered the inhibitory effect; the larger the alkyl group the greater the degree of inhibition. The most effective inhibitor was hexoestrol with a saturated αα'-ethylenic linkage.

Subsequently, the cis and trans isomers of dibutylstilboestrol and also the corresponding saturated analogue dihydrodibutylstilboestrol were synthesized [9]. Some of the results on the effect of these compounds on human prostatic tissue preparations are shown in Table 3. Whereas diethylstilboestrol and hexoestrol inhibited the enzyme to the same extent, dibutylstilboestrol generally had little effect. Dihydrodibutylstilboestrol, consistently and effectively inhibited the DNA polymerase. Further investigations on the uptake and localisation of dihydrodibutylstilboestrol in prostatic tissue are now developing, since this compound, with only 0.1% of the oestrogenic activity of diethylstilboestrol, may be of some value in the

TABLE 3

The effect of various stilboestrol compounds on DNA polymerase isolated from human prostate tissue.

<table>
<thead>
<tr>
<th>Stilboestrol compounds added to enzyme assay to give a final concentration of 40 μM</th>
<th>% Inhibition of DNA Polymerase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzymes isolated from neoplastic tissue</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>DIETHYLSTILBOESTROL</td>
<td>31</td>
</tr>
<tr>
<td>HEXOESTROL</td>
<td>41</td>
</tr>
<tr>
<td>DIBUTYLSTILBOESTROL</td>
<td>0</td>
</tr>
<tr>
<td>DIHYDRODIBUTYLSTILBOESTROL</td>
<td>41</td>
</tr>
</tbody>
</table>

90
treatment of prostatic cancer. Oestradiol-17β unlike diethylstilboestrol did not consistently inhibit the human prostatic DNA polymerase [9] which is in direct contrast to the results with the calf thymus DNA polymerase [6].

The stimulatory effect of testosterone and 5α-androstane-3α, 17α-diol but not 5α-dihydrotestosterone, on the canine prostatic DNA polymerase may be of significance in relation to preliminary experiments in which [7α-3H] testosterone was incubated for 1 hr with minced prostatic tissue from a young dog. After incubation, tissue was removed, rinsed and the radiometabolites analysed in the whole tissue and also in the nuclear preparation isolated from an aliquot [10]. Results are shown in Table 4. Although a large proportion of the testosterone taken up by the mince was converted to 5α-dihydrotestosterone (19.96%) the radioactivity associated with nuclear 5α-dihydrotestosterone was in similar proportions to testosterone, which contrasts with the results from a similar study on rat prostate by Anderson & Liao [5].

<table>
<thead>
<tr>
<th>Steroid Investigated</th>
<th>Whole tissue % Radioactivity</th>
<th>Nuclear fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>51.36</td>
<td>1.25</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0.62</td>
<td>0.59</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>19.96</td>
<td>1.30</td>
</tr>
<tr>
<td>5α-Androstane-3α, 17α-diol</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>5α-Androstane-3α, 17β-diol</td>
<td>0.86</td>
<td>0.03</td>
</tr>
<tr>
<td>5α-Androstane-3β, 17α-diol</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Androstane-3β, 17β-diol</td>
<td>0.30</td>
<td>0.02</td>
</tr>
</tbody>
</table>

1 g of minced prostate tissue was incubated with 50 μC [7α-3H] testosterone in 12.5 ml of Krebs-Ringer bicarbonate glucose at 38.5°C for 1 hr. The % radioactivity found in the nuclear fraction has been corrected for nuclear recovery based on DNA determinations. The nuclear fraction was obtained as described [10].

Radioactive epitestosterone and 5α-androstane-3α, 17α-diol were isolated from the nuclear preparation in the same proportion as from the whole tissue homogenate indicating their selective accumulation in the nucleus. Whereas small yields of all the 5α-androstanediols were obtained, no evidence was available for the formation of the 5β-epimers. The activity of the 3α- and 17α-hydroxysteroid dehydrogenases and their subcellular localisation may well be worth further study in relation to this observed effect on DNA polymerase and their possible biological function. Dr. Baulieu and Dr. Lasnitzki have already described certain biological effects of some of the androstanediols on preparations of rat prostatic tissue in organ culture. It may be that certain of these ‘diols could have, in different species, biological roles equally as important as that of 5α-dihydrotestosterone. Experiments in which isolated nuclei from the dog prostate were incubated with [7α-3H] testosterone and [1,2-3H] epitestosterone indicated that these preparations were capable of converting the substrates to the various diols (Table 5). A comparison of the ability of nuclear preparations from human hyperplastic prostatic tissue, rat ventral prostatic tissue and normal canine prostatic tissue to convert testosterone to 5α-dihydrotestosterone (Table 6) showed the canine preparation to be much less active than the tissue from man and rat. Noteworthy was the lack of effect of various other compounds (Table 6) on the ability of the rat prostatic nuclei to convert testosterone to 5α-dihydrotestosterone.

Dr. Franks has emphasized at this Workshop that further consideration must be given to the separation of the various cell types of the prostate, in particular the epithelial and fibromuscular tissues, in which steroid metabolism may be very different. Tissue culture studies could be of value in this respect and our own preliminary experiments with explants of rat prostatic tissue have indicated that valuable information is to be gained in this way. Maintained explants of rat ventral prostate in organ culture in the presence of [7α-3H] testosterone (Table
TABLE 5. Incubation of canine prostatic nuclei with [7α-3H] testosterone and [1,2-3H] epitestosterone

<table>
<thead>
<tr>
<th>Steroid Investigated</th>
<th>Testosterone Incubation</th>
<th>Epitestosterone Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>65.45</td>
<td>0.51</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0.55</td>
<td>36.00</td>
</tr>
<tr>
<td>Androstene-3,17-dione</td>
<td>0.37</td>
<td>0.69</td>
</tr>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>0.26</td>
<td>0.37</td>
</tr>
<tr>
<td>5α-Androstane-3α,17α-diol</td>
<td>0.01</td>
<td>0.80</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>0.33</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Androstane-3β,17α-diol</td>
<td>0.01</td>
<td>0.31</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>0.25</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Nuclei were obtained as described [10] and incubated with 10 µCi [7α-3H] testosterone and 10 µCi [1,2-3H] epitestosterone as described [3].

TABLE 6. Nuclear fractions incubated with [7α-3H] testosterone for 1 hr at 37°C.

<table>
<thead>
<tr>
<th>Rat Prostatic Nuclei</th>
<th>Testosterone</th>
<th>5α-Dihydrotestosterone</th>
<th>Epitestosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.7</td>
<td>33.0</td>
<td>0.15</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>56.0</td>
<td>27.9</td>
<td>0.14</td>
</tr>
<tr>
<td>Dihydriodihydro</td>
<td>52.3</td>
<td>25.9</td>
<td>0.16</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>52.9</td>
<td>27.1</td>
<td>0.27</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>50.6</td>
<td>29.9</td>
<td>0.30</td>
</tr>
<tr>
<td>DPB</td>
<td>49.3</td>
<td>32.7</td>
<td>0.66</td>
</tr>
<tr>
<td>Dog 1</td>
<td>59.0</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>Dog 2</td>
<td>65.5</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Human Prostatic Nuclei</td>
<td>40.5</td>
<td>15.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Nuclei were obtained as described [10] and incubated with 10 µCi [7α-3H] testosterone for 1 hr. in the medium described by Bruchovsky & Wilson [3]. 10 µg of various compounds were added to the rat nuclei incubations. Steroids were analysed as described [10]. DPB = 1,4-diphenyl-2,3-dihydroxybutane.

TABLE 7. Incubation of rat ventral prostate explants with [7α-3H] testosterone for 24 hr.

<table>
<thead>
<tr>
<th>Steroid investigated</th>
<th>Specific activities (dpm/mumol) in whole tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>650.2</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>4.5</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>613.2</td>
</tr>
<tr>
<td>5α-Androstane-3α,17α-diol</td>
<td>3.1</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>43.5</td>
</tr>
<tr>
<td>5α-Androstane-3β,17α-diol</td>
<td>15.2</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>41.8</td>
</tr>
</tbody>
</table>

The cultures were incubated with 10 µCi [7α-3H] testosterone for 24 hr in Eagles MEM supplemented with 10% calf serum, then pooled, washed, cold authentic steroid added and analysed as described [10].
HORMONAL EFFECTS IN THE PROSTATE

7) formed radioactive 3α,17α and 3β,17α-5α-androstane diols as well as their 17β-epimers, [11] which have been found by Baulieu and his colleagues [12, 13]. Other experiments in which oestradiol-17β and diethylstilboestrol were added to similar rat prostate cultures suggested that these oestrogens inhibited the uptake or retention of testosterone or 5α-dihydrotestosterone in the nuclei of the cells (Table 8). These observations in organ and tissue culture support similar results obtained from rat prostate incubation studies recently described by Liao and his colleagues [14].

TABLE 8

INCUBATION OF RAT AND CANINE PROSTATE EXPLANTS WITH \(^{3}H\) TESTOSTERONE IN THE PRESENCE AND ABSENCE OF VARIOUS OESTROGENS (1.7 X 10\(^{-5}\)M)

<table>
<thead>
<tr>
<th>Species examined and tissue fraction analysed</th>
<th>Steroids Investigated</th>
<th>Specific activities of steroids investigated (expressed as dpm/mmol) per 25 mg of tissue cultured</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT WHOLE TISSUE</td>
<td>TESTOSTERONE</td>
<td>253.0</td>
</tr>
<tr>
<td></td>
<td>5α-DIHYDRO-TESTOSTERONE</td>
<td>1334.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>227.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>226.3</td>
</tr>
<tr>
<td>RAT NUCLEAR FRACTION</td>
<td>TESTOSTERONE</td>
<td>248.4</td>
</tr>
<tr>
<td></td>
<td>5α-DIHYDRO-TESTOSTERONE</td>
<td>3075.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>78.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>DOG WHOLE TISSUE</td>
<td>TESTOSTERONE</td>
<td>424.5</td>
</tr>
<tr>
<td></td>
<td>5α-DIHYDRO-TESTOSTERONE</td>
<td>144.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>503.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>512.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>584.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>650.0</td>
</tr>
<tr>
<td>DOG WHOLE TISSUE</td>
<td>TESTOSTERONE</td>
<td>741.0</td>
</tr>
<tr>
<td></td>
<td>5α-DIHYDRO-TESTOSTERONE</td>
<td>174.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>708.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>661.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>773.0</td>
</tr>
</tbody>
</table>

DES = Diethylstilboestrol  DHBS = Dihydrodibutylstilboestrol

Cultures were incubated with 20 \(\mu\)C \(^{3}H\) testosterone, in the presence or absence of 1.7 X 10\(^{-5}\)M oestrogens, for 24 hr in Eagles MEM supplemented with 10% calf serum. They were then pooled, washed and homogenised and the nuclei obtained as described [10]; Nuclear results were corrected for recovery based on DNA estimations.

It is normally accepted that oestrogen, administered in vivo, indirectly affects the prostate by reducing testosterone secretion of the testis, via pituitary suppression and ICSH release. Such a decrease in plasma ICSH has been described [15]. A decreased 17β-hydroxysteroid dehydrogenase activity in homogenates of testes removed from a diethylstilboestrol-treated man with prostatic cancer has been reported [16]. Results shown in Table 9 indicate a decreased ability to convert pregnenolone and DHA to testosterone by minced testicular tissue from an oestrogen-treated dog, although when compared to the tissue from the untreated animal, the overall rate of steroid metabolism appeared higher in the treated tissue. Noteworthy was the relatively high capacity of testicular tissue from the 16 year old dog to synthesise testosterone. Axelrod [17] suggested that there was a relative deficiency of the 17,20-desmolase enzyme system in the testis from a 61 year old prostatic cancer patient as compared with that from a 16 year old boy, indicating reduced testosterone secretion with advancing age in the human male. Further analysis of his results, however, indicates more of the incubated substrate was converted to testosterone in the tissue from the older man than in that from the boy, thereby being in keeping with our observations.

The arbitrary nature of in vitro techniques makes it necessary to interpret the results from such investigations with some care. Essentially one hopes to apply such results obtained to the in vivo situation in the human as it relates to prostatic carcinoma. It was therefore encouraging that the radiometabolites isolated from prostatic tissue after the parenteral administration of
TABLE 9  STEROID METABOLISM IN THE TESTES FROM NORMAL AND OESTROGEN TREATED DOGS

<table>
<thead>
<tr>
<th>Testicular tissue studied (g tissue incubated)</th>
<th>Substrate</th>
<th>% Radioactivity associated with carrier steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pregnenolone</td>
</tr>
<tr>
<td>Untreated testis (2 g)</td>
<td>[7α-3H]pregnenolone</td>
<td>30.8</td>
</tr>
<tr>
<td></td>
<td>[4-14C]DNA</td>
<td>-</td>
</tr>
<tr>
<td>Testis removed from oestradiol-17β treated dog (2 g)</td>
<td>[7α-3H]pregnenolone</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>[4-14C]DNA</td>
<td>-</td>
</tr>
<tr>
<td>Young testis</td>
<td>[4-14C]17αOH-progesterone</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>[7α-3H]DNA</td>
<td>-</td>
</tr>
<tr>
<td>Old testis</td>
<td>[4-14C]17αOH-Progesterone</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>[7α-3H]DNA</td>
<td>-</td>
</tr>
</tbody>
</table>

Dashes indicate that the metabolites were not examined and zeros indicate that no radioactivity was found in those metabolites. The oestrogen-treated dog received 15 mg oestradiol implanted subcutaneously at 3 wk. and 5 wk. prior to incubation studies.

[3H] testosterone to patients with hypertrophy of the gland [18], were principally those described by Farnsworth & Brown [19], and it would seem that the in vitro investigations are closely related to the situation as it pertains in vivo.

REFERENCES—

DISCUSSION

Wilson: The reason for the discrepancy between the results which I presented yesterday and these presented this morning in regard to the quantitative importance of 5α-reductase in the nuclei of dog prostate, is not at all clear to me. We should exchange protocols, but I think it is likely that the reason for the apparent difference is a technical one rather than a fundamental one. The nuclei of the dog prostate are less dense than the nuclei from the prostate of any other species we have studied and consequently more difficult to isolate free of cytoplasmic tags.

Griffiths: There certainly may be differences in technique between the two laboratories. We do of course relate our nuclear preparation to total DNA levels, and the nuclear DNA recovery was of the order of 20%. The testosterone-5α-dihydrotestosterone conversions by canine prostatic nuclei preparations are certainly lower than those reported by Dr Wilson. We considered it was interesting that the level of 5a-reductase of the nuclei was so much lower than in the rat and human prostate and some work recently done by Migeon and his colleagues [1] suggests that in the dog, the site of formation of 5α-dihydrotestosterone was possibly microsomal. Little of their 5α-dihydrotestosterone isolated from the prostate, was found in the nucleus. We did in fact think that our studies were related to their findings, but obviously many more experiments are required, before the story is complete. We did consider however that the work suggested that 5α-dihydrotestosterone may not be quite so important in the dog as in the rat. A great deal of attention is being directed towards androgenic properties of 5α-dihydrotestosterone at this period, and we thought it reasonable at this stage, that other steroids as well should also be considered.

Ofner: A few words concerning a preliminary study of canine prostatic nuclei, with reference to testosterone 5α-reductase and 5α-dihydrotestosterone formation in the gland. We did a subcellular fractionation of the canine prostate after direct infusion of testosterone-4-14C into the arterial supply of the organ [2]. We used the fractionation procedure of Hussein and Kochakian who demonstrated the purity of their nuclear preparation with marker enzymes. 5α-Dihydrotestosterone was the predominant, and 5α-diols were the minor metabolites, in the “pure” nuclear fraction. We also noted that in vitro transformation of testosterone to 17-oxosteroids is greater in the canine than in the human gland.

King: About the stimulation of DNA polymerase by testosterone. How do the concentrations compare with those Dr. Lasnitzki uses to get stimulation in the culture? I get the impression they were much higher than those used by Dr. Lasnitzki.

Griffiths: I think Dr. Lasnitzki uses 6 μg. The final concentration of all compounds throughout our series of experiments was 40 μM. However, you obtain the same effect with lower concentrations (approx. 0.4μM).

Lasnitzki: 17.5 × 10⁻⁶ M maintained them well.

King: On one slide, you quoted 5 × 10⁻⁵ M.

Griffiths: There is certainly a lot of steroid in the incubations — it may be a pharmacological amount, but I think this the first time, as far as we know, that the DNA polymerase has been studied in this way. It is interesting that these compounds have different effects in the same enzyme system and that there appears some specificity to this effect. In the calf thymus DNA polymerase preparation, diethylstilboestrol inhibits and oestradiol stimulates the enzyme system.

Baulieu: Did dimethylstilboestrol inhibit too?

Griffiths: Yes, in the calf thymus system but not as well as diethylstilboestrol. Furthermore, you can take DNA polymerase preparations from other tissues such as renal tumours, and show that oestradiol-17β and diethylstilboestrol again have opposite effects.

Williams-Ashman: I should like to make a point about these experiments. My impression is that the tissue preparation you use for assay of mammalian DNA polymerase is rather crude, and may be contaminated with nucleases of one form or another. I wondered whether it’s possible that some of these stimulations you see, which are experimentally significant, of course, are due to inhibition of the nuclease activity rather than actual stimulation of DNA polymerase activity.

Griffiths: I think the more you try to purify this enzyme system, the less stable it becomes. Professor Keir [4] would I believe consider it better to use the semi-purified preparation to try to relate the hormone effects in vitro to the in vivo situation, than to purify further producing what he called a non-native system.

Williams-Ashman: I would agree that a case could be made out for this, but perhaps the big
DISCUSSION

difference you get from one tissue to another is simply that the radioactivity in the product is a measure of the balance between polymerase activity and nucleases activity.

Griffiths: This may be true of course and we do consider our results preliminary. The calf thymus enzyme has been further purified and we still obtain similar effects with diethylstilboestrol and oestradiol-17β. Possibly diethylstilboestrol stimulates the nuclease and oestradiol-17β inhibits it, we don’t know. We are currently working on the prostate preparations to see if further purification does provide us with the same type of result, and also to investigate further, the mechanism of the hormonal effect.

Eik-Nes: Have you tried your nuclear preparations for other steroid isomerases than testosterone → epitestosterone; have you tried oestradiol-17β→ oestradiol-17α.

Griffiths: No this is on our programme of work.

Eik-Nes: An old question — the pools of cold material and your isotope. I completely agree with you with regard to Axelrod’s publication on androgen production in one old man. It is very confusing, though if any point can be made from the data it is that “old testis” may be inferior in the 17β-hydroxy steroid dehydrogenase system and the C17,20 desmolase system. Did you present the tissue with 17α-hydroxypregosterone and DHA at the same time?

Griffiths: Yes, the two steroids were incubated simultaneously. The thing about the Axelrod paper was that he did the incubations, then worked out testosterone metabolism as a percentage of the products found rather than of the precursors incubated. If the calculations were redone, the impression was that the older testis made more testosterone than the younger one.

Baulieu: Continuing with epitestosterone. Did you get sufficient material to identify it properly? This is the first time I have seen some testosterone-epitestosterone conversion demonstrated in any tissue. Is there any effect of epitestosterone or any of the diols with the 17α-hydroxy group on the prostate.

Griffiths: As I said, the 5α-androstane-3α,17α-diol was the only compound which had an effect apart from testosterone, on the canine DNA polymerase but nothing has yet been done in vivo. I think one of the problems in studying epitestosterone previously has been its separation from testosterone on TLC, and Dr. Ofner and ourselves have found recently, that if you use alumina on TLC, you can get a very good separation. By our techniques we feel that it is well identified.

Renwick: I should like to support Dr. Williams-Ashman’s comments on 5α-reductases. It is essential to compliment metabolic studies with detailed investigations of purified enzyme systems particularly in respect of substrate specificities. One frequently sees reports of enzymes concerned in steroid metabolism based on histochemical evidence with total disregard for substrate concentration, specificity of reaction and other basic tenets of enzyme biochemistry.

Griffiths: I think we agree that the steroid histochemical work is extremely limited.

Ofner: I should like to congratulate Dr. Griffiths and his colleagues on assembling the results of so much interesting work for presentation. The prostatic 17α-hydroxy metabolites which the Tenovus group characterized are seen to be active in stimulating enzymes and the 3β-hydroxysteroid transformation products appear to be potent androgens in assays based on topical applications (chick comb and organ culture), yet both classes of C19-steroids have been shown to be inactive by the systemic route. We are indebted to Drs. Lasnitzki and Baulieu for making us take a new look at the bioassay literature.

Griffiths: When you administer a steroid you must remember that it may well be rapidly conjugated or metabolised.

Ofner: Yes, that is what I am saying. This is the advantage of organ culture tests for this work on tissue metabolism directing attention to various compounds.

Griffiths: I think one can bring up all sorts of interesting points from these in vitro studies. One thing which we found very recently was that human breast tumour tissue was shown to have an active 5α-reductase system. We have infused androstenedione through the human breast tumour and shown an accumulation of 5α-dihydrotestosterone in the tumour tissue. One wonders what this has to do with the function of female accessory sex organs.

The Effect of Oestrogen on the Prostatic Metabolism of Testosterone in Tissue Culture

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During the last few years a great deal of information has accumulated with regard to the action of androgens on target tissues, especially that of testosterone (17β-hydroxyandrost-4-en-3-one) on the prostate gland. Testosterone metabolism by the prostate both in vivo and in vitro (Parnsworth & Brown, 1963; Ofner, 1968; Gloyna & Wilson, 1969; Kowarski, Shaf & Migeon, 1969; Pike, Peeling, Harper, Pierrepoint & Griffiths, 1970) has been studied, and one of the most interesting observations has been the metabolism of testosterone, steroids such as 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and the 5α-androstanediols appear to have specific roles to play in eliciting the androgenic response (Baulieu, Lasnitzki & Robel, 1968a, b). Such investigations on the action of androgens must be relevant to the study of the aetiology and treatment of prostatic cancer, since an imbalance in the metabolism of testosterone by the prostate could induce the neoplastic state. Although the androgen-dependent nature of prostatic cancer and its treatment with oestrogens has been known for many years (Huggins, Scott & Hodges, 1941), little is known about the effect of oestrogen on prostatic metabolism. Oestrogens can influence the prostate indirectly via their action on the hypothalamus and testis, with a consequent decrease in testosterone secretion, but a direct action on the prostate is also probable (Parnsworth, 1969). Tissue-culture studies provide a means of investigating the action of compounds on tissue without the added complications of interference from the general metabolism. A study of the metabolism of testosterone by rat and canine prostatic explants, cultured in the presence and in the absence of natural and synthetic oestrogens, is reported in this paper.

Prostate glands from adult dogs and ventral prostates of 8–12-week-old Sprague-Dawley rats were dissected under sterile conditions from any surrounding fat and connective tissue. The tissue was then cut into 1-mm-cube explants and incubated in organ-culture dishes on lens-paper rafts supported by stainless-steel grids. Each culture dish contained 1 ml of Eagle’s minimal essential medium fortified with 10% (v/v) of calf serum, 2 mM-glutamine, 200 units of penicillin and 100 µg of streptomycin. [7α-3H]Testosterone (20 µCi; specific radioactivity 1.51 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to each culture, and to some oestradiol-17β, dibutylthiostabol or diethylstilbestrol to give a final concentration of 17 µM. Cultures were incubated in an atmosphere of air + CO2 (95:5) at 37°C for 24 h. After incubation, explants from identical incubations were pooled, washed in phosphate-buffered saline (137 mM-NaCl-2.68 mM-KCl-8.09 mM-Na2HPO4-1.47 mM-KH2PO4), blotted dry and weighed before homogenization. In whole-tissue experiments the homogenate was analysed after the addition of 500 µg of various carrier steroids. The individual steroids were purified by extensive t.l.c. and formation of derivatives, and measured either by absorption of u.v. light or by g.l.c. Radioactivity was determined by using a Nuclear-Chicago scintillation spectrometer as detailed by Harper, Pierrepoint, Fahmy & Griffiths (1971). Samples were taken for the determination of DNA (Burton, 1956) and protein (Lowry, Rosebrough, Farr & Randall, 1951) for the latter crystalline bovine serum albumin was used as the standard. In experiments where the nuclear fraction was examined, more cultures were pooled to obtain enough tissue for fractionation. The tissue was homogenized in 0.25 M-sucrose containing 3 mM-magnesium chloride and centrifuged at 700g for 10 min to obtain a crude nuclear pellet. This was resuspended in 0.88 M-sucrose and layered over a gradient of 1.8–2.2 M-sucrose containing 3 mM-magnesium chloride and spun at 33000g av. for 45 min. The nuclear pellet was then examined for radioactive steroids in the same manner as the homogenate, and DNA was determined to estimate nuclear recovery. Table 1 shows the results of incubating [7α-3H]testosterone with canine and rat prostatic explants in both the control and test cultures. The ratios of testosterone to 5α-dihydrotestosterone, the most abundant metabolite, are given. The means of the specific radioactivities of the parent steroid and two derivatives are given in Table 1, and have been adjusted to 25 mg of tissue. The specific radioactivities showed significant...
Table 1. Incubation of rat and canine prostate explants with \(^{3}H\)testosterone in the presence and in the absence of various oestrogens (17 \mu M)

<table>
<thead>
<tr>
<th>Species examined and tissue fraction analysed</th>
<th>Steroids investigated</th>
<th>Control</th>
<th>Oestradiol</th>
<th>Diethylstilboestrol</th>
<th>Dibutyldihydroestrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat: whole tissue</td>
<td>Testosterone</td>
<td>253.0</td>
<td>277.9</td>
<td>227.2</td>
<td>226.3</td>
</tr>
<tr>
<td></td>
<td>5x-Dihydrotestosterone</td>
<td>1334.3</td>
<td>742.4</td>
<td>869.3</td>
<td>296.3</td>
</tr>
<tr>
<td>Rat: nuclear fraction</td>
<td>Testosterone</td>
<td>248.4</td>
<td>78.5</td>
<td>24.8</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5x-Dihydrotestosterone</td>
<td>3075.7</td>
<td>376.1</td>
<td>198.0</td>
<td>—</td>
</tr>
<tr>
<td>Dog: whole tissue</td>
<td>Testosterone</td>
<td>424.5</td>
<td>508.7</td>
<td>584.9</td>
<td>650.0</td>
</tr>
<tr>
<td></td>
<td>5x-Dihydrotestosterone</td>
<td>144.7</td>
<td>121.2</td>
<td>54.2</td>
<td>68.4</td>
</tr>
<tr>
<td>Dog: whole tissue</td>
<td>Testosterone</td>
<td>741.0</td>
<td>708.0</td>
<td>661.0</td>
<td>773.9</td>
</tr>
<tr>
<td></td>
<td>5x-Dihydrotestosterone</td>
<td>174.7</td>
<td>79.7</td>
<td>51.3</td>
<td>76.4</td>
</tr>
</tbody>
</table>

Changes, depending on the co-incubated test compounds. The ratio of 5x-dihydrotestosterone to testosterone decreased in all cases, suggesting that 5x-dihydrotestosterone formation within the prostatic tissue had been directly suppressed by the oestrogens. Noteworthy was the apparent lower conversion of testosterone into 5x-dihydrotestosterone in the canine prostatic tissue, consistent with earlier studies (Harper et al. 1971). In experiments where the nuclei were examined the uptake of radioactive steroid into the nucleus was decreased in the presence of the oestrogens. However, whether this represents an effect of oestrogens on the binding of 5x-dihydrotestosterone to protein receptor (Fang & Liao, 1969) or the result of changes in the cytoplasmic metabolism of steroids induced by the compounds is not shown by these experiments. These compounds have no effect on the conversion of testosterone into 5x-dihydrotestosterone by rat nuclear preparations (Griffiths et al. 1970). Other metabolites of testosterone isolated from the rat prostate cultures were also examined. 5x-Androstenediols were formed by the whole tissue, but the 17\beta-diol were formed in approx. 4 times the quantity of the 17\alpha-diois. Mean specific radioactivities in the whole tissue were 43 and 41 d.p.m./nmol for the 3x,17\beta-diol and 3\beta,17\beta-diol respectively, and 3 and 15 d.p.m./nmol for the 3x,17\alpha-diol and 3\beta,17\alpha-diol respectively.

Baulien et al. (1968a,6) showed in tissue-culture experiments with rat prostate explants that, although 5x-dihydrotestosterone maintained the cells as well as testosterone, it also caused some hyperplasia, whereas the metabolite 5x-androstane-3\beta,17\beta-diol produced hypertrophy of the gland without hyperplasia. 5x-Dihydrotestosterone has been implicated in the aetiology of prostatic hyperplasia in both men and dogs (Siiteri & Wilson, 1970; Gloyna, Siiteri & Wilson, 1970). Of interest are the results with dibutyldihydroestrolboloster, previously shown to affect prostatic DNA polymerase (Harper, Fahmy, Pierrepoint & Griffiths, 1970), which seems as effective as diethylstilboestrol in interfering with prostatic metabolism of testosterone and yet possesses little oestrogenic activity. This compound could possibly be of value in the treatment of prostatic tumours.

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THE METABOLISM OF STEROIDS IN THE CANINE PROSTATE AND TESTIS

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SUMMARY

Prostatic tissue from young mature dogs was incubated in vitro with [4-14C]testosterone to assess androgen metabolism by this target organ. At the same time tissue from a dog previously treated with implants of oestradiol-17β and tissue from an older animal were also studied. Tissue from the oestrogen-treated dog showed considerable qualitative and quantitative differences from the normal in regard to metabolic activity. Fractionation of prostatic tissue after incubation with labelled testosterone indicated the localization of certain enzymes and products in the nucleus, and 5α-androstane-3α,17α-diol formation appeared particularly interesting. The relative activities of testicular tissue from the dogs in regard to the formation in vitro of androstenedione and testosterone from various radioactive precursors were measured.

INTRODUCTION

It is well known that the prostate gland requires the hormonal secretions of the testis for normal function. Abnormalities in the total or relative hormone content of these secretions might cause prostatic dysfunction. The prostate, however, by its metabolic activity might induce its own diseased state by the transformation of testicular products.

The canine prostate, which commonly undergoes hypertrophy and hyperplasia although rarely neoplasia, is useful for studying the relationship between testis and prostate in both normal and abnormal states. The canine testis uses C21- and C19-steroid substrates for the formation of testosterone and androstenedione (Eik-Nes & Kekre, 1963; Hagen & Eik-Nes, 1964; Pierrepoint, Galley, Griffiths & Grant, 1967; Pierrepoint, 1968) and these androgens are secreted by the canine testis (West, Hollander, Kritchevsky & Dobriner, 1952) and further metabolized by the prostate (see Ofner, 1969). One of the metabolic products, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) has been implicated in androgen action by its localization in prostatic nuclei (Bruchovsky & Wilson, 1968a, b; Anderson & Liao, 1968) and by its stimulatory effect on cell division and secretion in rat prostate explants.
maintained in culture media (Baulieu, Lasnitzki & Robel, 1968a, b). These authors also described the stimulation of growth and activity of these cultured cells by 5α-androstane-3β,17β-diol (Baulieu & Robel, 1970), although the 3α-epimer was inactive in this respect. Furthermore, the 5α-androstane-3α,17α-diol stimulates the activity of the DNA polymerase enzyme isolated from the dog prostate (Harper, Pierrepont, Fahmy, Groom & Griffiths, 1970b).

The present paper describes a series of experiments performed to investigate certain aspects of the formation of testosterone and androstenedione in the canine testis in vitro, and their further transformation in the prostate of the same animal. The effect of age and oestrogen administration on these reactions was also examined.

**MATERIALS AND METHODS**

**Chemicals.** Radioactive steroids were purchased from the Radiochemical Centre, Amersham; 5α-dihydroepitestosterone, 5α-androstane-3α,17α-diol and 5α-androstane-3β,17α-diol were gifts from Dr M. H. Briggs, Schering Chemicals Ltd, Burgess Hill, Sussex.

**Tissues.** Prostatic and testicular tissues were obtained from two healthy young (2-yr-old) dogs, an aged (16-yr-old) dog and one treated with 15 mg oestradiol s.c. 3 and 5 weeks before operation. Tissues were cooled to 0 °C after removal and retained at this temperature until processed approximately 1 h later.

**Preparation of tissues and conditions of incubation.** Prostatic and testicular tissues were finely chopped with a razor blade. The minces (2 g in each case) were incubated with various radioactive steroids in 25 ml Krebs-Ringer bicarbonate-glucose medium for 2 h at 38-5 °C for prostatic tissue and at 37 °C for testicular tissue, with agitation in an atmosphere of 95% O₂ : 5% CO₂. Reactions were stopped by the addition of acetone. In two preliminary experiments, minced prostatic tissue (1 g) was incubated with [7α-3H]testosterone in 12-5 ml Krebs-Ringer bicarbonate-glucose medium for 60 min at 38-5 °C. After incubation, the tissue was collected, washed twice in 0-25 m-sucrose and homogenized in this medium using a Potter-Elvyjem homogenizer. The homogenate was filtered through gauze and samples retained for DNA determination and steroid analysis. The remainder was centrifuged at 800g for 10 min and the pellet resuspended in 0-88 m-sucrose/1-5 mm-CaCl₂. The mixture was layered on to a double gradient of 1-5 m- and 2-2 m-sucrose, each containing 0-5 mm-CaCl₂. The solutions were centrifuged at 33000g for 45 min in the SW-50L rotor of a Beckman L2 ultracentrifuge. The nuclear band at the 1-5 m/-2-2 m-sucrose interface was taken, resuspended in 0-25 m-sucrose and centrifuged at 800g for 10 min to obtain the nuclear pellet. Samples were taken for electron microscopy to assess the nuclear preparation and also for DNA determination before the addition of 500 µg non-radioactive carrier steroid in acetone for the analysis of radioactive metabolites.

**Extraction and fractionation of steroids from minced prostate and testis incubations.** After the addition of acetone to the incubation and tissue preparations, non-radioactive carrier steroids (300 µg) were added in ethanol. Mixtures were homogenized in a Silverson mixer with 5 vol. acetone and filtered. The residue was washed twice with acetone and the pooled acetone extracts and washings dried in vacuo. Steroids were fractionated as described by Fahmy, Griffiths, Turnbull & Symington (1968).
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Chromatography of steroids. Thin-layer chromatography on Merck silica gel HF_{254} and on Woelm–Eschwege aluminium oxide was used to isolate the individual steroids in the various fractions. Steroids and derivatives were detected and extracted from the adsorbents as previously described (Fahmy et al. 1968). The following solvent systems were used (v/v): I, chloroform:acetone (37:3); II, chloroform:acetone (150:1); III, cyclohexane:ethyl acetate (7:3); IV, cyclohexane:ethyl acetate (9:11); V, chloroform:methanol:water (187:12:1); VI, chloroform:methanol:water (149:9:1); VII, hexane:ethyl acetate (1:1); VIII, cyclohexane:ethanol (9:1); IX, tert.-butanol:ethyl acetate:5M-NH₄OH (41:50:20); X, cyclohexane:ethyl acetate (4:1); XI, ether:ether acetate (19:1); XII, cyclohexane:ethyl acetate (1:1); XIII, benzene:ethyl acetate (9:1).

Separation of some individual carrier neutral steroids by thin-layer chromatography. Portions of the neutral steroid fraction were applied as bands to thin-layers of silica gel. After chromatography in solvent system I, bands corresponding in mobility to androstenedione ($R_f$ 0-60), androsterone and 5α-dihydrotestosterone ($R_f$ 0-46), testosterone and epitestosterone ($R_f$ 0-30), 5α-androstan-3α,17β-diol, 5α-androstan-3β,17α-diol and 5α-androstan-3β,17β-diol ($R_f$ 0-22), 5α-androstan-3α,17α-diol ($R_f$ 0-14) and 6β-hydroxyandrostenedione ($R_f$ 0-12) were eluted. Androsterone and 5α-dihydrotestosterone were acetylated and separated by re-running in solvent system II ($R_f$ 0-45 and 0-58 respectively). After hydrolysis, the free steroids were oxidised to 5α-androstan-3,17-dione and chromatographed in solvent system III on silica gel ($R_f$ 0-42). Testosterone and epitestosterone were separated by running on alumina plates in solvent system I ($R_f$ 0-45 and 0-33 respectively). Further purification was obtained by acetylation and rerunning on alumina in solvent system III.

The 5α-androstanediols, the 3α,17β, the 3β,17α and 3β,17β were chromatographed on silica gel plates in solvent system XI, separating the 3α,17β ($R_f$ 0-60) from the 3β,17α and 3β,17β ($R_f$ 0-55). The latter two were separated on Merck alumina plates in solvent system XIII [3β,17α ($R_f$ 0-17) and 3β,17β ($R_f$ 0-27)]. Further purification of the 5α-androstan-3α,17α-diol was obtained by acetylation and rechromatography on silica gel in solvent system III ($R_f$ 0-49). After hydrolysis and oxidation the 5α-androstanedione formed was chromatographed in system X on silica gel.

Measurement of steroids and radioactivity. Steroids were measured after elution and derivatives prepared by procedures already established (Griffiths, Grant, Browning, Cunningham & Barr, 1966). Androsterone, the androstanediols, 5α-androstan-3β,17β-dione, 5α-dihydrotestosterone and their derivatives were measured by gas chromatography at 200 °C using 1·524 m (5 ft) column of 1% XE-60 on Gas Chrom Q (Applied Science Laboratories, Philadelphia). Radioactivity was measured by a Nuclear–Chicago Liquid Scintillation spectrometer. Counting time was always sufficient to give a 2% standard error for net activity. The mean of the specific activities of the carrier steroid and two derivatives was used to calculate percentage conversion from the substrate incubated.

RESULTS

The percentage conversion of testosterone to various metabolites by minced canine prostatic tissue is shown in Table 1. Evidence for the identification of these metabolites and the substrate remaining at the end of the incubation period is given.
in Table 2. Of the metabolites studied, 5α-dihydrotestosterone was the major steroid formed in the normal tissue and evidence was obtained for the synthesis of both testosterone sulphate and epitestosterone. However, a large part (20–30%) of the radioactivity incubated with normal prostatic tissue was unaccounted for. Testosterone was converted to the 5α-androstane diols (Table 5), mainly the 3α,17β and 3β,17β epimers and there was also evidence for 3α,17α- and 3β,17α-diol formation; 5β-isomers were not detected. The extensive metabolism of testosterone by prostatic tissue from dogs pretreated with oestradiol-17β (Table 1) was particularly interesting.

Table 1. Incubation of [14C]testosterone with minced canine prostate tissue. Values are % of incubated radioactivity found in the carrier steroid; 2 g tissue were incubated with 2-5 μCi [4-14C]testosterone

<table>
<thead>
<tr>
<th>Steroid investigated</th>
<th>Normal tissue Expt 1</th>
<th>Normal tissue Expt 2</th>
<th>Normal tissue Expt 3</th>
<th>Normal tissue Expt 4</th>
<th>Old animal Expt 5</th>
<th>Oestrogen treatment Expt 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (unmetabolized)</td>
<td>62-8</td>
<td>81-7</td>
<td>66-8</td>
<td>59-4</td>
<td>58-8</td>
<td>3-3</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0-7</td>
<td>1-3</td>
<td>0-7</td>
<td>—</td>
<td>1-9</td>
<td>—</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>1-7</td>
<td>4-8</td>
<td>3-5</td>
<td>4-8</td>
<td>1-9</td>
<td>0-4</td>
</tr>
<tr>
<td>6α-Hydroxyandrostenedione</td>
<td>0-2</td>
<td>0-01</td>
<td>0-2</td>
<td>—</td>
<td>0-0</td>
<td>4-6</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0-0</td>
<td>0-9</td>
<td>0-0</td>
<td>0-5</td>
<td>0-0</td>
<td>0-3</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>3-1</td>
<td>5-3</td>
<td>0-0</td>
<td>8-7</td>
<td>0-0</td>
<td>—</td>
</tr>
<tr>
<td>Testosterone sulphate</td>
<td>0-0</td>
<td>0-01</td>
<td>0-0</td>
<td>0-1</td>
<td>0-0</td>
<td>—</td>
</tr>
</tbody>
</table>

Dashes indicate that the metabolites were not examined and zeros indicate that no radioactivity was found in those metabolites.

since most of the radioactivity was converted to unidentified, highly polar metabolites. Little 5α-dihydrotestosterone was formed compared to normal tissue. A smaller yield of 5α-dihydrotestosterone was obtained from the tissue of the 16-yr-old dog.

The results from the incubation of various steroid precursors with canine testicular tissue are given in Tables 3 and 4. There was a decreased synthesis of testosterone from both [7α-3H]pregnenolone and [4-14C]dehydroepiandrosterone (DHA) by tissue from the dog treated with the oestrogen, yet when compared with a normal gland, the results suggest that a higher rate of steroid metabolism had been induced. 17α-Hydroxyprogesterone and DHA were extensively metabolized by testicular tissue from the older dog. The overall metabolic pattern suggests that the pathway for testosterone synthesis in canine testicular tissue is via DHA.

Two preliminary experiments in which [7α-3H]testosterone was incubated with minced canine prostatic tissue produced results which may be of significance in relation to the DNA polymerase studies described elsewhere (Harper et al. 1970b). Analysis of the nuclear preparations isolated from the tissue showed that in one experiment 3-5% of the incubated radioactivity was associated with testosterone and 2% with epitestosterone. Only trace amounts of radioactivity were found in the carrier 5α-dihydrotestosterone. In a second experiment (Table 5), in which the androstane diols were also studied, radioactivity was found associated with nuclear 5α-dihydrotestosterone in similar proportions to testosterone. Epitestosterone and
Table 2. Incubation of $^1\text{H}$C\textit{testosterone} with minced canine prostatic tissue

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvent systems used for purification ($S =$ silica; $\text{Al} =$ alumina)</th>
<th>Specific activities (disintegrations/min/nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I (S), I (Al)</td>
<td>3451</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), X (S)</td>
<td>3455</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (Al), III (S)</td>
<td>3299</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>I (S), I (Al)</td>
<td>37-8</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), X (S)</td>
<td>37-0</td>
</tr>
<tr>
<td>Epitestosterone acetate</td>
<td>III (Al), III (S)</td>
<td>38-2</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), X (S)</td>
<td>88-6</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I (S), X (S)</td>
<td>87-8</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (S)</td>
<td>90-5</td>
</tr>
<tr>
<td>6$\beta$-Hydroxyandrostenedione</td>
<td>I (S)</td>
<td>12-7</td>
</tr>
<tr>
<td>6$\beta$-Acetoxyandrostenedione</td>
<td>I (S), IV (S)</td>
<td>13-0</td>
</tr>
<tr>
<td>6$\beta$-Hydroxytestosterone</td>
<td>V (S)</td>
<td>12-4</td>
</tr>
<tr>
<td>5$\alpha$-Dihydroxytestosterone</td>
<td>I (S), X (S)</td>
<td>173-2</td>
</tr>
<tr>
<td>5$\alpha$-Androstan-3-17-dione</td>
<td>III (S), X (S), I (S)</td>
<td>168-7</td>
</tr>
<tr>
<td>5$\alpha$-Dihydrotestosterone acetate</td>
<td>II (S), III (S)</td>
<td>165-4</td>
</tr>
<tr>
<td>Androsterone</td>
<td>I (S), X (S)</td>
<td>0-0</td>
</tr>
<tr>
<td>Androsterone acetate</td>
<td>II (S), III (S)</td>
<td>48-3</td>
</tr>
<tr>
<td>5$\alpha$-Androstan-3,17-dione</td>
<td>III (S), X (S)</td>
<td>47-9</td>
</tr>
<tr>
<td>Testosterone sulphate</td>
<td>IX (S)</td>
<td>0-0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I (S), I (Al)</td>
<td>0-0</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (S)</td>
<td>0-0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S)</td>
<td>0-0</td>
</tr>
</tbody>
</table>

Dashes indicate that the metabolites were not examined and zeros indicate that no radioactivity was found in those metabolites. Solvent systems used in the purification of derivatives are numbered according to the list in the text.
Table 3. Steroid metabolism in the testes from normal and oestrogen-treated dogs

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Testicular tissue studied (g tissue incubated)</th>
<th>Steroid precursors incubated</th>
<th>% Incubated radioactivity found in carrier steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Young testis (2 g)</td>
<td>[7α-3H]Preg, 15 μCi (sp.act. 254 mCi/mmol)</td>
<td>Preg 30-8 DHA 35-2 17αOH-Prog 2-3 Test 9-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4-14C]DHA, 2 μCi (sp.act. 34 mCi/mmol)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Testis from oestradiol-17β-treated dog (2 g)</td>
<td>[7α-3H]Preg, 15 μCi (sp.act. 254 mCi/mmol)</td>
<td>Preg 19-8 DHA 18-6 17αOH-Prog 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4-14C]DHA, 2 μCi (sp.act. 34 mCi/mmol)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Young testis (2 g)</td>
<td>[4-14C]17αOH-Prog, 2-15 μCi (sp.act. 36.5 mCi/mmol)</td>
<td>Preg 0 DHA 61-6 Test 2-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[7α-3H]DHA, 8-53 μCi (sp.act. 144 mCi/mmol)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Old testis (1 g)</td>
<td>[4-14C]17αOH-Prog, 0-6 μCi (sp.act. 37-5 mCi/mmol)</td>
<td>Preg 0 DHA 0 Test 71-9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[7α-3H]DHA, 7-0 μCi (sp.act. 500 mCi/mmol)</td>
<td></td>
</tr>
</tbody>
</table>

And = androstene-3,17-dione; DHA = dehydroepiandrosterone; Preg = pregnenolone; 17αOH-Prog = 17α-hydroxyprogesterone; Test = testosterone. Dashes indicate that the metabolites were not examined and zeros indicate that no radioactivity was found in those metabolites.
Table 4. Evidence for the formation of various metabolites from radioactive steroids incubated with canine testicular tissue in vitro

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvent systems used for purification (S = silica; Al = alumina)</th>
<th>Young testis</th>
<th>Testis from dog treated with oestradiol-17β</th>
<th>Young testis</th>
<th>Old testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone</td>
<td>I (S)</td>
<td>12556</td>
<td>24834</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnenolone acetate</td>
<td>XIII (S)</td>
<td>12836</td>
<td>24720</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preg-5-ene-3β,20β-diol</td>
<td>I (S)</td>
<td>11886</td>
<td>25346</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHA</td>
<td>I (S)</td>
<td>364-5</td>
<td>2127</td>
<td>26-8</td>
<td></td>
</tr>
<tr>
<td>DHA acetate</td>
<td>III, (S)</td>
<td>385-9</td>
<td>2018</td>
<td>26-8</td>
<td></td>
</tr>
<tr>
<td>Androstenediol</td>
<td>VII, (S)</td>
<td>348-7</td>
<td>2085</td>
<td>25-8</td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>I (S)</td>
<td>455-6</td>
<td>0</td>
<td>33-8</td>
<td>3129-5</td>
</tr>
<tr>
<td>17α,20β-Dihydroxypregn-4-en-3-one</td>
<td>IV, I (S)</td>
<td>495-2</td>
<td>0</td>
<td>32-3</td>
<td>3384-2</td>
</tr>
<tr>
<td>17α-Hydroxy-20β-acetoxyprogren-4-en-3-one</td>
<td>IV (S)</td>
<td>487-9</td>
<td>0</td>
<td>34-3</td>
<td></td>
</tr>
<tr>
<td>Androstenediol</td>
<td>I, III (S)</td>
<td>3505-2</td>
<td>1161-7</td>
<td>45-1</td>
<td>30-6</td>
</tr>
<tr>
<td>Androstenediol diacetate</td>
<td>III (S)</td>
<td>3410-4</td>
<td>1116-4</td>
<td>46-2</td>
<td>30-3</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I, XII (S)</td>
<td>863-7</td>
<td>323-3</td>
<td>0</td>
<td>125-0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I (S)</td>
<td>866-4</td>
<td>336-4</td>
<td>0</td>
<td>130-6</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (S)</td>
<td>814-5</td>
<td>328-1</td>
<td>0</td>
<td>131-9</td>
</tr>
<tr>
<td>Testosterone</td>
<td>I (S), I (Al)</td>
<td>3501-8</td>
<td>1541-4</td>
<td>25-5</td>
<td>117-3</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), XII (S)</td>
<td>3550-5</td>
<td>1502-5</td>
<td>24-5</td>
<td>116-8</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (Al), III (S)</td>
<td>3303-0</td>
<td>1601-8</td>
<td>24-4</td>
<td>111-0</td>
</tr>
</tbody>
</table>

Specific activities (disintegrations/min/nmol)

Dashes indicate that those metabolites were not examined and zeros that no radioactivity was found in those metabolites. Solvent systems used in the purification of derivatives are numbered according to the list in the text. For definition of abbrevations see Table 3.
Table 5. Normal dog prostate mince incubated with 46-5 μCi [7α-3H]testosterone

(1 g of minced prostate tissue was incubated with 46-5 μCi [7α-3H]testosterone (sp.act. 1.63 Ci/mmol) in 12.5 ml Krebs–Ringer bicarbonate–glucose at 38.5 °C for 1 h. The % radioactivity found in the nuclear fraction has been corrected for nuclear recovery based on DNA determinations. The homogenate values represent the whole tissue content of 3H metabolites.)

<table>
<thead>
<tr>
<th>Steroid investigated and derivative formed</th>
<th>Solvent systems used for purification (S = silica; Al = alumina)</th>
<th>Homogenate</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>I (S), I (Al)</td>
<td>51.36</td>
<td>1.25</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), XII (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>III (Al), III (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>I (S), I (Al)</td>
<td>0.62</td>
<td>0.59</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>I (S), XII (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epitestosterone acetate</td>
<td>III (Al), III (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3α,17α-diol</td>
<td>I (S), IV (S)</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>5α-Androstene-3,17-dione</td>
<td>X (S), I (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3β,17β-diol</td>
<td>IV (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3β,17β-diacetate</td>
<td>III (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3α,17β-diol</td>
<td>I (S), XI (S)</td>
<td>0.86</td>
<td>0.03</td>
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<tr>
<td>5α-Androstene-3,17-dione</td>
<td>X (S), I (S)</td>
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<td></td>
</tr>
<tr>
<td>5α-Androstene-3β,17β-diol</td>
<td>IV (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3β,17β-diacetate</td>
<td>III (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3β,17α-diol</td>
<td>I (S), XI (S), XII (Al)</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Androstene-3,17-dione</td>
<td>X (S), I(S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3β,17β-diol</td>
<td>IV (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Androstene-3β,17β-diacetate</td>
<td>III (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>I (S), XII (S)</td>
<td>19.96</td>
<td>1.30</td>
</tr>
<tr>
<td>5α-Androstene-3,17-dione</td>
<td>X (S), I(S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Dihydrotestosterone acetate</td>
<td>II (S), III (S)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

small amounts of the 5α-androstanediols were also found in the nuclear fraction. It was interesting that in the case of epitestosterone and 5α-androstane-3α,17α-diol all the radioactivity associated with these steroids was found in the nuclear fraction. This is in contrast to the large proportion of the radioactivity in the prostatic tissue homogenate (Table 5) before fractionation, which was associated with 5α-dihydrotestosterone but which was subsequently shown not to be predominantly bound to the nucleus.

**DISCUSSION**

The incubation studies with normal canine prostatic tissue indicated that many of the metabolites previously shown to be formed by the human hypertrophic prostate from testosterone in vitro (Farnsworth & Brown, 1963; Chamberlain, Jagarinec & Ofner, 1966), were also synthesized in these experiments. Consistent with the results of these earlier investigations was the finding that one of the major metabolites of testosterone was 5α-dihydrotestosterone, although testosterone sulphate and epitestosterone
were also present. Apart from the demonstration in canine tissue of 17z-hydroxysteroid dehydrogenase, possibly localized in the nucleus, and 17β-hydroxysteroid sulphotransferase, analysis of the carrier androstanediols in one of the incubations (Table 5) indicated the presence in canine prostatic tissue of 3z- and 3β-hydroxysteroid dehydrogenase and 5z-reductase enzyme systems. No 5β-androstanediols could be identified. The activity of the 3z- and 17z-hydroxysteroid dehydrogenases and their subcellular localization may well be worth further study in relation to their possible biological function, since 5z-androstane-3z,17z-diol is the only other compound, in addition to testosterone, which will stimulate the DNA polymerase of dog prostate (Harper, Fahmy, Pierrepoint & Griffiths, 1970a). It may be significant that whereas with human hyperplastic prostatic tissue, both testosterone and 5z-dihydrotestosterone stimulated the isolated DNA polymerase (Harper et al. 1970a), 5z-dihydrotestosterone was found to be ineffective in the canine prostate. Furthermore, although Anderson & Liao (1968) showed that after incubation of rat prostatic tissue with [7z-3H]testosterone, 5z-dihydrotestosterone was selectively retained by the prostatic cell nuclei, similar (but preliminary) experiments with canine prostatic tissue, described in this communication, indicate that there was less 5z-dihydrotestosterone present in the preparations than in those from the rat (Table 5). Radioactive epitestosterone and various 5z-androstanediols, principally the 3z,17z and 3β,17β isomers, were also present in the nuclear preparation. Further consideration must be given to separation of the various cell types of the prostatic tissue, in particular the epithelial and fibromuscular tissues, in which steroid metabolism is probably very different.

Experiments with explants of rat and dog prostatic tissue in organ culture in the presence of [7z-3H]testosterone (Griffiths, Harper, Groom, Pike, Fahmy & Pierrepoint, 1970) showed that, whereas 5z-dihydrotestosterone was the principal steroid isolated from the nuclear preparation of the rat tissue, a similar preparation from the dog prostate showed testosterone and 5z-dihydrotestosterone to be present in approximately equal amounts. When Kowarski, Shalf & Migeon (1969) infused [1,2-3H]-testosterone into dogs for 120 min until a state of equilibrium between endogenous and exogenous hormone had been established, testosterone and 5z-dihydrotestosterone were found in a higher concentration in prostate than in other organs examined and were localized predominantly in the microsomal fraction of the gland, although 5z-dihydrotestosterone was also found in the nuclear fraction. These investigations suggest that despite the relatively large yield of 5z-dihydrotestosterone formed from testosterone by the canine prostate in vitro, this steroid may not be so important in the cell nucleus of this tissue. Comparison of the ability of prostatic nuclei to convert testosterone to 5z-dihydrotestosterone showed that preparations from the dog are much less active than those from rat or man (Griffiths et al. 1970).

Relatively consistent metabolic patterns were obtained by incubation of testosterone with normal canine prostatic tissue (Table 1). However, with tissue from the oestradiol-treated dog, only 3·3% of the incubated testosterone remained at the end of the incubation period and little could be accounted for in the radiometabolites studied. A small yield of 5z-dihydrotestosterone was obtained but the major proportion of radioactivity was found associated with a highly polar steroid fraction.

It is normally accepted that administered oestrogen indirectly affects the prostate
by reducing testosterone secretion from the testis by suppressing interstitial cell-stimulating hormone (ICSH) release. Such a decrease in plasma ICSH has been described (Alder, Burger, Davis, Dulmanis, Hudson, Sarfaty & Straffan, 1968). Slaunwhite, Sandberg, Jackson & Staubitz (1962) demonstrated a decreased 17β-hydroxysteroid dehydrogenase activity in homogenates of testes removed from diethylstilboestrol-treated men with prostatic cancer. Similar effects of hexoestrol on rat testis have been described (Oshima, Wakabayashi & Tamaoki, 1967). Results summarized in Table 5, indicate that in testicular tissue from an oestrogen-treated dog, there is a decreased ability to synthesize testosterone from both pregnenolone and DHA. The overall rate of steroid metabolism did, however, appear to be higher in the tissue of the treated animal, when compared with the tissue from the normal young dog. Evidence is accumulating to suggest that oestrogens may also have a more direct action on the prostate itself. Goodwin, Rasmussen-Tandal, Ferreira & Scott (1961), for example, described the inhibition of prostatic secretion by diethylstilboestrol in the androgen-maintained, but hypophysectomized dog, and evidence that oestradiol in vitro affects testosterone metabolism by human hyperplastic prostate has been presented (Farnsworth, 1969, 1970).

Testicular tissue from the 16-yr-old dog synthesized large amounts of testosterone, but the yield of 5α-dihydrotestosterone from incubation of testosterone with prostatic tissue from this animal was the lowest yield of this series of incubations. Obviously, these findings need further confirmation. The higher activity observed with the older dog’s testicular tissue is noteworthy in relation to earlier studies of Axelrod (1965), who investigated the biosynthetic activity of testicular tissue from a 16-yr-old boy and a 61-yr-old patient with prostatic cancer: this investigator suggested that a relative deficiency of the 17,20-desmolase enzyme system in the older tissue may account for the reduced testosterone secretion in advanced age in the human male, but analysis of his results indicates that more of the substrate was converted to testosterone by tissue from the older man than by that from the boy. Such results are in keeping with our own observations.

The authors wish to record their appreciation of the generous financial support from the Tenovus Organization. One of them (C.G.P.) is also supported by a research grant from the Wellcome Trust. They are also grateful to Mr Adrian Pike for his excellent technical assistance.

REFERENCES
Steroid metabolism in canine prostate


THE EFFECT OF STILBOESTROL ANALOGUES ON THE METABOLISM OF STEROIDS BY THE TESTIS AND PROSTATE OF THE RAT IN VITRO

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(Received 14 April 1973)

SUMMARY

Male Sprague-Dawley rats were injected (i.m.) daily for 10 days with 100 µg of either oestradiol-17β, diethylstilboestrol (DES), dl-dihydrodibutylstilboestrol (dl-DHBS) or meso-dihydrodibutylstilboestrol (meso-DHBS) in 0.2 ml sesame oil. After 10 days, the testicular tissue was removed and incubated simultaneously with [7α-3H]dehydroepiandrosterone and [4-14C]17α-hydroxyprogesterone. Less testosterone was synthesized by the testicular tissue from animals treated with oestradiol-17β, DES and meso-DHBS than by the controls or animals treated with dl-DHBS. The decreased synthetic activity was related to the decreased activity of both the 17β-hydroxysteroid dehydrogenase and 17α-pregnene-C17,20-lyase enzyme systems.

Prostatic tissue was also incubated with [7α-3H]testosterone. Administration of DES, oestradiol-17β or meso-DHBS increased the metabolism of testosterone by the prostatic tissue with a marked effect on the 5α-reductase enzyme system.

INTRODUCTION

The administration of diethylstilboestrol (DES) has been for a number of years a generally accepted form of treatment for carcinoma of the prostate. The use of such antiandrogenic therapy resulted from the investigations of Huggins, Scott & Hodges (1941), which clearly established the androgen-dependent nature of human prostatic cancer. DES is considered to exercise its main effect indirectly via the pituitary, thereby inhibiting the release of interstitial cell-stimulating hormone (ICSH), which thereby decreases testosterone synthesis (Slaunwhite, Sandberg, Jackson & Staubitz, 1962; Alder, Burger, Davis, Dulmanis, Hudson, Sarfaty & Straffon, 1968).

A recent report from this laboratory (Danutra, Harper, Boyns, Cole, Brownsey & Griffiths, 1973) compared the effects of DES on organ weights and plasma hormone concentrations in male rats with those produced after treatment with oestradiol-17β and meso-dihydrodibutylstilboestrol (meso-DHBS). Although all three compounds effectively decreased prostatic weight, lowered plasma testosterone and increased plasma prolactin concentrations, only DES significantly lowered plasma ICSH concentration at a dose of 100 µg/day/animal for 10 days. The results suggested that
a major part of the anti-androgenic effect of oestradiol-17β and meso-DHBS might have been due to a direct action on the synthesis of testosterone by the testis. A study of the metabolism of dehydroepiandrosterone (DHA) and 17α-hydroxyprogesterone by testicular tissue in vitro, together with an investigation of the metabolism of testosterone in vitro by prostatic tissue were therefore also undertaken with tissue from treated animals.

MATERIALS AND METHODS

Male Sprague-Dawley rats, aged 16-17 weeks, were given a commercial rat diet and tap water ad libitum. They were injected (i.m.) daily for 10 days with 100 µg of one of the following: oestradiol-17β, DES, meso-DHBS or DL-dihydrodibutylstilbestrol (DL-DHBS) in 0-2 ml sesame oil. Control animals were injected with vehicle alone. Oestradiol-17β and DES were purchased from Koch-Light Laboratories, Bucks., U.K., and the meso-DHBS and DL-DHBS were prepared for these studies with the kind co-operation of Dr A. L. Walpole and Dr D. N. Richardson, I.C.I. Limited, Alderley Edge, Manchester. The animals were killed on the 11th day of treatment by cervical dislocation and the testes and the prostates were removed. The glands were freed from surrounding tissue. Testes obtained from six animals were combined, minced and 2 g of the minced tissue was incubated simultaneously with [7α-3H]DHA (10 µCi, sp. act. 179-5 mCi/mmole) and [4-14C]17α-hydroxyprogesterone (2 µCi, sp. act. 35-9 mCi/mmole) for 1 h at 37 °C in 25 ml of Krebs–Ringer bicarbonate–glucose solution, pH 7-4, in an atmosphere of O2:CO2 (95:5 %). Ventral prostates obtained from 15 animals were combined and 1 g of the minced tissue was incubated with [7α-3H]testosterone (10 µCi, sp. act. 5-4 Ci/mmole) in 12-5 ml of Krebs–Ringer bicarbonate–glucose solution as described. All three radioactive steroids were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. Purity of the precursor steroids was checked by isotope dilution studies and extensive thin-layer chromatography (t.l.c.) before use. Reactions were stopped by the addition of 5-10 ml acetone containing 300 µg each of the non-radioactive carrier steroids to be investigated. Five carrier steroids were added to the incubations of testicular tissue: 17α-hydroxyprogesterone, DHA, androstenediol (androstan-5-ene-3β,17β-diol) androstenedione and testosterone. The following carrier steroids were added to the prostatic tissue incubations: testosterone, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), 5α-androstane-3β,17β-diol, 5α-androstane-3β,17α-diol, 5α-androstane-3β,17β-diol, 5α-androstane-3β,17α-diol. After the addition of carrier steroids, the mixtures were homogenized. The filtrates and acetone washes of the mixtures were combined and dried in vacuo. Steroids were fractionated as described by Fahmy, Griffiths, Turnbull & Symington (1968) and the individual carrier steroids were isolated by extensive t.l.c. on silica gel (Merck Kieselgel HF254+366, alumina (PF254+366, type T; E. Merck) or alumina F254 (Woelm, neutral TLC).

Solvent systems for the separation of the various carrier steroids and the procedures for the determination of the specific radioactivity of these steroids and their derivatives have previously been described (Pike, Peeling, Harper, Pierrepoint & Griffiths, 1970; Harper, Pierrepoint, Fahmy & Griffiths, 1971). The four androstane-3α-diol epimers were not isolated separately but were analysed as a group.
Table 1. Incubation of rat testicular tissue with $[7\alpha-3\text{H}]$dehydroepiandrosterone (DHA) and $[4-14\text{C}]7\alpha$-hydroxyprogesterone, and prostatic tissue with $[7\alpha-3\text{H}]$testosterone

<table>
<thead>
<tr>
<th>Steroid investigated</th>
<th>Specific activity of steroid investigated (d.p.m./nmol)</th>
<th>Control</th>
<th>Oestradiol-17(\beta)</th>
<th>DES</th>
<th>DL-DHBS</th>
<th>meso-DHBS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue: testis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17(\alpha)-Hydroxyprogestosterone</td>
<td>$^{14}\text{C}$</td>
<td>3492</td>
<td>3988</td>
<td>3912</td>
<td>3145</td>
<td>3462</td>
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<tr>
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<td>$^{3}\text{H}$</td>
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<td>3371</td>
<td>4185</td>
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<td>3929</td>
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<td>3319</td>
<td>4051</td>
<td>3746</td>
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<td>444-8</td>
<td>173-9</td>
<td>164-4</td>
<td>135-5</td>
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<tr>
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<td>$^{3}\text{H}$</td>
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<td>158-8</td>
<td>131-6</td>
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<td>0</td>
</tr>
<tr>
<td>Androst-5-ene-9(\beta),17(\alpha)-dione</td>
<td>$^{14}\text{C}$</td>
<td>509-7</td>
<td>513-6</td>
<td>293-2</td>
<td>303-1</td>
<td>367-9</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>524-5</td>
<td>312-9</td>
<td>381-4</td>
<td>38-7</td>
<td>390-3</td>
</tr>
<tr>
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<td>$^{14}\text{C}$</td>
<td>379-1</td>
<td>102-0</td>
<td>154-4</td>
<td>433-8</td>
<td>301-8</td>
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<td></td>
<td>$^{3}\text{H}$</td>
<td>366-0</td>
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<td>105-7</td>
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<td>150-7</td>
<td>397-2</td>
<td>292-9</td>
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<tr>
<td>Testosterone</td>
<td>$^{14}\text{C}$</td>
<td>872-8</td>
<td>829-6</td>
<td>887-4</td>
<td>854-2</td>
<td>615-6</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>715-0</td>
<td>684-1</td>
<td>9993</td>
<td>854-2</td>
<td>615-6</td>
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<td></td>
<td>$^{3}\text{H}$</td>
<td>667-0</td>
<td>1006-8</td>
<td>8641</td>
<td>825-2</td>
<td>794-8</td>
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<td>544-8</td>
<td>277-2</td>
<td>264-1</td>
<td>609-8</td>
<td>497-1</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>532-4</td>
<td>544-4</td>
<td>269-9</td>
<td>275-0</td>
<td>589-9</td>
</tr>
<tr>
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<td>$^{3}\text{H}$</td>
<td>558-1</td>
<td>278-0</td>
<td>265-5</td>
<td>565-3</td>
<td>479-7</td>
</tr>
<tr>
<td><strong>Tissue: prostate</strong></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>$^{3}\text{H}$</td>
<td>315-2</td>
<td>63-2</td>
<td>103-7</td>
<td>220-2</td>
<td>241-1</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>319-2</td>
<td>319-0</td>
<td>642-9</td>
<td>638-1</td>
<td>103-5</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>3195</td>
<td>635-3</td>
<td>9999</td>
<td>9999</td>
<td>2201-1</td>
</tr>
<tr>
<td>17(\beta)-Hydroxy-5x-androstan-3-one</td>
<td>$^{3}\text{H}$</td>
<td>8022</td>
<td>482-0</td>
<td>696-5</td>
<td>771-3</td>
<td>862-5</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>723-2</td>
<td>759-8</td>
<td>455-2</td>
<td>671-3</td>
<td>796-4</td>
</tr>
<tr>
<td>5x-Androstan-3,17-dione</td>
<td>$^{3}\text{H}$</td>
<td>755-1</td>
<td>437-9</td>
<td>6005</td>
<td>8447</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>129-6</td>
<td>469-1</td>
<td>374-7</td>
<td>121-2</td>
<td>146-0</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>125-1</td>
<td>128-7</td>
<td>514-1</td>
<td>496-6</td>
<td>127-4</td>
</tr>
<tr>
<td>Androst-4-ene-3,17-dione</td>
<td>$^{3}\text{H}$</td>
<td>139-9</td>
<td>13-49</td>
<td>15-26</td>
<td>5-60</td>
<td>12-69</td>
</tr>
<tr>
<td></td>
<td>$^{3}\text{H}$</td>
<td>13-43</td>
<td>13-66</td>
<td>14-22</td>
<td>13-45</td>
<td>15-88</td>
</tr>
</tbody>
</table>

Individual specific activities for the derivatives are shown, together with the mean specific activity. A zero indicates that radioactivity was not significantly above background. DES = diethylstilboestrol; DL-DHBS = DL-dihydrodibutylstilboestrol; meso-DHBS = meso-dihydrodibutylstilboestrol.

Animals were treated as described in the Methods section. Testicular tissue (2 g) was incubated simultaneously with equimolar amounts of $[7\alpha-3\text{H}]$dehydroepiandrosterone (10 µCi) and $[4-14\text{C}]7\alpha$-hydroxyprogesterone (2 µCi) in 25 ml of Krebs–Ringer bicarbonate–glucose solution for 1 h at 37 °C. Prostatic tissue (1 g) was incubated with $[7\alpha-3\text{H}]$testosterone (12.5 µCi) in 10 ml solution under similar conditions.
RESULTS AND DISCUSSION

Testis

Table 1 gives the specific activities of steroids investigated and their derivatives formed. The mean specific activities were used to calculate the total radioactivity associated with each steroid investigated, which is shown in Fig. 1. It can be seen

![Graph](https://example.com/graph.png)

Fig. 1. (a) Rat testes incubated with (i) [7α-3H]dehydroepiandrosterone (DHA), (ii) [4,14C]17α-hydroxyprogesterone (17-P). Animals were treated as described in the Materials section. Testicular tissue (2 g) was incubated simultaneously with [7α-3H]DHA (10 μCi) and [4,14C]17-P (2 μCi) in Krebs–Ringer bicarbonate–glucose solution for 1 h at 37 °C.

(b) Rat prostate incubated with [7α-3H]testosterone. Animals were treated as described in the Materials section. Prostatic tissue (1 g) was incubated with [7α-3H]testosterone (10 μCi) in Krebs–Ringer bicarbonate–glucose solution for 1 h at 37 °C. The calculation for androstanediol formation is based on one specific activity of the 5α-androstanediol fraction after conversion to 5α-androstane-3,17-dione.

Abbreviations: A = androst-4-ene-3,17-dione; T = testosterone; 5α-T = 5α-dihydrotestosterone; 5α-A dione = 5α-androstane-3,17-dione; 5α-A diols = 5α-androstane-3,17-diols. For other abbreviations see footnote to Table 1.
that the synthesis of testosterone in vitro from DHA and 17α-hydroxyprogesterone was less in testicular tissue obtained from meso-DHBS-treated, oestradiol-17β-treated and DES-treated animals. In particular, oestradiol-17β and DES were most effective in decreasing the activity of the 17β-hydroxysteroid dehydrogenase enzyme system in this tissue. A similar observation was also made by Slaunwhite et al. (1962) using homogenates of testes removed from patients with prostatic cancer who had also been treated with DES.

Tamaoki & Shikita (1966) indicated that the formation of testosterone from 17α-hydroxyprogesterone via androstenedione was the principal pathway for the biosynthesis of testosterone in the rat testis. The results shown in Fig. 1 suggested that under the conditions used in these studies, the synthesis of testosterone from the 17α-hydroxyprogesterone was also decreased in the testes of animals treated with oestradiol-17β, DES or meso-DHBS. Fig. 1(a), ii, also showed that these oestrogens affected the activity of the enzyme 17α-pregnene-C17,20-lyase, since the yield of androstenedione was also decreased. An earlier report by Oshima, Wakabayashi & Tamaoki (1967) suggested that this enzyme was unaffected after treatment with hexoestrol in vivo. The results described here suggest that the low plasma testosterone concentration together with unchanged ICSH levels (Danutra et al. 1973) in animals treated with either oestradiol-17β or meso-DHBS could be due to a direct effect of these compounds on the testes. It would appear, therefore, that DES differs from the other oestrogens in that it can affect the pituitary–testicular system with a decrease in ICSH levels, while also having a direct action on the testis.

Prostate

Fig. 1(b) shows the effect of the administered compounds on the metabolism of testosterone by the ventral prostatic tissue from the same animals. As suggested previously from studies with the dog prostate (Harper et al. 1971), oestrogen administration increased the rate at which testosterone was metabolized by prostatic tissue. In the DES- and oestradiol-17β-treated animals, the prostatic 5α-reductase activity was increased although the formation of 5α-dihydrotestosterone at the end of the incubations was lower in these groups due to the formation of increased amounts of other 5α-reduced metabolites. Shimazaki, Kato, Nagai, Yamanaka & Shida (1972) have shown that neither castration nor addition of oestradiol-17β in vitro to the incubated tissue affected the rate of reduction of 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol. It was obvious, however, from our studies that prolonged oestrogen treatment in vivo did affect testosterone metabolism by prostatic tissue in vitro.

The authors wish to acknowledge the generous financial support of the Tenovus Organization. One of them (V.D.) is grateful to the British Council for a Scholarship to enable her to study in this country. They are also grateful for the kind co-operation of Dr A. L. Walpole and Dr D. N. Richardson, I.C.I. Ltd., for the supply of dihydrodibutylstilboestrol used in these studies.
REFERENCES


STEROIDS OF ADRENAL ORIGIN METABOLIZED BY HUMAN PROSTATIC TISSUE BOTH IN VIVO AND IN VITRO

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SUMMARY

The pattern of metabolites present in human benign prostatic tissue after infusion of either [7α-3H]testosterone, [7α-3H]androstenedione or [7α-3H]-dehydroepiandrosterone (DHA) sulphate 30 min before prostatectomy were investigated. Infusion of [7α-3H]testosterone yielded 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) as the major metabolite present in the prostatic tissue together with other 5α-androstan compounds. The major radioactively labelled steroids identified in the prostatic tissue after [7α-3H]androstenedione infusion were epiandrosterone and androsterone, and dehydroepiandrosterone after [7α-3H]DHA sulphate infusion, although various other 5α-androstan compounds were also present after both infusions. The proportion and identity of radioactive steroids present in the epithelial and stromal portions of the tissue after in-vivo infusion of [7α-3H]testosterone was investigated. Also the ability of separated epithelial and stromal elements of prostatic tissue to metabolize steroids in vitro was studied. Dehydroepiandrosterone sulphate and DHA metabolism in vitro by minced human prostatic tissue yielded various 5α-androstan compounds in addition to DHA and androstenediol (andrast-5-ene-3β,17β-diol).

INTRODUCTION

It is well established that prostatic tissue depends for its growth and normal function on the secretion of testosterone from the testes. There is now, however, considerable evidence to indicate that 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), a metabolite of testosterone in the prostate, is the active androgen in the gland (Anderson & Liao, 1968; Bruchovsky & Wilson, 1968a, b). Furthermore, it would also appear that other metabolites of testosterone within the target tissue have specific roles to play in eliciting the androgenic actions (Baulieu, Lasnitzki & Robel, 1968a, b; Farnsworth, 1970; Harper, Pierrepoint, Fahmy & Griffiths, 1970). Plasma hormones other than testosterone could therefore affect prostatic metabolism if taken up by the gland and converted to these particular active metabolites.

An earlier report indicating a relationship between the adrenal gland and the
prostate (Tullner, 1963) has received considerable support from subsequent investigations (Fingerhut & Veenema, 1967; Tisell, 1970, 1972; Münzting, 1971). It would seem that a number of the adrenal hormones, cortisol, progesterone or the adrenal androgens may influence prostatic functions. This paper describes the analysis of radioactive steroids in prostatic tissue taken from men undergoing prostatectomy for benign prostatic hypertrophy into whom either [7α-3H]dehydroepiandrosterone sulphate (DHA sulphate) or [7α-3H]androstenedione had been infused. The analyses have been compared with those obtained after infusion of [7α-3H]testosterone. Dehydroepiandrosterone sulphate is a major secretory product of the human adrenal cortex and is present in a high concentration in plasma. The metabolism in vitro of DHA sulphate and also DHA by human benign hypertrophic prostate tissue is also described together with studies on the radioactive steroids present in separated epithelial and stromal tissue removed after [7α-3H]testosterone infusion. The ability of the individual epithelial and stromal elements to metabolize testosterone and androstenedione was also compared.

MATERIALS AND METHODS

Materials

Non-radioactive steroids were purchased from Sigma London Ltd and Steraloids Ltd, Croydon. All reagents including ethanol were analytical grade. Other solvents of laboratory reagent grade were redistilled before use.

Radioactive substrates [7α-3H]androstenedione (sp. act. 3.1 Ci/mmol), [4-14C]-androstenedione (sp. act. 60 mCi/mmol), [7α-3H]testosterone (sp. act. 5.4 Ci/mmol), [7α-3H]DHA sulphate (sp. act. 290 mCi/mmol) and [7α-3H]DHA (sp. act. 16 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks. The purity of these materials was checked by reverse isotope dilution, with chromatography on thin-layer silica gel in a number of solvent systems before and after derivative formation.

Methods

Infusion of radioactive steroids

Human subjects undergoing Millen retropubic prostatectomy for benign prostatic hypertrophy were infused through the cephalic vein with 50 µCi of either [7α-3H]-testosterone, [7α-3H]androstenedione or [7α-3H]DHA sulphate, 30 min before removal of the prostatic adenoma. The enucleated tissue was immediately frozen and transported to the laboratory for analysis.

Separation of stromal and epithelial tissue

The prostate tissue was sliced into pieces approximately 3 mm thick and passed through a Latapie press. The brei obtained was suspended in a known volume of 0.25 M-sucrose containing 0.12 M-nicotinamide and passed through one layer of gauze. Tissue retained in the gauze was resuspended in the sucrose-nicotinamide solution and re-filtered through gauze. The combined filtrates consisted of epithelial tissue, while the material retained in the press comprised mainly stromal tissue. The remainder of the brei that did not pass through the gauze consisted of a mixture of both epithelial and stromal elements and was not used in the experiments. The
histology of each sample was checked by light microscopy. Micrographs of the separated tissue (Pls 1 and 2) provide some indication of the degree of separation achieved.

**Incubation studies**

Adenomatous tissue from patients with benign prostatic hypertrophy was obtained at operation and kept at 4 °C until processed in the laboratory within 30 min of removal.

For whole tissue incubations, the prostatic tissue was finely chopped with a razor blade and added to Krebs–Ringer bicarbonate–glucose medium, pH 7.4, in a tissue buffer ratio of 1:12.5 before addition to the flask.

Homogenates (20%, w/v) of separated stromal and epithelial cells were prepared in 0.25 M sucrose containing 0.12 M nicotinamide, using a Silverson mixer for the stromal tissue, and a Philpot–Stanier homogenizer for epithelial cells. These homogenates were mixed with an equal volume of an incubation medium (Griffiths & Glick, 1966) containing cofactors such that the final concentration of each was as follows: Tris-HCl buffer, pH 7.4, 43.02 mmol/l; KCl, 42.82 mmol/l; MgSO₄, 3.16 mmol/l; potassium fumarate, 8.57 mmol/l; ATP, 0.94 mmol/l; NADP⁺, 0.18 mmol/l; glucose-6-phosphate, 17.60 mmol/l; glucose-6-phosphate dehydrogenase, 0.5 Kornberg unit. These mixtures were then added to the incubation flask containing radioactive precursor.

A solution of the radioactive precursor steroid in ethanol was placed in an incubation flask, together with two drops of propylene glycol. After evaporation of the ethanol in a stream of air, the buffer and tissue, or homogenate mixture were added to the flasks. Incubations were performed in a shaking water bath at 37 °C, in an atmosphere of either 95% air (homogenates) or O₂ (minced tissue): 5% CO₂. Reactions were stopped by addition of acetone (10 ml) containing 500 μg each of the non-radioactive steroids investigated.

**Extraction and fractionation of steroids**

Whole prostatic tissue and the separated stromal and epithelial tissue from the perfusion experiments in vivo were minced before being homogenized using a Silverson mixer in 5 vol. acetone containing 500 μg of each non-radioactive carrier steroid investigated. The acetone mixtures from these experiments, and those from the incubations were filtered, the residue extracted twice more with acetone and again filtered. The pooled acetone extracts were evaporated to dryness under reduced pressure, and the steroids fractionated using methods described by Fahmy, Griffiths, Turnbull & Symington (1968).

The steroids present in the neutral and conjugate fractions were subjected to extensive thin-layer chromatography. Solvent systems used in the separation and determination of the individual steroids were those described previously (Harper, Pierrepoint, Fahmy & Griffiths, 1971). Δ⁴-3-Oxosteroids were measured by their selective absorption of light at 240 nm and the remaining steroids and their derivatives were determined by g.l.c. Radioactivity was measured using a Mark II Nuclear-Chicago liquid scintillation counter.

The specific radioactivities of the carrier steroids and their derivatives were used
to calculate the total radioactivity associated with each steroid investigated. Differences in the specific activities of the carrier steroid and two of its derivatives were always less than 10%.

RESULTS

In-vivo studies

Infusion with $[\text{7a-3H}]$testosterone

Five patients were infused with $[\text{7a-3H}]$testosterone (50 µCi) 30 min before prostatectomy. The radioactive steroids present in the enucleated tissue were determined. The following steroids (500 µg) were added to the acetone mixture: testosterone, androstenedione, 5α-dihydrotestosterone, androsterone, epiandrosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. Table 1 shows that the major metabolite found in the tissue was 5α-dihydrotestosterone. Small amounts of radioactivity were present in androsterone, epiandrosterone, androstenedione and 5α-androstanedione.

<table>
<thead>
<tr>
<th>Steroid investigated</th>
<th>% Total radioactivity associated with the carrier steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testosterone infusion Patient no.</td>
</tr>
<tr>
<td></td>
<td>31</td>
</tr>
<tr>
<td>Testosterone</td>
<td>9:2</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>51:2</td>
</tr>
<tr>
<td>Androsterone</td>
<td>8:7</td>
</tr>
<tr>
<td>5α-Androstenedione</td>
<td>1:5</td>
</tr>
<tr>
<td>Androstanedione</td>
<td>6:8</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>8:9</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>5:7</td>
</tr>
<tr>
<td>DHA</td>
<td>—</td>
</tr>
<tr>
<td>DHA sulphate</td>
<td>—</td>
</tr>
</tbody>
</table>

Dashes indicate steroid was not investigated in these experiments.

Prostatic tissue from another patient was separated into stromal and epithelial elements, which were then analysed separately for radioactive steroids; Table 2 gives the results of this analysis. Again, 5α-dihydrotestosterone represented a large percentage of the radioactivity, 75% in the case of the epithelial tissue and 35% in the stromal tissue. Androsterone and epiandrosterone represented most of the remaining radioactivity in the epithelial tissue. Although 5α-androstanediols were found in the whole tissue analyses, their identification in the separated tissue was difficult because of the extremely low levels of radioactivity.
Infusion with $[7\alpha-^3H]$androstenedione

Four patients were infused with 50 $\mu$Ci $[7\alpha-^3H]$androstenedione 30 min before the removal of the prostatic adenoma. The following non-radioactive carrier steroids were added to the tissue homogenate: testosterone, androstenedione, 5$\alpha$-androstenedione, 5$\alpha$-dihydrotestosterone, androsterone, epiandrosterone, 5$\alpha$-androstane-3$\alpha,17\beta$-diol and 5$\alpha$-androstane-3$\beta,17\beta$-diol. Table 1 shows the percentages of the total metabolites found in the prostatic tissue. The major radioactive metabolites of androstenedione were epiandrosterone and androsterone. Both 5$\alpha$-androstane-3$\alpha,17\beta$-diol and the -3$\beta,17\beta$-diol were present. In all prostatic tissue examined, some of the infused radioactivity was present as testosterone and 5$\alpha$-dihydrotestosterone.

Table 2. Radioactive steroids present in separated stromal and epithelial portions of human prostatic tissue after in-vivo infusion of 50 $\mu$Ci $[7\alpha-^3H]$testosterone

<table>
<thead>
<tr>
<th>Steroid investigated</th>
<th>% Total radioactivity present in the tissue identified in the carrier steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stromal tissue</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0</td>
</tr>
<tr>
<td>5$\alpha$-Dihydrotestosterone</td>
<td>35-71</td>
</tr>
<tr>
<td>Androsterone</td>
<td>15-84</td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>19-35</td>
</tr>
<tr>
<td>5$\alpha$-Androstenedione</td>
<td>0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0</td>
</tr>
<tr>
<td>5$\alpha$-Androstane-3$\alpha,17\beta$-diol</td>
<td>0</td>
</tr>
<tr>
<td>5$\alpha$-Androstane-3$\beta,17\beta$-diol</td>
<td>0</td>
</tr>
</tbody>
</table>

Infusion of $[7\alpha-^3H]$DHA sulphate

Three patients were infused with 50 $\mu$Ci $[7\alpha-^3H]$DHA sulphate before the removal of the prostatic adenoma. The following non-radioactive carrier steroids (500 $\mu$g) were added to the acetone mixture: DHA, DHA sulphate, androstenediol, 5$\alpha$-androstane-3$\alpha,17\beta$-diol, 5$\alpha$-androstane-3$\beta,17\beta$-diol, androstenedione, testosterone, 5$\alpha$-dihydrotestosterone, 5$\alpha$-androstenedione, androsterone and epiandrosterone. Table 1 gives the distribution of radioactivity in these carrier steroids. Most of the radioactivity in the prostate was present as DHA sulphate. Radioactive DHA, androstenedione, testosterone and 5$\alpha$-dihydrotestosterone were also identified in the tissue.

In all the in-vivo infusions approximately 0.1% of the injected radioactive steroid per 30 g prostatic tissue was recovered in the tissue.

In-vitro studies

Homogenates of stromal tissue (1 g) and epithelial tissue (1 g) were incubated with $[7\alpha-^3H]$testosterone (7.52 $\mu$Ci, 33.06 nmol) and $[4-^{14}C]$androstenedione (2.75 $\mu$Ci, 33.29 nmol) for 90 min at 37 °C. At the end of the incubation carrier steroids were added in acetone, separated and isolated as described. Table 3 gives the steroids isolated together with their percentage conversions from the two precursor steroids in both tissues. Stromal tissue metabolized testosterone to 5$\alpha$-dihydrotestosterone
and 5α-androstane-3z,17β-diol but a large proportion of the radioactivity was not accounted for. Most of the [14C]androstenedione was left unmetabolized, although androsterone appeared to be the major metabolite with the formation of 5α-androstenedione. Epithelial tissue also metabolized testosterone to 5α-dihydrotestosterone and 5α-androstane-3z,17β-diol. No androstenedione or 5α-androstenedione was formed from testosterone. Epithelial tissue actively metabolized androstenedione; 40% was converted to 5α-androstenedione. Both androsterone and epiandrosterone were also formed from androstenedione by this tissue but little of the 5α-androstane-diols was formed.

Table 3. Incubation of human prostatic stromal and epithelial cells with 7.52 μCi [7α-3H]-testosterone (33.06 nmol) and 2.75 μCi [4-14C]androstenedione (33.29 nmol) in vitro for 90 min

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>34.75</td>
<td>4.40</td>
<td>31.94</td>
<td>0.40</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0</td>
<td>75.11</td>
<td>0</td>
<td>34.35</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>18.20</td>
<td>1.50</td>
<td>36.60</td>
<td>2.80</td>
</tr>
<tr>
<td>5α-Androstenedione</td>
<td>0</td>
<td>10.90</td>
<td>0</td>
<td>46.70</td>
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<tr>
<td>Androsterone</td>
<td>0</td>
<td>19.80</td>
<td>0</td>
<td>10.40</td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>0.12</td>
<td>1.15</td>
<td>0</td>
<td>5.60</td>
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<tr>
<td>5α-Androstane-3z,17β-diol</td>
<td>14.26</td>
<td>0.52</td>
<td>15.96</td>
<td>0.63</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>1.74</td>
<td>0.00</td>
<td>3.86</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Table 4. Incubation of human benign hypertrophic prostate tissue in vitro with either [7α-3H]dehydroepiandrosterone sulphate or [7α-3H]dehydroepiandrosterone (DHA)

<table>
<thead>
<tr>
<th>Steroid investigated</th>
<th>Incubation with [7α-3H] DHA sulphate</th>
<th>Incubation with [7α-3H] DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHA sulphate</td>
<td>42.50</td>
<td>37.51</td>
</tr>
<tr>
<td>DHA</td>
<td>31.60</td>
<td>19.88</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.91</td>
<td>0.32</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0</td>
<td>0.63</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>98.96</td>
<td>11.13</td>
</tr>
<tr>
<td>5α-Androstenedione</td>
<td>0</td>
<td>0.63</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>2.15</td>
<td>5.08</td>
</tr>
<tr>
<td>Androsterone</td>
<td>3.50</td>
<td>11.13</td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>0.88</td>
<td>1.60</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>7.41</td>
<td>18.95</td>
</tr>
<tr>
<td>5α-Androstane-3z,17β-diol</td>
<td>1.06</td>
<td>8.21</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>3.51</td>
<td>13.90</td>
</tr>
<tr>
<td>5α-Androstane-3z,17α-diol</td>
<td>0</td>
<td>0.63</td>
</tr>
<tr>
<td>5α-Androstane-3β,17α-diol</td>
<td>0</td>
<td>0.20</td>
</tr>
</tbody>
</table>

[7α-3H]DHA sulphate and [7α-3H]DHA incubation with minced tissue

Minced human benign prostatic tissue (2 g) was incubated separately with [7α-3H]DHA sulphate (11.39 μCi, sp. act. 290 mCi/mmol) and [7α-3H]DHA (14.12 μCi, sp. act. 241 mCi/mmol). Non-radioactive carrier steroids (500 μg) were added in acetone to the incubations. The percentage of total radioactivity incubated, found in
the individual carrier steroids can be seen in Table 4. Dehydroepiandrosterone sulphate was metabolized by the prostatic tissue to DHA (31%) and androstenediol (7%). Small amounts of radioactive 5α-dihydrotestosterone, androsterone and 5α-androstan-3β,17β-diol were also formed. Dehydroepiandrosterone was actively metabolized to androstenediol, androsterone, 5α-dihydrotestosterone, 5α-androstan-3α,17β-diol and 5α-androstan-3β,17β-diol.

**DISCUSSION**

Many studies have intimated that the adrenal gland may exert some influence on the normal function of prostatic tissue although the adrenal secretions concerned with this response have not yet been established with certainty. Tullner (1963) found that administration of adrenocorticotropic hormone affected the ventral prostate of immature castrated rats, but there was no response when the animals had been adrenalectomized. Experiments of Berswordt-Wallrabe and his colleagues (Berswordt-Wallrabe, Bielitz, Elger & Steinbeck, 1970) showed that progesterone administration stimulated the growth of the ventral prostate of castrated rats and prevented involution of the gland in castrated, hypophysectomized, adrenalectomized animals. Lostroh & Li (1957), however, showed that corticosteroids were without effect on the prostate of the adult hypophysectomized, castrated rat.

Dehydroepiandrosterone sulphate is a major secretory product of the human adrenal cortex, and is present in large quantities in plasma. There is the possibility, therefore, that if it was taken up by the prostate and metabolized to more active androgenic steroids, DHA sulphate might play some role in the control of prostate function, particularly in castrated or oestrogenized men with prostatic cancer. Results presented in this paper indicated that human benign prostatic tissue could metabolize DHA sulphate and DHA in vitro to 5α-androstenediols and 5α-androstanolones and also that radioactive DHA sulphate infused into patients undergoing prostatectomy was taken up by the benign hypertrophic tissue. The relatively small proportion of the infused DHA sulphate present in the prostatic tissue in the form of active 5α-dihydrotestosterone may well be significant considering the large concentration of DHA sulphate in the blood.

Androstenedione, a C₁₉-steroid partly of adrenal origin, is another plasma steroid which may under certain conditions be concerned in eliciting an androgenic response in the prostate. Acevedo & Goldzieher (1965) showed that human pathological prostatic tissue could metabolize androstenedione in vitro to a variety of compounds including testosterone, androsterone, and 5α-androstenedione but not 5α-dihydrotestosterone. The results for the infusion of [3H]androstenedione in vivo indicated that radioactivity was taken up by human prostatic tissue and was associated with the biologically active 5α-reduced metabolites, although not to the extent observed after testosterone infusion. The pattern of radioactive metabolites found in the prostate tissue after infusion of [3H]testosterone agrees well with that previously reported from this laboratory (Pike, Peeling, Harper, Pierrepont & Griffiths, 1970). In contrast to the infusion with androstenedione and DHA sulphate however, a large proportion of the radioactivity was associated with 5α-dihydrotestosterone and small amounts with the 5α-androstenediols. Although the results indicate that human
prostatic tissue possesses the ability to take up and metabolize these adrenal steroids, it cannot be assumed that the radioactive metabolites identified in the prostatic tissue after infusion of the radioactive precursors were all formed there. Systemic interconversion of the steroids is quite possible. It is still apparent however that active 5α-androstane compounds originating from androstenedione and DHA sulphate are present in the prostate which might account for the adrenal influence on this tissue. Conversion to 5α-dihydrotestosterone is small especially when compared with the amounts formed from testosterone but might become more significant in circumstances where plasma testosterone levels are lowered, as in diethylstilboestrol treatment or castration.

Incubation of separated epithelial and stromal tissue showed that both types of prostatic tissue metabolized testosterone and androstenedione, although differences in their metabolic activity were observed. Epithelial tissue actively metabolized androstenedione, and the conversion of testosterone to 5α-dihydrotestosterone was greater than in the fibro-muscular tissue. This is in accord with the results of Hansson and colleagues (Hansson, Tveter, Attramadal & Torgersen, 1971) who found a pronounced uptake of [3H]testosterone into benign nodular prostatic tissue with a significantly lower accumulation in the muscular tissue. Becker, Kaufmann, Klosterhalfen & Voigt (1972) also noted a difference in the uptake and metabolism of testosterone in adenomatous benign hypertrophy specimens compared with samples composed mainly of fibrous tissue. Care should be taken, however, in interpretation of these incubation studies with stromal and epithelial elements since the conditions of incubation were not comparable, the stromal tissue being extremely difficult to homogenize. The results are however of interest when compared with the steroids isolated from stromal and epithelial tissue removed from a patient after [3H]testosterone infusion before the operation.

The authors wish to record their appreciation of the generous financial support from the Tenovus Organization. They are also indebted to Dr Audrey Peeling for her valuable assistance and co-operation throughout the programme.

REFERENCES


C19 steroids in human prostate gland


DESCRIPTION OF PLATES

PLATE 1

Fig. 1. Human prostatic tissue showing stromal and epithelial elements. Magnification ×278: stained with haematoxylin and eosin.

Fig. 2. Separated epithelial cell fraction showing epithelial cells maintaining some associations as seen in whole tissue lining the acini. Magnification ×111: stained with haematoxylin and eosin.

PLATE 2

Fig. 3. Separated epithelial cells at higher magnification ×695: stained with haematoxylin and eosin.

Fig. 4. Separated stromal tissue, magnification ×278: stained with haematoxylin and eosin.
Studies on the Steroid Metabolism of Normal and Pathological Prostatic Tissue in Organ Culture

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Tenovus Institute for Cancer Research, Welsh National School of Medicine, The Heath, Cardiff.

SUMMARY

The steroid metabolism in organ cultures of human benign hyperplastic prostatic tissue and normal rat and canine prostatic tissue was studied. In the presence of oestradiol-17β or either of the synthetic oestrogens diethylstilboestrol and meso-dihydrodibutylstilboestrol a decrease in the conversion of testosterone to the 'active androgen' 5α-dihydrotestosterone in the explants was observed. Prolactin added to the cultures also produced changes in the testosterone metabolism of the tissue. The relative importance of these direct actions of oestrogens and prolactin in vivo has still to be established. Diethylstilboestrol administration to patients with prostatic cancer not only results in decreased plasma testosterone concentrations which obviously inhibits prostatic growth, but also an increase in plasma prolactin levels which may also modulate the response of the prostatic tissue to the treatment.

INTRODUCTION

The androgen-dependent nature of human prostatic cancer and its treatment with the synthetic oestrogen diethylstilboestrol (DES) has been established since the early 1940's as a result of the pioneering studies of Huggins and his colleagues (Huggins and Hodges, 1941; Huggins, Scott and Hodges, 1941). It is not completely understood however, by what processes oestrogens produce their effects on prostatic growth. Oestrogens can influence the prostate indirectly by decreasing testosterone secretion via their action on the pituitary and testes but a direct action on the prostatic tissue is also probable. Knowledge of the mechanism by which oestrogens act on the prostate is essential for the design of alternative compounds for clinical use, which while being as effective against the disease would have less of the unwanted side effects seen with DES.

Tissue culture studies provide us with the means of investigating the action of a compound on a specific tissue without the added complication of interference from the general metabolism, organ culture presents a further advantage of maintaining the anatomical relationships of the different tissues of the gland. This technique was therefore
used in our studies to investigate the direct effects of oestradiol-17β and the stilboestrol analogues diethylstilboestrol (DES) and meso-dihydrodibutylstilboestrol (DHBS) on prostatic tissue metabolism.

Studies during recent years have shown that the characteristic metabolism of testosterone by prostatic tissue is essential for hormone action and that one of the metabolites, 5α-dihydrotestosterone is the 'active' hormone at the cellular level (Bruchovsky and Wilson 1968a, b; Liao and Fang 1969). We decided to study the uptake of testosterone into the explants and its conversion to 5α-dihydrotestosterone in the presence of the various oestrogens. Oestrogens are known to increase prolactin levels in vivo which could also affect prostatic biochemistry therefore the action of this protein hormone on testosterone metabolism in tissue cultures was also investigated.

METHODS

Essentially the procedure employed was a modification of the Trowell technique (1954; 1959). The prostatic tissue was removed from dogs and rats under aseptic conditions and from patients undergoing open prostatectomy and transported on ice in sterile containers to the laboratory. All further manipulations were performed under sterile conditions using a 'Microflow' tissue cabinet. Tissues were dissected free from surrounding fat and capsular tissue and any areas of necroses were removed. The tissue, moistened with buffered saline, was cut into explants approximately 1 mm cubed and cultured in plastic dishes. Two types of media were employed which gave identical results:

(a) Eagle’s minimum essential medium (MEM) fortified with 10% calf serum, 200 units/ml penicillin, 100 μgs/ml streptomycin and 2 mM glutamine.

(b) 199 medium supplemented in the same way.

Radioactive steroids in ethanol were dissolved in the medium prior to its addition to the culture dishes. Compounds under investigation were either dissolved directly in the medium, if water soluble, with subsequent sterilization by passage through a millipore filter, or added in ethanol (5 μl) directly to the medium—in the dishes. The explants (6 to 12 per dish) were placed on lens paper rafts supported by stainless steel grids situated at the surface of the medium (1 ml) contained in the well of the dish. The surrounding trough contained moist filter paper. The culture dishes were placed in sealed plastic boxes in an atmosphere of 95% air : 5% CO₂ and transferred to an incubator at 37°C. At the end of the incubation period (24 or 48 h), explants from identical cultures were pooled, washed twice with phosphate buffered saline (137 mM NaCl, 2.68 mM KCl, 8.09 mM Na₂HPO₄, 1.47 mM KH₂PO₄), blotted dry with filter paper and quickly weighed. For the analysis of the steroid metabolites in the cultures, explants were homogenized in phosphate buffered saline (1 ml) using a Griffiths ground glass homogenizer. Carrier steroids (300 μgs or 500 μgs in
acetone, 5 ml) were added to the homogenate. Steroids were extracted by mixing with acetone (25 ml), centrifuging the precipitated proteins and removing the acetone layer. The pellet was further extracted with acetone, and the acetone extracts pooled. Fractionation, separation and determination of the specific radioactivities of the individual steroids were carried out by procedures described by Harper, Pierrepoint, Fahmy and Griffiths (1971).

In experiments in which the radioactive steroid content of the nuclear fraction of the prostatic explants was also analysed, more cultures were pooled and homogenized in 0.25 M sucrose containing 3 mM MgCl₂ and fractionated by sucrose density centrifugation (Harper et al., 1971). The final nuclear preparation was resuspended in 0.25 M sucrose, homogenized, and a known fraction removed for DNA determinations which was used to estimate nuclear recovery. Carrier steroids were added to the remainder of the homogenized nuclear preparation and acetone extracted. Specific radioactivities of the individual steroids were determined by identical procedures used for the whole tissue homogenates.

Results

(a) Effects of Oestrogens

Rat prostatic explants were cultured for 24 h with [7α-³H]testosterone (10 μCi/dish; specific radioactivity 5.3 Ci/mmol) and one of the following compounds at a final concentration of 1.7 × 10⁻⁵ M, oestradiol-17β, DES and meso-DHBS in ethanol (5 μl). Control cultures contained 5 μl ethanol. The specific radioactivities of both testosterone and 5α-dihydrotestosterone determined after purification and derivative formation are shown in Table 1. It can be seen that the conversion of testosterone to 5α-dihydrotestosterone was decreased in the presence of all three compounds under investigation, both in the whole tissue and in the nuclear fraction.

Canine prostatic explants were cultured for 24 h with [7α-³H]testosterone (10 μCi/dish; specific radioactivity 5.3 Ci/mmol) with either oestradiol-17β, DES or meso-DHBS at a final concentration of 1.7 × 10⁻⁵ M. The specific radioactivities of both testosterone and 5α-dihydrotestosterone for both the whole tissue and the nuclear fraction is shown in Table 2. As with the rat prostatic tissue the conversion of testosterone to 5α-dihydrotestosterone is decreased in the presence of oestradiol and the stilboestrol analogues even though the 5α-reductase activity appears to be much lower in this species.

Similarly when human benign prostatic tissue was cultured under identical conditions the oestrogens produced a reduction in the conversion of testosterone to 5α-dihydrotestosterone which is indicated by the ratio’s of testosterone and 5α-dihydrotestosterone seen in Table 3.

The results indicate that the testosterone metabolism of human, dog and rat prostatic tissue can be directly affected by oestradiol and the stilboestrol analogues, the meso-DHBS being equally as effective as DES.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Oestradiol-17β</th>
<th>DES</th>
<th>(meso) DHBS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole tissue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>2601.6</td>
<td>2772.6</td>
<td>2754.6</td>
<td>2640</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>17481</td>
<td>11072</td>
<td>12592</td>
<td>12542</td>
</tr>
<tr>
<td>Ratio: T/5αT</td>
<td>0.148</td>
<td>0.250</td>
<td>0.218</td>
<td>0.210</td>
</tr>
<tr>
<td><strong>Nuclear fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>148.6</td>
<td>72.1</td>
<td>62.6</td>
<td>62.3</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>1067.5</td>
<td>301.4</td>
<td>345.0</td>
<td>372.2</td>
</tr>
<tr>
<td>Ratio: T/5αT</td>
<td>0.139</td>
<td>0.239</td>
<td>0.181</td>
<td>0.167</td>
</tr>
</tbody>
</table>

Corrected for nuclear recovery, standard tissue weight and total fraction content. Nuclear recovery:- control cultures 27.2%, oestradiol-17β cultures 23.7%, diethylstilboestrol cultures 28.8%, dihydrodibutylstilboestrol cultures 22.2%. 
### TABLE 2
Mean specific radioactivities of testosterone and 5α-dihydrotestosterone after culturing [7α-3H]testosterone with canine prostatic tissue in the presence of various oestrogens

<table>
<thead>
<tr>
<th></th>
<th>Mean specific radioactivity (dpm/nmol/25 mg tissue)</th>
<th>(m esc o)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>E₂-17β</td>
</tr>
<tr>
<td>Whole tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>1738.7</td>
<td>1874.7</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>1250.5</td>
<td>695.2</td>
</tr>
<tr>
<td>Ratio: T/5αT</td>
<td>1.39</td>
<td>2.69</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>22.52</td>
<td>41.27</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>34.51</td>
<td>42.12</td>
</tr>
<tr>
<td>Ratio: T/5αT</td>
<td>0.65</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Corrected for nuclear recovery, standard tissue weight and total fraction content. Nuclear recovery: control cultures 22.5%, oestradiol-17β cultures 25.3%, diethylstilboestrol cultures 27.7%, dihydrodibutylstilboestrol cultures 27.0%.

### TABLE 3
Mean specific radioactivity of testosterone (T), and 5α-dihydrotestosterone (5αT) isolated from human prostatic explants cultured in the presence of oestrogens with [7α-3H]testosterone

<table>
<thead>
<tr>
<th>Tissue protein (mg)</th>
<th>Specific radioactivity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T dpm/nmol</td>
<td>5αT dpm/nmol</td>
</tr>
<tr>
<td>Control</td>
<td>1.406</td>
<td>3090</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>1.813</td>
<td>3970</td>
</tr>
<tr>
<td>DES</td>
<td>1.850</td>
<td>3823</td>
</tr>
<tr>
<td>m esc o-DHBS</td>
<td>1.591</td>
<td>3533</td>
</tr>
</tbody>
</table>
(b) Effects of prolactin

Rat ventral prostate explants were cultured in medium 199 for 24 h with [7α-3H] testosterone (10 μCi/dish; specific radioactivity 5.3 Ci/mmol) in the presence of varying concentrations of ovine prolactin. Carrier steroids were added and purified as described in the methods and the specific radioactivities determined. The results shown in Table 4 indicate that only when a large concentration (5 IU) was present in the culture medium was testosterone uptake increased and a change in 5α-reductase activity observed.

TABLE 4

<table>
<thead>
<tr>
<th>Prolactin added (I.U./ml)</th>
<th>Tissue protein (mg)</th>
<th>Specific radioactivity dpm/nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T</td>
</tr>
<tr>
<td>0</td>
<td>1.850</td>
<td>35.06</td>
</tr>
<tr>
<td>0.001</td>
<td>2.017</td>
<td>30.92</td>
</tr>
<tr>
<td>0.01</td>
<td>2.165</td>
<td>43.24</td>
</tr>
<tr>
<td>0.1</td>
<td>1.906</td>
<td>22.61</td>
</tr>
<tr>
<td>1.0</td>
<td>2.368</td>
<td>44.30</td>
</tr>
<tr>
<td>5.0</td>
<td>1.739</td>
<td>129.4</td>
</tr>
</tbody>
</table>

Table 5 shows the results of culturing the ventral, dorsal and lateral lobes of rat prostate with [7α-3H] testosterone in the presence and absence of ovine prolactin (5 IU) under identical conditions. It can be seen that a slight increase in the uptake of radioactivity into the tissue occurs in the presence of prolactin. There was also a reduction in the 5α-reductase activity of the prostatic explants of prolactin containing cultures.

Explants of human benign prostatic tissue when cultured with prolactin showed slight changes in both the conversion of testosterone to 5α-dihydrotestosterone and uptake of radioactivity (Table 6).

DISCUSSION

These investigations show a direct action of oestradiol-17β and the stilboestrol compounds on the testosterone metabolism of prostatic tissue in vitro. Of interest is the decrease in 5α-dihydrotestosterone
TABLE 5

Uptake of radioactivity and mean specific radioactivity of testosterone (T) and 5α-dihydrotestosterone (5αT) isolated from rat prostatic explants cultures with [7α-3H]testosterone in the presence of ovine prolactin (5 IU)

<table>
<thead>
<tr>
<th></th>
<th>Tissue protein (mg)</th>
<th>Radioactivity present in tissue</th>
<th>Specific radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>dpm × 10^{-5}/mg protein</td>
<td>dpm/nmol/mg protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T</td>
</tr>
<tr>
<td>Ventral prostate</td>
<td></td>
<td></td>
<td>402.5</td>
</tr>
<tr>
<td>Control</td>
<td>0.981</td>
<td>1.8</td>
<td>675.8</td>
</tr>
<tr>
<td>Prolactin</td>
<td>0.888</td>
<td>2.7</td>
<td>560.1</td>
</tr>
<tr>
<td>Lateral prostate</td>
<td></td>
<td></td>
<td>689.5</td>
</tr>
<tr>
<td>Control</td>
<td>1.388</td>
<td>1.9</td>
<td>63.17</td>
</tr>
<tr>
<td>Prolactin</td>
<td>1.517</td>
<td>2.0</td>
<td>123.4</td>
</tr>
<tr>
<td>Dorsal prostate</td>
<td></td>
<td></td>
<td>1.073</td>
</tr>
<tr>
<td>Control</td>
<td>0.925</td>
<td>1.4</td>
<td>123.4</td>
</tr>
</tbody>
</table>
TABLE 6

Mean specific radioactivity of steroids isolated from human prostatic explants cultured with either \([7\alpha-^3H]\) testosterone or \([1, 2-^3H]\) androstenedione in the presence of ovine prolactin (5 IU).

<table>
<thead>
<tr>
<th>Precursor steroids</th>
<th>Compound added</th>
<th>Tissue protein (mg)</th>
<th>T</th>
<th>A</th>
<th>5αT</th>
<th>5αA</th>
<th>An</th>
<th>Total 5α-red. steroid/ precursor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>Control</td>
<td>1.730</td>
<td>203.1†</td>
<td>12.42</td>
<td>163.6</td>
<td>107.4</td>
<td>69.08</td>
<td>340.1</td>
</tr>
<tr>
<td></td>
<td>Prolactin</td>
<td>1.673</td>
<td>265.4†</td>
<td>6.48</td>
<td>111.1</td>
<td>45.56</td>
<td>30.19</td>
<td>186.9</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>Control</td>
<td>1.448</td>
<td></td>
<td>84.88†</td>
<td>47.02</td>
<td>428.7</td>
<td>238.7</td>
<td>714.4</td>
</tr>
<tr>
<td></td>
<td>Prolactin</td>
<td>1.861</td>
<td></td>
<td>99.46†</td>
<td>41.21</td>
<td>299.5</td>
<td>165.1</td>
<td>505.8</td>
</tr>
</tbody>
</table>

† precursor steroids — not investigated

\(T = \text{testosterone}\)  \(A = \text{androstenedione}\)  \(5\alpha T = \text{5\(\alpha\)-dihydrotestosterone}\)

\(An = \text{androsterone}\)  \(5\alpha A = 5\alpha\)-androstan-3,17-dione
Steroids of Cultured Prostate

in the whole tissue and perhaps more significantly in the nuclear fraction, the cellular location in which it is assumed to have its hormonal action by affecting nuclear RNA and DNA synthesis. The results with meso-DHBS were also encouraging as this compound was equally effective as DES in reducing the 5α-reductase activity in the cultures. Several investigations in our laboratories have indicated that it will effect other prostatic enzyme systems similarly to DES (Harper, Fahmy, Pierrepoint and Griffiths 1970; Davies, Fahmy, Pierrepoint and Griffiths 1972). As it possesses only 1/2 of the oestrogenic activity of DES it may prove an alternative compound for use in the treatment of prostatic cancer.

As oestrogens are known to increase prolactin levels in vivo (Frantz; 1973) it is important to consider the direct and indirect actions of this protein hormone on prostatic tissue. Evidence has accumulated which suggest that prolactin effects prostatic biochemistry. Changes in zinc uptake and accumulation in the prostate with prolactin have been observed (Gunn, Gould and Anderson 1965), and changes in prostatic adenyl cyclase activity (Golder, Boyns, Harper and Griffiths 1972). Alterations in vivo of the prostatic tissue growth are dependent on changes in androgen synthesis. Evidence for a synergistic action of prolactin with LH on testicular synthesis of androgens is suggested by the work of Bartke (Hafiez, Lloyd and Bartke 1972). Furthermore work in our laboratories suggests that prolactin can affect the synthesis of androgens by the adrenal (Boyns et al. 1972). The results of culturing prostatic explants with prolactin indicate that this hormone might influence the uptake of testosterone into the tissue in agreement with the findings of Farnsworth (1972).

Patients who initially respond to DES treatment often relapse after several months. It is possible that this phenomena is related to increased prolactin opposing the main DES action. Therefore it is necessary to consider if joint therapy with DES and a prolactin inhibitor such as CB154 would be more successful.

REFERENCES


Thin-Layer Chromatography and High Resolution Selected Ion Monitoring for the Analysis of C_{19} Steroids in Human Hyperplastic Prostate Tissue

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Abstract—Interfering nonpolar lipid material was removed from the acetone extracts of several samples of benign hyperplastic prostate tissue. Endogenous steroids were separated by preparative thin-layer chromatography into fractions containing testosterone, 5a-dihydrotestosterone and the 5a-androstan-3-17-diol, and concentrations of these steroids were measured by molecular ion monitoring of suitable derivatives at a resolution of 8500 to 10,000 during combined gas chromatography mass spectrometry. The sensitivity and specificity of this procedure allowed unequivocal detection of the steroids of interest at the femtomole level and the incorporation of epimers as internal standards enabled accurate quantitative measurements. The results indicated that 5a-dihydrotestosterone is usually the most predominant of the steroids measured, and that of the 5a-androstanediols, only the 3a, 17β and 3β, 17β isomers occur in measurable concentration, with the former usually predominant.

Introduction

The prostate gland depends on an adequate plasma level of testosterone for its maintenance and growth. However, in recent years, evidence has accumulated to indicate that 5z-dihydrotestosterone (17β-hydroxy-5z-androst-3-one), a metabolite of testosterone in prostatic tissue, is the active androgen in accessory sex organs.1-4 Studies both in vitro5-7 and in vivo8 have clearly shown that extensive metabolism of testosterone occurs in prostatic tissue, forming not only 5z-dihydrotestosterone (5z-DHT) but also the various isomeric 5a-androstanediols (Fig. 1). Studies on cultured rat prostate tissue9 have also suggested that these diols may have a biological role in the cell and it would seem reasonable to consider the thesis5 that some imbalance in the metabolism of testosterone in the ageing prostate of man may be responsible for the development of benign hyperplasia, a common condition of the elderly.

Therefore, it is of interest to compare endogenous concentrations of C_{19} steroids in both normal and pathological prostatic tissue. An earlier study on this topic10 indicated that 5z-DHT levels are elevated in benign hyperplastic prostates compared with normal tissue. Here a new method for measurement of endogenous testosterone, 5z-DHT and the 5a-androstanediols in prostatic tissue is reported for the first time. The procedure was based on high resolution selected ion detection (s.i.d.) with epimers as internal standards.

Materials

All gas chromatographic columns and the reagent bis(N,O)trimethylsilyl acetamide (BSA) were provided by Jones Chromatography Ltd, Llanbradach, Glam., UK. Hydroxylamine hydrochloride and methoxylamine hydrochloride from Eastman Kodak Ltd were stored in pyridine solution (20 mg/ml) at −20 °C. Epitestosterone (17α-hydroxy-androst-4-en-3-one) and 5α-dihydroepitestosterone (17α-hydroxy-5α-androstan-3-one) were obtained from Steraloids, Inc., USA, while radiochemically pure tritiated standards were supplied by the Radiochemical Centre, Amersham, Bucks., UK. Precoated silica gel plates (Polygram Sil G/UV 254) were obtained from Macherey, Nagel and Co., Duren, Germany. All solvents were a redistilled analytical grade.

EXTRACTION OF THE TISSUE

Prostatic tissue stored at 4 °C was dissected free of capsule, minced, weighed and homogenized in acetone (20 vols) using a Silverson mixer. The internal standards, epitestosterone (20 ng), 5α-androstane-3β, 17β-diol (20 ng) and 5α-dihydroepitestosterone (20 ng), were normally added at this stage. After centrifugation, the supernatant was decanted and the residue extracted twice more with 10 ml acetone.

The combined supernatants were evaporated to dryness in vacuo and the interfering nonpolar lipid material removed by a procedure previously described.11
THIN-LAYER CHROMATOGRAPHY

The residue in ethanol was applied as a thin band on a prewashed silica gel coated t.l.c. plate which was then developed in chloroform + acetone (185:10 v/v). High specific activity tritiated steroids (testosterone, 5α-DHT and 5α-androstane-3β, 17β-diol) were used as chromatography markers. Corresponding areas were removed and eluted with acetone + methanol (1:1, 10 ml) using a Zander all glass elution apparatus. The eluates were evaporated to dryness in a stream of dry nitrogen and the residues stored in ethanol (0.5 ml) at −20°C prior to analysis.

DERIVATIVE FORMATION

Fractions from the t.l.c. separations were transferred to glass tubes (30 mm × 4 mm) each having a ground glass neck. The samples were blown to dryness and desiccated in vacuo over a phosphorus pentoxide for 1 h; then bis(trimethylsilyl)acetamide (BSA; 15 µl) was added to the residues containing 5α-androstanediols.

Those containing testosterone and 5α-dihydrotestosterone were treated, respectively, with 30 µl of a pyridine solution of hydroxylamine hydrochloride (20 mg/ml) and 30 µl of methoxylamine hydrochloride (20 mg/ml) in pyridine. The tubes were stoppered, the top part of each stopper being lightly greased to prevent ingress of moisture, and left overnight at 20°C. The tubes containing the oxime reagents were dried down and BSA (15 µl) added to each. After 12 h at 20°C, analyses were carried out on 1.5 µl aliquots within 48 h of complete derivatization, although the solutions remained stable for several weeks in the absence of moisture.

HIGH RESOLUTION MOLECULAR ION MONITORING

Full details of the procedure for selected ion monitoring at high resolution have been described previously.12–14

A Varian 2700 gas chromatograph fitted with a 2 m × 2 mm i.d. helical glass column containing 3% OV-17 on Gaschrom Q (100 to 120 mesh) was inter-
faced to the Varian MAT 731 mass spectrometer by a two stage Watson-Biemann type separator followed by a glass lined probe. The ion source temperature was 150 °C, while the g.c. injector, separator and probe temperatures were 275, 250 and 245 °C, respectively. The electron beam energy was 100 eV with the resolving power at 8500 to 10 000 (10% valley) at the mass to be monitored, which was located with the aid of the reference compound PFK. Approximately 1.5 µl of each sample solution was injected for each measurement and the output of the mass spectrometric detector was displayed on a potentiometric recorder. The respective peak heights of the desired compound and its internal standard were determined and compared with a standard curve for quantitation.

Results and Discussion
In principle, single ion monitoring at a resolution of around 10 000 during g.c.m.s. is usually sufficient for unequivocal detection of a particular g.c. component, regardless of the complexity of the mixture containing it and provided that the gas chromatographic column is capable of resolving isomers. The technique has been successfully employed in our laboratory to measure endogenous steroid concentrations in crude acetone extracts of tumour tissue from human mammary13,14 and adrenal15 glands. Unfortunately, crude acetone extracts of hyperplastic prostate tissue yielded residues of at least 20 mg/g wet weight of tissue. Since the concentration of any C19 steroid was not expected to exceed 10 ng/g wet weight,10 a high degree of purification was required in order to enhance the concentration of C19 steroids prior to g.c.m.s.

The extraction and purification procedures used gave steroid-enriched fractions which were easily taken up in small reagent volumes (15 µl) prior to analysis. In addition, these procedures enabled the judicious choice of reagents added to each fraction to provide the optimum assay conditions for the steroids of interest, using the same gas chromatographic column throughout (vide infra).

MEASUREMENT OF THE 5α-ANDROSTANEDIOLS
Satisfactory derivatives for g.c.m.s. analysis of the four isomeric 5α-androstane-3α,17β-diol were the bis-(TMS) ethers, since these are fully resolved on gas chromatographic columns containing OV-1716 and exhibit intense molecular ions peaks in their mass spectra. High resolution molecular ion detection therefore provided the basis for a specific and sensitive assay for the diols, as indicated by the mass fragmentogram of a standard mixture reproduced in Fig. 2(a). After recovery from t.l.c. plates, the ratios of peak heights recorded for the diols present in the standard mixture were reproducible to within an accuracy of ±5%, and 20 pg (70 fmol) of 5α-androstane-3β,17β-diol was detectable with a signal-to-noise ratio of better than 3:1 under the usual operating conditions.

Examination of the diol fractions from eight specimens revealed only 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol in measurable concentration, and 5α-androstane-3β,17α-diol was therefore selected as an internal standard for future measurements. (The revised procedure for analysis of diols in prostatic tissue is summarized in Fig. 3). This involved addition of 2 ng of the internal standard prior to purification of the tissue extracts, then comparing ratios of the high resolution s.i.d. signals from the 3α,17β and 3β,17β-diols to that of the internal standard with calibration graphs, such as that shown in Fig. 4 for 5α-androstane-3α,17β-diol. A typical example of a mass fragmentogram obtained from these experiments is reproduced in Fig. 2(b). The results of the diol fraction analysis of prostatic tissue samples are given in Table 1. Where the internal standard was not added, the values were calculated using an external standard calibration procedure9 and the recoveries estimated from a parallel extraction, containing radioactively labeled diol, of

FIG. 2. (a) Separation and detection of the isomeric 5α-androstane-3α,17β-diol (0.2 ng each approx.) as their TMS derivatives, by g.c. high resolution s.i.d.; (b) Detection of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol in a typical extract from benign hyperplastic prostatic tissue to which 2 ng of internal standard (5α-androstane-3β,17α-diol) was added before extraction; (c) Detection of 5α-DHT and 5α-epi-DHT (internal standard) as methyloxime-TMS derivatives in a prostatic tissue sample by high resolution s.i.d.; (d) Detection of testosterone and epitestosterone as oxime-TMS derivatives in a prostatic tissue sample by high resolution s.i.d.
molecular ion intensity [Fig. 5(a)] and a single gas chromatographic peak, enabling detection in concentrations as low as 20 pg/μl. Analysis of the fractions containing 5α-DHT from several specimens of tissue did not show appreciable concentrations of androsterone, epiandrosterone or 5α-dihydroepiandrosterone, and since the latter also gives a single gas chromatographic peak after formation of the methoxyline-TMS ether, which is fully resolved from that of 5α-DHT and has an almost identical mass spectrum [Fig. 5(b)], it was selected as an internal standard for further assays of 5α-DHT. The procedure employed follows that described in the foregoing section on 5α-androstenediols and one of the original traces obtained is reproduced in Fig. 2(c). Calculation of the 5α-dihydroepiandrosterone concentration is then straightforward with the aid of a calibration graph. The results of the analysis of five different samples, shown in Table 1, indicate an average value of 12.7 ± 7.1 ng/g which compares with the value quoted in an earlier investigation10 of 6.0 ± 1.0 ng/g, (n = 10).

**TESTOSTERONE**

Neither the TMS ether nor the methoxyline-TMS ether derivatives of testosterone were entirely satisfactory for subnanogram level analysis of testosterone: the former because of low molecular ion intensity15 and gas chromatographic column absorption; the latter owing to partial separation of the isomeric pair of 3-methoxyines on OV-17. The oxime-bis(TMS) ether displayed good characteristics, however, giving rise to only one gas chromatographic peak and a high molecular ion intensity [Fig. 6(a)]. This allowed detection at the same level as the other steroids mentioned above. Analysis of the fractions containing testosterone from several specimens revealed only testosterone and lesser amounts of the isomeric dehydroepiandrosterone, which is largely separated out by t.l.c. Therefore, epitestosterone, which gives an almost identical mass spectrum [Fig. 6(b)] and yet is

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**Fig. 3.** Summary of the procedures for extraction, purification and assay of C19 steroids in human benign hyperplastic prostate tissue.

**Small aliquots of the tissue extracts. The results indicate mean values (±standard error of mean) of 1.06 ± 0.23 ng/g (n = 8) and 0.53 ± 0.12 ng/g (n = 7) for 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol, respectively, after discounting the highest and lowest values.

**5α-DIHYDROTESTOSTERONE**

The methoxyline-TMS ether derivative of 5α-dihydrotestosterone displays excellent characteristics for high resolution s.i.d. In particular, it has a high

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**Fig. 4.** Calibration graph for 5α-androstane-3α,17β-diol against 5α-androstane-3β,17α-diol (2 ng) as internal standard (minimum concentration 200 pg in total sample).
Fig. 5. Comparison of the mass spectra of (a) 5a-DHT and (b) 5a-epi-DHT as their methylloxime-TMS derivatives obtained during g.c.m.s.

Fig. 6. Comparison of the mass spectra of (a) testosterone and (b) epitestosterone as their oxime-bis(TMS) derivatives obtained during g.c.m.s.
fully resolved from testosterone on g.c., was selected as the internal standard for further accurate analyses. Testosterone was measured in seven specimens of benign prostatic hyperplasia (Table 1) and the mean value of 1.7 ± 0.6 ng/g compared with the value of 0.9 ± 0.2 ng/g (n = 10) obtained in an earlier study.10

In conclusion, the combined techniques of preparative t.l.c. and high resolution s.i.d. allow specific detection and quantitation of a wide range of C19 steroids in human prostatic tissue. These procedures will be extended to determine the endogenous concentrations of other C19 and C18 steroids and their conjugates in normal and malignant prostatic tissue in addition to the benign hyperplastic prostate now described.

Since high resolution s.i.d. does not appear to have been routinely employed in other laboratories, some general comments should be made.

First, it is clear that high resolution s.i.d. is the most specific method available for detection of trace components in biological samples and only one characteristic ion per compound need be monitored; preferably the molecular ion, since this is the most characteristic and is not likely to be confused with fragment ions from other compounds. Second, very high sensitivity can be achieved despite narrowing of the ion beam required for high resolution owing to enhanced signal-to-noise ratio, the baseline being generally devoid of signal. In some double focusing mass spectrometers, the drop in overall sensitivity with increase in resolution may be too severe to permit satisfactory operation at a resolution of 8500 to 10 000, but in many cases a lower resolution will still permit the required specificity. Third, less extensive purification of the sample is required if high resolution s.i.d. is to be applied, the main criterion being the total amount of residue from the sample in relation to the final volume of the sample solution required for analysis, which one would not like to exceed 50 µg/ml. Finally, it is better to choose an internal standard that is either a closely related homologue or isomer for each steroid to be assayed rather than a stable isotope derivative, since switching between different positions in the mass scale at high resolution leads to lower sensitivity and precision. This fact also limits the number of components which can be monitored simultaneously in a single sample injection. This technique is therefore well suited to applications where a detailed study of a particular system is required or a small commitment to a routine program is envisaged and accurate, reliable data are essential.

ACKNOWLEDGEMENTS

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Calculation and statistical assessment of kinetic data from infusion experiments. By D. W. Wilson, C. G. Pierrepoint and K. Griffiths. Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XX

The administration of tracers as a single dose or by constant infusion provides useful information concerning the distribution, metabolism and generalized rates of transfer in open pool systems. However, with few exceptions (DiStefano, Durando, Jang, Jenkins, Johnson, Mak, Marshall, Mons, Warsavsky & Fisher, 1973) statistical assessment of infusion data has been inadequate or indeed non-existent.

Therefore a computer program has been devised to calculate, inter alia, the rates of transfer, secretion and interconversions of steroid hormones together with their fiducial limits from either single or dual label infusions. Particular attention has been focused on known sources of experimental error. These include the treatment of errors primarily associated with the measurement of plasma levels of product and precursor (Rodbard & Hutt, 1974), and those incurred in the corresponding determinations of plasma label concentrations. Provision has also been made for accommodating fluctuations in specific activities around the steady-state condition. The program has both deterministic and stochastic analytical options as well as various print-out formats, depending on the nature of the infusion and the statistical method of analysis employed.

These analytical techniques have been applied to studies on androgen metabolism in man and dog to evaluate prostatic function. The rates of secretion and transfer of corticosteroids have also been investigated in foetal and maternal compartments of the pregnant sheep.

REFERENCES


The elucidation of the biochemistry and aetiology of benign prostatic hypertrophy (BPH) and hyperplasia has been hindered by the lack of a suitable animal model which spontaneously develops prostatic tumours. Certain strains of golden hamster (Mesocricetus auratus), such as the BIO 87.2 strain used in this study, have been shown by gravimetric and histological data to develop benign tumours of the prostate gland spontaneously between 90 and 125 days of age.

Since an imbalance in the androgen : oestrogen ratio or a change in the androgen status with ageing has been implicated in the development of BPH, it seemed pertinent to measure the concentrations of endogenous androgens in the prostatic tissue and plasma of hamsters of the BIO 87.2 strain and a control strain throughout the life span.

Known amounts of non-endogenous internal standards were added to tissue homogenates and C-19 steroids were isolated using an acetone extraction. Non-polar and neutral lipids were removed using aqueous calcium chloride (Ismail, Love and McKinney, 1972).

Thin-layer separation on silica flexiplates in chloroform : acetone (185 : 10, v/v), yielded separate 5a-androstanediol, testosterone and androstanolone fractions, each with its own internal standard.

Gas chromatography with a column of 3% OV17 on Gaschrom Q and high-resolution single-ion-detection mass spectrometry, with a Varian 731 mass spectrometer of the trimethylsilyl ether derivatives of the androgens, allowed specific quantitation of three 5a-androstanediol epimers, testosterone, 5a-dihydrotestosterone and androsterone.

In hamsters of the BIO 87.2 strain, concentrations of endogenous total 5a-androstanediol and testosterone increased progressively to reach peak values at 200 days of age. Increases in the levels of total 5a-androstanediols originated ostensibly from 5a-androstane-3a,17β-diol. This is in agreement with stimulated 3x-hydroxysteroid oxidoreductase activity noted by Jacobi & Wilson (1976, 1977) in hypertrophied human and canine prostate glands. By contrast, the control strain showed no significant change in the concentration of total 5a-androstanediol nor testosterone for the same time course.

A surprisingly stable profile was obtained for 5a-dihydrotestosterone concentrations in both strains. Little fluctuation was noted from 60 to 250 days, although the values for BPH animals were uniformly higher than those for control animals throughout the time course studied.

Plasma concentrations of these same six androgens were also measured by high-resolution single ion detection. Data for this part of the work are preliminary but there does appear to be a progressive rise in the concentrations of total 5α-androstanediols and testosterone in the BIO 87.2 strain. The BPH animals showed increased tissue levels of 5α-androstane-3α,17β-diol and testosterone, compared with control values, whereas preliminary values for the plasma concentrations appeared to vary in the opposite manner. This apparent paradox could be attributable to the retention of 5α-androstanol-3α,17β-diol by the prostatic tissue of the BPH animals, possibly resulting in a lower contribution to the plasma pool.

Increases in the levels of steroids in the tissue occur at the time of tumour development, but the extremely high levels are detectable only when tumours are well established.

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TISSUE ANDROGEN CONCENTRATIONS IN GOLDEN HAMSTERS WITH BENIGN PROSTATIC TUMOURS

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SUMMARY

Using gas chromatography–high resolution mass spectrometry with selected ion detection, the concentrations of testosterone, 5α-dihydrotestosterone, androsterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17α-diol and 5α-androstane-3β,17β-diol were measured in the dorsal and ventral prostates of two strains of golden hamster (Mesocricetus auratus) at various ages from 60 to 250 days. The tissue concentrations from a control strain were compared with those of the BIO 87-2 strain of hamster, the latter developing benign hyperplasia of the prostate between 90 and 120 days of age.

Marked increases in the concentration of total 5α-androstanediols in both prostatic lobes of the BIO 87-2 strain were detected, with the highest level of over 345 nmol/g protein being found at 200 days of age. In comparison, the control strain showed a less pronounced increase in concentration at 150 days. Increases in total 5α-androstanediols were mainly associated with increases in the concentration of 5α-androstane-3α,17β-diol.

Concentrations of testosterone in prostatic tissue were also found to increase in the BIO 87-2 animals with peak values being seen at 200 days. Increases in concentration of androsterone were observed by 150 days and these levels were maintained up to 250 days of age. Control animals showed no comparable increases in testosterone and androsterone during the time-course studied.

Surprisingly, no significant variation in 5α-dihydrotestosterone concentration was detected in either strain from 60 to 250 days although levels were slightly but consistently higher in the BIO 87-2 strain.

There were no significant fluctuations in any of the androgens assayed either before or during tumour development. The increases in 5α-androstane-3α,17β-diol, testosterone and androsterone were detected only after tumours had become established.

INTRODUCTION

The high incidence of prostatic hyperplasia in both man and dog has stimulated research into the endocrinology and biochemistry of the condition, especially in relation to its aetiology (Coffey, 1975). The species specificity and age-related occurrence are well established. The growth, maintenance and functional activity of the prostatic tissue, both normal and abnormal, appear to be dependent upon androgenic stimulation. This has resulted in investigations into the changes in plasma hormones with ageing (Vermeulen, Rubens & Verdonck, 1972; Pirke & Doerr, 1973; Rubens, Dhout & Vermeulen, 1974; Shearns, MacDonell, Kaufman, Padua, Lucman, Winter & Faiman, 1974) and the comparison of the endocrine status of the clinically normal man with that of patients with prostatic disease (Harper, Peeling, Cowley, Brownsey, Phillips, Groom, Fahmy & Griffiths, 1976; Hammond, Ruokonen, Kontturi, Koskela & Vihko, 1977). Although it is generally assumed that there is
a hormonal role in the pathogenesis of prostatic hyperplasia, there are few marked differences between the concentrations of plasma hormones in the normal and diseased conditions.

The possibility, however, that hyperplasia may be related to abnormal metabolism of testosterone by prostatic tissue, with an increased production and accumulation of 5α-dihydrotestosterone, received support from earlier observations of Siiteri & Wilson (1970) showing that the endogenous concentrations of this steroid were five times greater in benign prostatic adenomas than in normal post-mortem prostatic tissue. Subsequent investigations have shown a relationship between prostatic size and 5α-dihydrotestosterone content and have demonstrated the effect of various steroids on prostatic growth in the dog (Gloyna, Siiteri & Wilson, 1970). Studies in this laboratory (Millington, Buoy, Brooks, Harper & Griffiths, 1975) using high resolution–mass spectrometry with selected ion detection for the analysis of endogenous steroids in human prostatic adenomas have indicated concentrations of 5α-dihydrotestosterone similar to those reported by Siiteri & Wilson (1970). Levels of 5α-androstane-3,17α-diol were consistent with those recently described by Albert, Geller, Geller & Lopez (1976) who suggested that a decreased 3-hydroxysteroid dehydrogenase activity could be responsible for 5α-dihydrotestosterone accumulation with its consequent growth-promoting effect.

Whether this abnormal metabolic profile relates to the course of hyperplasia or represents an effect of the prostatic dysfunction is difficult to establish in man. Studies have been further hindered by the lack of an appropriate animal model system which spontaneously develops benign tumours with ageing.

Following our preliminary report (Finney, Harper, Gaskell & Griffiths, 1978), studies are now presented on two strains of golden hamster, a control strain and the BIO 87-2 strain, the latter having been shown by histological and gravimetric studies to develop a benign cystic hyperplasia of the prostate (Homburger & Nixon, 1970; Weerawutananamoop, 1976).

The use of the hamster model is advantageous in that it allows a comparison of androgen concentrations in normal and hypertrophic prostatic tissue during an entire life-span of animals. Compared with the canine prostate in which hypertrophy does not become evident until 8 or 9 years of age, tumours are well established in a high proportion of BIO 87-2 hamsters by 120 days. In man, of course, benign prostatic hypertrophy is relatively rare before the sixth decade of life.

MATERIALS AND METHODS

Chemicals
Radioactive [1,2,6,7-3H]testosterone (94 Ci/mmol), [1,2,4,5,6,7-3H]5α-dihydrotestosterone (119 Ci/mmol) and [1,2,5H]5α-androstan-3α,17β-diol (41 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks. Unlabelled steroids were purchased from either Sigma Fine Chemicals, Poole, Dorset or Steraloids Incorporated, Wilton, New Hampshire, U.S.A. Packing materials for gas chromatography columns, bis-(N,O)-trimethylsilylacetamide and trimethylchlorosilane, were from Jones Chromatography Ltd, Llanbradach, Glamorgan and hydroxylamine hydrochloride and methoxylamine hydrochloride were from Eastman Kodak Ltd, Rochester, New York, U.S.A. Precoated silica gel flexiplates (Polygram Si G/uV254) were purchased from Camlab Ltd, Cambridge. All organic solvents were of redistilled analytical grade.

Animals
Control and BIO 87-2 hamsters were bred in the Institute animal unit and maintained on a constant schedule of 13 h light : 11 h darkness. Some animals were castrated by the scrotal route under ether anaesthesia when 65 days old.

After dissection, all tissue was placed on crushed ice until use.
Androgens in hamster prostatic tumours

Extraction of tissue

Dorsal and ventral prostates from groups of approximately 15 animals were dissected free of capsular tissue and fat, weighed and then homogenized in 100 mmol Tris–HCl buffer, pH 7.4. A portion was taken for protein estimation by a modification of the Lowry method (Hartree, 1972). The remainder of the homogenate was then extracted with 40 vol. acetone using a Silverson mixer.

To the acetone extract were added the internal standards 4-androsten-17α-ol-3-one (epitestosterone; 2 ng), 5α-androst-17α-ol-3-one (5α-dihydroepitestosterone; 20 ng) and 5α-androstan-3α,17α-diol (2 ng) in 100 μl ethanol and the whole was left to equilibrate for 30 min. Preliminary experiments had shown that none of the internal standards used were present endogenously in the tissue.

After centrifugation at 800 g for 10 min, the solvent was removed from the extract under reduced pressure. This was followed by an alkali wash with 1 m-NaOH and neutral and non-polar lipids were removed by differential solubility in 1% CaCl₂ (Ismail, Love & McKinney, 1972).

Thin-layer separation of steroids

After filtration through a sintered-glass funnel, the aqueous fraction was shaken with 2 × 20 ml diethyl ether. The direct organic extract was then resuspended in ethanol and applied as a thin band to a prewashed silica gel ‘flexiplate’, which was developed in chloroform : acetone (185 : 10, v/v). Radioactive steroids of high specific activity were used as chromatography markers for the fractions containing testosterone, androstanolones and total 5α-androstanediols. Since investigations with authentic steroids had shown the 5α-androstanediols to be eluted in a single band, only [³H]5α-androstane-3α,17β-diol was used as marker for this fraction. After scanning for radioactivity (Berthold LB 2722 Radiocchromatogram Scanner II, Camlab Ltd), the relevant areas were removed and eluted with methanol.

The separated steroid fractions were dried in a stream of nitrogen, transferred to screw-capped 2 ml vials, vacuum desiccated and derivatized for subsequent gas chromatography–high resolution mass spectrometry with selected ion detection.

Derivative formation

To the dried residues containing 5α-androstanediols was added bis-(N,O)-trimethylsilylacetamide (20 μl) containing 10% trimethylchlorosilane.

The residues containing testosterone and 5α-dihydrotestosterone were treated with 20 μl hydroxylamine hydrochloride in pyridine (20 mg/ml) and 20 μl methoxylamine hydrochloride in pyridine (20 mg/ml) respectively and they were left overnight at room temperature. The samples were then vacuum desiccated and 20 μl bis-(N,O)-trimethylsilylacetamide added to each.

Gas chromatography–high resolution mass spectrometry

All analyses were performed within 2 days of formation of derivatives and 4 days of extraction of tissue. Gas chromatography–high resolution mass spectrometry was undertaken using a Varian 2700 gas chromatograph fitted with a helical glass column (2 m × 2 mm, internal diameter) containing 3% OV 17 on Gas Chrom Q (100–120 mesh), interfaced to a double-focussing Varian MAT 731 mass spectrometer by a two-stage, all glass Watson–Biemann separator.

Details of the technique for selected ion monitoring mass spectrometry at high resolutions (m/Δm = 8500–10 000, where m is the mean mass of two adjacent ions separated by a 10% valley and Δm is the difference in mass) in tissue extracts have been described previously (Millington, 1975; Millington et al. 1975; Maynard, Pike, Weston & Griffiths, 1977; Gaskell & Pike, 1978; Gaskell, Finney & Harper, 1979).
The bis-trimethylsilyl (TMS) ethers of the 5α-androstanediols yielded suitably intense molecular ions, of mass : charge ratio \((m/z)\) 436-319, to permit sensitive selected ion detection. The use of high-mass spectrometric resolution affords great analytical specificity by virtue of the separation of ions of the same nominal mass but differing elemental composition. Furthermore, discrimination of isomeric 5α-androstanediols is achieved by the gas chromatographic separation which permits the use of a non-endogenous isomer as internal standard. The mass spectrometric detection limit for the 5α-androstanediol–bis-TMS ethers was approximately 30 pg.

![Typical gas chromatography traces for derivatized fractions from hamster prostatic tissue chromatographed on columns of 3% OV 17 at 280 °C](image)

(a) androstanolone fraction using 5α-dihydroepitestosterone as internal standard, \(m/z\) 391-291; (b) 5α-androstanediol fraction using 5α-androstane-3α,17α-diol (5ααα) as internal standard, \(m/z\) 436-319; (c) testosterone fraction using epitestosterone as internal standard, \(m/z\) 447-299. 5αββ, 5α-Androstane-3β,17β-diol; 5αβα, 5α-androstane-3β,17α-diol; 5αββ, 5α-androstane-3β,17β-diol.

The TMS oxime, TMS ether derivative of testosterone, on electron impact yielded a molecular ion, \(m/z\) 447-299, of sufficient abundance to detect levels of 20 pg. The intensities of the molecular ions at \(m/z\) 391-291 of the methyloxime, TMS ether derivatives of 5α-dihydrotestosterone and androsterone allowed the detection of amounts as low as approximately 100 pg. The gas chromatographic traces (Fig. 1) show the use of various steroids as internal standards.
Androgens in hamster prostatic tumours

RESULTS

Tissue concentrations of 5α-androstanediols

There was a marked increase in the concentrations of total 5α-androstanediols in both dorsal and ventral prostates of the BIO 87-2 hamsters with increasing age (Fig. 2a). Concentrations of 147 and 271 nmol 5α-androstanediols/g protein were found in dorsal and ventral glands respectively by 200 days of age. It may be seen (Fig. 2b) that the marked rise in total 5α-androstanediols particularly, reflected an increase in the tissue concentration of 5α-androstane-3β,17β-diol. This was found to be the most abundant isomer in both dorsal and ventral prostates of both strains, although the 5α-androstane-3β,17α-diol, not detected in human prostatic tissue (M. E. Harper, S. J. Gaskell & A. W. Pike, unpublished data) and the 5α-androstane-3β,17β-diol were together found to account for 56% of the concentration of total 5α-androstanediols in the group of animals at 200 days of age.

![Fig. 2. Tissue levels of (a) total 5α-androstanediols and (b) 5α-androstane-3α,17β-diol in the dorsal prostates of BIO 87-2 (open bars) and control hamsters (solid bars) and the ventral prostates of BIO 87-2 (hatched bars) and control hamsters (stippled bars). Due to the number of animals required for each extraction, most assays were performed only once. However, duplicate assays were carried out during periods of extensive change in steroid concentrations.](image-url)
Data for the control group of animals indicated that although the steroid levels increased with age the concentration was never as high as in the prostatic tissue from BIO 87-2 animals. The effects 24 h after castration on 5α-androstanediols appeared to be similar in both groups of animals (Table 1). The concentrations of total 5α-androstanediols and particularly that of 5α-androstane-3α,17β-diol were increased in both dorsal and ventral lobes of BIO 87-2 and control animals.

Table 1. Androgen concentration in the dorsal (D) and ventral (V) prostates of two strains of entire hamsters and of others 24 h after castration performed at 65 days of age

<table>
<thead>
<tr>
<th>Androgen</th>
<th>Prostate lobe</th>
<th>Tissue (nmol/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BIO 87-2</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Entire</td>
<td>Castrated</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>D</td>
<td>3.79</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>5.78</td>
</tr>
<tr>
<td>5α-Androstane-3β,17α-diol</td>
<td>D</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>5.50</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>D</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>5.92</td>
</tr>
<tr>
<td>Testosterone</td>
<td>D</td>
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<tr>
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<tr>
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<tr>
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<td>V</td>
<td>6.85</td>
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</table>

Concentration of testosterone in tissue

Figure 3a shows the increased tissue concentrations of testosterone at 150 days of age in the BIO 87-2 strain of hamster; peak values of 155 and 128 nmol/g protein were found at 200 days in the dorsal and ventral lobes respectively. There was no comparable increase in the concentration of testosterone in the control strain although there was a relatively higher concentration at 150 days of age.

There was an increase in the concentration of testosterone in the tissue of the prostatic lobes of both strains of animal 24 h after castration. The effect was more pronounced in the ventral prostate of the BIO 87-2 hamsters and the dorsal prostate of the control strain.

Concentration of 5α-dihydrotestosterone and androsterone in tissue

There was surprisingly little variation in the 5α-dihydrotestosterone concentration in the prostatic tissue from either group of animals during the time-course studied (Fig. 3b), although concentrations in the BIO 87-2 animals were consistently higher than those for age-matched control hamsters.

Androsterone concentrations showed a rapid increase in the prostate tissue from the BIO 87-2 hamsters, from approximately 35 nmol/g protein at 100 days to 258–361 nmol/g protein at 150 days, levels which were maintained up to 250 days (Fig. 3c).

Castration decreased the 5α-dihydrotestosterone and increased the androsterone concentrations in prostatic tissue of both strains after 24 h.
Androgens in hamster prostatic tumours

Fig. 3. Prostatic tissue concentrations of (a) testosterone, (b) 5α-dihydrotestosterone and (c) androstenedione in the dorsal prostates of BIO 87-2 (open bars) and control hamsters (solid bars) and the ventral prostates of BIO 87-2 (hatched bars) and control hamsters (stippled bars). Due to the number of animals required for each extraction, most assays were performed only once. However, duplicate assays were carried out during periods of extensive change in steroid concentrations.

DISCUSSION

The rapid increases in 5α-androstane-3α,17β-diol, testosterone and androstenedione which occur from 150 to 200 days in the prostates of BIO 87-2 animals suggest either a change in the biochemistry of the prostates themselves or an increase in the amount of plasma androgen available to the glands at these times.
The accumulation of 5α-androstenediols and particularly of 5α-androstane-3α,17β-diol in the tumour-bearing strain is especially interesting since it has been found that the formation of the 3α,17β- and 3β,17β-5α-androstenediols is significantly higher in the microsomal and cytosol fractions from hypertrophic human prostates when compared with normal tissue (Jacobi & Wilson, 1977). Also, the isomer has been implicated in the pathogenesis of prostatic hypertrophy in the dog. Whereas 5α-dihydrotestosterone and testosterone failed to produce hypertrophy of the prostate in castrated dogs (Wilson, Gloyna & Siiteri, 1975) 5α-androstane-3α,17β-diol, either alone or in conjunction with oestrogen, caused hyperplastic growth within 6 months (Walsh & Wilson, 1976). Enzymic rates of formation of 5α-androstane-3α,17β-diol have been found to be tenfold higher in hypertrophic compared with immature canine glands (Jacobi & Wilson, 1976).

The increased levels of androgens occurred only after the period of tumour development in the BIO 87-2 hamsters and so may be viewed as more of a consequence rather than an initiator of tumour growth. They may be accounted for by a change in the activities of those enzymes responsible for the metabolism of testosterone, notably Δ4-5-3-oxosteroid 5α-reductase and 3α/3β-hydroxysteroid oxidoreductase.

Since 5α-reduction rates in slices from the normal and hypertrophic prostates in man and dog have shown no significant differences, it has been deduced that the accumulation of 5α-dihydrotestosterone in hypertrophic tissue is partially accountable for by a less extensive metabolism to 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol (Gloyna et al. 1970; Siiteri & Wilson, 1970).

Preliminary steroid metabolism studies using the prostates of young hamsters have indicated considerable conversion of [3H]testosterone into 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol in both strains (R. W. Finney and M. E. Harper, unpublished data). An examination of the activity of 5α-reductase and 3α/3β hydroxysteroid oxidoreductases throughout an age range of animals of both groups is now in progress.

Increased concentrations of androgens detected in the BIO 87-2 strain may be related to the amounts of the various steroids available in the plasma pool. This may involve increases in total plasma androgen or in the amount not bound by plasma binding components and therefore metabolically available to the tissue. An examination of the plasma concentrations of the same six androgens for a similar time course will be reported elsewhere.

The stable profile obtained for 5α-dihydrotestosterone in both strains of hamster is very surprising since it is this androgen which has been widely implicated in the androgenic stimulation of prostatic tissue (Bruchovsky & Wilson, 1968a, b; Wilson & Gloyna, 1970; Mainwaring, 1975) and it has been detected in higher concentrations in hypertrophic than in normal human prostatic tissue (Siiteri & Wilson, 1970; Geller, Albert, Lopez, Geller & Niwayama, 1976).

That the observed increases in concentrations of androgen in the tissue after tumour development may be due to qualitative or quantitative change in the binding-protein content of the prostate of the BIO 87-2 hamsters cannot be discounted. These studies are at present being undertaken.

The authors wish to express their gratitude to the Tenovus Organization for the generous financial support. R. W. F. also wishes to thank Mr A. W. Pike for his expert advice.

REFERENCES


Androgens in hamster prostatic tumours


In a previous communication (Finney, Harper, Gaskell & Griffiths, 1978) we reported the detection of raised concentrations of various androgens in the prostatic tissue of tumour-bearing animals of the BIO 87.2 strain of hamster which spontaneously develops benign cystic hyperplasia.

These increases may be influenced by changes in the plasma hormone concentrations or alterations in the prostatic steroid metabolizing enzymes during the period of tumour development and these two possibilities have been investigated.

Plasma samples (1 ml) from BIO 87.2 and control hamsters were extracted with diethyl ether and known amounts of non-endogenous isomeric internal standards were added. Reversed and straight-phase gel chromatography of the extracts on Lipidex 5000 and Sephadex LH-20 microcolumns respectively (Gaskell & Pike, 1978) yielded an androgen-rich fraction which was derivatized for gas chromatography–high resolution mass spectrometry with selected ion detection.

Gas chromatography of the t-butyldimethylsilyl ether derivatives on a column of 1% OV 17 on Gaschrom Q (100–120 mesh) and high-resolution single-ion detection, using a Varian 731 mass spectrometer, allowed specific quantification of three 5α-androstanediol stereoisomers; testosterone, 5α-dihydrotestosterone and androsterone.

In the BIO 87.2 animals, plasma concentrations of testosterone were found to rise gradually from 17:36 nmol/l to values in excess of 35.00 nmol/l in animals bearing tumours, whereas those in control animals remained stable at 17:36 nmol/l. At all other times, no differences were detected between comparable age groups of the two strains.

Plasma concentrations of 5α-dihydrotestosterone, androsterone and 5α-androstane-3α,17β-diol in the two hamster colonies were found to be statistically different only when BIO 87.2 hamsters were bearing tumours. At these times, plasma concentrations were higher in tumour-bearing animals when compared with age-matched controls.

Simultaneous increases in the prostatic tissue and plasma concentrations of androgens in tumour-bearing hamsters might have been attributable to changes in the prostatic tumour production of these compounds, followed by their release into the blood. This might involve alterations in the activities of the 3α- and 3β-hydroxysteroid oxidoreductase enzymes. An examination of the metabolism of [3H]5α-dihydrotestosterone into [3H]5α-androstanediols at pH 7.0 and 37 °C with NADPH as cofactor, by cytosol fractions prepared from the prostates of BIO 87.2 and control hamsters aged 40–250 days, showed that, in the dorsal prostates, enzyme activities remained constant throughout the age range. In the ventral prostates, however, the enzyme activity increased at 90 days, coinciding with the onset of tumour development in the BIO 87.2 colony but no significant difference was seen in prostates of normal hamsters and those with benign prostatic hypertrophy.

It would appear that the increased tissue and plasma concentrations of these androgens were not a consequence of changes in the activities of the enzymes studied.

REFERENCES


PROLIFERATION OF HUMAN PROSTATIC EPITHELIAL CELLS IN CULTURE: ASPECTS OF IDENTIFICATION

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ABSTRACT

A reproducible explant culture technique has been developed to study in monolayer, the growth of human prostatic epithelial from specimens of tissue removed from patients with benign prostatic hyperplasia. The procedure was used to study hormone responsiveness of prostatic tissue. The cells appear to originate from the progenitors of glandular epithelium and possess epithelial characteristics, as assessed by histologic, electron microscopic and immunocytochemical procedures. Close cell-to-cell contacts, desmosomes and microvilli were visualized in electron micrographs. In addition, we observed immunocytochemical localization of epithelial antigens in the cells which constituted the monolayer by using antisera raised against purified prostatic epithelium. Prostatic acid phosphatase was not detected histochemically in the monolayers and may reflect the non-secretory state of the cells. Growth parameters under various hormonal conditions were assessed by counting metaphase arrested cells, total cell number and measurement of the area covered by the monolayers. Proliferation was significantly increased by the addition of physiological concentrations of either testosterone or 5α-dihydrotestosterone.

Although the androgen dependent nature of prostatic tissue is well accepted, the role of these steroids in the etiology of benign prostatic hyperplasia (BPH) has not been established. Factors affecting the growth of human prostatic epithelium are difficult to study in vivo, and proliferation of such tissue under various hormonal environments can only be investigated at present with culture techniques. Organ culture procedures have been extensively used to study the metabolism of steroids and their effects on RNA and DNA synthesis. Stathmokinetic drugs have been employed in the study of proliferation, but only in monolayers is this really feasible, because cell turnover rate is relatively slow in organ culture. The application of clonal growth assays to established prostatic cell lines was recently reported although short-term primary cultures, which may be more representative of the parent cells, do not lend themselves to this type of study. Proliferation of normal human prostatic epithelium in monolayer has also been studied with thymidine incorporation and area of cell growth.

The value of any culture system is completely dependent upon adequate characterization of the component cells. Electron microscopy was employed for identification in these studies, together with histochemical and immunocytochemical procedures, with antisera specific to epithelial cells. Although there is a lack of suitably specific markers for identifying human prostatic epithelium in culture, localization of the prostatic acid phosphatase has been the subject of intense investigation.

This communication describes the use of a primary monolayer culture technique for studying the proliferation of human BPH epithelial cells in response to steroids and also the identification of the cell types present in the culture.

MATERIALS AND METHODS

Cell culture. A sample of each BPH specimen studied was fixed in Bouins fluid prior to culture and saved for subsequent embedding and histologic analysis. Explants of tissue (0.5-1.0 mm.) were prepared as described by Stone and Hemp.

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† Recipient of Wellcome Trust grant. 10034/1.5.
EMMA-4 electron microscope-microanalyzer (Kratos, U.K.).

**Histology.** Prostatic acid phosphatase activity was studied by a modification of the Gomori lead-ion capture method 10 with phosphorycholine as substrate.14 Fresh, frozen, cryostat sections (5 μm) of BPH tissue and epithelial monolayers were fixed briefly in 70 per cent ethanol before incubation in sodium acetate buffer (0.05 M, pH 5.0) containing phosphorycholine (0.40 per cent) and lead nitrate (0.14 per cent) at 37°C. The reaction product was developed in fresh yellow ammonium sulphide (less than 1 per cent) for 1–2 minutes. Substrate was omitted in controls. Acid phosphatase activity was then detected by the presence of brown/black deposits.

**Preparation of prostatic epithelial antisera.** Pure prostatic epithelium was prepared by the method of Harper and associates.15 Tissue slices, 1–2 mm. thick, were scored with scalpels to expose glandular acini and passed through a Latapie press. The resulting cell suspension was pelleted and a portion assessed for purity by histologic examination while the remainder was stored at −20°C until sufficient material had been collected. Epithelial cell pellets were thawed, pooled and resuspended in 0.9 per cent saline and emulsified with an equal volume of Freund’s complete adjuvant. The emulsion was injected intracutaneously in rabbits such that each animal received 1 mg. cellular protein. After initial injection the animals were “boosted” every 4 weeks and bled at 2 weekly intervals. Immunodiffusion techniques were employed to ascertain the increase in titer by serial dilution of antisera and testing against a homogenate of purified epithelial cells until precipitation bands were not evident. The antisera titer gradually increased until the 12th week and remained constant thereafter. The 16-week bleed which provided the aHPE referred to in this text was used for immunocytochemical investigations.

**Immunocytochemistry.** Localization of the rabbit anti-human prostatic epithelial antisera (aHPE) was visualized by an unlabeled antibody enzyme method.16 Bouins fixed epithelial and fibroblastic monolayers and serial sections of BPH tissue (5 μm.) were incubated with aHPE (1:500, PBS v/v) followed by sheep antirabbit serum (1:20, PBS v/v), peroxidase antiperoxidase antisera (1:12, PBS v/v) and finally the substrate hydrogen peroxide and diaminobenzidine with PBS washes between each stage. Preincubation with nonimmune sheep serum was used to prevent nonspecific binding of sheep antirabbit serum. Rabbit serum obtained prior to the immunization schedule served as a control and PBS as a blank by replacing aHPE in the incubation sequence. Absorption controls were prepared by admixture of aHPE with purified epithelial cells. Cross reactivity with cultured prostatic fibroblasts was eliminated by absorption of aHPE with a suspension of the fibroblasts. Species and tissue specificity of aHPE was assessed by incubation of the antiserum on tissue sections at a variety of human tissues and prostates from different animals.

Localization of prostatic acid phosphatase was also investigated with identical procedures employing human prostatic acid phosphatase antisera. (Gift from Dr. R. Van Effen, Purdue University, Indiana).

**RESULTS**

The explant culture of over 50 specimens of human BPH tissue has been achieved, with more than 95 per cent of these giving rise to epithelial monolayers (fig. 1). In experiments undertaken to study the origin of the cells which constitute the outgrowths, explants were removed at daily intervals during the first 7 days in culture and subjected to histologic examination. Explants showed changes in general tissue architecture within 24 hours of culture, with differentiated epithelium being sloughed into the lumen. Multiplication of the underlying epithelial cells was seen between days 1–2 (fig. 2, a). These cells appeared to encapsulate the explants particularly if acini were exposed to the growth medium and progressive cell loss was observed in the stroma (figs. 2, b and c). After the explants become attached to the culture dishes (day 3), the encapsulating cells appear to multiply and migrate over the surface of the dish forming a monolayer. When acini were absent in the explants, encapsulation and subsequent monolayer formation did not occur.

**Growth assay.** A typical growth curve was obtained by measuring the area covered by the epithelial monolayers (fig. 3). After an initial lag phase of 2–3 days, the area increased linearly until day 15 when a plateau, indicating maximal outgrowth began to form.

When the results expressed as ‘metaphases scored’ in a given number of cells, were plotted against ‘metaphases counted’ in a given area and subjected to linear regression analysis, a significant correlation was obtained: r = 0.993 (fig. 4). Therefore, data can be presented as metaphases counted per mm².

**Influence of hormones on proliferation.** The effect of 5α-dihydrotestosterone over a concentration range of 1 × 10⁻¹⁰ to 1 × 10⁻⁵ M was performed in duplicate on 4 separate specimens of BPH tissue. Optimal stimulation of epithelial cell growth was observed between 1 × 10⁻⁹ and 1 × 10⁻⁷ M when metaphase arrested cell counts were used in the analysis and at 1 × 10⁻⁷ M when the area of outgrowth was considered (fig. 5). Addition of testosterone to the medium over the same concentration range and number of data points gave maximal stimulation at 1 × 10⁻⁷ M when either metaphase arrested cells were counted or outgrowth parameters were measured. A significant inhibition of growth was noted at 1 × 10⁻⁵ M testosterone (fig. 6).

**Ultrastructure.** The cells composing the monolayers were generally in close proximity, had tight cell-to-cell contacts and possessed numerous microvilli (fig. 7). Desmosomes, identified by the presence of a central lamella (fig. 8) and intermediate-type junctional complexes were observed between attached cells. Abundant tonofilaments and fibrils were also present. Ovoid nuclei with a thin rim of heterochromatin and prominent nucleoli were apparent in the cells together with elongated mitochondria, free ribosomes, lysosomes and glycogen deposits. Golgi structures and endoplasmic reticulum were sparse and secretory granules rarely encountered (fig. 7).

**Localization of prostatic acid phosphatase.** Acid phosphatase activity, as detected by histochemical localization of reaction product, was observed in frozen sections of BPH tissue in the apical portion of cytoplasm in epithelial cells, but not in the stromal components. Staining indicative of acid phosphatase was not seen in cultured epithelial cell monolayers.

The absence of acid phosphatase activity in the cultured cells was further supported by the negative results obtained when a specific antisera was used which was raised against the prostatic

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**Fig. 1.** Monolayer of BPH epithelial cells derived from explant, day 7 of culture. Hematoxylin and eosin stain. (×440).
acid phosphatase in the immunocytochemical procedure.

Detection of epithelial antigens. An indication of the purity of epithelial cells obtained after separation from the fibromuscular stroma, and which was subsequently used for raising aHPE, is shown in figure 9. Localization of aHPE as represented by the brown reaction product was observed clearly in BPH sections and epithelial monolayers whereas only a faint stain was apparent in fibroblastic cultures. Staining was cytoplasmic and found exclusively in epithelial cells of both the tissue sections and monolayers after absorption. When aHPE was absorbed with homogenized fibroblastic cells prior to use in the immunocytochemical procedure, no staining of fibroblasts was seen but epithelial cells were still intensely stained (fig. 11). In addition, a positive result was not observed in tissue sections of human breast, kidney, testes and adrenal gland or in mouse, rat, hamster, dog and monkey prostate. Replacement of aHPE...
logic examination, indicate that the presence of acini with glandular epithelium in the explants is a requisite for subsequent epithelial cell outgrowths. Initial loss of differentiated epithelium, followed by apparent replacement with underlying cells shown in this study, is of interest because Franks claimed that outgrowth cultures were derived from stem cells and were not fully differentiated.

Electron microscopy is also useful for identification of cells. Spot desmosomes, observed in the cells are characteristic of epithelial cells and are distinguished by the presence of a central lamella and 10 nm. tonofilaments. It has been reported that intermediate junctions found in both mesenchymal and epithelial cells have mistakenly been classified as desmosomes by some authors, but these never have a central lamella and are associated with 7.5 nm. filaments.

Immunocytochemical procedures have proved useful in the visualization of antigenic sites within a tissue. It is, however, important to include appropriate controls for validation of results. In this report specific staining was not detected when aHPE was tested on tissue sections of prostate from 5 different

in the immunocytochemical procedure by non-immune rabbit serum or a sample of aHPE that had been absorbed with epithelial cells resulted in negative staining of BPH sections and monolayers.

**DISCUSSION**

Production rates of epithelial cell monolayers derived from human BPH specimens have been measured by the use of the "metaphase-arrest" drug colchicine. The culture technique is reproducible in both initiation of monolayers and in replication of results.

The use of established cell lines offers ease of handling and repetition of results, but because of considerable cell selection, might not accurately reflect the parent cells. Therefore short term primary cultures, as employed in this study, are probably more representative of the original cell population and enable comparisons to be made between different specimens of tissue. It is important to identify the cells which have been propagated in culture. Webber's studies, together with the investigations now reported on the origin of the cells as assessed by morpho-

![Fig. 5](image_url)

**Fig. 5.** Influence of 5α-dihydrotestosterone on metaphase accumulation and area of monolayer outgrowth. Data expressed as mean ± standard deviation, n = 4. *2P = 0.28. Mann-Whitney U test.

![Fig. 6](image_url)

**Fig. 6.** Influence of testosterone on metaphase accumulation and area of monolayer outgrowth. Data expressed as mean ± standard deviation, n = 4. *2P = 0.28. Mann-Whitney U test.
HUMAN PROSTATE IN PRIMARY CULTURE

Fig. 7. Electron micrograph of BPH epithelial cell in monolayer showing tight junctional complexes, J.C.; microvilli, M.V., ovoid nuclei with a thin rim of heterochromatin, H.; elongated mitochondria, M.; fibrils, F.; Lysosomes, L.; and glycogen, G. (x4680).

Fig. 8. Higher magnification of desmosome showing the presence of a central lamella, CL; and tonofilaments, T (x153600).

Fig. 9. Histology of epithelial cells separated from stromal components. Azan stain (x440).

Fig. 10. Photomicrographs of consecutive sections of a single acinus from a specimen of human BPH (x290). a. Immunocytochemical staining obtained with aHPE (1:500). b. Hematoxylin and eosin stain.

species of animal and on a variety of human tissues. In addition, when non-immune rabbit serum was used to replace aHPE or if the aHPE had been absorbed with pure epithelial cells, staining was not observed in the epithelial cells. A positive response when unabsorbed antiserum was used can therefore be attributed to the presence of prostatic epithelial antigens. Whole cell homogenates used for raising antisera in this study contain a milieu of antigenic components and hence aHPE probably recognizes a number of different cellular components, only some of which are specific to prostatic epithelium. The
slight amount of staining seen in cultured prostatic fibroblasts, indicating the presence of some cross-reacting antigens with epithelial cells, was eliminated by admixture of the antiseraum with homogenized fibroblasts, and thus the removal of the antibodies common to both. The strong stain remaining in the epithelial cells is therefore not due to antigens found in fibroblasts.

Histochemical and immunocytochemical detection of prostatic acid phosphatase has been favored as a method for characterizing prostatic epithelium.10-12 Its presence, however, has not been shown to some prostatic epithelial cultures12 and might therefore limit its use as a specific marker. The reduction in Golgi structures and lack of secretory granules in cells forming the monolayers used in this study are indicative of the undifferentiated nature of the cells and perhaps explains why this enzyme was not detected.

The sensitivity of prostatic epithelium in vivo to changes in androgen concentration is well documented and it would be expected that these cells would respond to androgens in tissue culture. However, some studies in organ culture failed to show that growth of BPH tissue was affected by hormones6,14 although Lasnitzki15 has observed changes in DNA and RNA synthesis when studied by autoradiographic methods, with DNA synthesis being increased in the presence of 5α-androstane-3α,17β-diol. Some previous investigators have also failed to show that both testosterone and 5α-dihydrotestosterone influence the growth of epithelial cells in monolayers of many primary cultures and established cell lines.5,20-23 Difficulties encountered in obtaining such a response in vitro may be attributable to either the androgen independent nature of the cells or to the high concentrations (â¥1 µg/mL) of steroids used in the experiments. Webber,8 working on normal prostatic epithelium in monolayer, has observed stimulation of growth when using 5α-dihydrotestosterone at more physiologic levels.

Furthermore, results presented in this paper indicate that both testosterone and 5α-dihydrotestosterone stimulate cell division of prostatic epithelium grown in monolayer from BPH specimens at physiologic concentrations. The explant culture technique therefore appears to provide a model system for studying BPH epithelial cells in vitro and for assessing their sensitivity to various hormonal environments with a view to elucidating the role of such compounds in the etiology of the disease.

REFERENCES


I. INVESTIGATIONS RELATING TO CANCER OF THE PROSTATE.

C. PROLACTIN AND THE PROSTATE.
An Effect of Prolactin on Prostatic Adenylate Cyclase Activity

By Meriel P. Goldner,* A. R. Boyns,† Maureen E. Harper* and K. Griffiths*

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(Received 13 April 1972)

There is increasing evidence that prostatic growth can be directly affected by pituitary hormones. Grayhack & Lebowitz (1967) showed that pituitary prolactin and testosterone stimulate the growth of the lateral lobe of the prostate in hypophysectomized orchidectomized rats.

The recent isolation of human pituitary prolactin (Lewis et al., 1971) emphasizes the need to examine the role of this hormone in regulating prostatic metabolism. In the present study the effects of prolactin and other hormones on prostatic adenylate cyclase activity were examined. Adenylate cyclase activity was determined by measuring the conversion of [α-32P]ATP or [8-14C]ATP into cyclic [32P]AMP (adenosine 3':5'-cyclic [32P]monophosphate) or cyclic [8-14C]AMP (Krishna et al., 1968). DEAE-Sephadex was used to separate cyclic AMP from other nucleotides (Rosset et al., 1970), and this allowed sample processing by a batch procedure.

Materials and methods

Cyclic AMP (acid form), AMP, ADP and ATP were obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.). [α-32P]ATP and [8-14C]ATP (The Radiochemical Centre, Amersham, Bucks., U.K.) were diluted in distilled water and stored at −30°C before use. Cyclic [8-3H]AMP (ammonium salt) (The Radiochemical Centre) was stored at 4°C and diluted in distilled water immediately before use. DEAE-Sephadex A-25 (Pharmacia (G.B.) Ltd., London W.5, U.K.) was washed with 0.40m-NH₄HCO₃ until the washings were free of chloride (tested with AgNO₃), then washed with distilled water. Finally it was equilibrated with 2mM-NH₄HCO₃. Sheep prolactin (NIH-P-S8; potency 28I.u./mg), human luteinizing hormone (IRC-2 (10.12.64); potency 5xNIH-LH-S1), human growth hormone (MRC Research Standard A62/6) and human chorionic somatomammotropin (MRC human placental lactogen 70/194) were dissolved in 21mM-tris–HCl buffer, pH7.6, before addition to incubation medium. Testosterone [Sigma (London) Chemical Co. Ltd.] was dissolved in ethanol.

Prostatic tissue was taken from 14-week-old male rats (Sprague–Dawley). Fat was removed and the tissue minced finely. A 10% (w/v) homogenate was prepared in 0.25m-sucrose by using a Philpot Stainer homogenizer (12 strokes). The homogenate was filtered through cheesecloth before assay. Homogenate (20μl) was mixed with 10μl of hormone solution for 1 min at 0°C. Then 50μl of incubation medium was added (at room temperature) so that the final concentrations of reactants were as follows: MgCl₂ (3.1mM); ATP (2.5mM); cyclic AMP (1mM); bovine serum albumin (0.5mg/ml); [α-32P]ATP (25μCi/ml) or [8-14C]ATP (2.5μCi/ml); tris–HCl buffer, pH7.6 (21mM). The tubes were incubated at 37°C for 15min before the addition of 70μl of a solution containing ATP (8.7μM), cyclic AMP (3.5μM) and cyclic [8-14C]AMP (0.29μCi/ml). After the tubes had been heated in a boiling-water bath for 3min they were centrifuged at 1500g for 5min, and 100μl of the supernatant was mixed with 1.5ml of a 1:20 (w/v) suspension of DEAE-Sephadex A-25 in 2mM-NH₄HCO₃. The suspension was mixed for 1h at 4°C and then centrifuged at 2000g for 5min at 4°C. The supernatant was discarded and the sediment washed twice with distilled water before elution of the adsorbed cyclic AMP with two 1ml volumes of 64mM-NH₄HCO₃. Then 0.2ml of 0.17m-ZnSO₄ was added to the combined eluates followed by 0.2ml of 0.15m-Ba(OH)₂ (final pH 7.5–8.0) to remove traces of AMP. After centrifuging, the ZnSO₄–Ba(OH)₂ treatment was repeated, and 0.5ml of the supernatant was mixed with toluene scintillant containing solubilizer and its radioactivity counted. The homogeneity of the final supernatant after ZnSO₄–Ba(OH)₂ treatment was checked by descending chromatography on Whatman 3MM paper (Bär & Hechter, 1969) in the solvent system propan-2-ol–aq. NH₃ (sp.gr. 0.88)–water (7:1:2 by vol.). The chromatogram was developed for 15h at 23°C, and the spots were localized under u.v. light, cut out and placed in counting vials. Water (0.5ml) was added to elute radioactive material before its radioactivity was counted in scintillator as before. Enzyme activity was expressed as nmol of cyclic AMP formed/15min per mg of protein, after subtraction of the value of the blank (boiled tissue).

Results and discussion

DEAE-Sephadex A-25 removed over 95% of the added AMP, ADP, ATP and cyclic AMP from solution (Table 1). Treatment with 64mM-NH₄HCO₃ eluted a considerable fraction of the cyclic AMP, a small proportion of AMP but insignificant quantities
Experimental details

Concentrations

64 mm-\(\text{NH}_4\text{HCO}_3\).

hormone; HLH, using Student's t test (N.S., not significant). Abbreviations: OMtH, sheep prolactin; HGH, human growth hormone; HLH, human luteinizing hormone; HCS, human chorionic somatomammotrophin.

Results

Cyclic AMP formation (nmol/15 min per mg of protein) 

Table 1. Adsorption of nucleotides on DEAE-Sephadex A-25 and their subsequent elution

A 1.5 ml volume of gel suspension (1:20, w/v) in 2 mm-\(\text{NH}_4\text{HCO}_3\) was mixed with 0.1 ml of nucleotide solution (6 mm) for 1 h at 4°C. After washing with two 1 ml volumes of 2 mm-\(\text{NH}_4\text{HCO}_3\), nucleotides were eluted with 64 mm-\(\text{NH}_4\text{HCO}_3\). Concentrations were determined by measuring the absorbance at 270 nm or by counting the radioactivity.

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<th>(^{[\text{P}]}\text{ATP} )</th>
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<td>Amount adsorbed by DEAE-Sephadex (% of total)</td>
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<td>51.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

of ADP and ATP. ZnSO\(_4\)-Ba(OH)\(_2\) treatment removed over 99.5% of AMP but less than 2% of cyclic AMP from solutions of the nucleotides (1 mm).

Prostate homogenates were incubated with sheep prolactin at two different concentrations of the hormone. The low concentration (1.5 \(\mu\)g/ml) enhanced adenylate cyclase activity, but there was no effect at the higher concentration (60 \(\mu\)g/ml) (Table 2, Expt. 1). Testosterone had no effect on enzyme activity when added alone or in combination with prolactin. Human luteinizing hormone, chorionic somatomammotrophin and growth hormone stimulated adenylate cyclase activity (Table 2, Expt. 2).

Paper chromatography was performed on the 'cyclic AMP' fraction isolated from incubations of prostatic tissue and sheep prolactin. The increased radioactivity in this fraction produced by the addition of prolactin was shown to be due to a component having the mobility of cyclic AMP.

There is good reason to believe that the formation of cyclic AMP from ATP by adenylate cyclase is necessary for the mediation of protein-hormone action (Jost & Rickenberg, 1971). In the present study sheep prolactin stimulated adenylate cyclase activity when present in doses of 1.5 \(\mu\)g/ml (0.042 i.u./ml), but was ineffective at a higher concentration of 60 \(\mu\)g/ml (1.681 i.u./ml). This correlates with the results obtained by Lasnitzki (1970), who reported that prolactin at low doses (0.01-0.05 i.u./ml) would sustain the growth of rat prostate in vitro whereas
higher doses (0.10 i.u./ml) failed to maintain epithelial and secretory activity.

Testosterone did not stimulate adenylate cyclase activity in our homogenates, confirming the findings of Rosenfeld & O'Malley (1970). The steroid supports prostate growth in vitro, although this effect can be reversed by high doses of prolactin (Lasnitzki, 1970). Prolactin enhances the uptake of radioactive testosterone by the prostate (Farnsworth, 1970). In our experiments the addition of testosterone to prolactin did not augment the effect of the latter on adenylate cyclase activity. Testosterone stimulation of the prostate does not therefore appear to depend on adenylate cyclase activation per se, but its action may be potentiated by an effect of prolactin on the enzyme. In other tissues, e.g. chick oviduct, steroids can stimulate adenylate cyclase systems, at least in vivo (Rosenfeld & O'Malley, 1970).

Human chorionic somatomammotrophin stimulated adenylate cyclase activity in the prostate. Prolactin and the chorionic somatomammotrophin have a trophic effect on breast, and the latter hormone can displace prolactin from binding sites on breast tissue whereas human growth hormone is relatively inactive in this respect (Turkington, 1971). Human growth hormone stimulated prostatic adenylate cyclase activity in our experiments, a finding that is at variance with the failure of hormone to promote testosterone uptake by the prostate in vitro (Farnsworth, 1970).

Administration of luteinizing hormone in vivo stimulates prostatic growth partly by promoting testosterone secretion from the testes (Farnsworth, 1970). The present results suggest the possibility that it may work directly via the adenylate cyclase system.

Steroids have been utilized as the basis of the endocrinological treatment of prostatic neoplasia for many years (Huggins et al., 1941). The role of pituitary hormones, e.g. prolactin (Gala & Reece, 1964), released by these agents must also be considered in investigating their mechanism of action.

We thank Dr. V. C. Stevens, Dr. A. S. Hartree and Dr. P. M. Cotes for gifts of purified hormones. The generous support of the Tenovus Organization made this work possible.

Huggins, C., Scott, W. W. & Hodges, C. V. (1941) J. Urol. 46, 997
PROLACTIN STUDIES WITH THE PROSTATE


Although the androgen dependent nature of the prostate has been recognised for many years, the precise role which the pituitary protein hormones play in controlling prostatic metabolism is still far from certain. It is well established that luteinizing hormone (LH) is concerned in maintaining the weight and functional integrity of the prostate by regulating the synthesis of testosterone by the testis. However, there is also an increasing amount of evidence to suggest that a nongonadotrophin, possibly prolactin, may be directly involved in controlling the activity of the prostate. For example, there is a more marked prostatic atrophy after hypophysectomy than after orchidectomy [1,2] and the diminished prostatic response to administered androgen observed in hypophysectomized rats can be augmented by simultaneous treatment with prolactin [3,4,5]. Similarly, administration of prolactin and LH together into immature hypophysectomized rats is more effective in promoting prostatic growth than LH alone [6], although recent studies of Hafiez et al [7] suggest that prolactin acts synergistically with LH to promote testosterone synthesis and secretion by the testis. Some of these inter-relationships are illustrated in Fig.1.

![Hormones regulating prostatic growth and function](image)

Fig. 1. Hormones regulating prostatic growth and function. LH - ICSH, Luteinizing Hormone (Interstitial Cell Stimulating Hormone). ACTH, Adrenocorticotrophic Hormone. DHA, Dehydroepiandrosterone.
Lawrence and Landau [8] suggested that prolactin plays a permissive role in promoting testosterone localisation in the prostate and the report of Farnsworth [9] that prolactin increases the uptake of radioactive testosterone by slices of human prostate in vitro supports this concept. In our own studies, organ culture was used as a means of investigating the action of prolactin at the tissue level. Table 1 indicates that only comparatively high concentrations of prolactin (5 I.U./ml) were effective in increasing the uptake of [7α-3H] testosterone by rat ventral prostate in culture. At the same time within the tissue the distribution of radioactivity between testosterone and 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) changed, with a relatively decreased formation of the reduced compound. The uptake of other C19-steroids such as androstenedione, DHA or DHA-

<table>
<thead>
<tr>
<th>Prolactin (I.U. ml)</th>
<th>Specific activity (dpm/nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testosterone (T)</td>
</tr>
<tr>
<td>Control</td>
<td>35.1</td>
</tr>
<tr>
<td>5.0</td>
<td>129.4</td>
</tr>
<tr>
<td>1.0</td>
<td>44.3</td>
</tr>
<tr>
<td>0.1</td>
<td>22.6</td>
</tr>
<tr>
<td>0.01</td>
<td>43.2</td>
</tr>
</tbody>
</table>

Table 1 Effect of ovine prolactin (NIH-P-S8) on the uptake of [3H] testosterone (1.0 µCi; 5.4 Ci/mmol) by rat ventral prostate in culture. After 24 hours the cultures were washed and homogenised. Carrier steroids (300 µg) were added and the specific activity of the testosterone and 5α-dihydrotestosterone fractions determined [10].

<table>
<thead>
<tr>
<th>Steroids investigated</th>
<th>Incubation 1 (Substrate: DHA sulphate)</th>
<th>Incubation 2 (Substrate: DHA)</th>
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<tr>
<td>DHA sulphate</td>
<td>13,555</td>
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</tr>
<tr>
<td>DHA</td>
<td>7,732</td>
<td>31.60</td>
</tr>
<tr>
<td>Testosterone</td>
<td>221</td>
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</tr>
<tr>
<td>Epitestosterone</td>
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<td>0</td>
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<tr>
<td>Androstenedione</td>
<td>7.2</td>
<td>0.03</td>
</tr>
<tr>
<td>5α-Androstenedione</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>526</td>
<td>2.15</td>
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<tr>
<td>Androstosterone</td>
<td>856</td>
<td>3.5</td>
</tr>
<tr>
<td>Epianandrostosterone</td>
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<td>0.88</td>
</tr>
<tr>
<td>Androstenol</td>
<td>1,810</td>
<td>7.41</td>
</tr>
<tr>
<td>5α-Androstan-3α, 17β-diol</td>
<td>261</td>
<td>1.06</td>
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<tr>
<td>5α-Androstan-3α, 17α-diol</td>
<td>862</td>
<td>3.51</td>
</tr>
<tr>
<td>5α-Androstan-3α, 17α-diol</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2 Incubation of 2 g of minced human benign hyperplastic prostatic tissue for 2 hr. in Krebs-Ringer bicarbonate buffer at 37°C in 95% O2: 5% CO2: 11.39 µCi of DHA-sulphate were added to incubation 1, and 14.12 µCi of DHA to incubation 2. 300 µg carrier steroids were added to the incubations as before [10].
PROLACTIN & PROSTATE

Although the role of the adrenal androgens in the maintenance of prostatic function has always been considered to be of a relatively minor nature, they must assume greater significance in the castrated animal or orchidectomy patient with carcinoma of the prostate. Treatment of castrated rats with ACTH will increase the growth of the prostate [11], an effect also shown to be enhanced by prolactin. It has long been suspected that a pituitary factor other than ACTH may be concerned in the regulation of androgen synthesis and secretion by the adrenal. The possibility that prolactin may exercise such a control was studied by incubating explants of guinea pig and human adrenal tissue in organ culture (Table 3). The synthesis and secretion of androstenedione and testosterone into the medium by mixed fascicular and reticular tissue of guinea pig adrenal glands was stimulated by α²⁶⁴ACTH (1 μg/ml: 0.1 I.U./ml). Ovine prolactin (230 μg/ml: 5.0 I.U./ml) increased the secretion of testosterone but not androstenedione.

<table>
<thead>
<tr>
<th>Period</th>
<th>Hormone</th>
<th>Testosterone (pg/mg tissue)</th>
<th>Androstenedione (pg/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>39</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>ACTH (0.10 I.U.)</td>
<td>65</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.50 I.U.)</td>
<td>31</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.05 I.U.)</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>ACTH (0.10 I.U.)</td>
<td>11</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.50 I.U.)</td>
<td>11</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Prolactin (0.05 I.U.)</td>
<td>5</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 3: Effect of ovine prolactin (2nd IS ovine prolactin) on androgen production by guinea pig adrenal in organ culture. After 3 days (Period 1) the medium was removed, fresh medium added and the cultures incubated for a further 3 days (Period 2). Steroid concentrations were determined by gas liquid chromatography [12].

A similar experiment was performed with explants of separated fascicular and reticular tissue from a human adrenal gland (Table 4). Prolactin but not ACTH increased the output of androstenedione and testosterone from the fascicular tissue. However, the tissue from the zona reticularis appeared much more responsive to prolactin and ACTH with a marked effect on the secretion of testosterone. These results are of a preliminary nature and considerably more work is necessary to substantiate these observations. However, it is interesting that earlier work from this laboratory [14, 15] provided evidence that the zona reticularis may indeed be the site responsible for the synthesis and secretion of C₁₇-steroids by the adrenal cortex.

In this laboratory, further studies concerned with the elucidation of the action of prolactin at the target organ have been directed towards an investigation of the enzyme adenylate cyclase. Activation of this enzyme is one of the first steps in the mediation of protein hormone action at tissue level [16]. Specific receptors for protein hormones are found in hormone sensitive tissues and in close association with adenylate cyclase [17]. We have studied hormone responsive adenylate cyclase in tissue samples by measurement of the conversion of added [α³²P] ATP to cyclic AMP using tritium labelled cyclic-AMP to monitor losses during the procedure. Cyclic AMP was isolated from the incubation media by a two-stage batch procedure which allowed the simultaneous processing of large numbers of samples [18]. Homogeneity of the purified cyclic AMP fraction was monitored by paper chromatography [19].
Table 4. Effect of ovine prolactin (2nd IS ovine prolactin) on androgen production by the separated zones of the human adrenal cortex in organ culture. After three days the medium was removed and steroid concentrations estimated by gas liquid chromatography [12] and competitive protein binding [13].

The effect of protein hormones including prolactin on the adenylate cyclase of homogenates of whole prostates of rats is shown in Figs 2, 3 and 4. Significant stimulation of the enzyme activity was observed with the low dose of ovine prolactin (1.5 ng/ml) but not the higher dose (60 ng/ml). Significant stimulation of adenylate cyclase activity was also observed on addition of human luteinizing hormone, human growth hormone and human chorionic somatomammotrophin (Fig. 2). Testosterone did not affect the enzyme activity, (Fig. 4), thus confirming earlier observations of other investigators [20]. Furthermore,

![Image of a graph showing the effect of protein hormones on adenylate cyclase activity in an homogenate of rat prostate.](image-url)

Fig. 2. Effect of protein hormones on adenylate cyclase activity in an homogenate of rat prostate. (Mean ± SD): Estimations in quadruplicate. C, Control. OP, Ovine prolactin (NIH-P-S8). HLH, Human luteinizing hormone (IRC - 2 10.12.64). HGH, Human growth hormone (MRC Res. Std. A62/6). HCS, Human chorionic somatomammotrophin (MRC placental lactogen 70/194). NS, Non significant.
testosterone did not augment the stimulatory effect on the enzyme induced by the low doses of prolactin. It is perhaps significant that Lasnitzki [21] has shown that lower concentrations of prolactin are more effective in maintaining the prostate in tissue culture.

An insight into the range of physiological processes affected by prolactin can be gained by selectively altering the secretion of the hormone in vivo. The ergocornine derivative CB154 (Sandoz Ltd.) appears to reduce plasma concentrations of prolactin in this way [22].

Fig. 3. Paper chromatography [19] of purified 'cyclic AMP' fraction from incubations of rat prostatic homogenates and [α-32P] ATP. (a) Tissue alone. (b) Tissue + ovine prolactin (1.5 μg/ml).

Fig. 4. Effect of Testosterone and ovine prolactin (NIH-P-S8) on adenylate cyclase activity in an homogenate of rat prostate (mean ± SD). Same experiment shown in Figs. 2 and 3.
This compound has been used in these laboratories to investigate the action of prolactin in the male [24]. CB154 (100μg/day in sesame oil) was administered to groups of mature, male Sprague-Dawley rats for a period of 10 days. On the 11th day the animals were killed and tissues removed for analysis. Control groups were given injections of sesame oil alone. Other groups were adrenalectomized 48 hours before administration of CB154.

Rats that had been treated with CB154 showed a lower weight gain over the experimental period compared to the control animals [Fig. 5]. Treated and untreated adrenalectomized rats also had lower weight gains than the normal controls.

Adrenalectomy significantly increased the weight of the ventral and dorsal lobes of the prostate gland [Fig. 6]. CB154 tended to produce further weight increases although this was only shown to be significant in the dorsal lobe. This result was of course rather surprising in view of the proposed trophic action of prolactin on the lateral lobe of the prostate [23]. Possibly an eventual reduction in the total weight of the prostate induced by low prolactin levels is the final outcome of CB154 administration over longer periods and long term studies are now in progress to investigate this possibility.

![Graph](image)

Fig. 5. Effect of CB154 (100μg/day) on body weight in normal and adrenalectomized, mature, male Sprague-Dawley rats (mean ± SD). Animals killed after 10 days of treatment.

![Graph](image)

Fig. 6. Effect of CB154 (100μg/day) on prostatic weight in normal and adrenalectomized, mature, male Sprague-Dawley rats (mean ± SD). Animals killed after 10 days of treatment.
The concentration of prolactin in the plasma was measured by radioimmunoassay. Adrenalectomy alone induced a small elevation in plasma prolactin (Fig. 7). Treatment with CB154 however markedly depressed plasma prolactin in both normal and adrenalectomized groups. Plasma immunoreactive LH concentrations were also found to be elevated after adrenalectomy, with CB154 inducing further increases in both normal and adrenalectomized animals (Fig. 8). Despite considerably wide variations between individual rats, the mean concentration of testosterone was found in these experiments to be reduced by treatment with CB154 in both normal and adrenalectomized animals (Fig. 9).

Also of interest were experiments in which a group of rats had been treated with CB154 and were injected with 25 μCi[7α-3H] testosterone 1 hour before being killed. The prostates were removed and the corresponding lobes from different animals in a group pooled for analysis. After addition of carrier steroids the specific activity of extracted testosterone and 5α-dihydrotestosterone were determined [10] [Table 5]. CB154 appeared to have little overall effect on the specific activities of the isolated carrier steroids except perhaps a slight decrease in the 5α-dihydrotestosterone: testosterone ratio. However, bearing in mind the decreased levels of plasma testosterone due to CB154 administration, then it would appear that these results agree with those of Lawrence and Landau [8] that decreased levels of prolactin lead to a decreased localisation of testosterone in the prostate gland.

Physiological evidence has been reported for an increased secretion of gonadotrophins.
Table 5. Uptake of testosterone by the prostate of normal (non-adrenalectomized), mature, male Sprague-Dawley rats after 10 days of treatment with CB154 (100 µg/day). Animals injected with 25 µCi of [3H] testosterone 1 hour before being killed. Lobes from different animals pooled and homogenized. Specific activity of extracted steroid fractions determined as before [10].

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>CB 154</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>5α dihydro-testosterone</td>
<td>39.0</td>
<td>31.5</td>
</tr>
<tr>
<td>5α T/T</td>
<td>12.0</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Fig. 9. Effect of CB154 (100 µg/day) on plasma testosterone (mean ± SD) in normal and adrenalectomized, mature, male Sprague-Dawley rats. Animals killed 10 days after treatment. Testosterone concentration estimated by gas-liquid chromatography [12].

The results of the study now reported, showing increased levels of LH in the plasma of rats treated with CB154, provide direct support for these earlier findings. A direct effect of this drug on the hypothalamic-pituitary axis could be responsible for this observation since there appears to be, in certain circumstances, a reciprocal relationship between prolactin and gonadotrophin secretion [22]. Alternatively, the fall in the concentration of plasma testosterone could have been the determining factor.

Our investigations, and those reported earlier by other workers, suggest that prolactin may be concerned with the synthesis and secretion of androgens by the adrenal gland and the testis. If in fact, prolactin plays a role in androgen production, then the decreased levels of testosterone observed after CB154 administration may be the result of lowered plasma prolactin. A summary of the effects of CB154 is given in Fig. 10. Adrenalectomy produced small but significant changes in plasma LH levels and in the weight of the dorsal and ventral prostatic lobes. These observations provide additional evidence that pituitary hormones other than ACTH may be concerned in the regulation of adrenal metabolism.

In conclusion, our studies clearly suggest that the nature of the metabolic response of
the prostate to circulating prolactin may be dependent on the concentration of the hormone in the plasma. These and other results described in this report indicate that the end-organ effect mediated by prolactin may depend upon a relatively low hormone concentration. Furthermore, any consideration given to the treatment of carcinoma of the prostate with anti-prolactin drugs must take into account secondary effects induced in other hormonal systems.

Fig. 10. Effect of CB154 on hormones which regulate prostatic growth.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the generous financial support of the Tenovus Organisation. They are also grateful to the National Institute of Health, Endocrine Study Section, Bethesda, USA for kindly supplying kits for the determination of rat prolactin and LH, and also for purified ovine prolactin. They wish to thank the MRC Reference Laboratory, Mill Hill for a generous gift of placental lactogen, LH and GH, and Dr. E. R. Evans, (Sandoz Ltd.) for samples of CB154.
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22. Fluckiger, E. - this volume.
25. Fluckiger, E. (Personal communication).
Maternal plasma concentrations exhibited no significant change in basal values of approx. 15 μg/100ml from 116 days gestation until 1–2 days pre-partum.

At term the concentrations rose to at least five times the basal values, but accurate computation was not possible as term values were beyond the range of the standard curve employed in this study.

In summary, we have demonstrated the presence of prolactin in foetal and maternal plasma in the last fifth of gestation, the concentrations showing a tendency to increase towards term in the former. Prolactin, although in low amounts, was also measured in foetal fluids, with the greater concentration occurring in the allantoic compartment.

Any definite interpretation of these results must await the acquisition of more results and further investigation into the prolactin-like activity that has been measured.

The authors are grateful to the Wellcome Trust and Tenovus Organization for generous financial support and to the Len West Research Laboratory for surgical facilities. Thanks are due to Dr. H. Buttle and Dr. A. Cowie for the gift of plasma from hypophysectomized sheep and to Dr. R. Crighton for providing the sheep pituitary hormones. Dr. A. R. Boyns kindly prepared the rabbit anti-(ovine prolactin) serum. The Statistics Department of the Welsh National School of Medicine, Cardiff, gave considerable help in formulating the rejection criterion of the sample data.


The Effects of Certain Stilboestrol Analogues on Plasma Prolactin and Testosterone Concentrations in the Rat

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Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff CF4 4XX, U.K.

The androgen-dependent nature of human prostatic cancer was established by the classical experiments of Huggins & Hodges (1941). The treatment of this disease by anti-androgen therapy such as the administration of diethylstilboestrol followed from these studies (Huggins et al., 1941). It is not completely understood, however, whether the principal effect of oestrogen treatment is on the pituitary, thereby decreasing the secretion of interstitial-cell-stimulating hormone with a subsequent lowering of plasma testosterone concentration, or by a more direct action on either the testis or prostate. Studies in this laboratory (Harper et al., 1970) indicated that diethylstilboestrol and certain stilboestrol analogues, in particular dibutyldihydrostilboestrol, inhibited in vitro DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7) isolated from human neoplastic prostate tissue. Further, dibutyldihydrostilboestrol was shown to affect the formation of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) by explants of prostatic tissue in culture (Groom et al., 1971). Since dibutyldihydrostilboestrol possesses 0.1% of the oestrogenic activity of diethylstilboestrol (Grundy, 1957), it was suggested that it may well be of value in the treatment of prostatic tumours, and the present report describes further investigations on the comparative biological effects in the rat of oestradiol-17β, diethylstilboestrol, (±)-dibutyldihydrostilboestrol and mesto-dibutyl-dihydrostilboestrol.

Male Sprague–Dawley rats (aged 11–13 weeks) were given a commercial rat diet and tap water ad libitum. They were divided into five groups of six animals each and

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injected (intramuscularly) daily with 100 μg of oestradiol-17β, diethylstilboestrol, (±)-dibutylidihydrostilboestrol or meso-dibutylidihydrostilboestrol (the samples of dibutylidihydrostilboestrol were prepared by the courtesy of Dr. A. L. Walpole and Dr. D. Richardson, Imperial Chemical Industries Ltd., Alderley Edge, Cheshire, U.K.) suspended in 0.2 ml of sesame oil for 10 days. Control animals were injected with vehicle alone. On day 11 the animals were anaesthetized with ether, bled by cardiac puncture and killed by cervical dislocation. The organs were removed and weighed. Plasma samples were stored at -20°C. Plasma testosterone was measured in pooled plasma from each group of animals.

The plasma was made alkaline, then, after the addition of [1,2,6,7-3H4]testosterone to monitor losses during analysis, extracted with diethyl ether, and the extracts were washed with 1% acetic acid. The dried extracts were dissolved in light petroleum (b.p. 40–60°C) and chromatographed on a flexible silica-gel sheet with the solvent system chloroform-acetone (40:3, v/v). Testosterone was eluted with methanol, dried and converted into the iodomethylsilyl ether. The iodomethylsilyl ethers were partitioned between water and hexane, and the hexane extract was dried and chromatographed on a flexible alumina sheet with the solvent system benzene–cyclohexane–acetone (20:20:1, by vol.). The derivative was eluted with acetone and dried, and 17β-hydroxyandrosten-14-dien-3-one iodomethylsilyl ether in hexane was added for subsequent determination by g.l.c.

Immunoreactive prolactin and interstitial-cell-stimulating hormone were measured in plasma samples by double-antibody radioimmunoassays with kits distributed by the National Institute for Arthritis and Metabolic Diseases, Bethesda, Md., U.S.A.

Table 1 shows that, whereas oestradiol-17β, diethylstilboestrol and meso-dibutylidihydrostilboestrol decreased the weight of all three prostatic lobes, (±)-dibutylidihydrostilboestrol administration produced only a slight effect on the lateral lobes. The seminal vesicles were markedly decreased in weight by oestradiol-17β, diethylstilboestrol or meso-dibutylidihydrostilboestrol. Oestradiol-17β and diethylstilboestrol administration also decreased the weight of the testes. All the compounds studied increased the weight of the adrenal glands. Table 2 shows that diethylstilboestrol increased plasma prolactin concentration and decreased those of interstitial-cell-stimulating hormone and testosterone. Oestradiol-17β and meso-dibutylidihydrostilboestrol increased the plasma prolactin concentration and lowered the testosterone concentration without showing any significant effect on that of plasma interstitial-cell-stimulating hormone.

Although the decrease in weight of the male sex accessory organs after administration of oestradiol-17β, diethylstilboestrol and meso-dibutylidihydrostilboestrol may well be related to the observed decrease in plasma testosterone concentration, it is noteworthy that the concentration of interstitial-cell-stimulating hormone fell only after diethylstilboestrol treatment. This could indicate that oestradiol-17β and meso-dibutylidihydrostilboestrol have a more direct action on the testis, an effect shown previously by Slauwhte et al. (1962) and Harper et al. (1971). However, oestrogens have also been shown to have a direct effect on the prostate in vitro (Farnsworth, 1969; Groom et al., 1971), which in part may explain the decreased weight of the accessory organs. Further, since adrenocorticosteroids have been shown to decrease the effective action of testosterone on the accessory organs (Tveter & Aakvaag, 1969; Tisell, 1972), the observed hypertrophy of the adrenal glands after oestradiol-17β, diethylstilboestrol and meso-dibutylidihydrostilboestrol administration may also be concerned in the overall effect of these compounds on prostatic weight.

Further studies are obviously necessary to elucidate the precise role of prolactin in the control of prostatic growth and function. It is known that there is a more marked atrophy of the prostate after hypophysectomy than after orchidectomy (Huggins & Russell, 1946; Lostroh & Li, 1957), and the diminished prostatic response to administered androgen observed in hypophysectomized rats can be augmented by simultaneous treatment with prolactin (Vanderlaan, 1953; Grayhack, 1963). Evidence of a direct stimulatory action of prolactin in vitro has also been presented (Farnsworth, 1970; Lasnitzki, 1970). Of interest therefore was the observed decrease in prostatic growth.
Table 1. Effects of oestradiol-17β, diethylstilboestrol and its derivatives on the weight of certain organs (prostate, seminal vesicles, testes and adrenals) in mature male Sprague-Dawley rats

Group of rats were given daily intramuscular injections of 100μg of compound in 0.2ml of sesame oil or vehicle alone (controls). Organ weights (means±s.d.) are expressed as fractions of initial body weight. Statistical significance was determined by Student's *t* test: *P* (versus controls) <0.05; †*P* (versus controls) <0.005.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ventral prostate</th>
<th>Lateral prostate</th>
<th>Dorsal prostate</th>
<th>Seminal vesicles</th>
<th>Testes</th>
<th>Adrenals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.27±0.35</td>
<td>0.36±0.15</td>
<td>0.44±0.13</td>
<td>3.13±1.27</td>
<td>11.65±1.79</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>0.46±0.10†</td>
<td>0.16±0.05*</td>
<td>0.21±0.05†</td>
<td>0.74±0.20†</td>
<td>7.88±0.35†</td>
<td>0.24±0.07*</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>0.52±0.24†</td>
<td>0.17±0.04*</td>
<td>0.23±0.02†</td>
<td>0.80±0.12†</td>
<td>8.09±0.93†</td>
<td>0.25±0.04†</td>
</tr>
<tr>
<td>(±)-Dibutylidihydrostilboestrol</td>
<td>1.31±0.25</td>
<td>0.31±0.02*</td>
<td>0.39±0.10</td>
<td>2.80±0.63</td>
<td>9.84±0.99</td>
<td>0.17±0.01*</td>
</tr>
<tr>
<td>meso-Dibutylidihydrostilboestrol</td>
<td>0.66±0.33*</td>
<td>0.14±0.05*</td>
<td>0.18±0.09*</td>
<td>0.91±0.63*</td>
<td>10.69±0.81</td>
<td>0.19±0.01†</td>
</tr>
</tbody>
</table>
Table 2. Effects of oestradiol, diethylstilboestrol and its derivatives on the concentrations of plasma hormones in mature male Sprague-Dawley rats

Groups of rats were treated in the same way as described in Table 1. *P (versus controls) < 0.05; †P (versus controls) < 0.005. Values for prolactin and interstitial-cell-stimulating hormone are means ± s.d.; those for testosterone are (a) single measurements of pooled plasma from three rats or (b) averages of two measurements of two separate pooled plasma samples from four rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prolactin</th>
<th>Interstitial-cell-stimulating hormone</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.3 ± 5.5</td>
<td>93.7 ± 8.4</td>
<td>3.70 (a)</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>57.8 ± 19.3†</td>
<td>83.5 ± 16.1</td>
<td>0.20 (a)</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>41.0 ± 9.8*</td>
<td>76.2 ± 9.3*</td>
<td>0.30 (b)</td>
</tr>
<tr>
<td>(±)-Dibutyldihydrostilboestrol</td>
<td>31.6 ± 6.0</td>
<td>95.6 ± 10.8</td>
<td>3.43 (b)</td>
</tr>
<tr>
<td>meso-Dibutyldihydrostilboestrol</td>
<td>51.3 ± 10.9†</td>
<td>83.5 ± 11.0</td>
<td>0.25 (a)</td>
</tr>
</tbody>
</table>

weight in the presence of high prolactin concentrations after oestrogen administration in these studies now reported, and it would seem that the more direct action of the oestrogen overrides that mediated by prolactin.

The finding that the biological changes caused by meso-dibutyldihydrostilboestrol are similar to those produced by diethylstilboestrol provides additional evidence that this compound is worthy of further study.

The authors acknowledge the generous financial support of the Tenvous Organization. V. D. is grateful to the British Council for a scholarship to enable her to study in this country. They are also grateful to the National Institutes of Health Endocrine Study Section, Bethesda, Md., U.S.A., for kindly supplying kits for the determination of rat prolactin and interstitial-cell-stimulating hormone.

Huggins, C. & Hodges, C. V. (1941) *Cancer Res.* 1, 293–297
THE EFFECT OF CERTAIN STILBOESTROL ANALOGUES ON PLASMA PROLACTIN AND TESTOSTERONE IN THE RAT

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(Received 14 August 1972)

SUMMARY

Oestradiol-17β, diethylstilboestrol (DES), dl-dihydrodibutylstilboestrol (dl-DHBS) and meso-dihydrodibutylstilboestrol (meso-DHBS) were injected intramuscularly into male Sprague-Dawley rats in a daily dose of 100 μg for a period of 10 days. Oestradiol-17β and DES decreased the weight of the prostate and seminal vesicles to the same extent, whereas meso-DHBS was less effective. dl-DHBS was almost inactive. Only oestradiol-17β and DES caused a decrease in the weight of the testes. The adrenal glands increased in weight after administration of either oestradiol-17β, DES or meso-DHBS.

Four hormones in the plasma were measured: testosterone, androstenedione, prolactin and interstitial cell-stimulating hormone (ICSH). DES decreased the plasma concentration of both ICSH and testosterone. Oestradiol-17β and meso-DHBS administration resulted in a lowering of the plasma testosterone concentration with no effect on ICSH. Oestradiol-17β, DES and meso-DHBS markedly increased plasma prolactin concentrations. dl-DHBS appeared to have little biological effect causing only very small changes in all the parameters investigated.

INTRODUCTION

The treatment of carcinoma of the prostate by administration of diethylstilboestrol (Huggins, Scott & Hodges, 1941) followed from classical experiments of Huggins & Hodges (1941) which established the androgen-dependent nature of prostatic cancer. It is accepted that part of the antiandrogenic effect of oestrogen therapy is exercised indirectly on prostatic tissue via the pituitary, reducing testosterone secretion by decreasing the release of interstitial cell-stimulating hormone (ICSH). Such an effect has been described in patients with prostatic carcinoma (Alder, Burger, Davis, Dulmanis, Hudson, Sarafaty & Straffon, 1968). Studies in vitro (Slaunwhite, Sandberg, Jackson & Staubitz, 1962; Harper, Pierrepoint, Fahmy & Griffiths, 1971) showed a decreased capacity to synthesize testosterone in human and canine testicular tissue after oestrogen treatment in vivo. Other investigations have also indicated a direct action...
of oestrogens on the testis (Samuels, Short & Huseby, 1964; Oshima, Wakabayashi & Tamaoki, 1967).

However, oestrogen may have a direct action on the prostate itself. Goodwin, Rasmussen-Taxdal, Ferreira & Scott (1961) described the inhibition of prostatic secretion by diethylstilboestrol in the androgen-maintained hypophysectomized dog, and the addition of oestradiol-17β and diethylstilboestrol affects testosterone metabolism by prostatic tissue in vitro (Farnsworth, 1969; Farnsworth, 1970; Groom, Harper, Fahmy, Pierrepoint & Griffiths, 1971). Furthermore, following the report (Fahmy & Griffiths, 1968) that diethylstilboestrol and some of its analogues inhibited the activity in vitro of calf thymus DNA polymerase (DNA nucleotidyl-transferase EC 2.7.7.7), it was shown that these compounds had a similar inhibitory effect on the enzyme in human neoplastic prostatic tissue (Harper, Fahmy, Pierrepoint & Griffiths, 1970). Alkyl substitution in the αα'-position of stilboestrol appeared to increase the inhibitory effect on DNA polymerase while both diethylstilboestrol and dihydrodibutylstilboestrol, which contains a saturated αα'-'ethylenic linkage, consistently inhibited enzymic activity. Oestradiol-17β and cis- and trans- isomers of dibutylstilboestrol had little or no effect on the enzyme. Since dihydrodibutylstilboestrol has only 0.1% of the oestrogenic activity of diethylstilboestrol (Grundy, 1957) further studies on its biological action in the rat might be of value with regard to its possible use in the treatment of prostatic cancer. This report describes the effects in the rat of oestradiol-17β, diethylstilboestrol, DL-dihydrodibutylstilboestrol and meso-dihydrodibutylstilboestrol. The last two compounds were synthesized by ICI Ltd, Alderley Edge, with the kind co-operation of Dr D. N. Richardson and Dr A. L. Walpole.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were used. They were given a commercial rat diet (Pilsburys Ltd, Birmingham) and tap water ad libitum.

Experiment 1. Rats (11–13 weeks old) were divided into five groups of six and received daily intramuscular injections for 10 days of either oestradiol-17β, diethylstilboestrol (DES) (Koch-Light Laboratories, Bucks), DL-dihydrodibutylstilboestrol (DL-DHBS) or meso-dihydrodibutylstilboestrol (meso-DHBS), 100 μg of each suspended in 0.2 ml sesame oil. Control animals were injected with vehicle alone. On the 11th day the animals were anaesthetized with ether, bled by cardiac puncture and killed by cervical dislocation. The organs were removed and weighed. Plasma samples were stored at −20 °C.

Experiment 2. This was similar in design to experiment 1, although the rats were slightly older (16–17 weeks). There were 24 animals in the control group and 18 in each of the experimental groups. In this experiment the daily food intake of each cage of rats (six animals) was measured by the difference between a weighed amount of food and the food remaining on the following day. The change in body weight of each rat during the experimental period and the weight of each organ at the end of the experiment were expressed as a fraction of the initial body weight.

Statistical significance was determined by Student’s t-test.
Effects of oestrogens on hormone secretion

Plasma samples from the different experimental groups were pooled for steroid analysis. Plasma was made alkaline by the addition of 2 m-sodium hydroxide, and [1,2,6,7,2H5]testosterone and [1,2-3H]androstenedione were added to monitor losses during analysis. Plasma samples were twice extracted with 10 ml diethyl ether and the pooled ether extracts washed once with 5 ml 1 % acetic acid. The washed extracts were then evaporated and the residues dissolved in petroleum ether (b.p. 40–60 °C) and chromatographed on a flexible silica gel sheet (Macherey-Nagel and Co., Polygram Sil G/UV254 Camlab, Cambridge) using the solvent system chloroform: acetone (40:3, v/v). The testosterone (RF 0·40) and androstenedione (RF 0·80) were eluted with methanol. Androstenedione was reduced to testosterone by 17β-hydroxysteroid dehydrogenase, released from Pseudomonas testosterone by sonication at pH 6·0, and subsequently processed identically. Testosterone was converted to the iodomethylsilyl ether derivative (Thomas, 1971). The derivative was then partitioned between water and hexane, the hexane extracts were evaporated and the residue was chromatographed on a flexible alumina sheet (Macherey-Nagel and Co., Polygram Alox N/UV 254 Camlab, Cambridge) using the solvent system benzene:cyclohexane: acetone (20:20:1, by vol.). The testosterone iodomethylsilyl ether (RF 0·50) was eluted with acetone and 17β-hydroxyandrost-1,4-dien-3-one iodomethylsilyl ether added. An aliquot of the extract was removed for liquid scintillation counting; the remainder was evaporated and redissolved in hexane for gas–liquid chromatography on 1 % XE 60. Measurement was by pulsed (150 μs) electron capture detection.

Measurement of plasma prolactin and ICSH

Immunoreactive prolactin and ICSH were measured in plasma samples by double antibody radioimmunoassay using kits distributed by the National Institute for Arthritis and Metabolic Diseases (NIAMD), Bethesda, Md, U.S.A.

Prolactin. NIAMD rat prolactin-I-1 was labelled with [131I]iodine (Radiochemical Centre, Amersham) by the method of Greenwood, Hunter & Glover (1963) and separated from iodide on columns of Biogel P-60 (Bio-Rad Laboratories). The labelled hormone was diluted in assay buffer (50 mM-phosphate buffer, pH 7·3, containing 0·01 % merthiolate and 0·5 % human plasma albumin) to give a concentration of approximately 20000 c.p.m./100 μl (5 ng/ml). Antiserum (NIAMD anti-rat prolactin-S-1) was diluted (1/100000) in 50 mM-phosphate buffer containing 50 mM-EDTA and 1/400 non-immune rabbit serum. Standard rat prolactin (NIAMD rat prolactin-RP-1) was diluted in assay buffer to give standard solutions in the range 1–125 ng/ml. Antisera to rabbit serum globulins were raised in sheep by the intramuscular injection of the antigen emulsified in Freund’s complete adjuvant and were diluted (1:10) in assay buffer. Standard curves were prepared as follows: 100 μl prolactin standard were mixed with 200 μl of antibody to prolactin, 100 μl plasma from a hypophysectomized male rat and 300 μl assay buffer. After incubation for 24 h at 4 °C, 100 μl 131I-labelled prolactin were added and the solution incubated for 24 h at 4 °C. Finally, 200 μl sheep anti-rabbit globulin serum were added and the solution was incubated overnight at 4 °C. The tubes were centrifuged at 1500 g for 30 min at 4 °C and the supernatants decanted. After draining, the tubes were
counted in a Nuclear Chicago gamma counter. When plasma samples were assayed, a 100 µl sample replaced the standard prolactin solution and 100 µl assay buffer replaced plasma from a hypophysectomized male rat. Assays were carried out in duplicate, the radioactivity in each tube being expressed as a percentage of the radioactivity present in the tubes containing the zero standard.

**ICAL**. NIAMD rat LH-I-2 was labelled with [131I]iodine by the methods described above and used at a concentration of 5 ng/ml. Antiserum (NIAMD anti-rat LH-S-1) was diluted 1:20,000. Standard rat LH (NIAMD rat LH-R-P-1) was diluted to give standard solutions in the range 6–400 ng/ml. Other components of the assay system were similar to those used in the prolactin assay, except that the sample volume was 200 µl and horse serum was included instead of plasma from hypophysectomized rats. Standard solution, diluted antisera, horse serum and buffer were incubated at 4 °C for 2 days before addition of labelled hormone. Sheep antibodies were added after a further 3 days incubation at 4 °C.

**Table 1. Effect of oestradiol-17β, diethylstilboestrol (DES) and its derivatives, on the food intake and change in body weight in mature male Sprague–Dawley rats (means ± S.D.)**

(Groups of rats were given daily i.m. injections of 100 µg of compound in 0·2 ml sesame oil or vehicle alone (controls) for 10 days. Animals were killed on the 11th day.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Food intake (g/age of 6 rats/day)</th>
<th>Change in body weight (g/kg initial body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>184·6±12·0</td>
<td>+48·2±24·1</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>18</td>
<td>129·0±17·6***</td>
<td>+99·6±91·7***</td>
</tr>
<tr>
<td>DES</td>
<td>18</td>
<td>80·9±24·7***</td>
<td>-151·3±40·8***</td>
</tr>
<tr>
<td>DL-DHBS</td>
<td>18</td>
<td>167·3±11·1***</td>
<td>+30·9±18·9***</td>
</tr>
<tr>
<td>Meso-DHBS</td>
<td>18</td>
<td>158·4±13·8***</td>
<td>-7·7±22·3***</td>
</tr>
</tbody>
</table>

Significance of difference from controls: * P < 0·1; ** P < 0·05; *** P < 0·001. DL-DHBS = DL-dihydrodibutylstilboestrol; Meso-DHBS = Meso-dihydrodibutylstilboestrol.

**RESULTS**

**Food intake and body weight**

Table 1 shows the food intake and the change in body weight of animals during experiment 2. Rats in all the experimental groups ate less than the controls, the greatest effect being found with oestradiol-17β and DES. Animals treated with oestradiol-17β, DES or Meso-DHBS lost weight in comparison with the controls. Although the DL-DHBS-treated group gained body weight, the gain was significantly less than that of the controls.

**Organ weight**

Table 2 shows the weights (mean ± s.d.) of organs after 10 days of treatment with each compound.

**Prostate**

**Experiment 1 (Table 2)**

Oestradiol-17β, DES and Meso-DHBS reduced the weight of all three prostatic lobes. The effects of the compounds were not significantly different in any of the lobes. DL-DHBS had a small but significant effect on the lateral lobes only.
Table 2. Effect of oestradiol-17β, diethylstilboestrol (DES) and its derivatives, on organ weights in mature Sprague–Dawley rats (means ± s.d.)

(Groups of rats were given daily i.m. injections of 100 μg of compound in 0-2 ml sesame oil or vehicle alone (controls) for 10 days. Animals were killed on the 11th day.)

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Ventral prostate</th>
<th>Lateral prostate</th>
<th>Dorsal prostate</th>
<th>Seminal vesicles</th>
<th>Testes</th>
<th>Adrenals</th>
<th>Kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>6</td>
<td>1.27 ± 0.35</td>
<td>0.36 ± 0.15</td>
<td>0.44 ± 0.13</td>
<td>3.13 ± 0.27</td>
<td>11.65 ± 1.79</td>
<td>0.15 ± 0.02</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oestradiol-17β</td>
<td>6</td>
<td>0.46 ± 0.10</td>
<td>0.18 ± 0.05*</td>
<td>0.21 ± 0.05†</td>
<td>0.74 ± 0.20†</td>
<td>7.88 ± 0.35†</td>
<td>0.24 ± 0.07*</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>6</td>
<td>0.53 ± 0.24†</td>
<td>0.17 ± 0.04*</td>
<td>0.23 ± 0.02†</td>
<td>0.80 ± 0.12†</td>
<td>8.09 ± 0.93†</td>
<td>0.25 ± 0.04†</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>DL-DHBS</td>
<td>6</td>
<td>1.31 ± 0.25</td>
<td>0.31 ± 0.02*</td>
<td>0.39 ± 0.10</td>
<td>2.80 ± 0.63</td>
<td>9.84 ± 0.99</td>
<td>0.17 ± 0.01*</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>meso-DHBS</td>
<td>6</td>
<td>0.66 ± 0.33*</td>
<td>0.14 ± 0.05*</td>
<td>0.18 ± 0.09*</td>
<td>0.94 ± 0.63*</td>
<td>10.69 ± 0.81</td>
<td>0.19 ± 0.01†</td>
<td>—</td>
</tr>
</tbody>
</table>

| 2             | Control       | 24             | 1.49 ± 0.30      | 0.26 ± 0.04      | 0.37 ± 0.07     | 3.27 ± 0.55      | 7.40 ± 0.88 | 0.11 ± 0.01 | 6.69 ± 0.45 |
|               | Oestradiol-17β| 18             | 0.31 ± 0.10†     | 0.16 ± 0.04†     | 0.23 ± 0.06†    | 0.91 ± 0.22†     | 6.51 ± 0.90† | 0.15 ± 0.02† | 6.63 ± 0.58 |
|               | DES           | 18             | 0.40 ± 0.17†     | 0.16 ± 0.03†     | 0.24 ± 0.04†    | 1.03 ± 0.57†     | 6.27 ± 0.57† | 0.19 ± 0.05† | 6.38 ± 0.68 |
|               | DL-DHBS       | 18             | 1.36 ± 0.27      | 0.27 ± 0.05      | 0.37 ± 0.05     | 3.39 ± 0.48      | 7.46 ± 0.59 | 0.10 ± 0.01† | 6.28 ± 0.42 |
|               | meso-DHBS     | 18             | 1.09 ± 0.23†**   | 0.23 ± 0.04      | 0.32 ± 0.06**   | 2.64 ± 0.60†     | 7.17 ± 0.67 | 0.13 ± 0.01† | 6.42 ± 0.59 |

Significance of differences from:
- Controls: *P < 0.05, †P < 0.005;
- meso-DHBS treatment: **P < 0.01;
- Oestradiol-17β and DES treatments: ***P < 0.001;
- Oestradiol-17β, DL-DHBS and meso-DHBS treatments: $P < 0.005.

DL-DHBS = DL-dihydrodibutyroestrol; meso-DHBS = meso-dihydrodibutyroestrol.
Experiment 2 (Table 2)

Oestradiol-17\(\beta\) and DES consistently decreased the weight of all prostatic lobes. *meso*-DHBS was less effective in reducing the weight of the ventral and dorsal lobes when compared with experiment 1. Although *meso*-DHBS seemed to diminish lateral lobe weight, this change was not statistically significant.

**Seminal vesicles and testes**

In both experiments the weight of the seminal vesicles fell in the animals treated with either oestradiol-17\(\beta\), DES or *meso*-DHBS. The weight of this organ remained unchanged in the dl-DHBS-treated group. Testicular weight fell in animals which had received oestradiol-17\(\beta\) or DES but no significant change was observed in the other treated groups.

Table 3. Effect of oestradiol-17\(\beta\), diethylstilboestrol (DES) and its derivatives, on the plasma concentrations of hormones in mature Sprague–Dawley rats (means ± S.D.)

(Groups of rats were given daily i.m. injections of 100 \(\mu\)g of compound in 0-2 ml sesame oil or vehicle alone (controls) for 10 days. Animals were killed on the 11th day.)

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Prolactin (ng/ml)</th>
<th>ICSH (ng/ml)</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>6</td>
<td>24.3 ± 5.5</td>
<td>93.7 ± 8.4</td>
<td>3.70 (a)</td>
</tr>
<tr>
<td></td>
<td>Oestradiol-17(\beta)</td>
<td>6</td>
<td>57.8 ± 19.3(\dagger)</td>
<td>83.5 ± 16.1</td>
<td>0.20 (a)</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>6</td>
<td>41.0 ± 9.8(\ast)</td>
<td>76.2 ± 9.3(\ast)</td>
<td>0.30 (b)</td>
</tr>
<tr>
<td></td>
<td>dl-DHBS</td>
<td>6</td>
<td>31.6 ± 6.0</td>
<td>96.5 ± 10.8</td>
<td>3.43 (b)</td>
</tr>
<tr>
<td></td>
<td><em>meso</em>-DHBS</td>
<td>6</td>
<td>51.3 ± 10.9(\dagger)</td>
<td>83.5 ± 11.0</td>
<td>0.25 (a)</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>24</td>
<td>25.3 ± 9.3</td>
<td>93.8 ± 21.5</td>
<td>1.65 (c)</td>
</tr>
<tr>
<td></td>
<td>Oestradiol-17(\beta)</td>
<td>18</td>
<td>69.7 ± 13.9(\dagger)</td>
<td>98.9 ± 33.1</td>
<td>&lt; 0.50 (d)</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>18</td>
<td>55.8 ± 13.6(\dagger)</td>
<td>79.8 ± 11.8(\ast)</td>
<td>&lt; 0.50 (d)</td>
</tr>
<tr>
<td></td>
<td>dl-DHBS</td>
<td>18</td>
<td>26.8 ± 11.3</td>
<td>98.0 ± 20.0</td>
<td>0.95 (d)</td>
</tr>
<tr>
<td></td>
<td><em>meso</em>-DHBS</td>
<td>18</td>
<td>51.6 ± 15.6(\dagger)</td>
<td>107.5 ± 13.9(\ast)</td>
<td>0.63 (d)</td>
</tr>
</tbody>
</table>

dl-DHBS = dl-dihydrodibutylstilboestrol; *meso*-DHBS = meso-dihydrodibutylstilboestrol; ICSH = interstitial cell-stimulating hormone. Significance of difference from controls: \(\ast\) \(P < 0.05\); \(\dagger\) \(P < 0.005\). (a) Single measurement of pooled plasma samples from three rats; (b) average value of two measurements of two separate pooled plasma samples from four rats; (c) average value of four measurements of five separate pooled plasma samples from 24 rats; (d) average value of three separate pooled plasma samples from 18 rats.

**Adrenal glands**

In experiment 1, all the compounds studied significantly increased the weight of the adrenal glands. Although the mean weight of the adrenal glands in animals treated with oestradiol-17\(\beta\) or DES were higher than those in the *meso*-DHBS-treated group, only that of animals treated with DES was significantly higher \((P < 0.01)\). In experiment 2, the increase in adrenal weight produced by DES was significantly greater than that produced by oestradiol-17\(\beta\) or *meso*-DHBS \((P < 0.005)\). The effect of dl-DHBS was equivocal: in experiment 1 there was an increase, whereas in experiment 2 the weight was slightly but significantly decreased.

**Other organs**

The administration of the oestrogens had no effect on the weight of the kidneys.
Effects of oestrogens on hormone secretion

Plasma testosterone and androstenedione

Oestradiol-17β, DES and meso-DHBS markedly decreased the level of plasma testosterone in both experiments 1 and 2 (Table 3). Although dl-DHBS had no obvious effect on plasma testosterone in experiment 1, there was a slight decrease in experiment 2. Androstenedione was undetectable (< 0.2 ng/ml) in control and treated animals.

Plasma prolactin and ICSH

Prolactin concentrations were increased in the plasma of animals treated with oestradiol-17β, DES or meso-DHBS (Table 3). In the dl-DHBS-treated groups the concentration remained unchanged. Administration of DES decreased ICSH levels in both experiments. No effect was demonstrable with meso-DHBS in experiment 1, but in experiment 2 there was a significant increase in ICSH plasma concentrations.

DISCUSSION

The administration of oestradiol-17β and DES leads to a reduction in the weight of the male accessory organs (Woodruff & Umiker, 1960; Perklev, 1971; Schreiber, Pribyl & Rohačová, 1971). It is well known that oestrogens have an indirect inhibitory effect on the secretion of testosterone by the testes by means of pituitary suppression of ICSH release. Although results obtained in the rats injected with DES support this concept, animals which received either oestradiol-17β or meso-DHBS did not show a significantly lower ICSH level than the controls. However, there was a decrease in plasma testosterone after oestradiol-17β or meso-DHBS administration, despite the unchanged, apparently normal, level of ICSH. This may not be unreasonable since a direct inhibitory action of oestrogens on the testes has been suggested (Slaunwhite et al. 1962; Oshima et al. 1967; Harper et al. 1971).

Apart from their effect on the pituitary and testis, oestradiol-17β and DES have been shown to affect several steps concerned with the regulation of prostatic growth, inhibition of testosterone metabolism (Farnsworth, 1969; Groom et al. 1971), nucleic acid synthesis and metabolism (Mangan, Neal & Williams, 1967) and decreased uptake and retention of 5α-dihydrotestosterone by prostatic cell nuclei (Fang & Liao, 1969). The activity of DNA polymerase was shown to be inhibited by stilboestrol and some of its derivatives including DHBS (Fahmy & Griffiths, 1968; Harper et al. 1970). DHBS was as effective as DES and oestradiol-17β in inhibiting the conversion of [7α-3H]testosterone to 5α-dihydrotestosterone in organ cultures of rat and dog prostate (Groom et al. 1971).

The increase in adrenal weight after the administration of oestrogen has been shown by a number of investigators (Selye, Collip & Thomson, 1935; Kitay, 1963; Perklev & Groning, 1969; Perklev, 1971; Schreiber et al. 1971). It has been postulated that the oestrogen stimulates the pituitary gland causing hypersecretion of adrenocorticotropic hormone and adrenal hyperplasia (Selye & Collip, 1936). These mechanisms have been reviewed by Kitay (1968). Other investigators (Tveter & Aakvaag, 1969; Tisell, 1972) have shown that adrenocorticosteroids decrease the effect of testosterone on the male accessory reproductive organs. Therefore, the adrenal glands
may play a role in the regulation of steroid metabolism by the prostate although this might be of minor importance in the intact animal.

Following the report of Grayhack, Bunce, Kearns & Scott (1955) that prolactin augmented the prostatic response to testosterone, this anterior pituitary hormone has received attention from several workers who have investigated its role in the control of prostatic growth and function. Farnsworth (1970) stated that prolactin increased the uptake of radioactive testosterone by slices of human prostatic tissue. Since oestrogens stimulate prolactin release (Ratner, Talwalker & Meites, 1963; Ratner & Meites, 1964; van der Gugten, Sala & Kwa, 1970), it might be expected that oestrogen administration would increase prostatic weight. However, since there is usually a decrease in the weight of this organ, other effects of oestrogens must override the trophic effect of prolactin on the prostate. It must be noted that oestrogens induced loss of appetite and a decrease in body weight in our experiment. The smaller change in weight of the organs of animals given meso-DHBS as compared with the oestradiol-17β- or DES-treated groups may be due to the fact that the last two groups lost more weight than the former groups.

The administration of meso-DHBS caused changes similar to those of oestradiol-17β and DES in several parameters measured in this study. However, the smaller changes in prostatic weight of animals in the older group suggested that a higher dose of meso-DHBS is required in order to decrease the prostatic weight to the same extent obtained by oestradiol-17β or DES. The relatively unchanged level of plasma ICSH in the animals injected with oestrogen is in contrast to the decrease obtained by other investigators in studies with human beings (Alder et al. 1968). The results with meso-DHBS in rats provide additional evidence that the biological properties of the compound merit further study. In particular its effect on prostatic growth and metabolism should be investigated in order to evaluate its use in the treatment of prostatic dysfunction in man. DES has been used clinically for many years but it has the disadvantage of causing feminization and loss of libido in some patients. DHBS with its low oestrogenic potency may offer a better alternative and a study of its effectiveness in the treatment of prostatic dysfunction in the dog is in progress.

The authors wish to acknowledge the generous financial support of the Tenovus Organization. They would also like to thank the National Institutes of Health, Endocrine Study Section, Bethesda, U.S.A., for kindly supplying kits for the determination of rat prolactin and LH. V. D. is grateful to the British Council for a scholarship to enable her to study in this country.

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EFFECTS OF 2-Br-α-ERGOCRYPTINE (CB 154) ON PLASMA HORMONES AND ACCESSORY SEX ORGANS IN THE MALE RAT.


There are a number of studies indicating that prolactin may be concerned in the maintenance of normal growth and function of the prostate gland. The possible use of CB154 (2-Br-α-ergocryptine, Sandoz Ltd) to decrease prolactin levels in males with prostatic dysfunction is of particular interest and a series of studies were undertaken to investigate the biological effects of the drug in male rats.

Intact and adrenalectomised male Sprague-Dawley rats were injected (i.m.) daily with 100 μg CB154 in 0.2 ml sesame oil for 10 days. The animals were sacrificed on the 11th day and plasma prolactin, ICSH and testosterone were determined. The uptake of [7α-3H] testosterone in vivo, and the formation of 5α-dihydrotestosterone in different lobes of the prostate were also measured in one group of rats 60 min. before death.

There was a marked decrease in plasma prolactin in the CB154 treated animals. The testosterone concentration decreased but the plasma ICSH level was shown to increase. Despite the low plasma prolactin concentration of animals on CB154, there was little effect on the uptake of radioactive testosterone by the prostatic tissue, nor on the formation in vivo of 5α-dihydrotestosterone. Further studies on the synthesis of testosterone in vitro by minces of testicular tissue indicated that there was no effect of CB154 on this tissue, as far as this type of study could show.

These experiments will be discussed in relation to an investigation into the localisation of zinc in the prostate of CB154 treated animals, studied by use of the electron-microscope microanalyser (EMMA 4, AEI). The application of this analytical technique to measure the zinc concentration of the nucleolus and other intracellular organelles of the prostate will be discussed.
FAILURE OF PROLACTIN INHIBITION TO INFLUENCE THE CORPUS LUTEUM FUNCTION IN PRIMATES.

A nocturnal release of prolactin has been found to be luteotrophic in the rat (Freeman M.E. and Neill J.D., Endocrinology 90 (1972) 1292). In humans a nocturnal release of prolactin has also been found (Sassin J.F. et al. Science 177 (1972) 1205) but its relation to the survival of the corpus luteum is not known. L-dopa is a potent inhibitor of prolactin release in humans as demonstrated in several studies.

In this study L-dopa was given daily after ovulation to five rhesus monkeys (Macaca mulatta) and five women during a normal ovulatory cycle. The maximum daily dose was 1 g in the monkeys and 2.4 g in the women. The treatment was continued until the onset of menstrual bleeding. The drug was generally well tolerated.

It appears that L-dopa in the dose and schedule used in this study does not affect the function of the corpus luteum as reflected by the length of the luteal phase and the peripheral plasma levels of progesterone.
Studies on subcellular zinc distribution in relation to hormone levels in rat prostatic tissue using the electron microscope microanalyser, EMMA. By J. A. Chandler, M. E. Harper and K. Griffiths. Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XX

Although in both man and rat the prostate gland is known to concentrate high levels of zinc, the importance of this metal in the maintenance of cellular structure and function is not well understood. Previous investigations have shown that plasma testosterone is a major factor controlling the accumulation and maintenance of the high zinc concentration in the dorsolateral prostate of the rat (Gunn & Gould, 1956; Millar, Elcoate & Mawson, 1957). There is evidence to suggest that in the rat, at least, prolactin may also be concerned in controlling the concentration of zinc in the cells of the prostate (Gunn, Gould & Anderson, 1965).

The studies now reported describe the use of X-ray microanalysis to investigate further the influence of various endocrine changes on the prostatic subcellular zinc distribution. In particular, the effect of CB 154 (2x-Br-ergocryptine, Sandoz Ltd), a drug which effectively lowers prolactin levels in plasma, has been investigated in the rat. The morphology of the prostate gland, ultrastructural changes and subcellular zinc concentrations and distribution were determined using the electron microscope microanalyser (EMMA) (Chandler, 1973). This technique provides the capacity to measure quantitatively the subcellular content of most elements down to the level of $10^{-18} \text{g}$.

The reduction of plasma prolactin concentration by CB 154 was accompanied by a slight fall in the level of plasma testosterone (Boyns, Cole, Golder, Danutra, Harper, Brownsey, Cowley, Jones & Griffiths, 1972). Although the total glandular weight of the prostate changed only slightly after CB 154 administration (100 $\mu g$/day for either 10 days or 30 days, i.m. in sesame oil), major structural changes in the lateral lobe were evident after 10 days' treatment.

Partial disintegration of the alveoli could be seen after 10 days' treatment, with epithelial cells separating from the basement membrane. After 30 days virtually all epithelial cells had become necrotic. In control animals zinc was shown to be localized within the secretory material in the lumen, and also in nucleoli, nucleoplasm and secretory granules. During CB 154 treatment, the total zinc content of the cells was reduced and after 30 days, zinc was no longer localized within the various subcellular organelles. Cell debris and secretory material found within the lumen after 30 days' treatment were, however, still shown to contain relatively high concentrations of zinc.

It would seem, therefore, that CB 154 administration, with the resultant fall in plasma prolactin level, produced marked changes in the rat dorsolateral prostate gland. The technique of electron microscopy microanalysis using EMMA will obviously be of particular value in elucidating the role of various hormones in controlling zinc distribution in the prostate and the relationship of zinc levels to prostatic dysfunction.

The generous financial support of the Tenovus Organization is gratefully acknowledged.

REFERENCES


INTRODUCTION

Although testicular androgens are believed to be the principal factors in maintaining prostatic function, there is increasing evidence that pituitary, adrenal, and thyroid hormones are also important. Studies in the dog and rat\cite{18,26} have shown that hypophysectomy (H) results in more marked prostatic atrophy than that seen after simple castration (C). In hypophysectomized-castrate (HC) rats, the administration of growth hormone (GH) and ACTH was reported to stimulate prostatic growth, whereas LH, HCG, FSH, and prolactin were without effect.\cite{26} Other investigators\cite{3,30} have been unable to reproduce the action of GH on the prostate, but they showed that adrenalectomy would prevent the ACTH-stimulated response,\cite{30} which was also not mimicked by hydrocortisone.\cite{26} Synergism between pituitary hormones was demonstrated since the simultaneous administration of prolactin and ACTH to C rats produced greater prostatic growth that ACTH alone.\cite{30} Furthermore, ACTH and prolactin would only promote prostatic growth in HC rats when the hormones were supplemented with thyroxine.\cite{30}

From these experiments, it is not clear whether the synergistic effects of ACTH and prolactin in the C rat are exerted solely at the adrenal level. There is evidence that pituitary hormones may modify the uptake of androgen.
by the gland. The weights of prostates from C rats return to normal values following treatment with testosterone, whereas the weights of prostates from HC rats remain slightly, but significantly, lower than normal unless testosterone treatment is complemented by prolactin, thyroxine, or growth hormone. These effects are minimal, and there is also evidence of a lesser degree of synergism between testosterone and ACTH or gonadotrophin. In addition, the combination of testosterone, prolactin, and GH was much more potent in sustaining prostatic weight than testosterone plus prolactin. In all these experiments, the possibility that prolactin and GH may have adrenotrophic actions was not considered.

More sensitive indices than prostatic weight or trophic effects are available. Testosterone plus prolactin produces a greater increase in the fructose and citric acid content of the prostate of the HC rat than testosterone alone, the dorsolateral lobe being mainly affected. Testosterone uptake by the prostate is significantly lower in HC rats than in normal or C rats. After the initial observation that radioactive zinc is concentrated to a greater degree by the dorsolateral lobe than by the ventral lobe, it was found that testosterone treatment stimulates zinc uptake by the dorsolateral lobes of H and HC rats and that the stimulation is significantly enhanced by prolactin. In the H rat, prolactin, FSH, TSH, and ACTH do not affect zinc uptake; LH alone has this property.

PROLACTIN EFFECTS IN VITRO

In confirmation of the experiments of Farnsworth, we found that fairly high doses of ovine prolactin (180 μg/ml; 5 IU/ml) stimulate the uptake of radioactive testosterone by organ cultures of rat prostatic tissue, the largest increase being observed in the dorsal lobe (Table 24-1). Prolactin also enhanced the uptake of labelled testosterone into the testosterone and 5α-dihydrotestosterone fractions extracted from organ cultures of human benign hyperplastic prostatic tissue (Table 24-II). In attempting to understand the mecha-
Prolactin and the Prostate

TABLE 24-1
EFFECT OF OVINE PROLACTIN (2ND I.S.) ON THE UPTAKE OF [³H]TESTOSTERONE (10 μCi : 5.4 Ci/mmol) BY RAT PROSTATIC TISSUE IN 24 HR ORGAN CULTURE

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>SPECIFIC ACTIVITY (dpm/n mol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testosterone</td>
</tr>
<tr>
<td>CONTROL</td>
<td>402.8</td>
</tr>
<tr>
<td>PROLACTIN (5 I.U./ml)</td>
<td>675.8</td>
</tr>
<tr>
<td>CONTROL</td>
<td>560.2</td>
</tr>
<tr>
<td>PROLACTIN (5 I.U./ml)</td>
<td>689.2</td>
</tr>
<tr>
<td>CONTROL</td>
<td>63.2</td>
</tr>
<tr>
<td>PROLACTIN (5 I.U./ml)</td>
<td>123.2</td>
</tr>
</tbody>
</table>

nism of this phenomenon, we studied the effect of prolactin on prostatic adenylate cyclase activity because cyclic AMP is believed to mediate the protein hormone action in many tissues. Enhancement of adenylate cyclase activity may therefore be used as an indication that a hormone is physiologically active in a particular tissue. We were not able to

TABLE 24-II
EFFECT OF OVINE PROLACTIN (2ND I.S.) ON THE UPTAKE OF [³H]TESTOSTERONE (10 μCi : 5.4 Ci/mmol) BY HUMAN BENIGN HYPERPLASTIC PROSTATIC TISSUE IN 24 HR ORGAN CULTURE

<table>
<thead>
<tr>
<th>COMPOUNDS ADDED</th>
<th>SPECIFIC ACTIVITY dpm/n mole/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>CONTROL</td>
<td>105.3</td>
</tr>
<tr>
<td>PROLACTIN</td>
<td>160.6</td>
</tr>
</tbody>
</table>

Steroids isolated: T, testosterone; 5αT, 5α-dihydrotestosterone; 5α-A, 5α-androstane-3β, 17β-diol A-DIOL (ββ). 5α-androstane-3β, 17β-diol.
demonstrate any stimulation of enzyme activity by testosterone, a finding that is consistent with the idea that the main features of androgen action are not dependent on the generation of cyclic AMP (see papers by Singhal and by Mainwaring in this volume). Low doses of ovine prolactin (1.5 μg/ml: 0.042 IU/ml) stimulated adenylate cyclase activity in whole prostatic homogenates, while higher doses (60 μg/ml: 1.7 IU/ml) were ineffective. It is of interest in this connection that Dr. Lasnitzki has found that the histological structure of rat prostate organ cultures is better maintained in the prostate of low doses than of high doses of prolactin.\(^2\) Other protein hormones (HGH, HLH, and HCS) also stimulate prostatic adenylate cyclase activity;\(^9\) their function in the prostate is unknown.

**PROLACTIN EFFECTS IN VIVO**

To assess the importance of prolactin as a factor in promoting prostatic growth, it is useful to observe the effects of selectively lowering the blood level of the hormone. The

![Figure 24-1. Effect of CB154 (100 μg/day) on plasma prolactin (mean ± SD) in normal and adrenalectomized mature male Sprague-Dawley rats. Animals were killed after 10 days of treatment. Assay standard NIH - RP - 1 (11.1 IU/mg); NIAMD kit.](image-url)
ergocornine derivative 2-Br-α-ergocryptine (CB154, Sandoz) has this property,\(^6\) and rats treated with CB154 (100 \(\mu\)g/day for 10 days) show a marked fall in the concentration of prolactin in the plasma\(^1\) (Fig. 24–1). However, there was no reduction in prostatic weight (Fig. 24–2), on the contrary, there was a slight but significant increase. Longer term treatment (100 \(\mu\)g/day for 30 days) was also without effect. If weight is used as an index, it may be concluded that depletion of plasma prolactin has only a minor effect on prostatic growth, and the significance of the in vitro results is therefore difficult to understand.

PROLACTIN AND TESTICULAR ANDROGEN PRODUCTION

The work of Hafiez and his colleagues\(^{15,16}\) suggests that prolactin may synergize with LH in the regulation of testicular testosterone synthesis because both LH and prolactin
are required to maintain normal plasma levels of testosterone in H rats. It is of interest that rats treated with CB154 show a significant depression in plasma testosterone coincident with a "compensatory" rise in plasma LH. Furthermore, uptake of radioactive testosterone into the 5α-dihydrotestosterone and testosterone fractions extracted from the prostatic tissue of CB154-treated rats is lower than that of untreated rats. These results suggest that prolactin is necessary for testosterone synthesis and that it also promotes testosterone uptake by the prostate in vivo.

PROLACTIN IN THE HUMAN MALE

The recent isolation and purification of human pituitary prolactin has led to the development of sensitive and specific radioimmunoassays for the hormone in plasma. Extensive investigations have characterized alterations in plasma levels during pregnancy and lactation and following the administration of drugs (including phenothiazines). Prolactin concentrations in the plasma of the human male have been comparatively little studied.

In many species, there is a parallel fluctuation in the plasma levels of testosterone and LH indicating that testosterone production is predominantly under LH control in these species. No such relationship appears to exist as clearly in man. With the use of heterologous and homologous radioimmunoassays for human prolactin, we have been examining the interrelationship between gonadotrophins, testosterone, and prolactin in the human male (this work is being done in cooperation with Dr. H. Simpson of the Department of Pathology, University of Glasgow). Figure 24-3 shows changes in immunoreactive LH, FSH, prolactin, and testosterone in six normal young human males over a 24-hour period. Prolactin was measured by a heterologous radioimmunoassay that depends on labelled human pituitary prolactin and an antiserum raised against human amniotic fluid prolactin. There is some indication that plasma levels of prolactin fluctuate parallel with levels of testosterone, at least in some of the subjects. Table 24-11I shows the linear
Figure 24-3. Prolactin, FSH, LH, and testosterone in the blood plasma of six young normal human males during a 24-hour period. Hormone concentrations were measured by radioimmunoassay.
correlation coefficients for testosterone versus the corresponding LH or FSH or prolactin concentrations in each of the six subjects. A significant correlation existed between prolactin vs testosterone and LH vs testosterone in at least three subjects. In each case, prolactin gave a higher coefficient than LH, both being usually much higher than that of FSH. More detailed studies are now in progress, but these results suggest that prolactin may play some part in controlling testosterone production in man.

**TABLE 24-III**

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>LH</th>
<th>FSH</th>
<th>PROLACTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.425</td>
<td>0.180</td>
<td>0.399</td>
</tr>
<tr>
<td>B</td>
<td>0.650</td>
<td>0.178</td>
<td>0.830</td>
</tr>
<tr>
<td>C</td>
<td>0.524</td>
<td>-0.279</td>
<td>0.795</td>
</tr>
<tr>
<td>D</td>
<td>0.514</td>
<td>-0.572</td>
<td>0.318</td>
</tr>
<tr>
<td>E</td>
<td>0.415</td>
<td>0.409</td>
<td>0.803</td>
</tr>
<tr>
<td>F</td>
<td>-0.061</td>
<td>-0.652</td>
<td>-0.039</td>
</tr>
</tbody>
</table>

**PITUITARY HORMONES AND HUMAN PROSTATIC CANCER**

A rational approach to therapy for endocrine-related cancers would seem to require the repeated estimation of plasma hormone concentrations during the course of treatment. Figure 24-4 shows plasma concentrations of immunoreactive FSH, LH, GH, prolactin, and testosterone in a patient with prostatic cancer during treatment with estrogen. TSH has been omitted; the levels were uniformly low. On injection of the estrogen, there was a rapid fall in plasma testosterone, LH, and FSH; a sudden rise in plasma pro-
lactin; and a slower rise in plasma GH. This patient remains clinically normal at the present time. Figure 24–5 shows another patient who displayed similar hormonal patterns, but evidence of tumor recurrence, accompanied by a slow rise in plasma testosterone, was detected during treatment. Indeed, it has been suggested that a secondary rise in plasma testosterone may be responsible for this type of recurrence.\textsuperscript{28}

Plasma prolactin concentrations are known to undergo rapid fluctuations in response to stress, and it was important to confirm that the concentrations we measured represented a sustained change in hormone secretion. In cooperation with Drs. Shamanesh and Hartog of the University of Bristol, multiple hormone assays were performed in patients with
PATIENT D. P. (AGED 69)

Figure 24-5. Prolactin, FSH, LH, GH, and testosterone in the blood plasma of a patient with prostatic cancer who was being treated with estrogen.

prostatic cancer who were being treated with estrone sulphate (Premarin) and various doses of stilboestrol. Glucose tolerance tests were also performed at intervals as part of a separate study.\textsuperscript{29} It can again be seen (Fig. 24-6) that estrogen raised prolactin levels, and these remained elevated throughout the glucose tolerance test. Similarly, GH tended to rise, but there were erratic and wide oscillations in plasma levels of this hormone during tests. Calculation of mean GH levels in a number of such patients confirmed that there was a rise in the mean plasma GH concentration. The extent of the increase was directly related to the dose of estrogen administered (Table 24-IV).
Figure 24-6. Prolactin, FSH, LH, GH, and testosterone in the blood plasma of a patient with prostatic cancer who was being treated with estrogen. Glucose tolerance tests (sampling at 30-min intervals) were performed periodically. The values for the hormone concentrations in the blood during each test are shown by the continuous lines.

<table>
<thead>
<tr>
<th>TABLE 24-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EFFECT OF ESTROGEN TREATMENT ON GH CONCENTRATION IN THE BLOOD PLASMA OF PATIENTS WITH PROSTATIC CANCER</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TREATMENT (PATIENTS, SAMPLES)</th>
<th>PLASMA GH (μU/ml ± SEM)</th>
<th>P (V. CONTROL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNTREATED (7, 45)</td>
<td>11.02 ± 2.84</td>
<td></td>
</tr>
<tr>
<td>STILBOESTROL 1 mg/day (5, 19)</td>
<td>20.16 ± 6.13</td>
<td>N.S.</td>
</tr>
<tr>
<td>STILBOESTROL 7.5 mg/day (8, 48)</td>
<td>29.75 ± 3.68</td>
<td>0.001</td>
</tr>
<tr>
<td>STILBOESTROL 15 mg/day (9, 64)</td>
<td>33.84 ± 3.37</td>
<td>0.001</td>
</tr>
<tr>
<td>PREMARIN 15 mg/day (9, 58)</td>
<td>29.50 ± 3.05</td>
<td>0.001</td>
</tr>
</tbody>
</table>
NORMAL AND ABNORMAL GROWTH OF THE PROSTATE

SUMMARY

Estrogen treatment for carcinoma of the prostate leads to a fall in plasma testosterone and tumor regression. There is also a rapid, sustained increase in plasma prolactin and GH, hormones that can stimulate testosterone production by the adrenal and testes and can promote the trophic action of testosterone in the prostate. It would appear useful to monitor plasma levels of these hormones during the course of treatment and to study the effects on the progress of the disease of selectively reducing their rates of secretion.

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Prolactin and the Prostate


for testosterone in hypophysectomized rats: Endocrinol. 77, 1119 (1965).


THE EFFECT OF 2-BROMO-α-ERGOCRYPTINE (CB154) ADMINISTRATION ON THE HORMONE LEVELS, ORGAN WEIGHTS, PROSTATIC MORPHOLOGY AND ZINC CONCENTRATIONS IN THE MALE RAT

By

M. E. Harper, V. Danutra*, J. A. Chandler and K. Griffiths

ABSTRACT

2-Bromo-α-ergocryptine (CB154) administration to male rats produced a significant decrease in plasma prolactin levels without changing the LH and testosterone concentrations. The weights of the accessory sex tissues, testes, adrenals and kidney were unaltered by the treatment. Zinc concentration and distribution in the cell organelles of the prostatic tissue was markedly changed by CB154 treatment. No changes in the uptake of testosterone in vivo occurred in the treated animals. Prolactin did not consistently influence the prostatic adenyl cyclase activity in vitro and only at high concentrations was the testosterone uptake in vitro with cultures of prostatic tissue increased.

The precise mechanism by which pituitary hormones affect the prostate is uncertain although a great deal of evidence for their influence on this gland has accumulated. Following hypophysectomy in the dog a greater prostatic atrophy occurred than after castration (Huggins & Russel 1946). A diminished prostatic response to exogenous androgen was noted in the hypophysectomized rats (Van der Laan 1953). Subsequently prolactin was reported to augment...
the prostatic response to testosterone (Grayhack et al. 1955) although further studies (Huggins et al. 1955; Lostroh & Li 1957; Chase et al. 1957; Tullner 1963) suggested that growth hormone and ACTH might play a role. Gunn et al. (1965) showed that the uptake of \(^{65}\)Zn into the prostate gland was influenced by LH, prolactin and testosterone. Since the plasma prolactin concentrations are increased in patients undergoing oestrogen therapy for prostatic cancer (Harper et al. 1976) it is essential to define the role of prolactin in prostatic metabolism and growth. Also pertinent to this study are the effects in male animals of 2-Br-\(\alpha\)-ergocryptine (CB154), a known inhibitor of prolactin secretion (Flückiger & Wagner 1968; Heuson et al. 1970; Billeter & Flückiger 1971). This report describes some studies relating to the effect of prolactin on the rat prostate and changes accompanying CB154 administration to male rats.

**MATERIALS AND METHODS**

Male Sprague-Dawley rats (age 12 weeks) on a commercial diet (Pilsbury Ltd.) and tap water *ad libitum* with 10 animals per group, were injected (im) daily with 100 \(\mu\)g CB154 (Sandoz Ltd.) per animal for 10, 20 and 30 days. Injections were made in 0.2 ml of sesame oil with the controls receiving only the vehicle. On the day of the last injection, animals were anaesthetized, bled by cardiac puncture and killed by cervical dislocation. Plasma samples were kept at \(-20^\circ\)C until analysed for testosterone, prolactin and LH as previously described (Donutra et al. 1973).

Two other groups of intact rats (6 animals per group) were given either CB154 in sesame oil or vehicle alone for 10 days as described above. On the 11th day, 25 \(\mu\)Ci [\(7\alpha\)-\(^3\)H]testosterone (sp. radioactivity 5.4 Ci/mmol) was injected (im) in 0.3 ml 15% ethanolic saline 60 min before the animals were killed. The prostate lobes were removed, weighed, minced with scissors, 300 \(\mu\)g of both testosterone and 5\(\alpha\)-dihydrotestosterone (17\(\beta\)-hydroxy-5\(\alpha\)-androstan-3-one) added in 5 ml acetone and the mixture homogenized in a Silverson homogenizer. Steroids were extracted, purified by extensive thin chromatography and the specific radioactivities of the parent steroids and their derivatives determined (Fahmy et al. 1968).

**Tissue culture study**

Prostatic tissue was removed from rats killed under aseptic conditions and transferred to sterilised Petri dishes. Cultures were set up in a 'Microflow' tissue culture cabinet using a modification of the Trowell (1954, 1959) technique. The culture medium (1.0 ml) was contained in the central well of a plastic dish (Falcon Plastics). The explants, 1 mm cubes of tissue, were positioned on lens paper rafts (12 explants/dish) supported by stainless steel grids placed at the surface of the medium. The dishes were maintained in an atmosphere of 95% air : 5% \(\mathrm{CO}_2\) and cultured for 24 h at \(37^\circ\)C.

The culture medium 199, supplemented with 10% calf serum, 200 units/ml penicillin and 100 \(\mu\)g/ml streptomycin and with the various additions of [\(7\alpha\)-\(^3\)H]testosterone (10 \(\mu\)Ci, \(1.85 \times 10^{-6}\) M) or ovine prolactin, was sterilised by passage through Millipore filters.

After incubation, explants from 4 identical cultures were pooled, blotted with tissue
and washed in ice-cold phosphate-buffered saline. The tissue was blotted again and homogenised in buffer using a Griffiths ground-glass homogeniser. Homogenates were made up to 10 ml with distilled water, an aliquot taken for protein analysis (Lowry et al. 1951) and acetone containing 300 µg various carrier non-radioactive steroids added to the remainder before determining the specific radioactivity of the steroids by techniques previously described (Fahmy et al. 1968).

**Adenyl cyclase study**

In a series of 5 experiments, ventral, lateral and dorsal lobes from 6 rats (age 12 weeks) were minced, homogenised in 0.25 M sucrose containing 3 mM MgCl₂ using a Philpot and Stainer homogeniser and the homogenates (10% w/v) filtered through cheesecloth. Homogenates were assayed for adenyl cyclase activity by a slight modification of the procedure of Colder et al. (1972) measuring the conversion of [8-¹⁴C]ATP into cyclic [8-¹⁴C]AMP (adenosine 3',5'-cyclic monophosphate). All assays were in quadruplicate and cyclic [8-³H]AMP was used to check recoveries. The effects of prolactin, ACTH, LH and testosterone on adenyl cyclase activity were examined. Tissue from 3-day castrated rats was also used for studies of adenyl cyclase in a similar manner.

**Effect of CB154 on the morphology of the prostate**

Eighteen male Sprague-Dawley rats (age 12 weeks) were used to investigate the effect of CB154 administration on the morphology of the prostate and on the subcellular levels of zinc. Six animals received CB154, 100 µg daily, for 10 days, six for 30 days and six received vehicle alone as described earlier.

**Histology**

Tissue samples were fixed for 24 h in Bouins fixative, embedded in paraffin and 5 µm sections cut and stained with haematoxylin and eosin.

**Electron microscopy**

Tissue samples were fixed for 2.5 h in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, rinsed in buffer, post fixed in 1% osmium tetroxide in phosphate buffer for 1 h and rinsed again in phosphate buffer. Tissues were dehydrated through ascending grades of alcohol and embedded in araldite. Sections were then stained with uranyl acetate and lead citrate, mounted on electron microscope grids and examined in an AEI EM6B.

**Electron microscope microanalysis**

Tissue samples were fixed in a mixture of 4% glutaraldehyde and 2% potassium antimonate buffered to pH 7.4 with 0.1 M phosphate buffer. They were then dehydrated in alcohol and embedded in araldite. Sections (90 nm) were mounted on aluminium electron microscope grids and lightly coated with carbon for analysis using the electron microscope microanalyser, EMMA-4 (AEI, Manchester).

Conditions of analysis for subcellular zinc distribution were 0.1 µA at 80 KV with a probe diameter between 0.1 and 0.2 µm. Counting time was 40 seconds per point analysis. The principles of operation of EMMA-4 have been reviewed (Chandler 1973, 1975).
Table 1.
Effect of CB154 on the organ weights of intact 12 week old male Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Treatment period (day)</th>
<th>Treatment group</th>
<th>Organ weight (mg tissue/g body weight) (Mean ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment group</td>
<td>Ventral prostate</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.22 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>1.29 ± 0.25</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>1.32 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>1.33 ± 0.27</td>
</tr>
<tr>
<td>20</td>
<td>Control</td>
<td>1.62 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>1.50 ± 0.26</td>
</tr>
</tbody>
</table>

CB154 (100 μg in 0.2 ml sesame oil) injected im daily. Control group received injections of vehicle alone. Ten animals used in each group.
Nuclear preparations

Tissue from the ventral, lateral and dorsal lobes were collected from the control group and from animals which received CB154 for 10 days. Nuclear fractions were prepared by sucrose density gradient centrifugation (Groom et al. 1971). The nuclear pellets were fixed with 4%/ glutaraldehyde, washed in phosphate buffer and resuspended in deionised water. Droplets of the suspension (5 µl) were allowed to dry on electron microscope grids and the zinc contents of individual nuclei determined using EMMA-4.

RESULTS

Results shown in Table 1 clearly indicate that the administration of CB154 to male rats for periods up to 30 days did not affect the weights of the various lobes of the prostate, nor of the seminal vesicles, despite the decrease in the plasma prolactin concentration (Table 2) to a level undetectable by the procedures used. Furthermore the concentrations of LH and testosterone in the plasma did not change after CB154 administration. Low circulating plasma prolactin concentrations failed therefore to affect the weight of the lobes of the prostate gland.

Furthermore, administration of radioactive testosterone to control rats and to those on CB154 for 10 days again failed to indicate any effect of the low plasma prolactin on the uptake and metabolism of the steroid by the different lobes of the prostate (Table 3). Moreover, only the presence of a comparatively

<table>
<thead>
<tr>
<th>Treatment period (day)</th>
<th>Treatment group</th>
<th>Plasma hormone levels (ng/ml) (Mean ± sn)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prolactin</td>
<td>LH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>8.3 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>undetectable</td>
</tr>
<tr>
<td>20</td>
<td>Control</td>
<td>13.7 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>undetectable</td>
</tr>
<tr>
<td>30</td>
<td>Control</td>
<td>21.3 ± 17.5</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

NS = not significant. CB154 (100 µg in 0.2 ml sesame oil) injected im daily. Control group received vehicle alone. Ten animals used in each group.
Table 3.
Uptake of [\(7a-\text{H}\)]testosterone (25 \(\mu\)Ci) by the prostate of intact, 12 week old, male Sprague-Dawley rats after 10 days of treatment with CB154 (100 \(\mu\)g in 0.2 ml sesame oil).

<table>
<thead>
<tr>
<th>Prostatic lobes</th>
<th>Treatment group</th>
<th>Specific radioactivity (dpm/nmole/mg tissue)</th>
<th>Plasma prolactin concentration ng/ml (mean ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 animals per group</td>
<td>Testosterone (T)</td>
<td>5(a)-Dihydro-testosterone (5(a)T)</td>
</tr>
<tr>
<td>Ventral</td>
<td>Control</td>
<td>3.3</td>
<td>39.0</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>3.4</td>
<td>31.5</td>
</tr>
<tr>
<td>Dorsal</td>
<td>Control</td>
<td>1.8</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>2.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Lateral</td>
<td>Control</td>
<td>2.9</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>CB154</td>
<td>3.1</td>
<td>17.2</td>
</tr>
</tbody>
</table>

Controls received vehiclealone. Testosterone injected 1 h before sacrifice.

Table 4.
[\(7a-\text{H}\)]Testosterone uptake by rat prostatic lobes in organ culture.

<table>
<thead>
<tr>
<th>Prostatic tissue</th>
<th>Hormone added to culture</th>
<th>Specific activity of isolated steroid from prostatic explant homogenates (dpm/nmol/mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Testosterone (T)</td>
<td>5(a)-Dihydro-testosterone (5(a)T)</td>
</tr>
<tr>
<td>Ventral</td>
<td>Control</td>
<td>402.8</td>
<td>905.0</td>
</tr>
<tr>
<td></td>
<td>Prolactin (5 IU/ml)</td>
<td>675.8</td>
<td>1163.7</td>
</tr>
<tr>
<td>Lateral</td>
<td>Control</td>
<td>560.2</td>
<td>386.5</td>
</tr>
<tr>
<td></td>
<td>Prolactin (5 IU/ml)</td>
<td>689.2</td>
<td>508.3</td>
</tr>
<tr>
<td>Dorsal</td>
<td>Control</td>
<td>63.2</td>
<td>372.3</td>
</tr>
<tr>
<td></td>
<td>Prolactin (5 IU/ml)</td>
<td>123.2</td>
<td>551.2</td>
</tr>
</tbody>
</table>
high concentration of ovine prolactin (5 IU/ml medium) consistently increased the uptake and metabolism of [7a-3H]testosterone by explants of the various lobes of the prostate in culture (Table 4). Lower concentrations (up to 5 IU/ml) had no apparent effect on steroid metabolism. Only small amounts of radioactivity in the tissue was present in the metabolites of testosterone other than 5a-dihydrotestosterone.

These results would therefore suggest that prolactin does not have a direct major role in controlling or influencing androgen metabolism or action in the rat prostate gland. Such a conclusion may also be drawn from results obtained from studies with prostatic adenyl cyclase. A series of experiments were set

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Prostatic tissue</th>
<th>Concentration of hormone added (μg/ml)</th>
<th>nmols cAMP formed/30 min (Mean ± sd)</th>
<th>nmols cAMP formed/30 min/mg protein (Mean ± sd)</th>
<th>Significance of difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ventrал</td>
<td>Control —</td>
<td>5.74±0.48</td>
<td>25.40±2.12</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Prolactin 1.5</td>
<td>7.07±0.28</td>
<td>38.28±1.24</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>6.78±0.07</td>
<td>30.00±0.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lateral Control —</td>
<td>13.67±1.46</td>
<td>73.49±7.85</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Prolactin 1.5</td>
<td>14.29±0.88</td>
<td>76.83±4.73</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>13.87±0.77</td>
<td>74.57±4.14</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Dorsal Control —</td>
<td>5.62±0.08</td>
<td>27.28±0.39</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Prolactin 1.5</td>
<td>8.43±1.38</td>
<td>40.92±6.70</td>
<td></td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>6.74±0.68</td>
<td>32.72±3.50</td>
<td></td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>2 Ventrал</td>
<td>Control —</td>
<td>7.51±0.80</td>
<td>31.29±3.83</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Prolactin 1.5</td>
<td>8.28±0.51</td>
<td>34.50±2.13</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>8.67±0.22</td>
<td>36.13±0.92</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Lateral Control —</td>
<td>5.03±0.46</td>
<td>23.95±2.19</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Prolactin 1.5</td>
<td>5.42±0.24</td>
<td>25.81±1.14</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>5.32±0.83</td>
<td>25.33±3.05</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Dorsal Control —</td>
<td>4.85±0.36</td>
<td>18.51±1.37</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Prolactin 1.5</td>
<td>4.68±0.30</td>
<td>17.86±1.15</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>4.45±0.20</td>
<td>16.98±0.76</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are given as mean ± sd of quadruplicate determinations. 

P values are obtained using Student's t-test. 

NS = not significant.
Table 6.
Effect of CB154 on the zinc distribution of subcellular regions of epithelial cells from the lateral lobe of the prostate and on the zinc content of the nuclei from the three different lobes.

<table>
<thead>
<tr>
<th>Prostatic tissue</th>
<th>Control group mean ± SEM</th>
<th>CB154 treated group (10 days) mean ± SEM</th>
<th>CB154 treated group (30 days) mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei (gm x 10^-18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral lobe</td>
<td>31.4 ± 6.60</td>
<td>20.9 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>Dorsal lobe</td>
<td>125.2 ± 12.7</td>
<td>12.5 ± 2.5§</td>
<td></td>
</tr>
<tr>
<td>Lateral lobe</td>
<td>234.5 ± 25.4</td>
<td>68.1 ± 14.3§</td>
<td></td>
</tr>
<tr>
<td>Lateral lobe sections (weight fractions)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleolus</td>
<td>44.4 ± 5.19</td>
<td>21.8 ± 3.69§</td>
<td>31.4 ± 3.94</td>
</tr>
<tr>
<td>Nucleus</td>
<td>34.5 ± 2.60</td>
<td>14.4 ± 2.60§</td>
<td>10.0 ± 3.30*</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>6.6 ± 0.33</td>
<td>5.2 ± 2.05</td>
<td>Not detectable†*</td>
</tr>
<tr>
<td>Luminal secretory material</td>
<td>59.8 ± 3.38</td>
<td>Not detectable§</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>17.3 ± 2.59</td>
<td>18.4 ± 2.68</td>
<td>6.8 ± 1.49†*</td>
</tr>
<tr>
<td>Secretory granules</td>
<td>16.3 ± 1.28</td>
<td>29.8 ± 3.21§</td>
<td>17.8 ± 4.33†</td>
</tr>
</tbody>
</table>

Figures for the ultrathin sections are given in weight fractions (with SEM calculated by dividing characteristic X-ray counts of ZnKα by mass thickness). Mass thickness was monitored by measurement of white radiation (Chandler 1975) for each section analysed. Statistical significance ($P < 0.01$): § between control and 10 day treatment; * between control and 30 day treatment; † between 10 and 30 days treatment.

up to study the effect in vitro of prolactin and other protein hormones on the adenyl cyclase activity of the different lobes of the prostate. It was interesting that in some experiments prolactin in vitro significantly stimulated the adenyl cyclase activity of the homogenates prepared from the ventral and dorsal lobes, although not to any great extent (Experiment 1, Table 5). Similar preliminary results with whole prostate homogenates have been reported (Golder

Fig. 1.
Histology of lateral prostate of the rat:
(a) control;
(b) 10 days after CB154 treatment;
(c) and (d) 30 days after CB154 treatment; H and E stained.

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Fig. 2.
et al. 1972). However, in most other experiments, no such effect was found (Experiment 2, Table 5).

The histology of the prostatic tissue removed after CB154 administration for 10 or 30 days was examined. Although there was no apparent effect of CB154 on the ventral or dorsal lobes, parts of the lateral lobe were markedly changed by treatment over 30 days. After 10 days treatment, the epithelium generally remained intact (Fig. 1 b) although in places, some disintegration of alveoli was observed and cells had separated from the basement membrane. There was an apparent decrease in secretory material within the acini. After 30 days CB154 treatment, some alveoli had disintegrated (Figs. 1 c and 1 d) and normal secretory material was markedly reduced. These changes tended to occur more towards the centre of the lobe.

Few ultrastructural changes were observed in the tissue from the lateral lobes after CB154 for 10 days. There were no features characteristic of tissue removed after castration. After 30 days, however, there were many areas where the cells had separated from the basement membrane, nuclei were found to have disintegrated and there was an apparent increase in the lysosomal content of the cytoplasm and vacuolation of the Golgi apparatus (Figs. 2 a and 2 b). Analysis of the zinc concentration of the tissue sections of the lateral lobe using EMMA-4 showed that the zinc levels fell in most regions of the cell after CB154 treatment (Table 6). For example, the zinc content of the nucleoli and endoplasmic reticulum was significantly lower after 30 days CB154 administration and the luminal secretory material in the alveoli diminished. There was, however, more cell debris in the lumen during the period of treatment and the zinc content of this material was high.

Analysis of the isolated nuclei indicated a marked reduction in whole nuclear zinc after 10 days treatment in the dorsal and lateral lobes.

The histology and electron microscopy studies therefore clearly indicate that either the CB154 itself or the effects of the low plasma prolactin have a marked influence on the lateral lobe of the prostate gland.

---

**Fig. 2.**

Electron microscopy of lateral prostate:

(a) Control tissue showing intact columnar cells, well developed Golgi apparatus, secretory processes.

(b) Epithelium 30 days after CB154 treatment. The basement membrane has lost its integrity and the cells have sloughed off towards the lumen. Nuclei have become pyknotic.
DISCUSSION

It is evident from these studies that CB154 effectively inhibits prolactin secretion in the male rat, but there was no measurable effect on the plasma concentrations of testosterone and LH. Similarly in a clinical study of patients with carcinoma of the prostate gland (Harper et al. 1976) administration of 2.5 mg CB154/day to patients undergoing oestrogen therapy effectively decreased plasma prolactin concentrations without alteration to the testosterone and LH concentrations. These observations are, of course, relevant in relation to the report of Hafiez et al. (1972) that prolactin can synergise with LH in the control of testosterone synthesis and secretion by the rat testis.

The low plasma concentration of prolactin had no effect on the weights of the prostatic lobes or on the weights of the seminal vesicles, kidney, testes or adrenals. Furthermore, results from studies in which radioactive testosterone was injected into rats being treated with CB154 suggested that the absence of plasma prolactin did not markedly affect the metabolism of testosterone by the prostatic tissue, no significant differences being found in the specific radioactivities of testosterone and 5α-dihydrotestosterone in the gland of control and CB154 treated rats.

Farnsworth (1970) has reported, however, that ovine prolactin in vitro (2 IU/ml) increased the uptake of radioactive testosterone by slices of human benign hyperplastic prostate. Similarly, from the results now reported, a relatively high concentration of ovine prolactin (5 IU/ml) increased the uptake and metabolism of radioactive testosterone by explants of the various lobes of the rat prostate. Smaller doses were found to have no such effect and the significance of the prolactin effect must remain doubtful. Other studies (Damutra 1973) have failed to show any marked effect of prolactin on the metabolism of radioactive testosterone by explants of human prostatic tissue in culture. Similarly, although results from an earlier study (Golder et al. 1972) of prostatic adenyl cyclase were confirmed and some stimulation by prolactin of this enzyme in homogenates of prostatic tissue was observed in certain experiments, this stimulatory effect could not consistently be found with all prostatic homogenates. No effect of prolactin or testosterone on the adenyl cyclase enzyme system was found with prostatic tissue removed from castrated rats nor with tissue from animals which had received CB154 for 10 days and would therefore have had low levels of plasma prolactin.

In view of the reports from other groups (Grayhack et al. 1955; Gunn et al. 1965; Hafiez et al. 1972) that prolactin tends to act synergistically with other hormones, the inconsistent stimulation caused by prolactin on the adenyl cyclase activity could mean that the correct experimental system in vitro to study this effect had not been realised.

Although the experiments reported here tend to indicate that prolactin does
not itself have any trophic action on the prostate, histological and electron microscopic studies clearly showed that CB154 administration with concomitant decrease in plasma prolactin concentration markedly affects the lateral lobe of the rat prostate. Furthermore, microanalytical studies using EMMA-4 indicated that there were significant changes in the distribution and concentration of zinc in the lateral lobe. Analysis of individual nuclei prepared from homogenates of prostatic tissue from the various lobes of the prostate gland also indicated a decreased level of intranuclear zinc in these nuclear preparations from the dorsal and lateral lobes after CB154 treatment. Although it is well known that the prostate has a high concentration of zinc, the significance of this element in the biochemistry of the gland is unclear. Grant et al. (1971) have suggested that zinc may affect the binding of steroid to protein in prostatic cells and studies by Reed & Stitch (1973) have also been concerned with the levels of zinc binding protein.

These results tend to indicate that prolactin itself is not directly concerned with the steroid metabolic activity of the prostate of the rat nor in biological systems involved with tissue growth, but to be intimately involved in the elemental distribution in the lateral lobe of the rat prostate.

ACKNOWLEDGMENTS

The authors wish to acknowledge the generous financial support of the Tenovus Organization. One of them (Varapan Danutra) is grateful to the British Council for a Scholarship to enable her to study in this country. We are grateful to Dr. Flückiger, Santoz Limited, Basle, for the supplies of CB154 used in these studies.

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Immunocytochemical staining obtained with a rat prolactin antiserum in various rat tissues. By P. E. C. Sibley, M. E. Harper, B. G. Joyce and K. Griffiths. Tenovus Institute for Cancer Research, Welsh National School of Medicine, The Heath, Cardiff, CF4 4XX

The visual localization of polypeptide hormones in the pituitary gland by immunocytochemistry at the histological level is a well-established technique (Nakane, 1970; Sibley, Joyce, Groom, Chandler & Griffiths, 1978). Localization of prolactin in other tissues has proved more difficult due to the much lower prolactin content. In this study an antiserum raised against a rat prolactin standard (NIAMDD-Rat-Pr-RP-1) has been used in enzyme-conjugated and unlabelled antibody enzyme immunocytochemical staining procedures on various rat tissues. These tissues include the pituitary gland, 7,12-dimethylbenz(a)-anthracene (DMBA)-induced mammary tumours, lactating mammary gland (LMG) and the adrenal gland.

Tissues fixed in Bouin’s fluid were dehydrated and embedded in paraffin wax, and serial sections (5 μm) were cut. The use of serial sections enabled a comparison of the experimental and control immunocytochemical procedures. In the unlabelled antibody enzyme method (Sternberger, 1974), sections were incubated with rabbit anti-rat prolactin antiserum (aRPr) followed by sheep anti-rabbit serum (aRγG), peroxidase anti-peroxidase and finally the substrate to complete the staining sequence. Non-specific binding of aRγG was blocked by a preincubation with non-immune sheep serum.

In the anterior lobe of the pituitary gland, a clearly defined pattern of staining was observed when aRPr (1 : 1500) was used in the immunocytochemical procedure (Sibley et al. 1978). When aRPr (1 : 500) was used in the same sequence, although this pattern was visible, the result tended to be obscured by background staining. In the DMBA-induced mammary tumour, strong staining was observed in some cells, predominantly in the peripheral cell layer of the acini, together with staining in the stroma and also in the duct secretions. Staining in LMG was markedly different from that in the DMBA-induced mammary tumours, being mostly extracellular and confined to the stromal areas of the tissue. In the adrenal gland staining was notably confined to the cortex, particularly in longitudinally orientated sinusoids; however, in the zona fasciculata the nuclei of some cells gave strong staining. In all tissues, when the absorption control, prepared by admixing aRPr with the appropriate antigen, was included in the immunocytochemical sequence, no staining was observed.

Globulin fractions of the rat prolactin antiserum collected from ion exchange cellulose columns were also used in these procedures. The results were similar to those obtained with the whole serum except that the diffuse background staining was not observed.

REFERENCES
Protein Hormones and Prostatic Cancer


Tenovus Institute for Cancer Research, Welsh National School of Medicine, Cardiff CF4 4XX, Wales; and *Department of Urology, St. Woolos Hospital, Newport, Gwent, Wales

The basis of most endocrine studies concerning the prostate gland has been the fact that its growth, maintenance, and functional activity are largely dependent on androgenic hormones secreted by the testis. Despite experimental work with animals which has suggested that pituitary hormones may influence prostatic growth and function, their involvement in tumor development is unproven. Certainly there is little direct evidence to implicate hormones in the initiation of prostatic cancer (6,21). One approach to the study of the interrelationships of protein hormones to prostatic tumors has been an examination of their effects on prostatic tissue biochemistry. An alternative approach more amenable to studies with the human has been to compare plasma hormone concentrations and their changes in response to various stimuli in patients with benign and malignant prostatic tumors and in asymptomatic controls of similar age. Inherent in all such studies is the possibility that endocrine factors influencing the course of the disease may have been critically different at a time long before the patient is seen in the clinic.

DIRECT EFFECTS ON THE PROSTATE GLAND

Early studies in animals indicated that pituitary hormones could influence prostatic growth and functioning. A greater degree of atrophy of the gland was noted after combined hypophysectomy and castration than after castration alone (10,15). Impaired uptake of testosterone by the ventral prostate was observed in hypophysectomized rats (13). Some discrepancies then ensued as to which of the protein hormones were able to stimulate prostatic growth. Lostroh and Li (15) found growth hormone (GH) and adrenocorticotropic hormone (ACTH) to be active, but not luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin, while Tullner’s (19) experiments indicate a role for ACTH and prolactin as stimulators of rat prostatic growth. Grayhack and Lebowitz (5) observed an augmented increase in the fructose and citric acid content of rat prostate when prolactin and testosterone were administered compared with that caused by testosterone alone. GH appeared to be complementary to prolactin and testosterone in increasing prostate weight (2). It is possible
that the differences observed by the various investigators were a reflection of the heterogeneity of the pituitary hormone preparations used at that time. Experiments in our laboratory failed to show an effect of 30 days’ treatment with 2-Br-α-ergocryptine, a prolactin secretion inhibitor, on prostatic weight, but a marked decrease in prostatic zinc content and cellular distribution was noted. The latter observation was consistent with earlier work on the effect of prolactin on zinc uptake into the prostate gland (7). Studies by several groups (4,12,14) have indicated that prolactin could influence the uptake of steroids into prostatic tissue, a factor of some importance when treating prostatic cancer patients with various endocrine therapies. There is no evidence that LH or FSH directly effect the prostate gland, but they can indirectly alter its growth and maintenance through their influence on testosterone and estradiol-17β production. Prolactin also appears to work synergistically with LH to increase testicular androgen production (8) and, interestingly, prolactin receptors have been demonstrated in testicular tissue (1). Both prolactin and ACTH can increase the availability of steroids to the prostate by increasing adrenal steroid biosynthesis (3,11).

PLASMA HORMONE CONCENTRATIONS

Measurement of plasma hormone concentrations in patients with prostatic cancer or benign prostatic hypertrophy (BPH) and in asymptomatic controls of the same age have been carried out in an attempt to delineate an endocrine abnormality in the prostatic tumor patients if such should exist. In our original study (9) we noted no significant difference in the protein hormone concentrations of cancer patients when compared with controls. A significantly higher level of prolactin was seen, however, when the cancer patients were compared with the BPH group. Subsequent studies have not consistently shown elevated values of prolactin in the cancer group, so a more detailed study was undertaken in order to see if there was a relationship between the hormone values obtained and the clinical staging of the patients. Table 1 shows the results obtained when patients were staged according to the UICC classification (20). Prolactin values did not correlate with either the primary tumor stage or metastatic status. GH concentrations were significantly higher in the metastatic group, but whether this is a consequence of increased stress and debilitating disease in these patients or a preexisting condition conducive to metastatic spread warrants further study. A wide variation in hormone values was noticed in all these studies and might be due to single sample determination of parameters which exhibit circadian or periodic variations. Changes in hormone concentrations throughout the day or night were therefore undertaken in a few BPH and prostatic cancer patients, as few data are available in subjects of this age. Blood was withdrawn from patients via an indwelling catheter at 30-min intervals over 12- or 24-hr periods. Large subject-to-subject variation in hormone levels were also encountered in this multiple sampling study, even within the same age group and disease category. An example of the differences can be seen in Fig. 1 in which data for
TABLE 1. Plasma hormone concentrations in patients with prostatic carcinoma classified according to UICC

<table>
<thead>
<tr>
<th>Patient category</th>
<th>Statistic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Age (yr)</th>
<th>LH (U/liter)</th>
<th>FSH (U/liter)</th>
<th>GH (mU/liter)</th>
<th>Prolactin (U/liter)</th>
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<td></td>
<td></td>
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<sup>a</sup> N = number of patients.
two patients with BPH are plotted. As prostatic tumor development is an age-dependent process, profound changes in hormone concentrations will occur because of testicular senescence, which in the human occurs over a large age range. Perhaps as a consequence of this, a comparison of rhythms and periodicity of plasma gonadotrophins in patients with prostatic cancer and BPH revealed no significant differences. This was also true of these patients’ responses to LHRH injection.

**PLASMA HORMONE CHANGES WITH ENDOCRINE THERAPY**

Another aspect to be considered in the relationship of pituitary hormones and prostatic cancer is the alteration induced by various endocrine therapies which are designed to reduce androgen levels and thereby prostatic tumor growth (9). Typical profiles of testosterone and pituitary concentrations obtained with various therapies are illustrated in Fig. 2. It is interesting to consider that on one regimen, namely estrogen therapy (Fig. 2b), prolactin and GH levels are increased and gonadotropin levels are decreased, while on another regimen, orchidectomy (Fig. 2a), normal prolactin and GH concentrations exist but gonadotropins are significantly raised. The clinical response of patients in these two groups are similar, despite their vastly differing protein hormone milieux.

**PROTEIN HORMONE LOCALIZATION IN PROSTATIC TISSUE**

Another aspect of the association of pituitary hormones and prostatic tissue can be explored using immunocytochemistry. Visual localization of polypeptide
hormones in the pituitary gland by employing this technique is well established (16,17). Localization at the target tissue level is more difficult because of the much lower concentrations and success will largely depend on the specificity and titer of the antisera used. Results obtained using the method described by Sternberger (18) on sections of human prostate tumor with antisera raised against human FSH or GH or prolactin can be seen in Figs. 3 and 4. The staining obtained with the various antisera was different in tissue distribution. Staining with the FSH antiserum was mostly confined to the epithelial cell secretory margins and the prostatic secretion, while staining with the prolactin antiserum was present in some epithelial cells, and in stromal and blood cells. Predominantly stromal cell staining was obtained with the GH antiserum, but occasionally a few cells were stained in the epithelial region. Only with the advent of monoclonal antisera raised against highly purified antigens will it be possible to equate staining with the presence of antigen at that site.
FIG. 3. Photomicrographs of consecutive sections of a single acinus from a patient with BPH (%289). a: Stained with hematoxylin and eosin. b—d: Immunocytochemical staining obtained with antisera raised against: (b) h.FSH (1/100), staining (arrow) seen predominantly in the epithelial cell region; (c) h.GH (1/100), staining seen in stromal cells; (d) h.Pr (1/500), staining appears in the cytoplasm of epithelial cells lining the acinus and also in the stroma.
FIG. 4. Photomicrographs of consecutive sections of a block of tissue from a patient with benign, predominantly fibromuscular hypertrophy of the prostate (x289). a: Stained with hematoxylin and eosin. b—d: Immunocytochemical staining obtained with antisera raised against (b) h.FSH, staining (arrow) associated with stromal areas and with the cell membrane; (c) h.GH, staining predominantly seen in cells of the stroma and occasionally in epithelial cells (stain also appears in the secretions); (d) h.Pr, distribution of stain as in (c).
ACKNOWLEDGMENTS

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The Immunocytochemical Detection of Protein Hormones in Human Prostatic Tissues


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The immunocytochemical detection of four pituitary protein hormones in tissue from 13 patients with benign prostatic hyperplasia has been described. There have been marked differences in the distribution and intensity of reaction product attributable to the various hormonal antisera. The intracellular presence of endogenous prolactin and FSH in the epithelial cytoplasm has been suggested together with the stromal localization of growth hormone and prolactin. Minimal diffuse staining over most cellular components was observed with the LH antiserum. This technique has provided an invaluable means of studying the potential involvement of pituitary protein hormones in the control of prostatic function and disease.

Key words: immunocytochemistry, prostate, hormones, human, localization, tumor

INTRODUCTION

Although the androgen dependency of prostatic tissue is well documented [1,2], the role that pituitary protein hormones play in the growth and functioning of the gland is uncertain. The indirect effect of luteinizing hormone (LH) on prostatic growth by virtue of this hormone’s influence on testicular androgen synthesis [3] and of ACTH and prolactin on adrenal C19-steroid synthesis can easily be recognized [4,5]. The possibility that abnormal levels of pituitary protein hormones might be involved in the aetiology of prostatic hyperplasia and neoplasia has also received some attention [6–10]. Concentrations of various hormones in plasma from tumour-bearing patients and from normal subjects were not shown to be significantly different, however, probably due to the large age-related changes encountered in this population of men undergoing testicular senescence.

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Animal experimentation has indicated that prolactin has a direct, yet synergistic action with androgen on prostatic tissue, influencing both the growth and biochemistry [11–13]. Prolactin binding to prostatic tissue has been demonstrated by several groups [14,15], consistent with the suggested direct action of the hormone, but little attention has been directed to the possible binding of other protein hormones such as the gonadotrophins, LH, and follicle stimulating hormone (FSH), and also growth hormone. Growth hormone has to some extent been implicated in the pathogenesis of prostatic cancer [16], and there is a need to identify possible relationships that may exist between the localization of these hormones in prostatic tissue and biochemical effects. Such an objective may be facilitated using immunocytochemical techniques [17]. Identification and localization of the protein hormones in the pituitary gland have been well established by such procedures [18–20], although their application to the study of target tissues for hormones has met with greater problems, largely due to the lower hormonal concentrations in the tissues. There is good evidence to suggest that protein hormones elicit their effects by binding to membrane-bound receptors [21], and therefore it would be anticipated that the localization should be at these sites. Recent autoradiographic and biochemical studies have indicated, however, the presence of protein hormones within cells [22,23], which is in agreement with immunocytochemical data recently reported [24,25].

MATERIALS AND METHODS

Materials

Horseradish peroxidase (HRP:RZ:3.1–3.4) and 3,3′-diaminobenzidine-tetrahydrochloride were purchased from Sigma (London) Chemical Co., Poole, Dorset. Other reagents were obtained from British Drug Houses Ltd., Poole, Dorset.

Tissues

Benign prostatic hyperplastic (BPH) tissue from 13 patients was obtained by open prostatectomy operations. Confirmation of the histological diagnosis of BPH was obtained prior to inclusion in this study. Bouin’s fixed, wax-embedded serial sections allowed direct comparison of all experimental and control immunocytochemical procedures. Tissues from two patients were divided: one portion was snap frozen in liquid nitrogen, the other processed routinely. Serial frozen sections were cut and fixed in Bouin’s fluid prior to inclusion in the immunocytochemical procedure. Serial sections of formal-saline-fixed human pituitaries were also used to test the antisera. In each experiment, one of the serial sections was stained with haematoxylin and eosin for morphological comparison.

Antisera

Antisera raised in rabbits against human growth hormone, FSH and LH, with excellent radioimmunoassay qualities were used in the immunocytochemical procedure. Cross reactivities of the antisera with other hormones were less than 1%, as assessed by radioimmunoassay, except the human LH antisera,
which had a 30% cross reactivity with human chorionic gonadotrophin. An antiserum (7110) raised in the Tenovus Institute to a pituitary extract, and used to assay prolactin, had less than 1% cross reactivity with LH and FSH but up to 40% with growth hormone, depending on the label used [26]. The dilutions of the antisera used in this study were: LH antiserum 1/50, FSH antiserum 1/100, growth hormone antiserum 1/100, and prolactin antiserum 1/500.

The wide-spectrum second antibody, sheep anti-rabbit serum, was used [27], and the peroxidase anti-peroxidase was prepared as outlined by Mason and Sammons [28].

**Antigens**

Purified preparations of protein hormones extracted from human pituitary glands were used in absorption control experiments. Human prolactin (WHO hPRL 75/504) and human growth hormone (MRC 69/46) were obtained from the National Institute for Biological Standards and Control, and human FSH was the gift of Professor W. Butt, Birmingham and Midland Hospital for Women.

**Immunocytochemical Staining Procedures**

The unlabelled antibody enzyme method [17] was used in this study. This involved the overnight incubation of tissue with either the antiserum under investigation or control preparations, followed by incubation with sheep anti-rabbit serum and peroxidase anti-peroxidase. Finally the substrate containing 3,3'-diaminobenzidine-tetrahydrochloride (0.05% w/v) and hydrogen peroxide (0.01%) in Tris-HCl buffer (0.05 M, pH 7.6) were added to complete the staining sequence, the former being converted to a dark brown reaction product. Prior incubation with nonimmune sheep serum was used to block nonspecific binding of the sheep anti-rabbit serum. Each addition was preceded by copious washing procedures. A more detailed outline of the methodology is being reported [29].

**Controls**

Methodological controls involved replacement of the appropriate antiserum with phosphate-buffered saline or a nonimmune rabbit serum and the endogenous peroxidase activity was monitored by incubating sections with enzyme substrate alone. Specificity or “absorption” controls were prepared by admixing the hormonal antiserum and a purified hormone preparation for 24 hours at 4°C, prior to inclusion in the immunocytochemical procedure. A rat pituitary section with a rat prolactin antiserum [29] was included in each experiment as a reagent control.

An evaluation of the specificity of the hormonal antisera was also gained from a series of experiments using serial human pituitary sections. Due to the high concentration of hormones within this gland, very dilute antisera could be utilized to demonstrate staining which consequently required small amounts of purified protein hormones for the absorption controls. Specific cell populations were stained by the individual hormonal antisera and those cells stained by the FSH antiserum could be absorbed by FSH but not by the growth hormone and prolactin preparations. Cells stained by the growth hormone antiserum could be absorbed with growth hormone but not with prolactin or FSH preparations.
The prolactin antiserum (7110) stained two populations of cells in the pituitary, one of which was identical to growth-hormone-containing cells and could be absorbed with growth hormone, but neither were absorbed with FSH.

RESULTS

A minimal amount of staining was apparent when the LH antiserum was used in the immunocytochemical procedure in all 13 specimens (Figs. 2,8). Diffuse staining was discernible in both stromal and epithelial regions and an artefactual precipitate was found above the plane of the section, which was not seen with other antisera examined; therefore, it was difficult to estimate the significance of the results obtained. The nonimmune rabbit serum and buffer control sections were essentially blank, except for a small amount of reaction product visible in capillaries (Figs. 6,12). Endogenous peroxidase activity as measured by incubation of the sections with substrate alone was not detected in any of the tissues.

Staining, attributable to the FSH antiserum, was demonstrated in the cytoplasm of epithelial cells lining acini and also in their secretions (Figs. 3,9). The majority of cystic acini were intensely stained, but only a proportion of the epithelium in glandular acini displayed immunoreactive FSH localization. The amount of staining observed in the cytoplasm varied, even between adjacent epithelial cells (Fig. 3). Stromal areas, except for the occasional capillary, were blank. This pattern of cytoplasmic epithelial FSH localization was present in the tissue from all 13 patients. A small amount of stromal staining, mostly of capillary origin, was observed in tissue from four of the prostatic tumours.

Figs. 1–6. Consecutive sections from a patient (D.P.) with pathologically diagnosed fibroepithelial hyperplasia of the prostate (× 400).

Fig. 1. Haematoxylin and eosin: Two glandular acini can be seen, surrounded by stroma and infiltrating blood cells.

Figs. 2–6. Staining obtained with particular antiserum or control serum in the immunocytochemical procedure.

Fig. 2. Human LH antiserum (1/50): Diffuse stain discernible in epithelium and stroma. Acini secretion more markedly stained.

Fig. 3. Human FSH antiserum (1/100): Strong stain visible in cytoplasm of epithelial cells with nuclei clearly unstained. Little reaction product in the stroma.

Fig. 4. Human growth hormone antiserum (1/100): The staining seen in epithelial areas is due to blood cell infiltration (arrowed). Other reaction product is in the stroma or in acini secretion.

Fig. 5. Human prolactin antiserum (7110) (1/500): Intense stain intracellularly located in the cytoplasm of epithelial cells with the nuclei clearly unstained. Acini secretions and elements in the stroma, particularly a capillary, contain reaction product.

Fig. 6. Nonimmune rabbit serum (1/100): In the control section, some capillary staining is just discernible (arrowed).
When the growth hormone antiserum was included in the immuno-cytochemical procedure, the staining observed was mostly confined to stromal areas (Figs. 4, 10). The reaction product was located in capillaries, lymphocytes, plasma cells, and in acellular elements in the stroma. Staining seen in the epithelial region of a small number of acini in tissue from a few tumours was attributable to lymphocytic infiltration in this area (Fig. 4). The epithelium was predominantly unstained, a characteristic of all 13 specimens examined.

An apparent intracellular localization of prolactin was observed in the vast majority of epithelial cells (Figs. 5, 11). The epithelial localization was cytoplasmic and not confined to any particular region of the cytoplasm, with the nuclei noticeably unstained in ten of the specimens examined. One specimen showed a weakly discernible staining of both epithelial and stromal areas. Reaction product was also seen in acini secretions. Intense stromal staining was noted in capillaries, blood cells, and fibromuscular elements similar to those results obtained with the growth hormone antiserum. The most extensive distribution of reaction product, in both epithelial and stromal areas of all the BPH specimens studied, was obtained with the prolactin antiserum.

Identical results were obtained in frozen sections of tissue from patients with BPH as had been observed in the wax-embedded tissues. Recently, a second human prolactin antiserum (G/R/51-IIAB) was utilized which did not contain a separate population of growth hormone antibodies. When used in both wax-embedded and frozen sections of BPH tissue, albeit at a low dilution (1/10), the immunolocalization of reaction product was seen in the epithelial cells and not in the stroma.

Absorption control experimentation indicated that the stromal localization observed with both the prolactin (7110) and the growth hormone antisera could

Figs. 7–12. Consecutive sections from a patient (W.J.) with pathologically diagnosed fibroepithelial hyperplasia of the prostate (× 400).

Fig. 7. Haematoxylin and eosin: Two glandular acini are visible, surrounded by stroma.

Figs. 8–12. Staining obtained with particular antiserum or control serum in the immunocytochemical procedure.

Fig. 8. Human LH antiserum (1/50): Diffuse staining visible over both stroma and epithelium. A precipitation artefact (arrowed) was also observed.

Fig. 9. Human FSH antiserum (1/100): Staining is located intracellularly in the epithelium but not in the stroma.

Fig. 10. Human growth hormone antiserum (1/100): Reaction product is confined to fibromuscular and cellular elements in the stroma and not in the epithelium, although some staining is visible in acini secretions (arrowed).

Fig. 11. Human prolactin antiserum (7110) (1/500): Stain is observed in the epithelium intracellularly, in secretions, and in the stroma.

Fig. 12. Nonimmune rabbit serum (1/100): Control section is unstained.
be absorbed by growth hormone (100 μg/ml), leaving the epithelial staining of the former preparation unaffected. The epithelial staining attributable to the FSH antiserum could be absorbed by FSH (20 μg/ml) but was not absorbed with growth hormone. Absorption of the antisera with prolactin at the dilutions used in the target tissue was not possible with the limited availability of purified prolactin.

DISCUSSION

Antisera to protein hormones were used in this study to locate the presence of the endogenous hormones in sections of prostate tissue removed from patients with BPH. The problems concerned with the interpretation of immunocytochemical data are mainly due to the relatively poor specificity of the available antisera, coupled with the lack of definitive controls [30]. The preparation of antisera by monoclonal methods would probably ensure a greater specificity of the results. Using the antisera presently available, however, protein hormones appear to have been located in a variety of cellular components of prostatic tumours.

The immunocytochemical detection of prolactin in the cytoplasm of epithelial cells was interesting, as several reports have described the internalization of hormones or hormone receptor complexes in the cytoplasm of particular target tissues [31,32]. Prolactin has been shown to have direct effects on prostatic growth and metabolism and the cellular localization is in agreement with these biochemical data. Although membrane localization of the protein hormones may have been anticipated, this was not apparent in the present study, probably due to the low concentration of endogenous hormone present. In the limited information previously available on the visualization of prolactin in human prostatic tissue [24], reaction product was only detected in epithelial cytoplasm after a prior incubation with prolactin.

Studies with a growth hormone antiserum on tissue from the dog prostate have indicated membrane staining [25], but the method again included preincubation of the section with growth hormone and in consequence, more than endogenous hormone has been detected. In contrast, the studies now reported have shown a complete lack of growth hormone staining in epithelial cells in BPH tissue, although the staining of many of the stromal components was a characteristic of the specimens examined. Stromal localization of protein hormones in human and rat prostatic tissue was not observed using prolactin or human placental lactogen antisera [24,33]. The results reported here indicate that although the prolactin antiserum (7110) produced staining in both epithelial cells and some stromal components, the latter was possibly due to growth hormone antibodies in the preparation, as these were absorbed by growth hormone. Also, immunolocalization of the second prolactin antiserum (G/R/51-IIAB) was observed only within epithelial cells. Studies on the dog prostate have indicated prolactin in some fibromuscular elements, but the presence of growth hormone in these areas was not reported [25].

The presence of growth hormone in the stromal areas may be of importance when considering the interrelationships of the stroma and epithelium in the growth and dissemination of prostatic tumours. Of interest in this respect is the reported increased levels of growth hormone in prostatic cancer patients with metastases compared to those without metastatic spread [16].
Detection of immunoreactive FSH in prostatic epithelium was surprising, since neither receptors for FSH, nor autoradiographic localization of this hormone have previously been reported. Furthermore, direct effects of FSH on prostatic biochemistry have not been described, and a possible role for FSH in the prostate might be worth further investigation, especially when considering the increasing concentration of this hormone in the elderly male. LH, which also increases with advancing age, was not visualized to any great extent in the epithelium or stroma of these BPH specimens.

Immunocytochemical procedures to localize more precisely both endogenous hormone and also hormone receptor distribution, offer advantages over most other methods involving cell-free systems, especially when dealing with the heterogeneous population of cells generally present in most tumours. It is clear that such data could provide a valuable insight into the role of protein hormones in prostatic pathogenesis. These procedures are currently being used to study prostatic cancer tissue and the extension of this technique to detect steroid hormone receptors in this and other hormone-responsive tissues is underway.

**CONCLUSIONS**

Using two prolactin antisera there appeared to be localization of this hormone in the cytoplasm of epithelial cells in all of the BPH tissues examined. The stromal localization observed with one prolactin antiserum preparation (7110) was possibly due to growth hormone antibodies, as a growth hormone antiserum had given similar stromal staining and the second prolactin antiserum (G/R/51-IIAB) showed no stromal localization. The gonadotrophic hormones, LH and FSH, did not have similar distribution, minimal diffuse staining being seen with the LH antiserum over most tissue components. Intense FSH staining was observed in the epithelial cytoplasm of approximately one third of the glandular and in all of the cystic acini. These results are consistent with the internalization of fragments or whole protein hormone molecules in prostatic epithelium and their significance awaits further investigation.

**ACKNOWLEDGMENTS**

The authors would like to acknowledge the generous financial support of the Tenovus Organization. We are grateful for the generous donation of human FSH, LH, and growth hormone antisera from Professor W. Butt, Department of Clinical Endocrinology, Birmingham and Midland Hospital for Women. Human prolactin antiserum (7110) was obtained from Dr. G.V. Groom at the Institute, and human prolactin antiserum (G/R/51-IIAB) from Professor V. Marks, Department of Biochemistry, University of Surrey, Guildford. We would also like to thank Mr. C. Smith for excellent photographic assistance.

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The Immunocytochemical Detection of Growth Hormone and Prolactin in Human Prostatic Tissues


INTRODUCTION

Although the androgen dependency of prostatic tissue is well documented [1,2], the role that pituitary protein hormones play in the growth and functioning of the gland is uncertain. The possibility that abnormal levels of pituitary protein hormones might be involved in the aetiology of prostatic hyperplasia and neoplasia has also been intimated [3-7]. Concentrations of various hormones in plasma from tumour-bearing patients and from normal subjects were not shown to be significantly different.

Animal experimentation has indicated that prolactin has a direct, yet synergistic action with androgen on prostatic tissue, influencing both growth and biochemistry [8-10]. Prolactin binding to prostatic tissue has been demonstrated by several groups [11,12], consistent with the suggested direct action of the hormone, but little attention has been directed to the possible binding of other protein hormones such as growth hormone and follicle stimulating hormone (FSH). Growth hormone has to some extent been implicated in the pathogenesis of prostatic cancer [13], and there is a need to identify possible relationships that may exist between the localization of these hormones in prostatic tissue and any consequent biochemical effects. Such an objective may be facilitated using immunocytochemical techniques [14]. Identification and localization of the protein hormones in the pituitary gland has been well established by such procedures [15-17], although their application to the study of target tissues for hormones has met with greater problems, largely due to the lower hormonal concentrations in these tissues. There is good evidence to suggest that protein hormones elicit their effects by binding to membrane-bound receptors [18], and therefore it would be anticipated that the localization should be at these sites. Recent autoradiographic and biochemical studies have indicated, however, the presence of protein hormones within cells [19,20], which is in agreement with immunocytochemical data recently described [21,22].
Studies on the topographical distribution of protein hormones by immunocytochemical techniques are presently limited by the availability of specific antisera and highly purified antigens. The immunocytochemical specificity of the antisera was checked by localizing protein hormones in specific cells of the human pituitary gland. Possible changes in the antigenicity of the tissues during routine histological processing have been investigated by comparing results obtained in both frozen and wax-embedded tissue sections. A further indication of the specificity of protein hormone localization in prostate tissues has been gained from absorption control experiments by admixing the antisera with pure hormonal preparations.

**MATERIALS AND METHODS**

**Tissues, Antisera, Antigens**

Benign prostatic hyperplastic (BPH) tissue was obtained by open prostatectomy operations, and prostatic carcinoma specimens were acquired by means of cold punch or ‘Trucut’ needle biopsies. Confirmation of the histological diagnosis of BPH or prostatic carcinoma was obtained prior to inclusion in this study. Bouin’s fixed, wax-embedded serial sections allowed direct comparison of all experimental and control immunocytochemical procedures. Some tissues were divided into portions and snap frozen in liquid nitrogen or processed routinely. Serial frozen sections were cut and fixed in Bouin’s fluid prior to inclusion in the immunocytochemical procedures. Serial sections of formal-saline fixed human pituitaries were also used to test the specificity of the antisera. In each experiment, one of the serial sections was stained with haematoxylin and eosin for morphological comparison.

Antisera raised in rabbits against human growth hormone (M153) and FSH (M91/1), both gifts of Professor W. Butt, Birmingham and Midland Hospital for Women, U.K., were used in these studies. The cross reactivities of these antisera with other hormones were less than 1%, as assessed by radioimmunoassay. An antiserum (7110) raised in the Tenovus Institute to a pituitary extract and used to assay prolactin had less than 1% cross reactivity with LH and FSH [23]. This antiserum contained two separate populations of antibodies, one specific for prolactin and one specific for growth hormone. The dilutions of these antisera for immunodetection ranged from 1/100 to 1/500. Another prolactin antiserum (G/R/51-IIAB), provided by Professor V. Marks, University of Surrey, Guildford, U.K., which crossreacted less than 1% with LH, FSH, and growth hormone was utilized in later experiments. Additional methodological requirements included a wide spectrum second antibody, sheep anti-rabbit serum [24] and peroxidase antiperoxidase (PAP), which was prepared as outlined by Mason and Sammons [25].
Protein Hormone Localization in the Prostate

Purified preparations of protein hormones extracted from human pituitary glands were used in absorption control experiments. Human prolactin (WHO hPRL 75/504) and human growth hormone (MRC 69/46) were obtained from the National Institute for Biological Standards and Control, and purified human FSH was the gift of Professor W. Butt, Birmingham and Midland Hospital for Women, U.K.

Immunocytochemical Staining Procedures

The unlabelled antibody enzyme method [14] was chosen for these studies; the methodological procedures are outlined in Table I. This involved the overnight incubation of tissue with either the antiserum under investigation or control preparations, followed by incubation with sheep anti-rabbit serum and PAP. Finally the substrate containing 3,3'-diaminobenzidine-tetrahydrochloride (DAB 4HC1; 0.05% w/v) and hydrogen peroxide (0.01%) in Tris-HCl buffer (0.05 M, pH 7.6) were added to complete the staining sequence, the former being converted to a dark brown reaction product. Prior incubation with nonimmune sheep serum was used to block nonspecific binding of the sheep anti-rabbit serum. Each addition was preceded by copious washing procedures.

Controls

Methodological controls involved replacement of the appropriate antiserum with phosphate-buffered saline or a nonimmune rabbit serum, and the endogenous peroxidase activity was monitored by incubating sections with enzyme substrate alone. Specificity or “absorption” controls were prepared by admixing the hormonal antiserum and a purified hormone preparation for 24 hours at 4°C, prior

<table>
<thead>
<tr>
<th>TABLE I. Sequence of incubations for immunocytochemical localization: Unlabelled antibody enzyme method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Preincubation with nonimmune sheep serum</td>
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<tr>
<td>2) Incubation of protein hormone antiserum (or control serum preparations)</td>
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<tr>
<td>3) Three washes with fresh PBS</td>
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<tr>
<td>4) Incubation of sheep anti-rabbit serum</td>
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<tr>
<td>5) Three washes with fresh PBS</td>
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<tr>
<td>6) Incubation of PAP</td>
</tr>
<tr>
<td>7) Three washes with fresh PBS</td>
</tr>
<tr>
<td>8) Incubation of substrate (DAB4HCl + H2O2)</td>
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<tr>
<td>9) Final wash with PBS to terminate reaction</td>
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<tr>
<td>10) Counterstaining at this stage, if required, prior to mounting sections using UV inert aqueous mounting medium</td>
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to inclusion in the immunocytochemical procedure. A rat pituitary section with a rat prolactin antiserum [26] was included in each experiment as a reagent control.

An evaluation of the specificity of the hormonal antisera was also gained from a series of experiments using serial human pituitary sections. Due to the high concentration of hormones within this gland, very dilute antisera could be utilized to demonstrate staining which consequently required small amounts of purified protein hormones for the absorption controls. Specific cell populations were stained by the individual hormonal antisera, and those cells stained by the FSH antiserum could be absorbed by FSH but not by the growth hormone and prolactin preparations. Cells stained by the growth hormone antiserum could be absorbed with growth hormone but not with prolactin or FSH preparations. The prolactin antiserum (7110) stained two populations of cells in the pituitary, one of which was identical to growth hormone-containing cells and could be absorbed with growth hormone, but neither were absorbed with FSH.

RESULTS

BPH Tissues

Prostatic tissue sections from 13 patients with BPH were examined. Staining, attributable to the binding of the growth hormone antiserum was predominantly confined to stromal areas, particular stromal regions showing more intense staining than others (Fig. 1c). The reaction product was located in capillaries, some blood cells, and in acellular elements of the stroma. Epithelial cells were rarely stained.

When the prolactin antiserum (7110) was included in the immunocytochemical procedure, apparent intracellular staining of prolactin was observed in the vast majority of epithelial cells (Fig. 1d). The epithelial localization was cytoplasmic and not confined to any particular region of the cytoplasm, with the nuclei noticeably unstained. Reaction product was also seen in the acini secretions. Intense stromal staining was noted in capillaries, blood cells, and fibromuscular elements, similar to the results obtained with the growth hormone antiserum.

Fig. 1. a–d) Consecutive sections of an acinus taken from a patient with BPH (×405). a) Haematoxylin and eosin (H & E) showing a glandular acinus, surrounded by stroma. b) Human FSH antiserum (1/100): Strong intracellular cytoplasmic staining of particular epithelial cells. c) Human growth hormone antiserum (1/100): Reaction product is visible only in the stroma. d) Human prolactin antiserum (7110) (1/500): Reaction product (arrowed) observed solely in the stroma. e,f) Consecutive frozen sections taken from a patient with BPH, counterstained with haematoxylin to ascertain the tissue morphology (×160). e) Human growth hormone antiserum (1/100): Reaction product (arrowed) observed solely in the stroma. f) Human prolactin antiserum (7110) (1/500): Staining is located intracellularly in the cytoplasm of epithelial cells and also in the stroma (arrowed).
Recently, a second human prolactin antiserum (G/R/51-IIAB) was utilized which did not contain a separate population of growth hormone antibodies. When used in both wax-embedded and frozen sections of BPH tissue, albeit at a low dilution (1/10), the immunolocalization of reaction product was seen in the epithelial cells and not in the stroma.

Staining, attributable to the FSH antiserum, was demonstrated in the cytoplasm of epithelial cells lining acini and also in their secretions (Fig. 1b). The majority of cystic acini were intensely stained, but only a proportion of the epithelium in glandular acini displayed immunoreactive FSH localization. The amount of staining observed in the cytoplasm varied, even between adjacent epithelial cells. Stromal areas, except for the occasional capillary, were blank. This pattern of cytoplasmic epithelial FSH localization was present in the tissue from all 13 patients. A small amount of stromal staining, mostly of capillary origin, was observed in tissue from four of the prostatic tumours.

Identical results were obtained in frozen sections of tissue from patients with BPH as had been observed with the various protein hormone antisera in the wax-embedded tissues. Staining was detected in stromal areas of the sections in the presence of both the growth hormone and prolactin antiserum (7110) (Fig. 1e, f), but was only seen in the epithelial cell cytoplasm with the prolactin antiserum (7110) (Fig. 1f). The nuclei of these frozen sections were counterstained with haematoxylin for ease of histological examination.

Only a limited number of absorption control experiments were undertaken on BPH sections due to the scarcity of purified protein hormone preparations. Prior absorption of the FSH antiserum with FSH (20 μg/ml) resulted in negative staining of epithelial cells in an acinus, which in a serial section exhibited positive staining with the same dilution of nonabsorbed FSH antiserum (Fig. 2a, b). Admixing the FSH antiserum with growth hormone (100 μg/ml) did not affect the result. The stromal staining observed with the growth hormone antiserum (Fig. 2c) could be eliminated by prior absorption of the antiserum with growth hormone (100 μg/ml) (Fig. 2d). Similarly this amount of growth hormone absorbed the stromal but not the epithelial cell staining obtained with the prolactin antiserum (7110) (Fig. 2e, f).

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**Fig. 2.** a-f) Absorption control experiments on sections taken from a patient with BPH (× 405): a) Human FSH antiserum (1/250): Intracellular, cytoplasmic staining of particular epithelial cells. b) Human FSH antiserum (1/250) absorbed with FSH (20 μg/ml): The specific immunolocalization has been completely absorbed. c) Human growth hormone antiserum (1/250): Reaction product is located in the stroma (arrowed). d) Elimination of staining by application of human growth hormone antiserum (1/250) absorbed with growth hormone (100 μg/ml). e) Human prolactin antiserum (7110) (1/500): Epithelial cytoplasm and stroma are stained. f) The stromal-staining component is absent when the human prolactin antiserum (7110) (1/500) is absorbed by growth hormone (100 μg/ml).
Prostatic Carcinoma Tissues

Cold punch or 'Trucut' needle biopsy prostatic tissue specimens from eight patients with histologically proven prostatic cancer were examined in this study. The majority of the tumours demonstrated marked variation in differentiation which necessitated evaluation of tissue sections from different regions of the specimen. Some characteristics of the protein hormone antiserum binding which had been observed in BPH specimens were present in the carcinomas, but additional morphological features were stained in several tumours.

Growth hormone antiserum gave positive stromal staining which was often more intense in areas adjacent to infiltrating carcinoma cells. Additional staining of epithelial cell nuclei and some epithelial cell cytoplasms was encountered in particular specimens (Fig. 3c). Even within a small area of tumour, marked variation in immunocytochemical staining was observed: Adjacent cells exhibited negative staining, cytoplasmic, nuclear, or cytoplasmic and nuclear staining (Fig. 4e). In another area of the same specimen only stromal staining was apparent (Fig. 4a).

Use of the prolactin antiserum (7110) in the prostatic carcinoma specimens resulted in intense staining of many areas. Positive staining was seen in the epithelial cytoplasm of all eight tumours (Fig. 3d, Fig. 4b, f). Nuclear staining of epithelial cells was noted in some regions particularly in the more anaplastic areas. Stromal staining of the tumours was extensive and often made the precise location of the reaction product difficult to ascertain (Fig. 4f).

Positive staining with the FSH antiserum was rarely encountered, but when present it was located in epithelial cell cytoplasm (not illustrated).

Occasional capillary staining was observed in the carcinoma tissues with nonimmune rabbit serum used at a similar dilution to the hormonal antisera in the immunocytochemical procedure (Figs. 3b, 3f, 4d).

DISCUSSION

Antisera to protein hormones were used in this study to locate the presence of the endogenous hormones in sections of prostatic tissue removed from patients with BPH or carcinoma of the prostate. The problems concerned with the interpretation of immunocytochemical data are mainly due to the relatively poor specificity of the available antisera, coupled with the lack of definitive controls.

Fig. 3. a–d) Consecutive sections taken from a prostatic cancer patient (H.H.) (x 260). Sections counterstained with haematoxylin for morphological comparison. a) H & E. b) Nonimmune rabbit serum (1/100) control section: No reaction product visible. c) Human growth hormone antiserum (1/100): Cytoplasm, nuclei, stroma stained (arrowed). d) Human prolactin antiserum (7110) (1/500): Cytoplasm, nuclei, stroma stained (arrowed). e,f) Prostatic cancer patient (A.R.) (x 350). e) H & E. f) Nonimmune rabbit serum (1/100) control counterstained with fast green (see also Fig. 4 a,b).
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[27]. The preparation of antisera by monoclonal methods would probably ensure a greater specificity of the results. Using the antisera presently available, however, protein hormones appear to have been located in a variety of cellular components of prostatic tumours.

An evaluation of the immunocytochemical specificity of the antisera was obtained by the positive staining of specific cells of human pituitary glands, which could be absorbed only with their appropriate antigens. The results described in this communication include data obtained from both frozen and wax-embedded BPH tissues. In addition, the specificity of FSH and growth hormone staining in prostatic tissues was demonstrated by prior absorption of the antisera with purified protein hormones.

The immunocytochemical detection of prolactin in the cytoplasm of epithelial cells was interesting, as several reports have described the internalization of hormones or hormone receptor complexes in the cytoplasm of particular target tissues [28,29]. Prolactin has been shown to have direct effects on prostatic growth and metabolism, and the cellular localization is in agreement with these biochemical data. Although membrane localization of the protein hormones may have been anticipated, this was not apparent in the present study, probably due to the low concentration of endogenous hormone present on such structures. In the limited information previously available on the visualization of prolactin in human prostatic tissue [21], reaction product was only detected in epithelial cytoplasm after a prior incubation with prolactin.

Nuclear localization of staining product was rarely encountered and was not seen in the BPH specimens with any of the protein hormones antisera tested, ie, prolactin, growth hormone, FSH, LH, TSH, and βHCG. Particular areas of prostatic carcinoma tissues, however, exhibited positive nuclear staining with prolactin and growth hormone antisera. This intriguing observation obviously requires further investigation as there is little evidence for direct interaction of protein hormones on nuclear function. These data would agree with the nuclear immunocytochemical localization of growth hormone and prolactin described in canine prostate tissue [22].

Studies with growth hormone antiserum on tissue from the dog prostate have indicated membrane staining [22], but the method again included preincubation of the section with growth hormone and, in consequence, more than endogenous

Fig. 4. a,b) Prostatic cancer patient (A.R.). Sections counterstained with fast green (× 350). a) Human growth hormone antiserum (1/100): stromal staining. b) Human prolactin antiserum (7110) (1/500): Intracellular, cytoplasmic, and stromal staining (see also Fig. 3 e,f). c–f) Different area of same tumour (× 350). c) H & E. d) Nonimmune rabbit serum control (1/100). e) Human growth hormone antiserum (1/100): Stained nucleus and cytoplasm (arrowed). f) Human prolactin antiserum (7110) (1/500): Stained nucleus and cytoplasm (arrowed).
hormone has been detected. In contrast, the studies now reported have shown a complete lack of growth hormone staining in epithelial cells in BPH tissues, although the staining of many of the stromal components was a characteristic of all the BPH specimens examined. Although the stroma exhibited positive growth hormone localization in the carcinoma specimens, some tumour cells also displayed diffuse cytoplasmic staining. In a study using prolactin or human placental lactogen antisera in human and rat prostate, stromal localization of these hormones has not been reported [21,30].

Studies on the dog prostate have indicated prolactin in some fibromuscular elements but the presence of growth hormone in these areas was not observed. The results reported here indicate that although the prolactin antiserum (7110) produced staining in both epithelial cells and some stromal components, the latter was possibly due to growth hormone antibodies in the preparation, as these were absorbed with growth hormone. Also, immunolocalization with a second prolactin antiserum (G/R/51-IIAB) was observed only within epithelial cells of BPH tissues.

The presence of growth hormone in the stromal areas may be of importance when considering the interrelationships of the stroma and epithelium in the growth and dissemination of prostatic tumours. Of interest in this respect is the reported increased levels of growth hormone in prostatic cancer patients with metastases compared to those without metastatic spread [13].

The heterogeneity of prolactin and growth hormone antiserum staining in the prostatic carcinomas may be an expression of tumour cell clones having different abilities to bind and perhaps respond to these hormones.

Detection of immunoreactive FSH in prostatic epithelium was surprising, since neither receptors for FSH, nor autoradiographic localization of this hormone have previously been reported. Furthermore, direct effects of FSH on prostatic biochemistry have not been described and a possible role for FSH in the prostate might be worth further investigation, especially when considering the increasing concentration of this hormone in the elderly male.

Apart from the accompanying changes in protein hormone concentrations which occur in the aging male, many of the major therapies for prostatic cancer drastically alter plasma protein hormones [5]. It would therefore seem pertinent to investigate their binding and significance in prostatic tumours. Immunocytochemical procedures to localize more precisely both endogenous hormone and also hormone receptor distribution, offer advantages over most other methods involving cell-free systems, especially when dealing with the heterogeneous population of cells generally present in most tumours. It is hoped that such data may provide a valuable insight into the role of protein hormones in prostatic pathogenesis.
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CONCLUSIONS

The immunocytochemical detection of prolactin, growth hormone, and FSH in both BPH and prostatic carcinoma tissues has been described. Limitations imposed on the interpretation of results due to the specificity of the antisera have been discussed. The distribution of staining in the BPH tissue was consistently found in particular cellular components. Growth hormone localized in stromal regions: FSH in the epithelial cell cytoplasm of approximately 30% of the acini and prolactin uniformly present in epithelial cell cytoplasm and in various stromal components. Prostatic carcinoma tissues displayed more diverse staining of cellular elements. Tumour cell nuclei and cytoplasm often showed reaction product deposition attributable to growth hormone and prolactin in addition to stromal staining. FSH was rarely found in the carcinomatous areas. The heterogeneity of the localization might be a consequence of the degree of tumour cell differentiation and this requires additional investigation. The immunocytochemical results are consistent with internalization of fragments of whole protein hormone molecules within prostatic cells and their significance needs further evaluation.

ACKNOWLEDGMENTS

The authors would like to acknowledge the generous financial support of the Tenovus Organization. We would also like to thank Mr. C. Smith for excellent photographic assistance.

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I. INVESTIGATIONS RELATING TO CANCER OF THE PROSTATE.

D. SOME CLINICAL ASPECTS OF PROSTATIC DISEASE.
Steroids and the Prostate

Mr W B Peeling
(The Royal Gwent Hospital,
Newport, Monmouthshire)
and Dr K Griffiths
(Tenovus Institute for Cancer Research, Cardiff)

Testosterone was first isolated in the mid 1930s, and since then has been regarded as the principal androgenic hormone responsible for developing and maintaining the activity of male accessory sex organs, such as the prostate. Recent studies in rats have questioned this concept because administration of tritium-labelled testosterone did not result in the accumulation of testosterone in the ventral prostatic tissue, but of a metabolite of this steroid, 5α-dihydrotestosterone (5αT) (Wilson 1970). Furthermore, 'receptor' proteins which specifically bound 5αT were demonstrated in the cytoplasm of rat prostatic cells, and these were absent from non-target tissues that were examined. 5αT was probably transferred to the nuclei of prostatic cells by a process which involved these receptors.

These are important new observations, and some attempt must be made to relate them to the problems of human prostatic disease.

Studies on rat prostatic tissue have been carried out on normal animals, and it is difficult to compare these with studies of normal human tissue as this is only obtainable from autopsy specimens and prostates removed at total cystoprostatectomy. However, our studies have shown that the metabolism of testosterone in benign hyperplastic prostatic tissue in humans is similar to that in rats. Tritium-labelled testosterone (10 μCi) was administered intravenously to men undergoing open prostatectomy, and the principal radio-
labelled steroid isolated from the removed tissue was 5αT (Pike et al. 1970). These observations relate well to those of Siiteri & Wilson (1970) who found that the concentration of 5αT was on average five times greater in hyperplastic than in normal prostatic tissue in men, and that it was sited mostly in the peri-urethral zone of the gland. Possibly 5αT plays a role in the etiology of benign hyperplasia of the prostate in humans; the observation that some degree of prostatic hyperplasia was also induced when young castrated dogs were treated with 5αT (Gloyna et al. 1970) supports this idea.

In relation to these observations the tissue culture studies of Baulieu and his colleagues (Baulieu & Robel 1970, Lasnitzki 1970) are particularly relevant. They showed that not only did testosterone and 5αT play some role in the maintenance of the functional integrity of rat prostate cells in culture, but also that other testosterone metabolites, the 5α-androstanediols which are also found in prostatic tissue, may control some aspects of cell metabolism. This suggests that testosterone and its various metabolites may possibly each have a particular function in the prostate, and that a change in the balance or relative concentrations of these steroids could be concerned with the induction of hyperplasia or neoplasia. A possible role of oestrogens in the control of these metabolic patterns requires further investigation.

Experiments conducted in our laboratories with rat, canine and human prostatic tissue in culture indicated that oestrogens affected testosterone metabolism by the tissue. Oestradiol-17ß and diethylstilbestrol decreased both the uptake of radioactive testosterone by the tissue and the 5αT formed. The amount of radioactive 5αT isolated from nuclear preparations of culture tissue decreased when either oestradiol-17ß or diethylstilbestrol was present in the medium. Moreover, an identical effect was obtained with dihydrodiethylstilbestrol, an analogue of diethylstilbestrol. This compound, synthesized in our laboratories (Harper et al. 1970), has 0-1% of the oestrogenic activity of oestradiol-17ß, and further studies on its biological activity in the rat and dog are currently in progress to assess any possible application to the treatment of prostatic dysfunction in men. One interesting series of experiments in this respect is concerned with the activity of the nuclear enzyme system, DNA polymerase, isolated from human prostatic tissue. When this isolated enzyme system was incubated with the appropriate co-factors, it was shown that 5αT and testosterone were capable of stimulating the activity of certain of the enzyme preparations from human hyperplastic prostatic tissue. Conversely, both diethylstilbestrol and dihydrodiethylstilbestrol inhibited the enzyme system isolated from both hyperplastic and malignant prostatic tissue, whereas other alkyl substituted derivatives of stilbestrol, such as dipropylstilbestrol and dibutylstilbestrol, had little or no effect. It is possible, therefore, that dihydrodiethylstilbestrol exercises a direct action on this nuclear enzyme system in vivo, and further studies are in progress to ascertain the mechanism of this inhibitory effect and any possible application to the treatment of benign hyperplasia and carcinoma of the prostate in men. The low oestrogenicity of this compound could be a crucial factor in any practical considerations, particularly in view of the recent reservations about the place of diethylstilbestrol in the treatment of prostatic cancer (Arduino et al. 1967).

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Plasma Prolactin, GH, LH, FSH, TSH and Testosterone During Treatment of Prostatic Carcinoma with Oestrogens*

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Abstract—Fourteen patients with prostatic carcinoma were treated with various doses of diethylstilboestrol (DES) and Premarin (conjugated equine oestrogens). DES (15 mg/day) produced a significant increase in plasma concentrations of growth hormone and prolactin and a decrease in plasma concentrations of testosterone, luteinizing hormone and follicle stimulating hormone. Premarin (15 mg/day) reduced plasma concentrations of testosterone and luteinizing hormone but had no effect on plasma concentrations of growth hormone and prolactin. Plasma levels of thyroid stimulating hormone were unaffected by treatment. The oral administration of glucose reduced plasma levels of prolactin.

INTRODUCTION

The early work of Huggins and his colleagues showed that the growth of prostatic carcinoma is stimulated by androgens, and is inhibited by oestrogens [1]. Oestrogen therapy lowers plasma concentrations of testosterone and its success appears to be related to the depressive effects on androgen metabolism in general [2-4]. Although the majority of cases respond to treatment, recurrence frequently occurs and there is evidence that this is accompanied by a gradual rise in plasma levels of testosterone [5]. This may result from stimulation of the testis or adrenal.

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Oestrogen increases adrenal weight in the rat in addition to lowering plasma concentrations of luteinizing hormone and raising circulating levels of prolactin and growth hormone [6, 7]. Prolactin has been shown to stimulate the release of testosterone by the human adrenal in vitro [8] and it may have an ancillary function in maintaining normal androgen synthesis by the testis [9]. However, no studies have yet been reported on the effects of long-term oestrogen treatment on plasma levels of prolactin in the human male.

A previous investigation was made by some of us into the changes in carbohydrate and lipid metabolism during the oestrogen therapy of prostatic cancer [10]. Plasma samples were obtained during glucose tolerance tests as part of that study. In the work reported here, we have used the same samples to examine the
effect of treatment with diethylstilboestrol (DES) and Premarin (conjugated equine oestrogens) on plasma levels of prolactin and other pituitary hormones in patients with carcinoma of the prostate.

MATERIAL AND METHODS

Fourteen patients with histologically-proven carcinoma of the prostate were studied. Their ages ranged from 49 to 83 years (mean 71 years). The patients had the nature of the study explained to them and had agreed to take part; they were treated with either DES (7·5 or 15 mg/day) or Premarin (15 mg/day). One patient was also treated with DES (1 mg/day). Not every patient underwent all the treatment regimens and the order in which the drugs were administered was varied. Six patients were also studied before starting therapy.

With most patients, 50-g oral glucose tolerance tests were carried out during a short hospital stay; the remainder underwent the tests as outpatients. The tests were started between 6·00 and 9·00 hours, blood being taken at 0, 30, 60 90 and 120 min after glucose ingestion.

One or more glucose tolerance tests were carried out after a patient had been on a particular treatment regimen for 6–90 weeks.

Plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured by double antibody radioimmunoassays shown to be specific for these hormones [11]. The sensitivity of each assay was 0·5 m.i.u./ml of 2nd IRP HMG and MRC 63/15 respectively. Plasma thyroid stimulating hormone (TSH) was measured by a double antibody radioimmunoassay using reagents distributed by the Medical Research Council. In this assay system, negligible cross-reaction was observed with FSH, LH, growth hormone and prolactin.

The sensitivity of the assay was 0·5 µu/ml of MRC 68/38 TSH. Plasma growth hormone (GH) was estimated by a specific double antibody radioimmunoassay [12] using purified human growth hormone (MRC 69/46) and rabbit antiserum to human growth hormone (Wellcome Reagents Ltd). Plasma prolactin was measured by a homologous double antibody radioimmunoassay in which there was negligible cross-reaction with FSH, LH, TSH, GH and chorionic somatomamtomotrophin [13]. The sensitivity of the assay was 10 milliamoulpes/ml of MRC 71/222. Total plasma testosterone was measured by a specific radioimmunoassay, the sensitivity of the method being 0·015 ng/ml [14].

Statistical analyses were performed by non-parametric methods.

RESULTS AND DISCUSSION

Oestrogen treatment produced a fall in plasma testosterone concentrations (Table 1). However, measurable amounts of the hormone were still detectable in the plasma even at the highest dose of DES. Similar findings were reported by Mackler et al. [15], showing that

<table>
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<tr>
<th>Treatment</th>
<th>T (ng/ml)</th>
<th>GH (m.i.u./ml)</th>
<th>PRL (m.i.u.)</th>
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<th>FSH (m.i.u.)</th>
<th>TSH (m.i.u.)</th>
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</tbody>
</table>

Table 1. Effect of diethylstilboestrol (DES) and Premarin on plasma concentrations of prolactin, growth hormone, testosterone, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone in patients with prostatic carcinoma.

T, testosterone (ng/ml); GH, growth hormone (m.i.u./ml); PRL, prolactin (milliumoles MRC 71/222/ml); LH, luteinizing hormone (m.i.u./MRC A 63/15/ml); FSH, follicle stimulating hormone (m.i.u./2nd IRP–HMG/ml); TSH, thyroid stimulating hormone (m.i.u./MRC 68/33/ml); N, number of patients. Values shown are the median and range of hormone concentrations found in plasma taken from patients after an overnight fast. P (v. controls): a<0·05; b<0·03; c<0·02; d<0·008; e<0·002; (Mann–Whitney two-tailed U-test).
large doses of oestrogen, while significantly suppressing LH and FSH release, do not succeed in completely abolishing testosterone synthesis.

It was of interest that the highest doses of DES (15 mg/day) stimulated growth hormone secretion, whereas the lower doses of DES and Premarin were without effect (Table 1). Premarin has only about half the oestrogenic activity of DES [16], probably accounting for the failure of the former drug to influence GH secretion. Although oestrogen has been shown to enhance the GH response to an infusion of arginine [17], our results (example Fig. 1) indicate that basal plasma levels of GH also increase after long-term administration of the drugs. Growth hormone has a powerful lipolytic action [18] and the stimulation of GH secretion may be responsible for the rise in plasma triglycerides that is observed during oestrogen therapy [10, 19].

Studies in women with breast cancer have shown that prolactin concentrations in the plasma rise after treatment with DES [20]. A similar increase in circulating prolactin was demonstrated in our patients treated with DES (15 mg/day). In contrast, DES (7.5 mg/day) or Premarin (15 mg/day) had no significant effect on plasma prolactin levels (Table 1).

The higher dose of DES (15 mg/day) and Premarin (15 mg/day) both significantly lowered LH concentrations in the plasma (Table 1). This confirms the reports of earlier workers [2, 5]. We also observed a reduction in plasma levels of FSH (Table 1), as has been noted in castrated human males after the administration of DES [21]. On the other hand, plasma concentrations of TSH were unaffected by oestrogens (Table 1). Such a finding was not unexpected as animal experiments suggest that oestrogens do not have a consistent effect on TSH secretion [22].

After glucose ingestion, plasma levels of prolactin fell by comparison with the fasting values (Table 2). A significant reduction in plasma levels of testosterone was also detected 120 min after glucose administration (Table 2). Since glucose is known to inhibit the secretion of GH and testosterone [23], these results indicate the possibility of a similar effect on prolactin secretion.

Prolactin and LH may have a synergistic action on testosterone synthesis in the testis.

---

**Table 2. Effect of glucose on plasma concentrations of prolactin (PRL), growth hormone (GH) and testosterone (T) in patients with prostatic cancer (all treatments)**

<table>
<thead>
<tr>
<th>Hormone</th>
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<tr>
<td></td>
<td>30</td>
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<tr>
<td>PRL</td>
<td>N=25</td>
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<tr>
<td></td>
<td>95(53-164)</td>
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<tr>
<td>GH</td>
<td>N=26</td>
</tr>
<tr>
<td></td>
<td>100(13-360)</td>
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<tr>
<td>T</td>
<td>N=17</td>
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<tr>
<td></td>
<td>109(39-169)</td>
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</table>

Values represent the hormone concentration (median and range) at each time interval expressed as a percentage of the hormone concentration at 0 min. *P* (v. 0 min): a <0.007; b <0.004; (Wilcoxon two-tailed matched-pairs signed-ranks test).

---

**Fig. 1. Plasma hormone levels in a patient with prostatic carcinoma being treated with oestrogens. GH, growth hormone; TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.**
[9], while the addition of prolactin to organ cultures of human adrenal tissue results in a stimulation of androgen synthesis [8]. It is therefore possible that the raised plasma concentration of prolactin contributes towards the gradual increase in plasma levels of testosterone that is often observed in patients on oestrogen therapy.

Although excessive stimulation of GH and prolactin secretion may be avoided by using doses of DES of less than 15 mg/day, consideration must now be given to the use of antiprolactin drugs (e.g. 2-Br α-ergocryptine) as part of the endocrine therapy of prostatic carcinoma. Such studies are now being undertaken.

Acknowledgements—We thank Ayerst Laboratories for the supply of Premarin tablets, Mr. R. G. Newcombe, Department of Medical Statistics, Welsh National School of Medicine, gave valuable advice.

REFERENCES

The Adrenal Cortex and Prostatic Cancer

by W B Peeling FRCS
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and K Griffiths Bsc PhD
(The Tenovus Institute for Cancer Research, Heath Park, Cardiff)

Testosterone in the plasma of normal men is mainly of testicular origin. After treatment for prostatic cancer by orchidectomy or suppression of testosterone synthesis by administration of compounds such as diethylstilboestrol, the main source of circulating testosterone is the adrenal cortex. Indirect evidence suggests that the adrenal cortex may be involved in some way with the natural history of prostatic cancer, and the possibility exists that it may be the direct or indirect source of secondary increases of plasma testosterone that is sometimes observed after a period of endocrine treatment in some men with this disease.

We have studied the patterns of response of plasma testosterone in men with prostatic cancer in relation to changes of adrenocortical function.

Clinical Material

Group 1: Fifteen newly referred patients with untreated histologically proven prostatic cancer were studied. Three received no further treatment because they were judged to suffer from asymptomatic Stage II disease. The remaining men experienced symptoms from Stage III or Stage IV disease. One of these underwent orchidectomy at his request, and the remaining 11 were treated with stilboestrol diphosphate (Honvan) 300 mg daily. Five men with advanced Stage IV disease failed to benefit from treatment and died within three to six months; 3 defaulted from follow up, and 3 attended regularly and were studied subsequently.

Group 2: Eleven patients with histologically proven prostatic cancer who had been treated with either diethylstilboestrol 15 mg a day or Honvan 300 mg a day for at least six months were studied. Three patients had been studied before treatment had commenced (from Group 1), and
3 had been castrated but had continued to take estrogenic compounds.

Group 3: Seventeen men in whom there was no evidence of benign or malignant prostatic cancer disease were studied. Their ages ranged from 60 to 75 years.

Methods

Adrenocortical stimulation: Adrenocortical stimulation was obtained by intramuscular injection of 1 mg of Depo-Tetracosactrin (Synacthen). Each test took two days – a control day and a test day. On the control day, venous blood samples were taken at 0900 and 1600, and on the following (test) day, a blood sample taken at 0900 was immediately followed by intramuscular injection of Synacthen. A further blood sample taken at 1600 on that day completed the test. In this way, it was hoped to minimize errors due to circadian variations of the levels of the hormones that were studied. There were no complications or side effects from these procedures.

Plasma testosterone measurements: The plasma levels of testosterone were measured by radioimmunoassay techniques at The Tenovus Institute for Cancer Research, Cardiff.

Adrenocortical suppression: Two patients with prostatic cancer under treatment with Honvan 500 mg a day were treated with dexamethasone 8 mg a day for three consecutive days. Daily venous blood samples were obtained at 0900 for five/six consecutive days for hormone analysis. The patients were volunteers for this test, and there were no side effects or complications.

Results

Adrenocortical stimulation: The mean plasma testosterone levels of three groups of patients that were studied are shown in Table 1. These observations showed that those patients with untreated prostatic cancer (Group 1) and those without evidence of prostatic disease (Group 3) exhibited a fall of mean plasma testosterone levels between 0900 and 1600 on the control day of study. This was a normal pattern and was not modified by injection of Synacthen. Men with prostatic cancer who had received treatment with estrogens for at least six months (Group 2) exhibited unvarying low mean plasma testosterone levels on the control day of study. After injection of Synacthen, there was a mean increase of their plasma testosterone levels by 93 ng/100 ml.

Adrenocortical suppression: The plasma testosterone levels of two men with treated prostatic cancer who underwent adrenocortical suppression with dexamethasone are shown in Table 2.

Discussion

Under normal conditions, stimulation of the adrenal cortex with ACTH does not result in increased testosterone synthesis. In this study the mean testosterone levels of men with untreated prostatic cancer and of men without evidence of prostatic disease behaved similarly and were not affected by adrenocortical stimulation with Synacthen. However, the pattern of response of testosterone in the plasma of men with treated prostatic cancer indicated an increase of this hormone in the circulation after adrenocortical stimulation (Table 1). This suggests that the response of the adrenal cortex to ACTH had been modified by Honvan used to treat these men, and that the adrenal cortex under these conditions can synthesize testosterone or its precursors. This view is supported by the observation that circulating testosterone was abolished by adrenocortical suppression with dexamethasone in 2 men receiving Honvan for prostatic carcinoma (Table 2). These findings are of a preliminary nature and indicate that more attention should be paid to the role of the adrenal cortex in prostatic cancer, particularly relating to its influence upon the androgenic environment of the treated patient and its relation to reactivation of controlled disease.

Table 1

<table>
<thead>
<tr>
<th>Mean plasma testosterone levels</th>
<th>Group</th>
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<th>Day</th>
<th>Mean plasma testosterone ng/100 ml</th>
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<td>1</td>
<td>15</td>
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<td>485</td>
<td>423</td>
<td>-62</td>
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Table 2

<table>
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<th>Case 2</th>
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PLASMA STEROID AND PROTEIN HORMONE CONCENTRATIONS IN PATIENTS WITH PROSTATIC CARCINOMA, BEFORE AND DURING OESTROGEN THERAPY

By


ABSTRACT

Plasma testosterone, androstenedione, oestradiol-17β, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were not significantly different in patients with prostatic cancer, with benign prostatic hyperplasia or in patients without prostatic disease. Plasma prolactin concentrations were significantly lower in the patients with benign disease than those with prostatic carcinoma. Endocrine therapy in the form of stilboestrol administration significantly decreased plasma levels of testosterone, oestradiol-17β, FSH and LH within 7 days of the treatment. After 7 days therapy prolactin levels increased significantly in all patients studied. Changes in growth hormone concentrations were more varied in response to stilboestrol, being elevated in several patients and remaining unchanged in others. Treatment of a few prostatic carcinoma patients who were receiving stilboestrol therapy with CB154, an inhibitor of prolactin secretion, brought an immediate decrease in prolactin levels which was sustained. Plasma testosterone, androstenedione and growth hormone were unchanged in these patients but a significant decrease in plasma oestradiol-17β was noted in two patients during CB154 administration.

department of Surgery, Royal Gwent Hospital, Newport, Gwent NPT 2UB, U.K.
Although endocrine therapy in the form of castration or administration of oestrogen is now widely accepted as the most effective form of treatment for carcinoma of the prostate in man, the role of androgens in the aetiology of the disease remains unknown. The clinical data indicate that the carcinoma functionally depends upon androgenic stimulation yet Isurugi (1967) showed that there was no evidence of an elevated testosterone production rate in patients with prostatic cancer. It is interesting that although prostate disease generally occurs at a time when testicular function is declining, Kent & Acone (1966) have reported that the plasma concentration of testosterone in the male is maintained at a relatively constant level from the age of 20 to the ninth decade, resulting, however, from a decrease in the metabolic clearance rate of testosterone with age. Vermeulen et al. (1972) in contrast, found in their study that testosterone decreased after the 6th decade although the range in concentration were very large. Plasma concentration of oestradiol-17β increases with increasing age in the normal healthy adult male (Pirke & Doerr 1973) and there has been speculation that changes in the androgen-oestrogen balance in elderly men may be implicated in the aetiology of prostatic disease. Somme (1957) considered that oestrogens were concerned in the pathogenesis of benign prostatic hypertrophy. At the same time, experimental studies in animals have suggested that pituitary hormones may influence prostatic growth and function Hypophysectomy for example, results in a more marked prostatic atrophy than that seen after simple castration (Huggins & Russel 1946; Lostroh & Li 1957) Furthermore, testosterone and prolactin produce a greater increase in the fructose and citric acid content of the prostate of the hypophysectomized castrated rat than testosterone alone (Grayhack & Lebowitz 1967). Little is known, however, about the relationship between protein hormones and prostatic disease in man.

As part of an investigation into the endocrinology of patients with prostatic dysfunction, certain steroid and protein hormones were determined in the plasma of a control group of patients, in a group with benign prostatic hyperplasia, and before and during treatment in patients with prostatic carcinoma The change of plasma hormone levels after administration of compounds such as Synacthen (Tetracosactin, Ciba), dexamethasone and CB154 (2-bromo α-ergocryptine, Sandoz) was investigated in certain patients during treatment with oestrogen or after castration.

As part of a major clinical research study, the plasma hormone concentrations of individual co-operative patients with prostatic cancer are being carefully monitored in relation to the progression of the disease. There may be an association between these plasma hormone changes and the clinical responses to therapy and the studies may provide insight into the relationship between the recurrence of the disease and the endocrine status of the patients.
PATIENTS AND METHODS

Patients

Thirty-three patients with carcinoma of the prostate, histologically-proven, but at various stages of the disease, forty-one patients with benign prostatic hyperplasia and thirty-five hospitalized patients without evidence of prostatic disease were studied. The control patients had no history of urinary difficulties nor a high plasma acid-phosphatase level. Ages ranged from 55–83 years, mean 73 years for the prostatic cancer group; 52–81 years, mean 71 years for the patients with benign disease; 59–79 years, mean 70 years for the control group. Blood samples were taken in the morning, as near to 09.00 h as possible. Twenty patients with carcinoma of the prostate were treated with either Honvan (diethylstilboestrol diphosphate; 100 mg b. d.) or diethylstilboestrol (1 mg t. d. s.) and their plasma hormone concentrations measured prior to therapy and at 3 and 7 days and 1 and 3 months after the commencement of endocrine therapy. Another patient was treated with bilateral orchidectomy and plasma hormone concentration subsequently determined over several months. Four patients with prostatic carcinoma who had been treated with Honvan for at least six months were given CB154 2.55 mg daily for periods up to 6 weeks. Plasma samples were obtained for analysis before and during CB154 treatment.

Most of the patients, controls, those with benign prostatic hypertrophy and those with prostatic carcinoma were subjected to an adrenal stimulation test. Patients with prostatic carcinoma were subsequently given a second test during the period of the endocrine therapy. On two consecutive days, at 09.30 and 16.30 h, blood was taken from patients by venepuncture. Synaethen depot (β1–24-corticotrophin zinc complex, Ciba, 1 mg) was given im immediately after the 09.30 h blood sample had been taken on the second day. Some of the patients also received dexamethasone (8 mg daily for 3 days).

All tests were undertaken after the consent of the patient had been received. Although reasonable volumes of plasma were taken, occasionally insufficient material was available to undertake all assays.

Assays

Plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured by double antibody radioimmunoassay shown to be specific for these hormones (Groom et al. 1971). The sensitivity of each assay was 0.5 mIU/ml of MRC 3/15 and 2nd-IRP-HMG, respectively. Plasma growth hormone (GH) was estimated by a specific double antibody radioimmunoassay (Schalch & Parker 1964) using purified human growth hormone (MRC 69/46) and rabbit antiserum to growth hormone (Wellcome Reagents Ltd.). Plasma prolactin was measured by a homologous double antibody radioimmunoassay in which there was negligible cross-reaction with FSH, H, TSH, GH and chorionic somatomammotrophin (Cole & Boyns 1973). The sensitivity of the assay was 10 milliampoules/ml (mamp/ml) of MRC 71/222. Total plasma androstenedione was measured by a specific radioimmunoassay, the sensitivity of the method being 15 pg/ml (Hillier et al. 1973). Plasma androstenedione was measured by a specific radioimmunoassay with a sensitivity of 110 pg/ml (Cassley et al. 1976). Plasma oestradiol was measured using the method of Cameron & Jones (1972) with a sensitivity of 3 pg/ml. Statistical analyses were performed using the multiple test.
<table>
<thead>
<tr>
<th></th>
<th>Control groups of mean value</th>
<th>Patients with mean value</th>
<th>Patients with mean value</th>
<th>Patients with mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n SEM</td>
<td>n SEM</td>
<td>n SEM</td>
<td>n SEM</td>
</tr>
<tr>
<td>Plasma hormone concentrations in patients with and without prostatic disease.</td>
<td>CONTROL GROUPS</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>TESTOSTERONE (ng/ml)</td>
<td>ANDROSTENEDIONE (ng/ml)</td>
<td>OESTRADIOL (pg/ml)</td>
<td>LH (mIU/ml)</td>
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<td>7.9 1.06</td>
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<td>of patients without</td>
<td>0.5 0.06</td>
<td>1.2</td>
<td>34.2</td>
<td>1.4 35</td>
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<td>35 35</td>
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<td>11.1 1.6</td>
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<td>1.6</td>
<td>1.6</td>
<td>1.5 37</td>
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<td>41</td>
<td>41 35</td>
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<td>38.5</td>
<td>8.2 2.5</td>
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<td>prostatic carcinoma</td>
<td>0.46 0.11</td>
<td>0.70</td>
<td>0.11</td>
<td>0.16 30</td>
</tr>
<tr>
<td>n = number of patients, SEM = standard error of the mean.</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

* Benign v. prostatic carcinoma P > 0.01.
STILBOESTROL
THERAPY

Fig. 1.
Changes in plasma testosterone in twenty patients with prostatic carcinoma before and after treatment with stilboestrol. Ten patients received diethylstilboestrol 1 mg t.d.s. (o) and ten received Honvan 100 mg b. d. (●).

RESULTS
Control and pre-treatment concentrations of plasma hormones
The plasma concentrations of testosterone, androstenedione, oestradiol-17β, H, FSH and prolactin for control hospitalized patients, and patients with benign or malignant prostatic disease are given in Table 1. With the exception
of prolactin, no significant difference was noted in the concentration of the hormones between the control, benign and malignant groups. However, in the case of these particular patients with benign prostatic hypertrophy, the mean plasma concentration of prolactin was lower than that found in patients with malignant disease ($t = 2.66; P < 0.01$).

**Changes in hormone concentrations after stilboestrol therapy**

The concentrations of plasma testosterone in patients with prostatic carcinoma, measured before and during treatment with either diethylstilboestrol or Honvan are shown in Fig. 1. In the majority of patients, testosterone concent-

**Fig. 2.**

Changes in plasma oestradiol-17β concentrations in twenty patients with prostatic carcinoma before and after treatment with stilboestrol. Ten patients received diethylstilboestrol 1 mg t.d.s. (○) and ten received Honvan 100 mg b.d. (●).
Changes in plasma FSH (a) and LH (b) concentrations in twenty patients with prostatic carcinoma before and after treatment with diethylstilboestrol (o) 1 mg t.d.s. or Honvan 100 mg b. d. (●).
Fig. 4.
Changes in plasma prolactin and growth hormone concentrations in twenty patients with prostatic carcinoma before and after treatment with diethylstilboestrol (○) 1 mg t. d. s. or Houvan 100 mg b. d. (●).
rations were below 100 ng/100 ml plasma within 7 days of therapy. There was no apparent difference in the rate of decrease of these testosterone levels between patients treated with either diethylstilboestrol or Honvan. Plasma oestradiol-17β concentrations were also significantly decreased after only 3 days of therapy. The 50% decrease in plasma concentration of oestradiol-17β was maintained 3 months after treatment (Fig. 2).

As might be expected, both the concentrations of plasma FSH (Fig. 3 a) and LH (Fig. 3 b) decreased after 7 days treatment to levels below the limits of detection of the methodology. Plasma prolactin concentration was found to significantly increase after treatment with either diethylstilboestrol or Honvan, but the latter therapy produced higher plasma prolactin levels than the diethylstilboestrol (Fig. 4 a). Within 7 days of therapy the majority of patients had plasma prolactin concentrations above the normal range (0–25 mamp/ml). Growth hormone concentrations were also found to rise during the period of treatment with some patients but remained unchanged in others, and the mean values after treatment were not significantly different from the pre-treatment levels with this group of patients (Fig. 4 b).

Monitoring of hormone concentrations of individual patients

A typical profile of hormone levels during treatment of patient W. J. during a 12 month period is shown in Fig. 5. This patient was treated with Honvan during the period shown. It was observed that the rise in plasma testosterone concentration at midsummer in this patient did not relate to any progression of the disease as far as was clinically manifest. The patient remained fit and well during this whole period. However, the rise of plasma testosterone seen in patient D. P. after 6 months treatment with Honvan coincided with recurrence of the clinical symptoms due to disseminated disease in particular, severe back pain, which subsequently subsided coincidently with a decrease in plasma testosterone (Fig. 6). Other hormone concentrations in both patients present a similar pattern throughout the period studied.

One patient (W. R. J.) was initially treated by orchidectomy, at his request, and the resultant hormone changes are shown in Fig. 7. Testosterone and oestradiol levels decreased whereas the concentration of prolactin and growth hormone remained within the normal range. FSH concentrations increased significantly and remained at an elevated level. On the other hand the LH concentration did not rise above the pre-orchidectomy level.

Treatment of patients with CB154

The effect of CB154 treatment of patients with carcinoma of the prostate being treated with Honvan (100 mg/b. d.) was studied. Patients were given .5 mg CB154 daily and plasma hormone concentrations determined during
An immediate decrease in plasma prolactin was observed after CB154 administration (Fig. 8), and the concentration fell to below 20 mamp/ml within 2 days. These low levels were sustained for the 2-6 weeks of CB154 administration. Testosterone, androstenedione and growth hormone concentrations did not change during CB154 treatment whereas a fall in plasma estradiol-17β concentrations was observed in two of the patients (Fig. 9).

Adrenal function tests
An adrenal stimulation test on controls, patients with benign prostatic hyperplasia and those with malignant disease prior to treatment, showed a significant fall (P < 0.05) in plasma testosterone concentration after Synacthen administration. Results shown in Fig. 10 a for the pre-treatment malignant group were typical values for all patients studied. After treatment, however, with either Honvan or diethylstilboestrol for > 6 months a rise in plasma testosterone concentration was observed following Synacthen injection (Fig. 0 b). It was of interest that three patients receiving Honvan treatment, were given dexamethasone, 8 mg daily for 3 days, after which their plasma testosterone values decreased from 50-100 ng/100 ml to below the limits of detection.

DISCUSSION
These studies indicate that there is no significant difference in the plasma concentration of testosterone and androstenedione in patients with either benign prostatic hyperplasia, prostatic carcinoma or control patients and therefore agrees well with the results of Moon & Flocks (1970). In contrast, however, Kaufmann (1968) reported a lower secretion of urinary testosterone glucuronide with benign prostatic hyperplasia when compared to controls. Furthermore, Gandy & Peterson (1968) described an elevated peripheral concentration of androstenedione in six patients with prostatic carcinoma but gain in our study of twenty-three patients, no such elevation was detected. Although it is well established that the growth of prostatic tissue is androgen dependent, opinions differ as to whether a reduced androgenic status in the male with associated prostatic atrophy precede the development of carcinoma (Franks 1954a,b) or whether prostatic tissue is subjected to increased androgenic stimulation prior to the development of neoplasia (McNeal 1965). Although alterations in testicular function are evident in older men (Kent &

Fig. 5. Patient W. J. aged 61 with carcinoma of the prostate Stage IV commenced Honvan therapy on 17.11.73 blood was withdrawn (20 ml) at approximately 09.00 h in the periods indicated.
<table>
<thead>
<tr>
<th>Year</th>
<th>Testosterone ng/ml</th>
<th>Oestradiol pg/ml</th>
<th>Progesterone ng/ml</th>
<th>FSH mIU/ml</th>
<th>LH mIU/ml</th>
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<tr>
<td>1972</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<tr>
<td>1974</td>
<td></td>
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**Honvan Therapy Commenced**

**Patient D.P. Orchidectomy**
Acne 1966; Isurugi 1967; Ismail & Harkness 1967) the plasma testosterone concentration remains unchanged until the age of 90. The concept that the pathogenesis of benign prostatic disease is associated with a decreased androgenic stimulation of the gland was not supported by the results from the analysis of plasma steroid concentration.

However, changes in the steroid metabolic activity of the prostate may well be responsible for the development of the diseased state and further studies are obviously required along these lines. The possible involvement of oestrogen in the pathogenesis of prostatic disease is suggested from early observations of changes in prostatic tissue after oestrogen administration to animals (Lacsagane 1933; Korenchevsky & Dennison 1934). An increase in the fibromuscular stroma was observed together with metaplastic changes in the epithelium. Patients in this study now described, were all found to have similar plasma oestradiol-17β concentrations which would agree with the urinary oestrogen measurements obtained by Marmorston et al. (1965) from a study of similar groups of patients. A rise in plasma oestrogen concentration with age has, however, been described (Nagai & Longcope 1971; Pirke & Doerr 1973) and one must consider that the relevant time to study such oestrogen changes may be years earlier before the patient enters the clinic.

Moreover, measurements of the endogenous levels of steroid in the tissue and steroid receptor concentration may provide more convincing evidence for a role of steroids in the pathogenesis of benign and neoplastic prostatic disease. The influence of the pituitary on prostatic growth and function has been quite extensively studied in animal experiments (Grayhack et al. 1955; Tullner 1963; Lawrence & Landau 1965; Gunn et al. 1965; Asano 1965) but there is little data on the plasma protein hormone levels in relation to prostatic cancer. Asano (1962), using a bioassay, claimed an increased urinary excretion of prolactin in prostatic cancer patients, results now reported reveal no discrimination between the malignant group and the control patients although the patients with benign disease were found to have lower plasma concentrations than patients with carcinoma of the prostate. Baseline plasma LH and FSH were found to be similar in our groups, but Geller et al. (1970) however, have reported a decreased LH reserve in elderly males with benign disease although no age matched controls were used in their study.

The marked decrease in LH, FSH and testosterone seen with stilboestrol therapy is consistent with the results obtained by Alder et al. (1968) and Baker et al. (1973). Only the preliminary studies from these laboratories on

Fig. 6.
Patient D. P. aged 69 with carcinoma of the prostate Stage IV commenced Honvan therapy (200 mg b.d.) on 2.10.72 and plasma hormones measured in the months indicated. On 28.10.74 bilateral orchidectomy was performed.
Patient W. R. J. aged 67 with carcinoma of the prostate Stage II had an orchidectomy on 25.10.72 and his plasma hormones measured in the months indicated.
Fig. 8.
Changes in plasma prolactin concentration in response to CB154 administration 2.5 mg daily for 2 to 6 weeks in four patients with prostatic carcinoma receiving Honvan therapy (100 mg b. d.). Blood was withdrawn as near to 09.00 h as possible.

Fig. 9.
Changes in plasma oestradiol-17/β concentration in response to CB154 administration 2.5 mg daily for 2 to 6 weeks in four patients with prostatic carcinoma receiving Honvan therapy (100 mg b. d.). Blood was withdrawn as near to 09.00 h as possible.
Changes in plasma testosterone following Synacthen administration (1 mg im) to patients with prostatic carcinoma (a) before and (b) after stilboestrol therapy. Columns 1 and 2 represent the 09.30 h and 16.30 h plasma values respectively on the control day. Columns 3 and 4 represent the 09.30 h and 16.30 h plasma values on the test day. Synacthen was administered immediately after the 09.30 h specimen on the test day.

the prolactin and growth hormone concentrations of patients with prostatic cancer undergoing therapy have been reported (Boyns et al. 1974). The rise in prolactin concentration was rapid and significant. Such an elevated level of prolactin may well be implicated in the eventual relapse of the patient especially in view of the reports of indirect and direct effects of this hormone on the prostate (Boyns et al. 1972). Studies indicate that it synergises with LH to increase testicular androgen synthesis (Hafiez et al. 1972), with ACTH to increase prostatic growth in the hypophysectomized castrated rat (Tullner 1963), with testosterone to increase fructose and citric acid levels in the prostate, and increases testosterone uptake in the prostate in vitro (Farnsworth 1972).

The possibility therefore that the high levels of plasma prolactin should be avoided during oestrogen therapy should receive some attention. The treatment with CB154 in a few patients with high plasma prolactin levels resulting from stilboestrol therapy, produced a marked decrease in concentration,
despite continued oestrogen treatment. Testosterone and androstenedione levels remained unchanged which would suggest that neither adrenal nor testicular synthesis of these androgens were affected by the high prolactin concentrations. It should be noted that these male patients found CB154 difficult to take and often complained of sickness.

The observed decrease in plasma oestradiol during stilboestrol treatment probably resulted from decreased testicular oestrogen synthesis. It was, however, interesting to note that treatment with CB154 reduced the remaining plasma oestradiol concentration in two of the patients studied.

ACKNOWLEDGMENTS

The authors wish to express their gratitude for the generous financial support of the Tenovus organisation. We are also indebted to Dr. E. R. Evans, Sandoz Ltd. for supplies of CB154.

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THE EFFECT OF ACTH ON PLASMA TESTOSTERONE AND ANDROSTENEDIONE CONCENTRATIONS IN PATIENTS WITH PROSTATIC CARCINOMA

By

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W. B. Peeling* and K. Griffiths

ABSTRACT

The effect of Synacthen (β1-24-corticotrophin) on plasma testosterone and 4-androstene-3,17-dione concentrations in untreated patients with prostatic carcinoma, and in patients receiving endocrine therapy is described. An established specific radioimmunoassay was used for the measurement of testosterone, and a radioimmunoassay for 3-androstene-3,17-dione using thin layer chromatography has been developed. Administration of Synacthen resulted in a fall in testosterone in untreated patients, but a rise in 4-androstene-3,17-dione was observed. The plasma concentration of testosterone in all treated patients increased after administration of Synacthen. An increased concentration of plasma 4-androstene-3,17-dione was also observed in these treated patients after Synacthen, but the magnitude of the response was not significantly different from that of untreated patients.

The work provides further evidence that in the patient being treated with oestrogen for carcinoma of the prostate a rise in plasma testosterone concentration will result from an increased secretion of ACTH.

It is well established that endocrine therapy in the form of castration or oestrogen administration provides the most acceptable treatment for the management of prostatic cancer in man. Despite the fact that approximately 80% of
patients responds favourably to this initial form of treatment (Ferguson 1972), a large proportion of them relapse at various periods of time after the initiation of the treatment. Such a relapse has often been considered to be due to secondary rise in the levels of plasma androgens of adrenal origin. Overall, there is a great deal of evidence which suggests that the adrenal gland may be responsible for this secondary androgen secretion. Scott & Verulen (1942) found in men, orchiectomised for carcinoma of the prostate, that after the initial post-operative fall in urinary 17-oxygenoid output, thecretion of these steroids increased to above pre-operative levels. These findings were confirmed by Burt et al. (1957) who also showed an enhanced effect of ACTH on the output of 17-oxygenoids after orchiectomy. Although Brook et al. (1959) also observed an increase in 17-oxygenoid excretion following diethylstilboestrol therapy, subsequent measurement of plasma testosterone in such patients, or in those after orchiectomy (Young & Kent 1968) failed to demonstrate a secondary rise in steroid concentration. More recently, wever, Robinson & Thomas (1971) reported a small but significant rise in plasma testosterone in patients during the first six months of oestrogen therapy. Sciarra et al. (1973) indicated that both plasma androstenedione (4-androstene-3,17-dione) and testosterone concentrations were high in a group of orchiectomised patients who had made unfavourable clinical progress. Administration of dexamethasone depressed these androgen levels.

These laboratories have undertaken an investigation into the endocrinology of patients with prostatic dysfunction. As part of this investigation, the effect of Synachten® (β-24-corticotrophin, Ciba) on plasma androstenedione and testosterone concentrations in patients with carcinoma of the prostate, both before and after treatment, has been studied. Further evidence is offered for renal involvement in the production of increased plasma androgen concentrations during oestrogen therapy.

MATERIALS AND METHODS

Solvents, reagents and materials

The sources of solvents and reagents, details of the preparation of the gelatin solutions and of the dextran-coated charcoal suspension, have been previously published (Amerson & Jones 1972; Hillier et al. 1973). [1,2,6,7-3H]testosterone was purchased from the Radiochemical Centre, Amersham, Bucks., U.K. and the Zander all-glass fractionation apparatus from Cam Lab., Cambridge, U.K.

Androstenedione

Androstenedione was stored for use as 10 ng/ml stock solution in benzene at 4°C. [2,6,7-3H]androstenedione was prepared by chromic acid oxidation of [1,2,6,7-3H]testosterone (S. A. 100 Ci/mmole) using Kiliani reagent (Griffiths et al. 1963) and
purified by thin layer chromatography on an alumina precoated plastic sheet in the solvent system cyclohexane:benzene:ethanol (100:95:5 v/v/v). A 10 μCi [1,2,6,7-3H4]androstenedione stock standard was prepared in benzene. [1,2,6,7-3H4]androstenedione was prepared from stock in two dilutions. (i) At approximately μCi/ml in benzene for use as the radioligand. Before each assay a further dilution was made in 0.1% gelatin in phosphate buffered saline such that 50 μl contain 20,000 dpm/30 pg androstenedione. (ii) At approximately 0.02 μCi/ml in benzene a radioactive tracer.

Antiserum

Anti-androstenedione-11α-bovine serum albumin (BSA) which was raised in she was kindly donated by Dr. R. J. Scaramuzzi, M. R. C. Reproductive Physiology Unit, Edinburgh. This was used at a final dilution of 1:3000.

Measurement of plasma testosterone

A procedure, developed earlier in these laboratories, was used for the determination of testosterone. The radioimmunoassay, using an antiserum raised against testosterone-3-BSA in rabbits, has been described (Hillier et al. 1973).

Measurement of plasma androstenedione

A radioimmunoassay for androstenedione, involving thin layer chromatography, has been developed and is described below.

Extraction of androstenedione from plasma

[1,2,6,7-3H4]androstenedione (2000 dpm/3 pg) was added in 50 μl benzene to plasma samples (0.5–1 ml). At this stage, 50 μl samples of radioactive tracer were also placed in counting vials containing 0.1% gelatin in phosphate buffered saline (0.2 ml) at +4°C and placed in a deep freezer at −15°C for 20 min. The ether layer was then decanted and the methanol/water phase dried under a N2 stream at 50°C. The residue was dissolved in 90% methanol/water (2 ml) at 45°C for 5 min followed by agitation using a vortex mixer. An aliquot (200 μl) was removed for assay and a 200 μl sample placed in a vial containing 5 ml ‘Aquasol’ for determination of recovery in a Nuclear Chicago (Mark I) liquid scintillation counter, the efficiency of which was 40% under these conditions. Recovery of labelled tracer added to plasma was usually 50–75%.

Chromatography of plasma extracts

Extracts were applied as 1 cm “streaks” to alumina precoated plastic sheets and developed in the solvent system cyclohexane:benzene:ethanol (100:95:5 v/v/v). Deoxycorticosterone acetate (DOC acetate) was used as a chromatography marker. Areas on the chromatogram containing androstenedione were eluted with 2 ml ethanol using Zander all-glass elution apparatus. The ethanol was then evaporated at 50°C in stream of N2.

The residue was dissolved in 0.1% gelatin in phosphate buffered saline (400 μl) by warming the mixture to 45°C for 5 min followed by agitation using a vortex mixer. An aliquot (100 μl) was removed for assay and a 200 μl sample placed in a vial containing 5 ml ‘Aquasol’ for determination of recovery in a Nuclear Chicago (Mark I) liquid scintillation counter, the efficiency of which was 40% under these conditions. Recovery of labelled tracer added to plasma was usually 50–75%.
Standards were prepared in duplicate by dilution of the 10 ng/ml stock androstenedione solution in benzene to a series of concentrations. Eluate residue from the alumina depressed the standard curve slightly, and to compensate for this effect it was necessary to add to the standards, eluate residue equivalent to that in the test samples. Standards and unknown samples were mixed with suitably diluted antiserum and \([2,6,7-3\text{H}_2]\text{androstenedione (20 000 dpm)}\) and incubated for at least 2 h at 4°C. Free steroid was removed with dextran-coated charcoal. After centrifugation, supernatant (1.5 ml) was transferred to a counting vial to which 5 ml 'Aquasol' was added. The radioactivity was again determined by liquid scintillation counting.

Evaluation of the androstenedione assay

The method blanks as determined by taking 1 ml distilled water through the method were invariably less than 5 ng/100 ml when corrected for losses.

Figures for percentage cross-reactivity of the antiserum are presented in Table 1. Androsterone (3α-hydroxy-5α-androstan-17-one), 4-androstene-3,11,17-trione and 5α-androstane-3,17-dione showed significant cross-reactivity. Furthermore although the cross-reaction with cholesterol was very low (0.0001 %), this becomes significant when the high plasma concentration of cholesterol is considered and was the basis for the introduction of the petroleum ether – 90 % methanol partition. The chromatographic step effectively removed cross-reacting steroids (Table 2).

The precision in the range 0–100 ng/100 ml was determined from duplicate measurements of plasma from patients with prostatic carcinoma the mean of the plasmas

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross-reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>11β-Hydroxy-4-androstenedione-3,17-dione</td>
<td>7.5</td>
</tr>
<tr>
<td>4-Androstene-3,11,17-trione</td>
<td>50.0</td>
</tr>
<tr>
<td>5α-Androstan-3,17-dione</td>
<td>47.0</td>
</tr>
<tr>
<td>Androsterone</td>
<td>97.0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.0</td>
</tr>
<tr>
<td>4-Androstene-3β,17β-diol</td>
<td>0.1</td>
</tr>
<tr>
<td>3β-Hydroxy-5-androsten-17-one (DHA)</td>
<td>1.9</td>
</tr>
<tr>
<td>3α-Hydroxy-5β-androstan-17-one</td>
<td>0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

% cross-reactivity = 100 \times \text{mass of androstenedione required to displace 50\% of }^3\text{H androstenedione}/\text{mass of cross-reacting steroid required to displace 50\% }^3\text{H androstenedione.}
Table 2.
Mobility of steroids relative to androstenedione on alumina precoated plastic sheet in the system cyclohexane:benzene:ethanol (100:95:5 v/v/v).

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-Androstane-3,17-dione</td>
<td>1.25</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.12</td>
</tr>
<tr>
<td>Deoxycorticosterone acetate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.04</td>
</tr>
<tr>
<td>4-Androstene-3,17-dione</td>
<td>1.00</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.72</td>
</tr>
<tr>
<td>4-Androstene-3,11,17-trione</td>
<td>0.69</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0.52</td>
</tr>
<tr>
<td>3β-Hydroxy-5-androsten-17-one</td>
<td>0.36</td>
</tr>
<tr>
<td>3α-Hydroxy-5β-androstan-17-one</td>
<td>0.36</td>
</tr>
<tr>
<td>11β-Hydroxy-4-androstene-3,17-dione</td>
<td>0.33</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Androstenedione = 1.00.  *Marker for androstenedione.

68.4 ± 16.34. Standard deviation, s, was ± 5.2 ng (n = 15). The sensitivity of the assay was calculated from the formula \( \frac{ts}{\sqrt{N}} \) (Brown et al. 1957) to be 11 ng/100 ml (P = 0.01, n = 2).

Within-assay reproducibility was determined by repeated assay of a pooled plasma, 138 ng/100 ml ± 8.4 sp (n = 8). Between-assay reproducibility, determined using another plasma pool, was 105 ng/100 ml ± 12.1 sp (n = 6).

Some indication of the accuracy of the method, assessed by determining the recovery of androstenedione added to water and plasma is given in Table 3.

Table 3.
Recovery of androstenedione added to water and plasma.

<table>
<thead>
<tr>
<th>Sample</th>
<th>A added (pg)</th>
<th>Total A (pg) mean ± sd</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (1 ml)</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>257 ± 39</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>524 ± 21</td>
<td>105</td>
</tr>
<tr>
<td>Plasma (0.5 ml)</td>
<td>0</td>
<td>654 ± 17</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>882 ± 10</td>
<td>91</td>
</tr>
<tr>
<td>Plasma (0.5 ml)</td>
<td>0</td>
<td>380 ± 27</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>771 ± 38</td>
<td>98</td>
</tr>
</tbody>
</table>

(Determinations were in quadruplicate).
A comparison of the radioimmunoassay method with a gas liquid chromatography method for androstenedione performed in these laboratories (electron capture detection the iodomethylsilylether derivative of testosterone after the enzymic reduction, ng hydroxysteroid dehydrogenase, of androstenedione extracted from plasma and rified by thin layer chromatography [Danutra et al. 1973], was made by analysis 31 male plasma samples by both procedures. Results gave a correlation coefficient 0.86.

**RESULTS**

Patients under investigation were divided into groups according to the form treatment they received. Fourteen patients were receiving no endocrine therapy; seven patients were treated with Honvan (dose range 300–600 mg/day); three received DES (dose range 30–100 mg/day); one patient was orchiectomised and one patient, who had been treated with Honvan (200 mg/day) was subsequently orchiectomised. The period of time during which patients had been rated before a Synacthen test was performed varied from 2 months to 10 years. Plasma testosterone and androstenedione concentrations on the control day and on the day of the Synacthen test for the untreated group are illustrated in Fig. 1. Three of the patients included in this group (R. S., W. J., W. R. J.) were later treated and a second Synacthen test was performed on these. The sal testosterone levels of the 14 untreated patients as determined from the control day values were within the range 148–950 ng/100 ml. As determined ing the Wilcoxon matched pairs signed rank test (Siegel 1956) the plasma testosterone levels at 9.30 a.m. (mean 516 ± 249 sd ng/100 ml) did not differ significantly from levels at 4.30 p.m. (mean 461 ± 172 sd ng/100 ml; P = 0.1074). After Synacthen administration on the following “test day” a
Plasma testosterone and androstenedione in untreated patients with carcinoma of the prostate at 9.30 a.m. and 4.30 p.m. on a control day (plain bars) and the following "test day" (stippled bars). Synacthen Depot (1.0 mg) was given immediately after taking the morning blood sample on the "test day".

A significant fall in plasma testosterone occurred (mean decrease 102 ± 8 ng/100 ml; $2P = 0.0014$).

The results of the Synacthen tests in the treated groups are shown in Fig. 316. The seven patients receiving Honvan therapy had testosterone levels in the range 14–70 ng/100 ml on the control day. The 9.30 a.m. levels (mean 38 ± 19 sd ng/100 ml) were not significantly different from the 4.30 p.m. levels (mean 29 ± 13 sd ng/100 ml) on this day ($2P = 0.0930$). After Synacthen there was a significant rise in plasma testosterone (mean 67 ± 40 ng/100 ml; $2P = 0.0178$). Of the three patients treated with DES the basal testosterone levels were suppressed below 50 ng/100 ml. These patients showed a poor Synacthen rise comparable to that in Honvan treated patients.

Both the orchiectomised patient (W. R. J.) and the orchiectomised patient who also received Honvan (H. Y.) responded to Synacthen with an increased testosterone level as did the other treated groups.

In untreated patients basal afternoon androstenedione levels (mean 71 ± 3 sd ng/100 ml) did not differ significantly from morning values (mean 77 ± 3 sd ng/100 ml; $2P = 0.9044$). Androstenedione levels measured in these patients on the control day were within the range 28–223 ng/100 ml. In contrast the testosterone in these patients, androstenedione was significantly elevated after Synacthen (mean increase 152 ± 76.2 ng/100 ml; $2P = 0.0010$).
Androstenedione levels in the Honvan treated group on the control day were in the range 13–99 ng/100 ml with 9.30 a.m. mean of 65 ± 26 sd/100 ml and a 4.30 p.m. mean of 38 ± 26 sd ng/100 ml. Although the 4 p.m. androstenedione levels were lower than the 9.30 a.m. levels on day, the difference was not statistically significant ($2 P = 0.0810$). After Nahten, on the following day, there was a significant rise in androstenedione (mean increase 167 ± 78.9 ng/100 ml; $2 P = 0.0178$). All three of the S treated patients responded to Synacthen with an increase in plasma androstenedione. Similarly the orchiectomised patient and the orchiectomised uvan treated patient had elevated androstenedione levels in response to nacthe.

Using the Mann-Whitney U test (Siegel 1956) there was a significant difference in the response of testosterone to Synacthen between Honvan treated and untreated patients ($2 P < 0.002$). Similarly the DES treated group differed significantly from the untreated group in the response of testosterone to nacthe ($2 P < 0.02$). There was no significant difference in the response of drostenedione between untreated and Honvan treated patients ($2 P > 0.100$) between untreated and DES treated patients ($2 P > 0.100$).

Fig. 2.

Plasma testosterone and androstenedione in treated patients at 9.30 a.m. and 4.30 p.m. in a control day (plain bars) and the following "test day" (stippled bars). Synacthen depot (1.0 mg) was given immediately after taking the morning blood sample on the test day". The period of time patients had received treatment before Synacthen was given is indicated.
Plasma concentrations of testosterone and androstenedione have been determined in patients with carcinoma of the prostate treated with either Honv, DES or orchiectomy, or by Honvan and orchiectomy combined. In these patients, some of whom have been treated for several years, testosterone levels were maintained below 70 ng/100 ml. This maintenance of low testosterone concentrations is in agreement with other investigations (Robinson & Thon 1971; Shearer et al. 1973).

Plasma androstenedione concentrations in untreated patients did not differ significantly from concentrations in elderly hospitalized men (10–125 ng/100 ml as determined in our laboratories. This is not in accordance with the results of Gaudy & Peterson (1968) who demonstrated elevated peripheral androstenedione levels in the range 410–910 ng/100 ml in 6 patients with carcinoma of the prostate. Androstenedione concentrations in treated patients were within the normal range for elderly hospitalized men. In no case was androstenedione elevated after prolonged treatment with oestrogen.

Administration of Synacthen to untreated patients resulted in a decrease in plasma testosterone concentrations, which is in agreement with the work of Rivarola et al. (1966) who administered ACTH to 6 normal males over seven days. This work was confirmed by Pizarro et al. (1970) who infused normal men with synthetic ACTH and Breitins et al. (1973) who administered ACTH to 8 normal men over 4 days.

Oestrogen treated patients, however, responded to Synacthen with a twoto ninefold increase in the plasma testosterone concentration. In the orchiectomised patient, levels rose from less than 5 ng/100 ml to 72 ng/100 ml after Synacthen. Testosterone rose in response to Synacthen irrespective of the dose of Honvan or DES administered to the patients.

The rise in peripheral testosterone concentrations observed in oestrogen treated patients after Synacthen could be a result of increased DHA (3β-hydroxy-5-androsten-17-one), DHAS (3β-hydroxy-5-androsten-17-one sulphate) and androstenedione secretion by the adrenal and their peripheral conversion to testosterone. There may, however, be an increased direct output of testosterone by the adrenal in response to ACTH. Evidence for direct secretion of testosterone was obtained by Baird et al. (1969). These workers observed a higher testosterone concentration in adrenal venous plasma in female patients. The adrenal synthesis of testosterone and its stimulation by Synacthen has been demonstrated in vitro on incubation of both male and female adrenal tissue in organ culture (Boyns et al. 1972).

In our series of patients, however, the treatment may not have enhanced the capacity of the adrenal to synthesize and secrete testosterone and/or its precursors, but having suppressed the secretion of testosterone by the testis, mere
masked the adrenal contribution. At the same time, the increased testosterone
iding capacity in the plasma of these oestrogen treated patients will un-
ubtly affect plasma testosterone concentration by influencing clearance rate.
Measurement of plasma cortisol concentrations by a specific radioimmuno-
say in six of the oestrogen treated patients (H. Y., R. S., R. R., A. F., E. W.
d J. R.) using the same plasma samples as for the estimation of testosterone
d androstenedione revealed high basal cortisol concentrations ranging from
to 92 µg per 100 ml plasma. It is also known that oestrogen treatment in-
eases the corticosteroid binding globulin in the plasma (Slaunwhite & Sand-
rg 1959), with a corresponding increase in total cortisol plasma concentra-
ion. Synaetthen stimulation caused an increase in cortisol concentrations
ranging from 25% to 170% in these six patients.
This study suggests therefore that the adrenal of the patient receiving endo-
ine therapy for carcinoma of the prostate is responsive to ACTH, and tends
support the observation that a secondary rise in plasma androgen concentra-
tion of some of these patients may be of adrenal origin (Birke et al. 1954;
ulbrook et al. 1959; Scott & Vermeulen 1942). Stimulation or stress will lead
an increase in plasma testosterone concentration and may result in a re-
umption of tumour growth with subsequent relapse.

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he authors wish to express their gratitude to the Tenovus Organization for generous
ancial support during this study.

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PLASMA SPERMINE CONCENTRATIONS OF PATIENTS WITH BENIGN AND MALIGNANT TUMOURS OF THE BREAST OR PROSTATE

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(Received September 4th, 1978)

Summary

A radioimmunoassay has been developed for the measurement of plasma spermine concentrations. The sensitivity of the method is 1 pmol spermine/100 μl plasma and the crossreactivity was 12% with spermidine and 0.18% with putrescine. Plasma spermine levels of patients with benign and malignant tumours of the prostate or breast were measured using this technique. Concentrations were only occasionally elevated in patients with prostatic tumours compared to normal individuals and there was no difference between those men with benign (mean concn. 0.21 ± 0.14 nmol/ml plasma) or malignant (mean concn. 0.21 ± 0.11 nmol/ml plasma) tumours. Only 17% of the patients with breast carcinoma had elevated levels of spermine, although there was a significant difference in the concentrations of the breast cancer group of patients compared to normals. No correlation was found between elevated plasma spermine concentrations and tumour grade or presence or spread of metastases in those patients.

Introduction

The concentrations of spermine and spermidine, short-chain aliphatic polyamines widely distributed in animals, plants, and bacteria, have been found to be high in actively proliferating animal tissues [1] and increase during early development of the embryo [2]. Since the concentration of polyamines was also found to be high in neoplastic tissue [2] it was reasonable to expect that patients with malignant tumours might have elevated levels in extracellular fluid such as plasma and urine and high levels were found in such patients [3–5]. It is however generally accepted that methods for the measurement

* To whom correspondence should be addressed.
of polyamines in biological fluids are tedious and/or lack sensitivity and in order to assess the value of polyamine analysis in the diagnosis of early cancer or in the assessment of the response of the tumour to therapy, it was considered necessary that a sensitive and relatively rapid radioimmunoassay was developed. Such a method has been described by Bartos et al. [7] for the analysis of spermine but with high-cross reactivity with spermidine.

This report now describes the development of another radioimmunoassay for spermine, but with greater specificity, which has been used to measure the concentration of spermine in patients with benign prostatic hyperplasia, with carcinoma of the prostate and in women with breast cancer. The levels have been compared with normal subjects with the aim of assessing the value of spermine analysis in the detection, staging and grading of these malignant tumours.

Materials and methods

Spermine, spermidine, cadaverine, putrescine and ornithine hydrochloride, bovine serum albumin, 1-ethyl-3-(3-dimethyl-amino propyl)carbodiimide hydrochloride (CDI) and activated charcoal were obtained from Sigma Chemical Co. [3H]Spermine tetrahydrochloride (44 Ci/mmol) was obtained from New England Nuclear, Boston, MA, and the radiochemical purity was checked at least every two months by thin-layer chromatography on cellulose plates in the solvent system 2-methoxyethanol/propionic acid/H₂O (saturated with NaCl) (70 : 10 : 20, by vol.); human normal immunoglobulin and human albumin were gifts from Blood Products Laboratory, Lister Institute, Elstree, Herts.; Dextran-150 was obtained from Pharmacia, Uppsala, Sweden.

Preparation of immunogen

The spermine/bovine serum albumin (BSA) conjugate was prepared by a modification of the techniques described by Skowsky et al. [8] and Goodfriend et al. [9] using the carbodiimide reaction. The reaction mixture, containing the reagents in the molar ratio (spermine : BSA : CDI) of 440 : 1 : 200 was performed at neutral pH. After 18 h, the reaction mixture was applied to a 2.2 by 30 cm Sephadex G-75 column to separate the conjugated and free spermine. The conjugate fractions were collected, dialysed and freeze-dried. A spermine/BSA conjugate, with an incorporation ratio of approximately 45 : 1, was obtained.

Immunization

Adult New Zealand white rabbits were immunized by a macrophage harvesting technique [10]. The rabbits were boosted at bi-weekly intervals with 1 mg of conjugate, dissolved in 1 ml of normal saline solution and emulsified with an equal volume of Freund’s complete adjuvant, until a sufficiently high titre of antibody was produced. The antibody titre in sera increased continuously, finally reaching a plateau after 2 months. Antisera collected between 2 to 4 months after first immunization were pooled and used for the assay. The association constant, Kₐ for spermine antibody was 0.72 × 10⁹ litres/mol.

Specificity of antiserum

The specificity of the spermine antiserum is shown in Fig. 1. The cross-
Fig. 1. Inhibition curves of related compounds with spermine antiserum. Compounds to be tested (1.98 to 31.64 pmol spermine (●), 2.75 to 352.6 pmol spermidine (○), 0.0045 to 72.6 nmol putrescine (▲), 0.0039 to 128.3 nmol cadaverine (■), and 0.0024 to 38.09 nmol ornithine (◊) per assay tube) were incubated with spermine antiserum (1:1000 dilution) and 0.56 pmol of [3H]spermine and percent bound of zero standard tube represents 100%. The cross reactivity is expressed as a percentage of the mass of each competitive compound required to replace 50% of bound [3H]spermine from its antibody.

reactivity, expressed as a percentage, is given as the mass of each competitive compound required to displace 50% of bound radio-labelled spermine from its antibody. The antiserum showed 11.7% cross-reaction with spermidine, 0.18% with putrescine, 0.04% with cadaverine and no cross-reaction with ornithine.

Radioimmunoassay

All assays were carried out in duplicate by a modification of the method of Bartos et al. [7]. Patients’ plasma without dilution, or diluted up to ten-fold, depending on plasma spermine concentrations, were added to each tube in 100-μl aliquots. Then 100 μl [3H]spermine (0.56 pmol; 30 000 dpm) were added to each tube, followed by 100 μl antiserum, diluted in 0.05 M borate buffer, pH 8.0, to a concentration at which 50–60% of the radio-labelled spermine was bound, (1:1000 final dilution). To prepare a standard curve, a series of tubes containing 100 μl solution of spermine in borate buffer, in con-
centrations ranging between 0.49 and 31.64 pmol together with 100 µl protein solution, prepared from human albumin and human normal globulin, as described by Bartos et al. [7], were set up. The blank and ‘total-count’ tubes were included in each assay. The total volume of the assay tube was 1.0 ml. The tubes were vortexed and allowed to equilibrate at 4°C for 4 h.

Antibody-bound and free spermine were then separated after addition of 100 µl of dextran-coated charcoal suspension (5% (w/v) charcoal, 0.1% (w/v) Dextran-150 in assay buffer) mixing, standing on ice for 10 min and centrifuging at 2000 rpm for 15 min. The supernatant was decanted as a 0.5-ml aliquot directly into scintillation vials containing 6 ml of scintillation fluid (10 g 2,5-diphenyloxazole, 1 l Triton X-100, 2 l toluene). Radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation spectrometer. Fig. 2 shows a characteristic standard curve for spermine.
### Recovery of Known Additions of Spermine from a Plasma Sample

<table>
<thead>
<tr>
<th>Added (pmol)</th>
<th>Measured (pmol)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.83</td>
<td>--</td>
</tr>
<tr>
<td>0.99</td>
<td>15.82</td>
<td>100</td>
</tr>
<tr>
<td>1.98</td>
<td>18.04</td>
<td>107</td>
</tr>
<tr>
<td>3.95</td>
<td>18.29</td>
<td>97</td>
</tr>
<tr>
<td>5.93</td>
<td>21.25</td>
<td>102</td>
</tr>
<tr>
<td>7.91</td>
<td>22.98</td>
<td>101</td>
</tr>
<tr>
<td>9.88</td>
<td>23.23</td>
<td>94</td>
</tr>
</tbody>
</table>

**Sensitivity, precision and accuracy**

The sensitivity of the assay was 1 pmol of spermine per assay tube. The intra-assay coefficient of variation was 6.4% for a mean value of 0.24 nmol/ml human plasma and the inter-assay coefficient of variation was 12.5% for a mean value of 0.19 nmol/ml plasma.

The accuracy of the spermine assay was reflected in the analytical recovery of spermine added to the plasma sample. There was a linear relationship between amount added and amount found (correlation coefficient = 0.974) as shown in Table I.

**Normal subjects**

Plasma was collected from a group of male volunteers, 18 to 79 years of age, and from a group of female volunteers 17 to 74 years of age all apparently in good health and free of any known disease. Plasma samples were stored at −20°C until analysed.

**Patients bearing benign or malignant tumours**

Samples were collected from patients with cancer before the initiation of therapy. Only histologically proven carcinoma of the prostate was taken to be an indication of malignant tumour and the clinical staging into T and M categories was assessed according to UICC classification [11]. Histological grading of the breast tumours was carried out according to Bloom and Richardson [12].

**Results**

Samples of plasma from 20 patients with benign prostatic hypertrophy and 14 patients with prostatic carcinoma were assayed for spermine. Table II shows he mean ± standard deviation and ranges of spermine concentrations in the plasma of these patients and normal male subjects. There was no significant difference in the spermine levels between patients with benign and malignant tumours of the prostate, nor between these and normal males. Of 54 prostatic cancer patients, only 3 had plasma spermine levels above the normal range.

The primary prostatic tumours were classified into T0, T1, T2, T3 and T4 categories and the metastatic status as M0 and M1, using the UICC system.
Fig. 3. Plasma spermine levels in normal and patients with benign or malignant tumours. Horizontal line represents the mean value of each population.
### TABLE II
**LEVELS OF PLASMA SPERMINE IN PATIENTS WITH PROSTATIC TUMOURS AND NORMAL SUBJECTS**
Elevated level is defined as a higher level than mean + 2 S.D. of normal male.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. of subjects</th>
<th>Spermine (nmol/ml)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>52—82</td>
<td>20</td>
<td>0.21 ± 0.14</td>
</tr>
<tr>
<td>Malignant</td>
<td>48—89</td>
<td>54</td>
<td>0.21 ± 0.11</td>
</tr>
<tr>
<td>Normal males</td>
<td>18—79</td>
<td>66</td>
<td>0.20 ± 0.10</td>
</tr>
</tbody>
</table>

### TABLE III
**THE RELATIONSHIP OF SPERMINE LEVELS IN PATIENTS WITH PROSTATIC TUMOURS STAGED ACCORDING TO VARIOUS T-CATEGORIES (UICC)**

<table>
<thead>
<tr>
<th>T-categories</th>
<th>No. of patients</th>
<th>Spermine (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>8</td>
<td>0.20 ± 0.09</td>
</tr>
<tr>
<td>T1</td>
<td>6</td>
<td>0.20 ± 0.06</td>
</tr>
<tr>
<td>T2</td>
<td>12</td>
<td>0.21 ± 0.08</td>
</tr>
<tr>
<td>T3</td>
<td>18</td>
<td>0.21 ± 0.12</td>
</tr>
<tr>
<td>T4</td>
<td>9</td>
<td>0.24 ± 0.16</td>
</tr>
</tbody>
</table>

### TABLE IV
**A COMPARISON OF PLASMA SPERMINE LEVELS IN THE PROSTATIC CANCER PATIENTS WITH AND WITHOUT METASTASES**
M0, without detectable metastases; M1, with detectable metastases, according to UICC classification.

<table>
<thead>
<tr>
<th>M-Categories</th>
<th>No. of patients</th>
<th>Spermine (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>24</td>
<td>0.23 ± 0.08</td>
</tr>
<tr>
<td>M1</td>
<td>19</td>
<td>0.23 ± 0.15</td>
</tr>
</tbody>
</table>

### TABLE V
**PLASMA CONCENTRATION OF SPERMINE IN PATIENTS WITH BREAST TUMOURS AND NORMAL SUBJECTS**
Elevated level is defined as a higher level than mean + 2 S.D. of normal female.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. of subjects</th>
<th>Spermine (nmol/ml)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>17—72</td>
<td>20</td>
<td>0.22 ± 0.09</td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-menopausal</td>
<td>24—55</td>
<td>27</td>
<td>0.27 ± 0.15</td>
</tr>
<tr>
<td>Peri-menopausal</td>
<td>44—56</td>
<td>6</td>
<td>0.23 ± 0.10</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>40—80</td>
<td>68</td>
<td>0.28 ± 0.19</td>
</tr>
<tr>
<td>Normal females</td>
<td>17—74</td>
<td>96</td>
<td>0.19 ± 0.09</td>
</tr>
<tr>
<td>Normal pregnancy</td>
<td>18—36</td>
<td>13</td>
<td>0.11 ± 0.04</td>
</tr>
</tbody>
</table>

**Difference from normal females is statistically significant, 2p = 0.0004 and 0, respectively.
Table III shows the spermine levels in relation to the T-categories of malignant tumours of the prostate. There was no significant correlation between spermine levels and clinical staging. Twenty-four patients with no evidence of distant metastases (M0) and 19 patients with distant metastases, were studied and there appeared to be no difference in spermine levels between those with localized malignant tumour and widespread metastatic disease as shown in Table IV.

A group of patients, with breast carcinoma, pre- and post-menopausal were studied, together with patients with benign tumours of the breast. There was no significant difference in plasma spermine levels between the patients with benign tumour and those with carcinoma of breast as shown in Table V. Breast cancer patients, both pre- and post-menopausal, however, had spermine levels significantly higher than normal females although only 5 out of 27 (19%) and

<table>
<thead>
<tr>
<th>Tumour grade</th>
<th>No. of patients</th>
<th>Spermine (nmol/ml)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.D.</td>
<td>Range</td>
</tr>
<tr>
<td>I</td>
<td>11</td>
<td>0.22 ± 0.09</td>
<td>0.13—0.44</td>
</tr>
<tr>
<td>II</td>
<td>21</td>
<td>0.28 ± 0.19</td>
<td>0.10—0.85</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>0.35 ± 0.18</td>
<td>0.23—0.75</td>
</tr>
</tbody>
</table>

Table VII
THE COMPARISON OF THE PLASMA SPERMINE CONCENTRATIONS BETWEEN PRIMARY AND ADVANCED CARCINOMA OF THE BREAST

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Spermine (nmol/ml)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Range</td>
</tr>
<tr>
<td>Primary</td>
<td>47</td>
<td>0.28 ± 0.20</td>
</tr>
<tr>
<td>Advanced</td>
<td>16</td>
<td>0.31 ± 0.24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oestradiol-17(\beta) receptor</th>
<th>No. of patients</th>
<th>Spermine (nmol/ml)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.D.</td>
<td>Range</td>
</tr>
<tr>
<td>Negative</td>
<td>36</td>
<td>0.32 ± 0.22</td>
<td>0.04—1.16</td>
</tr>
<tr>
<td>Positive</td>
<td>29</td>
<td>0.39 ± 0.66</td>
<td>0.13—3.71</td>
</tr>
</tbody>
</table>
Out of 68 (16%) of the patients respectively had elevated spermine levels. The relationship between plasma spermine levels and tumour grade [12] was studied. Grade I, represents the well differentiated carcinoma and Grades II and II the increasing degree of abnormality and undifferentiation. No correlation between spermine concentrations and the tumour grade was found (Table VI). Furthermore, no difference between spermine concentrations of patients with primary and advanced breast carcinoma was observed (Table VII) nor did the levels relate to the presence or absence of cytoplasmic oestradiol-17β receptor (Table VIII).

Discussion

Spermine antiserum used in this study provided the same sensitivity as the material previously described [7] but was more specific. Using this reagent, the normal range of plasma spermine was found to be similar to that described by Bartos et al. [7]. The normal range of plasma spermine in 66 male subjects was 0.05—0.52 nmol/ml (mean 0.20 ± 0.10) and in 96 female subjects was 0.05—0.48 nmol/ml (mean 0.19 ± 0.09). There appeared to be no age or sex difference, in agreement with earlier reports of spermine levels in whole blood [13] and urine [14]. In this study there was no elevation of plasma spermine concentrations in late pregnancy (Table V) contrary to previous reports [5,6,16]. The mean value of plasma spermine in patients with prostatic tumours was not significantly different from that in normal subjects, being elevated in only 3% of patients.

There was no difference between spermine concentrations in patients with localized malignancy of the prostate compared to those with widespread metastatic disease. Previous work of Fair et al. [17] showed how urinary spermine levels had no significant elevation in the patients with carcinoma of the prostate and it appears therefore that spermine assays are of little value in studies with human prostatic cancer, both for detection or monitoring response to therapy.

In the patients with breast tumours, both the pre- and post-menopausal groups of patients showed a higher mean spermine concentration than normal subjects. Approximately 17% of these patients had elevated plasma spermine concentrations. Only 1.3% had elevated urinary spermine levels in an early study [18]. It is possible however, that these high plasma values might be due to cross-reaction in the assay for high plasma spermidine concentrations, and spermidine analysis of the samples is at present underway. Breast tumour grading did not appear to correlate with the plasma spermine levels, although it is of interest that Fair et al. [17] reported that 30 of 34 patients with poorly differentiated carcinoma of the prostate (grades II, III and IV) had elevated urinary spermidine levels, whereas only 1 of 10 from patients with Grade I tumours had such an elevated concentration.

The plasma data show that no difference between plasma spermine levels in patients with primary breast carcinoma and those with advanced disease which agrees with the urinary analysis of Lipton et al. [14].

The study showed plasma spermine concentration will not provide a diagnostic index for the presence of prostatic cancer. It may however be of value as
a tumour marker in breast cancer in relation to other markers for this malignancy. It has been reported that urinary spermidine was significantly elevated in most patients with prostatic neoplasm [14] and the development of a radioimmunoassay to monitor plasma spermidine and putrescine in these patients is in progress in these laboratories.

Acknowledgements

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Prostatic Cancer

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Prostatic cancer is the fourth commonest cause of death from malignant disease of men in England and Wales (1). It claims about 4000 victims each year and despite this, rarely features prominently as a topic of interest or concern to the general public. At the other end of its pathological spectrum, prostatic cancer can be a most benign disease and poses the biological puzzle of a malignant tumour that can be detected histologically with increasing frequency as men get older (2, 3) and yet can show no evidence of aggressive activity.

In recent years, interest in prostatic cancer has accelerated almost to epidemic proportions because of the remarkable advances of technological expertise by cell biologists and biochemists, particularly in the field of radioimmunoassay by which accurate measurements of minute amounts of hormones and other substances in biological tissues can be made. This great expansion of information and knowledge has posed more questions about prostatic cancer than have been solved and it is particularly disappointing that, despite large amounts of new knowledge, no real advance in treatment has emerged since the early 1940s when Huggins laid the foundations of endocrine treatment for this disease (4, 5, 6).

Opinions about treatment methods for prostatic cancer now almost equal the number of urologists who treat the disease and as the present state of confusion must surely highlight our ignorance of the real nature of this disease, it is appropriate to pause and take a global view of various fundamental features relating to it. This will begin with a brief look at the basic construction of the normal prostate gland before proceeding to review some of the pathological and clinical features of the disease, and the investigations that precede treatment.
THE LOCATION OF CANCER IN THE PROSTATE

In recent years, a new understanding of the anatomy of the normal prostate has followed the elegant work of McNeal who re-examined the structure of this gland in relation to its developmental origins and its function (7, 8). To some extent, his views appear to conflict with the earlier work of Lowsley (9) and Franks (10), but he received recent support from Blacklock (11, 12). The key to McNeal's concept of the prostate is the course of the ejaculatory ducts through the prostate and their entry into the urethra at the verumontanum. He considers that the true prostate consists of a central zone (Fig. 1) which surrounds the ejaculatory ducts, and a peripheral zone whose ducts enter the urethra below the verumontanum whereas glands within the preprostatic sphincter, commonly described as 'periurethral glands', are unrelated to the functioning prostate. His studies suggest that the central zone is of-Wolffian origin for histologically it resembles the mucosa of seminal vesicles and ejaculatory ducts. The peripheral zone is thought to be of urogenital sinus origin and Blacklock (12) has reviewed evidence that suggests these zones may have a dual function as they appear to respond differently to endocrine stimulation, and it is the peripheral zone in particular that is affected by the changes in concentration of androgenic hormones that occur at puberty.

![Figure 1: Schematic drawing of McNeal's concept of normal prostatic anatomy showing a Central Zone (CZ) aligned with ejaculatory ducts and a Peripheral Zone (PZ) situated distal to the level of the verumontanum. The region of the Preprostatic Sphincter (PPS) in which the "periurethral glands" are situated lies above the level of the verumontanum.](image)

Prostatic ducts above and below the verumontanum are divided into two lines of development and the process of Wolffian descent probably begins with buds coming off the mesonephric tube that later become the verumontanum bud off the funnel into the ejaculatory ducts.

![Figure 2: Schematic drawing of prostatic zones.](image)
These new functional concepts of the prostate are probably the most significant advances in this field that have occurred in recent years and they become very convincing when they are examined in embryological terms. In the embryo, the ejaculatory ducts are derivatives of the mesonephric or Wolffian ducts (Fig. 2a) and they enter the endodermal tube that later becomes the urogenital sinus at the site of the future verumontanum. At a later stage of development, the seminal vesicles bud off the mesonephric ducts at which time the vasa deferentia and the ejaculatory ducts become structures in their own right (Fig. 2b).

Prostatic ducts develop from buds of urethral epithelium that sprout above and below the level of the verumontanum (Fig. 2b). These appear in two lines at the lateral angles of the posterior urethral wall (9, 13) and the process is induced by androgenic hormones, probably testosterone from the developing testes (14). Subsequent development probably depends upon the mesenchymal stroma with which these buds come into contact for in tissue cultures of embryonic urethral epithelium it is only stroma of urogenital sinus or Wolffian origin that will induce branching of prostatic glands (15, 16). Therefore, only the buds at the level of the verumontanum and distal to it will be in contact with stroma of Wolffian or urogenital sinus origin, and these develop into the functioning prostate. Glandular buds from the region of the verumontanum become aligned between and along the ejaculatory ducts to form a ‘central zone’ and those arising distal to this site develop into a ‘peripheral zone’. Urethral buds which develop above the level

Figure 2 Schematic drawing to show the development of the ‘true prostate’ distal to the level of the verumontanum; the preprostatic sphincter structure develops above this level.
of the verumontanum meet stroma from the trigonal area and as this is not of Wolffian or urogenital sinus origin, these buds do not develop functionally and become the 'periurethral glands' which later become incorporated within the preprostatic sphincter (7, 16).

It has been realised for a long time that benign hyperplasia of the prostate develops from periurethral glandular tissue and that prostatic cancer arises in the periphery of the gland (10, 17). In earlier descriptions of the histological features of the prostate (10), periurethral tissue merged physically with peripheral tissue and the occasional concurrence of focal or larger carcinomas with benign hyperplastic tissue was accepted as a result of this proximity. The fact that both conditions arise in older men did not, and has never, been considered as evidence that they are aetologically related, although in one study (18) it was found that there was a higher death rate from prostatic cancer of men who had been treated for benign hyperplasia which was taken to suggest that the benign disease could be an intermediate stage in the development of a malignancy in the prostate. In contrast to this, another study (19) in which 800 patients with benign hyperplasia were followed up for eleven years with matched controls, found that similar proportions of each group of patients later developed prostatic cancer. McNeal (20) defined the situation more clearly by showing that of 170 small carcinomas in 415 prostates that he examined, 148 occurred in the peripheral zone, 16 were situated at the junction between peripheral and central zones, only 6 were found in the central zone and none was present in the periurethral glandular tissue. From this work, there seems to be little doubt that prostatic cancer is a disease that arises in the 'true' prostate and it reinforces the likelihood that malignant and benign conditions of the prostate are independent conditions, probably with different aetiological backgrounds.

The cause of prostatic cancer is not known and the nature of factors that induce malignant change in normal prostatic epithelium is not understood. Until recently, it was believed that sclerotic atrophy of prostatic epithelium occurred in the periphery of the gland as a result of ageing—probably related to increased amount of circulating oestrogens—and that these changes were premalignant (3, 10, 21). In contrast to this idea, McNeal described an age-related increase in atypical hyperplasia of prostatic epithelium which could be diffuse or multifocal and he considered that these appearances were premalignant and indicated active androgenic stimulation (7). Focal atrophy he considered to be secondary to inflammation, not ageing, and his new concept, therefore, opposed the earlier idea that prostatic cancer develops in epithelium that has undergone atrophic changes as a result of increasing age.
result of reduced androgenic stimulus or increased oestrogenic stimulus
due to ageing of the individual. Whether McNeal's ideas will prevail,
only time and experience will show.

CLINICAL FEATURES OF PROSTATIC CANCER

Prostatic cancer is a silent disease that is often asymptomatic in patients
dying of old age or other causes. Even when relatively large, some
prostatic cancers produce remarkably few symptoms of urinary
obstruction. This may be due to the peripheral origin of these tumours
and contrasts with the symptoms from the benign hyperplasia that
develops from the preprostatic urethral glands situated so strategically
around the urethra. However, when patients with prostatic cancer
experience symptoms, they usually have features of urinary outflow
obstruction (22), often with a shorter and more relentless clinical
course than is seen with benign hyperplasia; it is also remarkable how
infrequently haematuria is associated with these problems (22). Local
invasion by tumour may involve the rectum producing features that
can be mistaken for rectal cancer and, occasionally, patients may
present with pain in the perineum that can be mistaken for prostatic
inflammation. Involvement of the ureters by infiltrating tumour can
cause upper urinary tract obstruction and uraemia, but this is often
difficult to distinguish from the back pressure effects that sometimes
follow the chronic outflow obstruction of benign hyperplasia which so
often coexists with prostatic cancer. Some patients may develop
swelling of the lower limbs or genitalia if pelvic lymphatics become
blocked by tumour and occasionally even inguinal nodes may become
involved.

Up to 20 per cent of patients present with symptoms caused by
metastatic tumour (22, 23) which often feature as pain in the
back or thighs, and in extreme situations paraplegia from vertebral
collapse may occur. Sometimes involvement of the bone marrow gives
rise to anaemia. Paramalignant syndromes are rare in prostatic cancer,
although one case of carcinomatous myopathy has occurred in our
personal series of 120 patients.

Physical examination of the prostate is limited to digital palpation
of the part of the gland lying next to the rectum. This is, of course, an
unsophisticated method of diagnosing prostatic cancer because only the
back of the prostate can be felt and the interpretation of the consistency
of lesions is so subjective that only about half of prostatic nodules
detected by rectal palpation prove to be malignant (24) and other
conditions like granulomatous prostatitis, calculous prostatitis and
tuberculosis can be mistaken for the stone-hard, irregular feel of larger cancers.

Metastatic lesions rarely are detectable by clinical examination, although tender spots in the ribs and over the spine may be present in some patients with actively progressing disease. Most lymphatic metastases cannot be felt as they are confined to the pelvis and paraortic regions, but occasional lymph node metastases appear in the neck or groin.

Apart from the primary lesion, physical signs are few and far between in prostatic cancer and detection of the presence of metastases requires investigations that are time consuming and expensive. Before embarking upon these the presence of cancer must be established in the patient otherwise some of them may be commenced on unnecessary treatment which can be hazardous. There is no excuse nowadays to treat a patient for prostatic cancer on the basis of a diagnosis made only by digital palpation of the prostate gland.

INVESTIGATION AND ASSESSMENT OF PROSTATIC CANCER

Biopsy and histological information

The most useful tool for biopsy of prostatic cancer is the Trucut needle (Travenol) which can be introduced into the prostate either transrectally or through the skin of the perineum. With this instrument, cores of tissue that should satisfy any pathologist are easy to obtain, and, although there is a very slight risk of implantation of tumour within the needle track (25) it has virtually no complications in practical use. Because prostatic cancer arises in the peripheral zone of the prostate, tumour tissue is most accessible to needle biopsy and is less likely to be obtained by transurethral resection unless it has infiltrated and distorted the tissues around the urethra. There is little point in carrying out needle biopsy to look for nonpalpable focal carcinomas as well under 10 per cent of these lesions are likely to be detected in this way (26, 27).

An alternative and increasingly popular method of biopsy of the prostate is by use of a fine Franzen needle which is introduced into the prostate transrectally for aspiration of prostatic fluid for cytological examination (28, 29). This technique is particularly suitable for outpatient use, but requires cooperation of an experienced cytologist.

There is no case in modern urology to use open perineal biopsy for prostatic cancer.
Pathologists have used several systems of histological grading to try to relate the cellular features of prostatic cancer with the apparent degrees of malignancy. It is now generally accepted, however, that apart from latent focal lesions, many active tumors show features of more than one histological pattern or grade (30), so that the least differentiated malignant cells seen in any specimen will determine the final grading assigned to that tumor. Since most cases of prostatic cancer are diagnosed from small cylinders of tissue obtained by needle biopsy or from chippings removed by transurethral resection, surgeons cannot expect pathologists to commit themselves to definite opinions about the malignant potential of prostatic cancer, because the sampling of the tumor by such methods leaves too much to chance.

When examined under the electron microscope, some interesting features about prostatic cancer cells are revealed that cannot be demonstrated by light microscopy. These have been described by Kirchheim and his colleagues (31) who emphasised that the characteristic electron microscopic feature of prostatic cancer was loss of secretory polarity and mature glandular features of the cells. In normal prostatic acini, secretory elements such as Golgi and secretory vacuoles were concentrated in the apical zones of epithelial cells and this pattern was retained in highly differentiated cancers even though by light microscopy changes were evident in the cell pattern. As differentiation decreased, the polarity of the secretory apparatus in prostatic cancer cells was lost, and these elements becoming displaced towards other parts of the cells, so that in anaplastic tumors they were virtually unrecognizable. Carcinoma cells seen within lymphatic channels showed total loss of polarity—in other words, were anaplastic cells according to those criteria—although it was not clear from their text whether only cells of this type were present in lymphatics.

Studies using light and electron microscopy describe only the appearance of pathological tissue, but histochemical procedures can to some extent examine the products of cell function and aim to relate abnormalities of cell metabolism with pathological changes. Acid phosphatase and aminopeptidase are present in normal and hyperplastic prostatic tissue, but they are less easily detectable in prostatic cancer tissue, particularly aminopeptidase which is not found even in well differentiated tumors (32, 33). Cooper and Parid (34) noted that citrate levels were reduced in prostatic cancer tissue, but that lactate was not affected, and they proposed that abnormal citrate/lactate ratios might indicate premalignant changes in the prostate before a histological or clinical diagnosis was possible.

It seems, therefore, that little reliable prognostic information can be
## Table 1 Comparison of UICC and VACURG clinical staging systems

<table>
<thead>
<tr>
<th>UICC</th>
<th>Primary tumour</th>
<th>VACURG</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>Incidental finding of carcinoma in an operative specimen</td>
<td>I (or A) No metastases normal S.A.P.</td>
</tr>
<tr>
<td>T1</td>
<td>No evidence of primary tumour, but evidence of metastases elsewhere</td>
<td>IV (or D) Distant metastases</td>
</tr>
<tr>
<td>T1 if under half the volume of the prostate</td>
<td>Tumour confined to the prostate</td>
<td>II (or B) No metastases normal S.A.P.</td>
</tr>
<tr>
<td>T2 if over half the volume of the prostate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 + involvement of seminal vesicles</td>
<td>Tumour extending beyond the prostate</td>
<td>III (or C1 if under 6cm diameter) (or C2 if over 6cm diameter)</td>
</tr>
<tr>
<td>T4 if tumour is fixed to pelvic wall or involving adjacent organs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Metastases**

<table>
<thead>
<tr>
<th>N (X to 2) Categories</th>
<th>Regional lymphatic spread</th>
<th>IV (or D) The prostate may vary from normal to fixed tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>M (X, 0 or 1) Categories</td>
<td>Skeletal and other metastases</td>
<td></td>
</tr>
</tbody>
</table>

**Clinical**

The purpose of this study is to evaluate the validity of the recently derived clinical staging system in the United Kingdom specifically for the management of prostatic cancer, with particular reference to the role of biochemical markers. The findings are intended to provide guidance for the clinician in the interpretation of these results.

Clinical staging is a critical component in the management of prostatic cancer, with the UICC and VACURG systems being the most widely used. The UICC system is based on the TNM classification, while the VACURG system focuses on the clinical extent of the disease. The comparison of these two systems is crucial for understanding the progression of the disease and the effectiveness of treatment.
expected from routine histological examination of tissue taken from a primary prostatic cancer, and it is unlikely that electron microscopy will remain more than a research tool unless electron microscopes become more generally distributed within the hospital service. It is interesting to speculate whether more insight into the malignant potential of prostatic cancer tissue could come from renewed interest in the histo-chemical features of the disease, for when related to routine histological examination of tumour tissue, such an approach might be profitable.

Clinical staging of prostatic cancer

The purpose of staging tumours is to create groups of patients that are categorised according to the degree of spread of their tumours, so that valid comparisons between different treatment regimes and other aspects of importance can be planned and assessed. Unfortunately, urologists in various parts of the world have not agreed to use one system of staging for prostatic cancer and this has restricted the value of direct comparisons of treatment and other data between different centres, particularly between those of North America and many centres in Europe.

The most commonly used systems nowadays are those advocated by the Union Internationale Contre le Cancer (UICC) and the Veterans Administrative Cooperative Urological Research Group (VACURG) and the relative features of these systems have been reviewed by Chisholm (35). Clinical staging of prostatic cancer according to the UICC recommendation takes into account the state of the primary tumour (T-Category), the presence of regional lymphatic spread (N-Category), and the existence of distant spread particularly in bone (M-Category). The VACURG system is primarily concerned with three stages of progressively extensive primary tumour growth with a fourth stage that includes all cases of metastatic disease irrespective of the nature of the primary tumour. Each system is based on different principles and they are, therefore, difficult to compare directly (Table I).

Despite its drawbacks, the UICC recommendation for TNM staging is becoming increasingly accepted and has the greatest flexibility for future adaptation into clinical practice and research, particularly as complex inter-relations of data can now be handled by computers.

Assessment of primary tumour (T-Category)

Sometimes a clinician can guess fairly accurately by rectal palpation whether a small prostatic cancer is confined to the prostate but no one
can dispute that the sides and deeper parts of larger tumours are simply not accessible in this way and their size cannot be assessed clinically at most stages in their existence. As the prostate cannot be outlined by radiological means there is an urgent need for reliable techniques to image the prostate accurately so that its volume can be determined at any stage, and also for techniques that can distinguish cancerous from benign tissue within it.

The first efforts to image the prostate were made in 1962 by Verrilli and his colleagues (36) who attempted prostatic scintiscanning in dogs with Zn\(^{65}\). These efforts were not successful for they could not distinguish the prostate from surrounding tissue. It had been known for many years that prostatic tissue has a particular affinity for zinc (37) and later it was shown that in human prostatic tissue there was less zinc present, in cancerous than normal tissue (38). Therefore, it was hoped that areas of prostatic tissue could be located by scintiscanning methods because of differential uptake of zinc isotope between malignant and other tissue within the prostate. Later studies used Zn\(^{69}\) and Zn\(^{65}\), which were found to be more effective than Zn\(^{65}\), but they were disappointing in clinical practice (39, 40, 41, 42). Perhaps this was due to the great variation of zinc content that exists in prostatic tissue (43), but it is also possible that account should have been taken of dynamic biological relationships between zinc in the prostate and factors such as I1, prolactin and testosterone which are known to influence the uptake of zinc by the prostate in animals (44, 45) and which might also cause wide daily fluctuations of zinc metabolism in human prostatic tissue.

In recent experimental work, labelled antiandrogens, oestrogens, and androgens have been used for scanning purposes in the hope that they would concentrate in the prostates of rats and dogs. These studies have also been disappointing because there was a high output of radio-active label in the urine and other tissues which limited the application of this potentially valuable technique (46).

It seems therefore, that at the present time prostatic scintiscanning has no place in clinical staging of primary prostatic cancer. However, within the past year the application of ultrasonography to prostatic imaging has been most encouraging. This technique is a most powerful diagnostic method, and has the advantage that it is noninvasive; unfortunately transabdominal ultrasound studies of the prostate are hampered by the symphysis pubis which limits the access of the bladder neck region to the investigations (47). The prostate can also be imaged by computerised tomography and recently Sukov and his colleagues (48) compared the relative merits of this technique with transabdominal
grey scale ultrasound scanning. They found that although tomography gave an excellent image of the prostate and pelvis, ultrasonography was more valuable in separating patients with carcinoma from those with benign conditions such as prostatitis, and that it was also more useful for volume determinations. Recently, problems of access to the prostate have been solved as the Aloka Company of Japan has developed an ultrasonic radial rectal scanner which is not impeded by the symphysis pubis or any other bony structure. By this technique the consistency of the prostate can be assessed, and it may even be possible to detect small carcinomas which cannot be felt by rectal examination; but in particular, it is also possible with this equipment to identify the spread of cancer through the prostatic capsule. It is likely that this technological development will play a most important part in future clinical practice because the possibility now exists for the first time to stage primary prostatic cancer accurately, and it may be possible for the first time to monitor the volume of the gland regularly during follow-up.

Assessment of regional lymph node involvement (N-Category)

In the UICC TNM classification for prostatic cancer, the N-Category related to 'Regional and Juxta-regional Lymph Nodes'; regional lymph nodes were defined as the pelvic lymph nodes below the bifurcation of the common iliac arteries, and juxtaregional lymph nodes were inguinal, common iliac and para-aortic nodes. It has been shown that the presence of lymph node metastases is an adverse prognostic feature in prostatic cancer (49) and other studies have indicated that metastatic spread of prostatic cancer to lymph nodes often occurs before vascular spread takes place to distant sites (50). Therefore, it is reasonable that clinicians should be anxious to establish as part of their initial evaluation of men with prostatic cancer the state of regional lymphatics to the prostate especially when surgical excision or radiotherapy for cure of the disease is being contemplated. Attempts to outline pelvic lymphatics by pedal lymphography are not backed up by commonsense for this technique does not demonstrate internal iliac lymph nodes which are known to be the main recipients of prostatic lymph (51) and, together with the obturator nodes, are the most frequent sites of lymphatic metastases from prostatic cancer (52). The results, and dangers, of pedal lymphography have been reviewed by Macdonald and Paxton (53), but it can only be concluded that this technique will not detect patients with early lymphatic spread of the
disease to regional lymph nodes and therefore cannot be used to identify men with truly localised disease.

As an alternative to lymphography, operative staging, biopsying obturator and internal iliac nodes, has been attempted. In general this has now been given up because complications such as lymphocele, lymphatic fistula, local oedema, iliac vein thrombosis, pulmonary embolism and even death are too high a price to pay for accurate staging of the tumour (54, 55, 56, 57).

Detection of metastatic disease (M-Category)

Prostatic cancer metastasises most commonly to skeletal structures, but unfortunately, methods to detect deposits of tumour tissue in bone are relatively crude. Most skeletal metastases only give features of pain and tenderness when they are well established, and it has been estimated that for these lesions to be identified radiologically, at least 50 per cent of the bone associated with them needs to be destroyed (58). Consequently much effort has been directed to earlier detection of metastatic lesions by looking for marker substances, produced by tumour tissue, that can be identified and measured in the plasma or urine of patients with prostatic cancer, and also by improved methods of imaging the lesions within skeletal tissue.

The classical 'marker' of prostatic tissue is a phosphatase (Phosphomonoesterase II) that is optimally active at a pH of 5.3 to 5.6—hence its name 'acid phosphatase'. Similar enzymes can be produced by other tissue such as erythrocytes, spleen, liver and kidney but the contribution from the prostate can be identified and measured for it can be inhibited by ethanol and tartrate (59, 60). Many prostatic cancers retain the ability of normal prostatic tissue to synthesise acid phosphatase, so that increased levels of this enzyme in the circulation of patients with carcinoma of the prostate are usually indicative of bone metastases, but high levels may also result from metabolically active large primary tumours (61). It is rare for serum acid phosphatase levels to be increased in relation to other diseases but this has been reported with conditions such as Gaucher's disease and myelomatosis (62) and the odd curiosity such as pancreatic islet cell carcinoma (63). The traditional view that rectal palpation of the prostate causes a transient increase of circulating acid phosphatase has in fact no foundation (64).

Not all prostatic cancers release acid phosphatase for a recent study from Leeds indicated that elevated levels of serum prostatic acid phosphatase were present in only 30-70 per cent of patients with demonstrable skeletal metastases (65). This group of workers also
studied a profile of acute phase reactant proteins, pre-albumin, α-antitrypsin, α-acidglycoprotein and haptoglobin, which when combined with serum prostatic acid phosphatase measurements increased their detection rate of metastatic disease to 88.6 per cent. Some workers have suggested that measurement of acid phosphatase levels in bone marrow might provide a method of early detection of bone metastases (66), but others have been more cautious about this approach, particularly as false positive results have been obtained when primary blood disorders were present (67). Recent studies using immunochromic methods of measuring serum acid phosphatase—which have a greatly increased sensitivity over the usual spectrophotometric methods—have indicated that circulating acid phosphatase may also be detectable in the presence of localised prostatic cancer (68). This observation reinforces the modern attitude held by many clinicians that the real value of serum prostatic acid phosphatase in clinical practice lies not in staging prostatic cancer, but in monitoring the response to treatment of those tumours that have the capacity to synthesise and release the enzyme. In fact, some recent studies have suggested that alkaline phosphatase is a better marker of the presence of bone metastases than acid phosphatase (61, 69).

Another marker of bone involvement is hydroxyproline which is a sensitive indicator of bone turnover, and when present in increased amounts in urine, often reflects the presence of skeletal metastases (69, 70).

Tumour markers cannot localise lesions to any particular site, but skeletal scintigraphy can do this by imaging the areas of increased bone turnover that surround active tumour deposits. The principles and features of this technique have been well reviewed by Merrick (71). Skeletal scintigraphy in the investigation of carcinoma of the prostate has been shown to increase the detection rate of bone metastases by 16 per cent over standard radiographic surveys (61), but it is important to exclude false positive scans due to benign bone disease by radiographic examination of regions of increased isotope uptake. In fact, many centres nowadays prefer to use skeletal scintigraphy as their first investigation of patients with prostatic cancer with radiographic examinations to investigate suspicious areas shown on the scintiscan.
TREATMENT OF PROSTATIC CANCER

Management of small cancers limited to the prostate (TX, T1, M0 or Stage I and II)

It has been assumed that early cancers can be cut out by big operations to achieve a total cure of the disease. Alternatively, radiotherapy to the primary tumour has aimed for the same result but its effectiveness has not yet been evaluated on a randomised basis for treatment of early localised prostatic cancer.

Undoubtedly, there are patients in whom prostatic cancer is confined to the prostate at the time of diagnosis, but most urologists who are not committed to any particular school of action in this regard are not convinced that the overall results of radical prostatectomy are better than other modes of treatment (72, 73, 74, 75, 76, 77). Each urologist must decide on this matter for himself but when doing so, it is relevant to consider a recent study of treatment of 33 men with multifocal Stage I tumours. They underwent radical prostatectomy and a five and ten year relative survival rate of 100 per cent was reported (78). This outstanding result should be tempered with the observation that men with untreated Stage I disease rarely die from prostatic cancer (79, 80) and on this evidence it would seem that radical surgery or indeed any other form of treatment is unnecessary to treat Stage I disease.

What about Stage II cancers? Flocks (81) pointed out that only about 5 per cent of Stage A and B or I and II lesions had either vascular or lymphatic spread at the time of diagnosis, and at first sight these tumours would appear to be ideal for total prostatectomy. Byar and his colleagues (82) noted in a pathological study of 208 prostates removed by total prostatectomy that staging of the tumour made by the pathologist was more advanced than that made by the clinician. In other words, clinical staging in prostatic cancer is generally too low and radical operations are therefore likely to be carried out on patients in whom the disease has already spread. This might account for Whitmore's findings that the five year survival rate of five series of men with Stage II prostatic cancer treated by total prostatectomy was only about 50 per cent (83).

In contrast to the results of radical surgery for Stage II prostatic cancer, Barnes and his colleagues (84) reported that of 115 patients treated with oestrogen therapy or bilateral orchidectomy for Stage II prostatic cancer and followed up over fifteen years, there was an overall five year survival rate of 71 per cent.

In general, radical prostatectomy as treatment of localised prostatic
cancer is not favoured in the United Kingdom firstly because it has not proved itself to be superior to endocrine therapy, but also because of its complications, the most feared of which is incontinence. In one series incontinence was complete in 15 per cent of patients and partial in another 42 per cent (78), and of course, the patients are all rendered impotent.

Management of large non-metastatic cancers (T2, T3, M0 or Stage II and III) and of enlarging smaller tumours

There is little disagreement that large primary cancers of the prostate causing symptoms, and small untreated cancers that are getting bigger, need treatment. Symptoms of bladder outflow obstruction or outright urinary retention should be treated straight away by transurethral resection to enable the patient to void urine again, but the choice for subsequent active measures lies between endocrine manipulation and radiotherapy. In recent years, cryosurgical treatment of primary prostatic cancer has also been proposed, but has not attracted support from urologists.

**Endocrine treatment**

For several centuries it has been known that the normal prostate depends upon testicular function if it is to grow satisfactorily (85) but it was not until 1941 that it was realised and shown by Huggins and his colleagues that many prostatic cancers retained some of the metabolic patterns of normal prostate and could be treated by orchidectomy or oestrogen therapy (5, 6). Coincidentally, the first nonsteroidal oestrogen, diethylstilboestrol (DES), was synthesised about the same time and this introduced a cheap orally active oestrogen at a time when the natural products were scarce and expensive. Since then, DES has remained the most commonly used oestrogen for the treatment of prostatic cancer. It has, however, always remained at the centre of controversy because initially there were those who regarded orchidectomy as a more reliable form of treatment (86, 87) and recently the VACURG studies have directed attention to its side effects which can include deaths from thromboembolic disasters (88, 89). Other effects, such as gynaecomastia, impotence, genital atrophy, nausea and occasional allergies, have added to its bad reputation but it is still used widely because there is, as yet, no better or cheaper drug. Its side effects are dose-related and it is now customary to prescribe DES in doses of 1 mg tds in the United Kingdom and 1 mg a day in the
USA. As it has salt and water retaining properties, many surgeons also prescribe a mild diuretic.

Some consider the disadvantages from diethylstilboestrol to be so serious that they advocate bilateral orchidectomy as the primary endocrine treatment for prostatic cancer (90), and recent studies have suggested that subcapsular orchidectomy is an adequate operation and is at least as effective as oestrogens (90, 91). Certainly, it is the treatment of choice for those who cannot be relied upon to take pills prescribed for them, for those who cannot tolerate oestrogens, and for those who have severe cardiovascular and pulmonary disease or are at risk from these conditions because they are very old.

Alternatives to DES are chlorotrianisene (Tace) 12 mg bd and the intramuscular preparation polyestradiol phosphate (Estradurin) starting at a dose of 160 mg monthly for three months and reducing to 80 or 40 mg per month subsequently. The natural oestrogens Premarin and ethinyloestradiol are not as effective as DES and are more expensive.

Progestogens such as hydroxyprogesterone caproate (Delalutin), chlormadinone acetate (Chlormadinone) and cyproterone acetate (Androcur) have been used to treat prostatic cancer and favourable results have been claimed (92, 93), but their place and relative effectiveness in the endocrine treatment of prostatic cancer have not been evaluated on a controlled, randomised basis.

Radiotherapy

In the early part of this century, attempts to treat prostatic cancer by irradiation were given up because the results were poor and also because of rectal and bowel side effects (94, 95). Then came Flock's report in 1959 of his work with injection of radioactive gold Au198 into the primary prostatic cancer (96) in which he claimed favourable results. In a later report, he implanted Au198 after total prostatectomy for Stage C (or VACURG III) lesions and noted that there was only a 4.4 per cent incidence of local recurrence of tumour after five years in his patients; this was in marked contrast with a recurrence rate of over 20 per cent when no adjuvant treatment had been given (50).

The work of Bagshaw (97, 98) and Budhraja and Anderson (99) rekindled interest in the place of external irradiation for the treatment of primary prostatic cancer and its regional lymph nodes, and a number of studies to evaluate the effectiveness of radiotherapy have been undertaken in recent years. Many of these have been reviewed by Morrison (100). Unfortunately, many patients treated with radiotherapy also receive other forms of treatment and until a controlled,
trial of radiotherapy alone against a policy of deferred treatment is undertaken, no clear statement is likely to be available to guide urologists about the real value of this form of treatment.

Management of advanced prostatic cancer (T0, T4, M1 or Stage III and IV)

Most patients with advanced or disseminated disease are treated primarily by endocrine methods either with diethylstilboestrol or by orchidectomy where indicated, and this should be preceded by transurethral resection when the primary tumour causes urinary outflow problems. Sometimes, external irradiation to the primary tumour helps.

Little is known about immunological aspects of prostatic cancer and although recent reports that cryosurgical attack on the primary tumour may induce and enhance immunological status (101, 102) in general, these have been received with some scepticism for Flocks (103) was unable to detect autoantibodies after perineal cryosurgery in eleven patients under his care. Adjuvant treatment with Bacille Calmette Guerin (BCG) has also been suggested and used for patients with advanced disease (104), but the place of this type of therapy has yet to be established.
treated men with reactivated prostatic cancer by bilateral adrenalectomy and pituitary ablation has been disappointing, although short term palliation undoubtedly sometimes occurred. The historical background and results of adrenalectomy and hypophysectomy have been reviewed by Hendry (107) but in general, enthusiasm for such treatment for advanced prostatic cancer has waned in recent years.

In the past eight years, the relationship between the adrenal cortex and prostatic cancer has been re-examined more accurately using sensitive and specific radioimmunoassays for the measurement of protein and steroid hormones in plasma. Some workers (108) noted that plasma testosterone levels always increased secondarily after treatment with oestrogens whereas others found no association between androgens and reactivation of prostatic cancer (109). The Tenovus Institute/Newport Group have extended this type of study with hormone profile measurements of patients treated with diethylstilboestrol for prostatic cancer. Increases of plasma testosterone levels of patients treated with oestrogens were rarely encountered in relation to reactivated disease but it was interesting that testosterone concentration in plasma could be increased by adrenocortical stimulation with Depot-tetracosactrin (‘Synacthen’, Ciba) of some patients under treatment. This effect was not observed in normal subjects, men with benign prostatic hyperplasia, and patients with prostatic cancer who had not commenced treatment with oestrogens. Administration of dexamethasone to oestrogen treated patients reduced the already low plasma levels of testosterone to undetectable values. Oestrogen treatment is known to increase plasma prolactin levels (110) and it was interesting that treatment of oestrogen treated patients with ergocryptin (CB 154, Sandoz), which is an inhibitor of prolactin synthesis by the pituitary, resulted in reduction of circulating testosterone levels in these patients (111, 112, 113). Early work from the Tenovus Institute (114) showed that administration of oestrogens to rats resulted in adrenal hypertrophy, but it is not known whether this occurs in humans. It is possible that the hyperprolactinaemia that is induced by oestrogen treatment may promote changes of responsiveness of the adrenal cortex to ACTH and so influence circulating androgens, but its place, if any, in the clinical management of prostatic cancer is not yet established. Further work is needed in this direction for circumstantial evidence suggests very strongly that there may be a relation between the pituitary and the adrenal cortex in the biology and control of prostatic cancer.

Chemotherapy

A number of agents (e.g. cyclophosphamide, triethylenemelamine, actinomycin-D, mitomycin-C) have been tried for advanced prostatic cancer.

Sometimes the initial response to treatment is dramatic, with a general improvement of the patient. In other cases, the response has been slight, and the disease has progressed. The overall results have been disappointing, with the exception of patients treated with high dose methylenitrosurea phosphate (ESMES) (106). This is a highly active antitumour drug with a high degree of specificity for the prostatic epithelium. The drug is linked to a nitrous acid group which is converted to the most active form, nitrosurea. A recent report from the Tenovus Institute clearly demonstrated that it produced a significant fall in prostate specific antigen levels in patients treated with ESMES. North American experience with the use of hormones for the treatment of prostate cancer has been disappointing. Some patients, however, were less sure as to the benefits of treatment, and reactivation of hormone sensitive disease was common. The American study of ESMES involved Phase 3 study (115), and was carried out at the University of California, USA, which included 100 patients.

Irradiation

When symptoms of prostatic cancer develop, or the disease spreads to other means, irradiation may be considered. The use of radioactive phosphorus (32P) for the treatment of prostate cancer offers an alternative, after a period of time, to some of the hormone sensitive patients. The rebound effect of hyperplasia in the prostate is increased by the radioactive phosphorus by natural means.

A recent trend has been the use of half-body irradiation. This offers worthwhile palliative benefit. When palliative is not achieved then reactivated disease is still present. It is possible to consider the use of actinomycin-D and cyclophosphamide for some patients, as the tumour is not hormone sensitive. Therefore, other means
Chemotherapy

A number of cytotoxic drugs have been used in the treatment of advanced prostatic cancer and these include bisulphan, cyclophosphamide, triethylene thiophosphoramide (thiotepa), 5-fluorouracil, actinomycin-D, Adriamycin, hydroxyurea and melphalan.

Sometimes these drugs have been given in combination, but in general the results from cytotoxic therapy have been poor and have been accompanied by the usual incidence of side effects especially suppression of bone marrow activity (115).

The exception to the general gloom in this field has been estramustine phosphate (Estracyt), which consists of a phosphorylated oestradiol linked to a nitrogen mustard. It is difficult to be sure which moiety is the most active against prostatic cancer as data from the Tenovus Institute clearly demonstrate high concentrations of plasma oestradiol—17β in patients treated with this drug. Reports from Scandinavia and North America have been encouraging and have even claimed that relief of pain may occur in about 50 per cent of patients, sometimes lasting up to three years (116, 117, 118, 119, 120). Chisholm and O'Donoghue (121) however, in a careful study of 30 patients with advanced cancer were less sure and recommended that the real place of this compound in the treatment of prostatic cancer could only be evaluated by comparative control trials and looked forward to the result of the current Phase 3 study of the National Prostatic Cancer Project Group in the USA, which might provide the answer.

Irradiation

When symptoms from widespread secondary deposits do not respond to other means, relief has been reported to follow administration of radioactive phosphorus $^{32}$ (122). The uptake of this isotope is increased after a period of treatment with Para-thor-mone for this causes a rebound effect when it is withdrawn and results in high uptake of phosphorus by bone (123).

A recent fearsome sounding alternative has been the introduction of half-body irradiation reported by Fitzpatrick and Rider (124) for which worthwhile palliation has been claimed.

Reactivated and uncontrolled prostatic cancer remains a considerable problem to the clinician as in most cases the patient's symptoms are considerable. Endocrine methods of management are probably irrelevant as the tumour is most likely to be autonomous in its behaviour. Therefore, other methods of treatment such as chemotherapy, systemic
irradiation and perhaps immunotherapy hold the best prospect for the future, but usually such patients are doomed and attempts to palliate their misery with cytotoxic drugs, operations and irradiation must be related to the reality of their future as individuals rather than to the aggression and zeal of the doctor.

FUTURE CONSIDERATIONS

The cause of prostatic cancer remains unknown and why it kills some men and not others is a mystery. There is no evidence to indicate that abnormalities of hormone balance induce this disease and epidemiological studies suggest that environmental factors might be important and could be related to the well known increased incidence of prostatic cancer in westernised society. It is most likely that hormones play a permissive role in the natural history of prostatic cancer and more must be learned about their relationship to the metabolic activity of prostatic epithelial and stromal cells. The epidemiological and endocrine aspects of this problem have recently been extensively reviewed by Griffiths et al (125).

From the clinical angle, the most pressing needs are for improved detection and imaging of the primary and secondary tumours so that staging of the disease can become an objective process based on accurate measurements rather than impressions. This should include improved imaging of the primary and secondary tumours, more specific markers of tumour metabolism, and also evaluation of the relevance of androgen and oestrogen receptors to endocrine methods of treatment.

Finally, unless agreement comes amongst urologists, to adopt one system of staging and assessment of patients with prostatic cancer, the present uncertainties about treatment of the disease will surely continue because of the difficulties of direct comparison of clinical results between centres.

The last major advance in the treatment of prostatic cancer was 40 years ago; let us hope that the next step forward is imminent.

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Evaluation of Plasma Hormone Concentrations in Relation to Clinical Staging in Patients with Prostatic Cancer

BRITISH PROSTATE STUDY GROUP*

Summary—Plasma concentrations of testosterone, oestradiol-17β, luteinising hormone (LH), follicle stimulating hormone (FSH), prolactin and growth hormone (GH) were measured in patients with histologically proven prostatic cancer, before any form of therapy was given for this disease. Patients were categorised according to UICC classification.

No systematic change in the group means of any of these hormones was associated with the progression of the disease from the T0 to the T4 stage. When multivariate analysis was applied to the combined intraprostatic (T0 + T1 + T2) and extraprostatic (T3 + T4) tumour category in patients without clinically evident metastases (M0) a discrimination was observed, GH substantially contributing to the separation of the 2 groups.

When plasma hormone data from patients classified as M0 (without metastases) were compared with M1 patients (with metastases), mean GH values were significantly larger (P<0.02) in patients with metastases. GH was also a major contributory factor to the discrimination between the M0 and M1 groups, using multivariate analysis. Testosterone group means for M0 versus M1 were also significantly different (P<0.02).

The basis of most endocrinological studies concerning the prostate gland has been the fact that its growth, maintenance and functional activity are largely dependent upon androgenic hormones secreted by the testes. The clinical behaviour of prostatic cancer also reflects androgen stimulation, although the endocrine factors concerned with the aetiology of the disease are little understood. It may well be that the hormonal role in the pathogenesis of prostatic cancer is more permissive than inciting, for present knowledge offers few leads that relate hormonal factors with the initiation of the condition.

Clinical data indicate that carcinoma of the prostate in man is, to some extent, functionally dependent upon androgenic stimulation and it is well established that the incidence of the disease increases with age during the period when testicular activity is declining. Although Kent and Acne (1966) reported that the plasma concentration of testosterone is maintained at a relatively constant level from the age of 20 to the ninth decade, this resulted from a decrease in the metabolic clearance rate of the hormone rather than from a sustained ability of the testes actively to synthesise and secrete testosterone. Conversely, studies by Vermeulen et al. (1972) and by other groups (Giusti et al., 1975; Baker et al., 1976) tend to suggest that plasma testosterone levels decrease with ageing, particularly after the sixth decade. This change, therefore, together with the observed increase with age in sex hormone binding globulin and plasma oestradiol-17β concentration (Pirke and Doerr, 1973) does direct attention to a possible imbalance in the androgen-oestrogen status of elderly men which may be implicated in the aetiology of prostatic disease. However, determination of the androgen and oestrogen levels in patients with prostatic cancer, benign prostatic hyperplasia and in age-matched controls has not shown significant differences between these 3 groups (Moon and Flocks, 1970; Harper et al., 1976; Bartsch et al., 1977).

Furthermore, despite experimental work in animals, which suggested that pituitary hormones may influence prostatic growth and function


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various groups in a manner. This research was undertaken at the Prostate Group and received little attention. It is possible that a particular endocrine milieu is conducive to the growth and spread of a primary tumour. The influence of prostatic cancer on the endocrine status of the host has also received little attention. Debilitating effects of advanced prostatic cancer have been reported to decrease androgen levels (Robinson and Thomas, 1971) but it is not known if tumour mass, grade or spread, either locally or distally, influence the secretion of hormones such as prolactin, growth hormone or cortisol. This study forms part of a major investigation into the endocrinology of prostatic cancer by the British Prostate Study Group. This group, consisting of urologists from various clinics in the United Kingdom, in association with the Tenovus Institute for Cancer Research, was established to promote co-operative studies on patients classified and managed in a consistent manner. An assessment of differences in the hormone concentrations between the various groups was undertaken using both non-parametric and parametric statistical methods.

Patients and Methods

One hundred and ninety-seven patients with ages ranging from 49 to 93 years were studied. The patients were those who had presented at one of the clinics associated with the Prostate Group and were selected, assessed and classified according to a standardised protocol accepted by the Group.

Patients had histologically proven carcinoma of the prostate and had not previously received any treatment. They were classified according to primary tumour site and metastatic status, using the T and M categories recommended by the Union Internationale Centre Cancer (UICC) (Wallace et al., 1975).

Blood samples, taken in the morning as near as possible to 0900 h, were obtained only after the consent of the patient had been received. Plasma was then stored, deep frozen, until assayed.

Hormone Assays

Total plasma concentration of testosterone was measured by a slight modification of a specific radioimmunoassay previously described (Hillier et al., 1973). Plasma oestradiol-17B was measured by a modified procedure of Cameron and Jones (1972).

Plasma LH and FSH were assayed by a double antibody radioimmunoassay shown to be specific for these hormones (Groom et al., 1971). Plasma GH was estimated by a specific double antibody radioimmunoassay (Schalch and Parker, 1964) using purified human GH (MRC 69/46) and rabbit antiserum to the hormone (Wellcome Reagents Ltd, England). Plasma prolactin was measured by a homologous double antibody radioimmunoassay in which there was a negligible cross-reaction with FSH, LH, TSH, GH and chorionic somatomammotrophin (Cole and Boyns, 1973). The sensitivities of the assay procedures for testosterone, oestradiol-17B, LH, FSH, GH and prolactin were 0.052 nmol/l, 11 pmol/l, 0.5 IU/l of MRC 63/15, 0.5 IU/l of 2nd-IRP-HMG, 0.5 mU/l and 0.03 U/l respectively as assessed by the method of Kaiser and Specker (1956), where the value of the constant (k) used in this study was 2.0.

All hormone measurements were undertaken by the Tenovus Institute for Cancer Research. Radioimmunoassay data-processing for the steroid and polypeptide hormones utilised the 4 parameter logistic (Rodbard and Hutt, 1974) and logit-log (Rodbard et al., 1970) models respectively. The quality control procedure used for the polypeptide hormones was that of Shewhart (1931). This technique was also used for the steroid hormones from 1974 until 1976. During the latter period (1976 to 1978) of the survey, the steroid assays have been monitored by more sophisticated cumulative sum
Table 1 Plasma Hormone Concentrations in Patients Classified According to the Primary Tumour Category in the Absence of Metastases (MO)

<table>
<thead>
<tr>
<th>Patient T category</th>
<th>Statistic</th>
<th>Age (years)</th>
<th>Plasma hormone concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T (nmol/l)</td>
</tr>
<tr>
<td>T0</td>
<td>Mean 74</td>
<td>14.6</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>SD 8.3</td>
<td>5.5</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td>Range 61-82</td>
<td>3.5-24.3</td>
<td>44-151</td>
</tr>
<tr>
<td></td>
<td>n 8</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>T1</td>
<td>Mean 67</td>
<td>13.5</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>SD 8.9</td>
<td>6.77</td>
<td>48.9</td>
</tr>
<tr>
<td></td>
<td>Range 56-80</td>
<td>1.4-22.9</td>
<td>51-195</td>
</tr>
<tr>
<td></td>
<td>n 9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>T2</td>
<td>Mean 71</td>
<td>15.6</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>SD 7.4</td>
<td>7.36</td>
<td>48.5</td>
</tr>
<tr>
<td></td>
<td>Range 50-82</td>
<td>6.9-27.1</td>
<td>59-254</td>
</tr>
<tr>
<td></td>
<td>n 22</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>T3</td>
<td>Mean 70</td>
<td>15.3</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>SD 8.7</td>
<td>5.49</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td>Range 50-88</td>
<td>5.9-30</td>
<td>44.1-184</td>
</tr>
<tr>
<td></td>
<td>n 34</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>T4</td>
<td>Mean 64</td>
<td>17.0</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>SD 8.2</td>
<td>3.61</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>Range 49-79</td>
<td>10.4-20.1</td>
<td>77-165</td>
</tr>
<tr>
<td></td>
<td>n 6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

T = testosterone, E = oestradiol-17β, LH = luteinising hormone, FSH = follicle stimulating hormone, GH = growth hormone, SD = standard deviation, n = number of patients, and ND = not detectable.

Statistical Analysis

The analysis of clinical and hormone data was performed using techniques similar to those reported previously (Griffiths et al., 1978; Wilson and Tan, 1978). Information from clinical and plasma hormone profiles was transcribed on to punched cards, verified and then validated for incorrect entries. Data were visually assessed in the form of graphic and histogram displays to establish interrelationships between variates and the nature of frequency distributions as required for multivariate analysis (Krishnaiah, 1969). All statistical analyses were performed on an ICL 4-70 computer.

Results

Plasma Hormone Concentrations in Various Groups of Patients

Plasma hormone concentrations were analysed in relation to the T categories of the prostatic cancer and to the presence or absence of metastases. Values for the plasma hormone concentrations for patients without evidence of metastases (M0) and those with (M1) are shown in Tables 1 and 2 respectively. Data for the comparison of M0 versus M1, irrespective of T category are shown in Table 3. Differences between groups were considered to be significant at the P<0.05 level using the Mann-Whitney U test.

The significant difference seen in the testosterone values (Table 2) for the (T0 + T1) group versus the (T3 + T4) group for the M1 category patients was not evident in the corresponding data from the M0 category patients (Table 1). A significant difference P<0.02 was observed however, in the testosterone concentrations in plasma from patients separated according to metastatic status, irrespective of T category of the primary tumour (Table 3).

Oestradiol-17β concentrations exhibited no significant difference either with respect to T category or metastatic status and the same observations were made for LH and prolactin, although a
Table 2 Plasma Hormone Concentrations in Patients With M1 Disease Classified According to Primary Tumour Category

<table>
<thead>
<tr>
<th>Patient T category</th>
<th>Statistic</th>
<th>Age (years)</th>
<th>Plasma hormone concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T (nmol/l)</td>
</tr>
<tr>
<td>T0</td>
<td>Mean</td>
<td>74</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>13.5</td>
<td>8.43</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>49-93</td>
<td>3.5-27.1</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>T1</td>
<td>Mean</td>
<td>75</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>3.7</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>71-80</td>
<td>1.0-13.2</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>T2</td>
<td>Mean</td>
<td>71</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>5.7</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>57-89</td>
<td>5.5-27.9</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td>T3</td>
<td>Mean</td>
<td>72</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>7.9</td>
<td>6.81</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>55-87</td>
<td>1.0-29.5</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>61</td>
<td>52</td>
</tr>
<tr>
<td>T4</td>
<td>Mean</td>
<td>68</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>9.7</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>55-90</td>
<td>1.4-29.2</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>25</td>
<td>20</td>
</tr>
</tbody>
</table>

T = testosterone, E₂ = oestradiol-17β, LH = luteinising hormone, FSH = follicle stimulating hormone, GH = growth hormone, SD = standard deviation, n = number of patients, and ND = not detectable.

Table 3 Plasma Hormone Concentrations in Patients With Prostatic Carcinoma With and Without Metastases

<table>
<thead>
<tr>
<th>Patient M category</th>
<th>Statistic</th>
<th>Age (years)</th>
<th>Plasma hormone concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T (nmol/l)</td>
</tr>
<tr>
<td>Patients with metastases</td>
<td>Mean</td>
<td>71</td>
<td>13.2*</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>8.4</td>
<td>6.18</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>49-93</td>
<td>1.04-29.5</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>118</td>
<td>99</td>
</tr>
<tr>
<td>Patients without metastases</td>
<td>Mean</td>
<td>70</td>
<td>15.3*</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>8.5</td>
<td>5.97</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>49-88</td>
<td>1.38-30.2</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>79</td>
<td>68</td>
</tr>
</tbody>
</table>

* P < 0.02.
Table 4  Correlation Tables for Age and Hormone Data

a. Primary tumour—T3 Category

<table>
<thead>
<tr>
<th>Age</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>0.30*</td>
</tr>
<tr>
<td>E2</td>
<td>0.23</td>
</tr>
<tr>
<td>LH</td>
<td>0.15</td>
</tr>
<tr>
<td>FSH</td>
<td>0.29*</td>
</tr>
<tr>
<td>GH</td>
<td>0.24*</td>
</tr>
<tr>
<td>Prolactin</td>
<td>0.11</td>
</tr>
</tbody>
</table>

b. Metastatic classification—M0 category

<table>
<thead>
<tr>
<th>Age</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>0.05</td>
</tr>
<tr>
<td>E2</td>
<td>0.02</td>
</tr>
<tr>
<td>LH</td>
<td>0.10</td>
</tr>
<tr>
<td>FSH</td>
<td>0.16</td>
</tr>
<tr>
<td>GH</td>
<td>0.10</td>
</tr>
<tr>
<td>Prolactin</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* P<0.05.
T = testosterone, E2 = oestradiol-17β, LH = luteinising hormone, FSH = follicle stimulating hormone, and GH = growth hormone.

consistent with the corresponding results for the M1 patients, who were also grouped according to T category (Table 2). Plasma GH concentrations appear, however, to be significantly higher at the P = 0.0239 level in those patients with metastatic disease (Table 3).

In summary, therefore, with the exception of GH, no differences between hormone concentrations or ages were obtained for the various clinical groups studied.

Interrelationships of Plasma Hormone Values and Age

The product moment correlation tables for plasma hormone concentrations and age were obtained for each group of patients, classified according to their T and M categories. The results for all the various T and M groups were essentially identical and only those obtained for the T3 and M0 categories are shown in Tables 4a and b. Significant values for the correlation coefficient (r) (P<0.05) were found for FSH and LH and were 0.51 for T0 (n = 24); 0.67 for T1 (n = 14); 0.72 for T2 (n = 31); 0.52 for T3 (n = 73); 0.81 for T4 (n = 22); 0.56 for M0 (n = 79) and 0.60 for M1 (n = 74). Table 4a also shows that testosterone, oestradiol-17β, FSH and GH correlated reasonably well (P<0.05) with age for all T3 category patients. This was not observed from the results shown in Table 4b where all patients in the M0 category were evaluated.

Canonical Variate Analysis

The plasma hormone values, together with the age of the patients in the various clinical groups, were subjected to canonical variate analysis. This type of multivariate statistical analysis can lead to the separation of groups of patients and consequently identifies those
variables which are likely to contribute most to the discrimination between the groups (Krzamowski, 1971).

When the hormone concentrations for patients without clinically evident metastases were grouped according to primary tumour T categories (T0 + T1 + T2) versus (T3 + T4) and compared by canonical variate analysis, separation of the variate group means was obtained.

Values for the variate group means for age, testosterone, oestradiol-17β, LH, FSH, GH and prolactin, denoted as $X_i$ (j = 1, . . . , 7), expressed in years, nmol/l, pmol/l, IU/l, mU/l and U/l were approximately 71.62 and 69.58; 15.9 and 14.0; 98 and 84; 4.59 and 7.75; 11.39 and 16.96; 2.22 and 0.96; 0.14 and 0.09 respectively. The corresponding loading factors were $-0.0223, -0.0363, -0.0172, 0.0571, 0.0302, -0.2495$ and $-2.5973$ and the values for the canonical variate means were $-0.49$ and $0.6533$ respectively. The latent root was significant at the $P<0.05$ level and the discriminant function was of the form:

$$y = 3.6822 + \sum_{j=1}^{7} L_j X_j,$$

GH contributed about 27% to the overall separation of the canonical variate means.

In the case of patients classified into 2 groups based on the presence or absence of metastases, the principal components in the separation of the canonical variate means were age (62%) and GH (31%), the remaining variates contributing very little to the degree of separation. Values for the variate group means were approximately 70.98 and 66.82 (age); 15.2 and 13.9 (testosterone); 92 and 91 (oestradiol-17β); 5.82 and 5.40 (LH); 13.59 and 11.73 (FSH); 1.69 and 2.68 (GH); 0.12 and 0.12 (prolactin) for the M0 and M1 groups respectively. Corresponding values for $L_j$ were $0.1063$, $0.0432$, $-0.004$, $-0.0264$, $0.0054$, $-0.22$ and $0.8906$ and the discriminant function was given by the expression:

$$y = -7.1694 + \sum_{j=1}^{7} L_j X_j.$$

The canonical variate means were $0.3181$ and $-0.3888$ for M0 and M1 respectively and the latent root was "significant" at the $P<0.10$ level.

Discussion

Although it is well established that the growth of prostatic tissue is androgen dependent and the clinical behaviour of carcinoma of the prostate indicates that the tumour is dependent upon the secretion of testosterone by the testes, there is little information to suggest that any abnormality in the endocrine status of the elderly man is related to the aetiology of the disease. Previous investigations into the plasma hormone concentrations of patients with prostatic carcinoma have generally failed to produce any marked differences between the determined values and those from normal men or men with benign prostatic hyperplasia (Harper et al., 1976). The present report, concerned with a more detailed survey of hormone levels of patients with untreated cancer in relation to various clinical parameters, has suggested that the use of multivariate analysis of the data may well offer a valuable guide to the interrelationship between the different factors concerned with the disease that are not apparent from the standard statistical treatment of such data.

Since primary tumour staging relates both to tumour size as well as to its intraprostatic localisation, any feedback of steroid metabolic products from the prostatic tissue, on the hypothalamic-pituitary axis, may be expected to be associated with alteration in plasma hormone concentrations as the disease progresses from stage T0 to T4. In the overall assessment of each hormone studied with respect to the different T categories of prostatic cancer, no progressive relationship was evident. Application of multivariate analysis to data from patients without clinically evident metastases produced a discrimination when patients were grouped according to the intraprostatic (T0 + T1 + T2) and extraprostatic (T3 + T4) spread of the primary tumour, with GH contributing most to the separation of the 2 groups.

The protein hormones, LH, FSH and prolactin, would seem to play a minimal role in the discrimination of the primary tumour groups and to be unrelated to the presence of metastatic disease. In animal studies, 5α-reduced metabolites of testosterone were shown to influence gonadotrophin secretion (Swerdloff et al., 1972; Kingsley and Bogdanova, 1973) and it has been reported that the androgen metabolism of a more differentiated carcinoma may differ from that of an anaplastic prostatic tumour (Morfin et al., 1977).

Wide variations in the plasma hormone concentrations were encountered in this extensive study. Many elderly men in their 70s and 80s had plasma testosterone concentrations which were comparable with those of younger men and yet others, aged 50 to 60, had low levels similar to those encountered in oestrogen-treated patients. It is also known that the protein hormones LH and FSH are released in a pulsatile manner and it is well established that stress can influence the
secretion of prolactin and GH. Furthermore, although blood was withdrawn between 0900 and 1000 h, variation in hormone values arising from circadian and other time-qualified rhythms (Aschoff et al., 1974) could still exert considerable influence on the statistical conclusions of this survey.

It was of interest, however, that when patients were grouped according to the presence or absence of metastases, GH was the major factor contributing to the separation of the 2 groups. In this study both testosterone and GH were significantly different at a 2% level despite the fact that no progressive relationship was observed with the primary T categories of the cancer.

The use of canonical variate analysis requires that the data are drawn from groups in which the variates are approximately normally distributed and that the variance-covariance matrices of each group are reasonably similar. To ensure that the basic assumptions in the use of canonical variate analysis were not violated, hormone values lying outside ±2 standard deviations from the mean were rejected as "outlying" values. This resulted in a more normal distribution of data. Data rejection must, however, be viewed with some degree of caution but it is pertinent to note that only 5% of the data for each hormone was rejected. Furthermore, GH was still implicated with respect to the above conclusions, when all data were included in the analysis.

These results suggest that the clinical stage of patients with carcinoma of the prostate may, in some cases, be accompanied by differences in their endocrine status and further studies are obviously required to substantiate the observation that GH may be more implicated in the disease than had hitherto been considered. Furthermore, the study has emphasised the potential value of a multicentre investigation to produce data from a large number of patients, clinically assessed in a standardised manner.

Acknowledgements

The Group gratefully acknowledges the financial support of the Tenvus Organisation and the Medical Research Council for a grant (G/976/123) to assist with the computer storage of data.

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sterone in human plasma by radioimmunoassay using antisera raised against testosterone-3-BSA and testosterone-11α-BSA. Steroids, 21, 735-754.


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THE ETIOLOGY AND ENDOCRINOLOGY OF PROSTATIC CANCER

K. Griffiths, P. Davies, M. E. Harper, W. B. Peeling and C. G. Pierrepoint

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I. INTRODUCTION

Prostatic cancer kills about 4000 men each year in England and Wales and is, according to the Registrar General's Statistical Review for 1968, the fourth commonest cause of death from malignant diseases in men. There has been a tendency to ignore these facts, partially because prostatic cancer is confined to men in their later years
and, therefore, lacks the drama of cancer of the breast and bronchus which can strike down people in their prime, but also because it occurs in what is, to the general public, a hidden and little known anatomical site inaccessible to all but the most meticulous clinician. The prostate is not a reality to patients and their relatives because it cannot be seen or felt like the breast, and it has no obvious function like the lungs or gastrointestinal tract, so they accept its presence as a mysterious influence that is likely to make micturition difficult when they get old. To many men, the inconvenience of prostatism is merely a barometer of their advancing years, which to them is a fact of life, like gray hair and decreasing exercise tolerance, and clinicians have similarly accepted that pathological changes giving rise to benign or malignant tumors in the prostate are also features of aging. This assumption tends to divert attention away from possible influences related to prostatic tumors that may occur in the early life of males when certain events would 'prime' the prostate and determine its eventual benign or malignant career. Therefore, any study concerning prostatic tumors should attempt to define clearly its relationship to benign or malignant diseases for, as discussed later, these are separate clinical conditions that arise in different parts of the prostate and are likely to have different biological characteristics. It is often difficult to do this, particularly in laboratory work, because the cornerstone of all endocrinological studies concerning the prostate has been the fact that its growth, maintenance, and functional activity are largely dependent upon androgenic hormones secreted by the testes, and this concept has been applied towards the treatment of both malignant and benign prostatic tumor: The relation between testicular function and the prostate was known 200 years ago when John Hunter reported that castration was followed by a decrease in size of the prostate gland.

The use of orchidectomy to treat prostatic hypertrophy followed some years later. Administration of diethylstilbestrol as a form of antiandrogen therapy for carcinoma of the prostate then developed from the classical laboratory experiments of Dr. Charles Huggins and colleagues, which established the concept that prostatic cancer cells also retained some degree of hormone dependence similar to that of the normal gland, so that antiandrogen therapy, either by estrogen administration or orchidectomy, is now the usual form of primary treatment for this disease. This is effective for the majority of patients, since up to 80% can be expected to improve clinically following treatment, although the extent and duration of response is unpredictable, and many of these men will eventually relapse. It would seem that relapse occurs because only a proportion of the neoplastic cells are hormone responsive, and progression of the disease results from the autonomous nature of the remainder which eventually kill the patient. It is obvious that the precise effects of endocrine therapy in the management of prostatic cancer are yet to be clearly established. Furthermore, the endocrine factors concerned with the etiology of the condition are also little understood. It may well be that the hormonal role in the pathogenesis of prostatic cancer is more permissive rather than inciting, for present knowledge offers few leads that relate hormonal factors with the initiation of neoplasia. Investigations related to endocrine status and etiology have, until now, been directed to the influence of androgens on prostate growth, the androgen-estrogen balance, the effect of estrogens on prostatic biochemistry and to pituitary, adrenal, and testicular activity. Certainly the clinical behavior of prostatic cancer tends to reflect androgen stimulation. The disease has not been reported in the prepubertally castrated male and evidence, currently available, tends to suggest a definite role for certain hormones in the promotion of abnormal prostatic growth.

A. Certain Aspects of the Pathogenesis and Epidemiology of Prostatic Cancer

Until quite recently, much of the research into the pathogenesis of prostatic cancer
has tended to be dominated by the observation of Franks\textsuperscript{8,9} that sclerotic atrophy, attributable to elevated estrogen levels, was probably a precancerous condition. Sommers\textsuperscript{10} supported this observation and Liavag\textsuperscript{11} has since described the association between atrophic prostatic epithelium and the presence of latent carcinoma. The concept that prostatic cancer originated from atrophic tissue, possibly resulting from a decreasing testicular activity of the aging male, has not, however, received much support from subsequent investigations of recent years.

A possible relationship between benign prostatic hypertrophy and carcinoma of the gland has also been a subject of controversy for many years. The high frequency of nodular hyperplasia in prostatic cancer, observed at autopsy,\textsuperscript{10} led Armenian and colleagues\textsuperscript{13} to reassess the concept that patients with benign prostatic hypertrophy might be at risk of developing malignant disease later in life. They reported a study in which 300 patients who had been treated for benign prostatic hypertrophy and a similar number of age-matched controls were traced until death. There was a 3.7 times higher death rate from prostatic cancer in the group that had a previous history of prostatic disease. They concluded from their data that hypertrophy may be a direct cause or an intermediate stage between causative factors and carcinoma. This information, together with the observation that the highest mortality rates for benign prostatic hypertrophy and cancer of the prostate are found in Iceland and the developed European countries\textsuperscript{12}—with the lowest rate for both conditions in Asian countries such as the Philippines, Singapore, and Japan—tends to favor the concept that the two conditions are related. The possibility that hyperplasia constitutes a premalignant condition cannot be summarily dismissed, although it would be reasonable to expect to find carcinoma and nodular hyperplasia in the same prostatic tissue since prostatic enlargement is common in the aging male. There are indeed, numerous reports of latent carcinoma associated with clinical benign hypertrophy of the prostate.\textsuperscript{14,15}

The consensus among urologists has generally been, however, that prostatic cancer is not directly related to hypertrophy and this is supported by another report, that of Greenwald et al.,\textsuperscript{16} who studied 800 patients with benign prostatic hypertrophy, again with matched controls. They were followed for 11 years and the results indicated that a similar proportion from both groups subsequently developed prostatic cancer. This study, therefore, offers support for the general opinion that benign prostatic hypertrophy and carcinoma are independent conditions and suggests that research must be based on the concept that the diseases have separate etiologies. Although some of the problems associated with this type of study have been discussed,\textsuperscript{17,18} the importance of investigations into the natural history of prostatic disease and of careful epidemiological studies cannot be overemphasized.

In trying to understand the relationship between the etiology of prostatic carcinoma and the endocrine status of patients with the disease, many investigators have attempted to define the effective hormonal disturbance from detailed study of various histological changes observed in tissue removed from the patients. Considerable controversy has arisen in recent years\textsuperscript{19} concerning the precancerous changes observed in resected prostatic tissue. Franks has consistently maintained\textsuperscript{8,9,20} that a particular type of focal atrophy could be recognized, often associated with lymphocytic infiltration and fibrosis of the peripithelial stroma, which could be considered precancerous. Definite areas of proliferation which develop from this atrophic epithelium have a pattern closely resembling the structure of small acinar carcinoma. McNeal, with a newly developed concept on the anatomy of the prostate gland in which function, morphology, and pathology have been effectively interrelated,\textsuperscript{19,21} has clearly indicated\textsuperscript{19} that he is diametrically opposed to such views.

An appreciation of McNeal's thoughts on the development of prostatic cancer in
certain regions of the human prostate is best seen in relation to his concept of the anatomy of the gland. This is considered a composite of three distinct, separate glandular structures within a single capsule,22 with two, the central zone and the peripheral zone, forming the "true functional prostate" (Figure 1). The third glandular structure is the periurethral gland, a series of small ducts opening into the upper segment of the urethra which lies above the upper end of the verumontanum and extends upwards to the bladder neck. This upper section of the urethra is enclosed in a cylinder of muscle tissue that functions as a urinary preprostatic sphincter.

Unlike the fully developed glandular tissue of the central and peripheral zones, the periurethral glands are small, simple structures which are not thought to contribute to the production of seminal fluid. In McNeal's view, the periurethral zone of the prostate is a distinct element in its own right, and previous descriptions that considered it to be part of the true prostate are not correct. Lowsley23 from a study of the anatomy of the fetal prostate also considered that these glands were not part of the prostate proper. The central and peripheral zones of the prostate generally undergo progressive but slow atrophy with increasing age, although with considerable individual variation such that older prostates can display glandular morphology comparable to that seen in glands from younger men.22 McNeal, however, described an age-related increase in atypical hyperplasia, diffuse or multifocal proliferation of ductal and epithelial tissue from "persistently active glandular tissue"—premalignant changes which were considered closely associated with prostatic carcinoma. A high incidence of carcinoma was found in the presence of atypical hyperplasia and, on occasion, continuity between
this premalignant tissue and the origin of small foci of invasive cancer. Focal atrophy, described by Franks, was considered a condition secondary, not to aging, but to inflammation.19

In McNeal's study22 170 small carcinomas (under 3 mm in diameter) were identified in the 415 prostates examined, and, of these, 148 were in the peripheral zone and none were found in the periurethral gland. This glandular structure McNeal considers the unique site of origin of benign nodular hypertrophy. Blacklock,24 in an excellent review of the surgical anatomy of the human prostate, strongly supported the views of McNeal from his own personal observations. Koppel and colleagues,25 in a retrospective study of the pathology of prostatic tissue, have also indicated that diffuse hyperplasia was found significantly more often in tissue from prostatic cancer patients than in controls and they considered the relevance of their observations in relation to pathogenesis of the disease.

It is interesting that Reischauer in 192526 also believed that the earliest hyperplastic changes occurred in the fibromuscular stroma, with associated epithelial proliferation, in the preprostatic urethral sphincter.

Small, asymptomatic latent carcinoma is relatively common in prostatic tissue, particularly in regions of the peripheral zone furthermost from the urethra. Their prognosis poses a difficult question and the proportion becoming clinically manifest has yet to be decided. The latent period before these tumors become more aggressive is probably considerable and the biochemical or endocrine conditions which promote the changes are unknown. Some believe27,28 that these microscopic foci of differentiated carcinoma rarely become clinically manifest. The time course is certainly difficult to establish and extensive neoplastic change could occur in these peripheral sites of the carcinoma, with early capsular involvement and spread of the disease outside of the prostate taking place before clinical signs were evident. From an autopsy series in which the occurrence of prostatic carcinoma in routinely sectioned glands was considered,29 the frequency, on routine sectioning at 75 years of age, was found to be the same as that from serial sections taken at 55, and it was suggested, therefore, that there was at least a 20-year lapse between the initial development of the neoplasm and its clinical manifestation.

Promotion of prostatic neoplasia may, therefore, be dependent upon an abnormal endocrine status of certain older males. At present, there are little data on even epidemiological characteristics relating to this disease. It was always hoped that factors which might reflect the hormonal status of a man prior to cancer development would assist in delineating a high-risk group, but studies of demographic features such as age, socioeconomic class, marital status, sex drive, etc. of subjects who presented with prostatic carcinoma have generally failed to consistently produce significant differences from control groups.30-32 Often conflicting reports in the literature have tended to cloud the issues. Kessler,33 for example, failed to demonstrate increased prostatic cancer mortality among diabetics despite Lea's observation34 that a high death rate from prostatic cancer relates to a high mortality from diabetes. Although Bourke and Griffin35 reported a high incidence of diabetes mellitus in an English population with benign prostatic hyperplasia, Greenwald et al.36 were unable to support the claim.

It is obvious, however, from studies of migrating populations that environmental and socioeconomic factors can influence the etiology of prostatic cancer.36 Death from the disease is rare in the Japanese as it is in other oriental populations, yet this rate increases to nearly half that of the indigenous American for those Japanese that become domiciled in the U.S. A number of epidemiological studies have been concerned with prostatic cancer in the Japanese male.37,38 It is interesting that the low incidence of prostatic cancer in Japan relates only to clinical cancer, since autopsy data show
that latent carcinoma is present as much as in Caucasians of corresponding ages. Investigations have obviously been directed to the evaluation of endocrine differences between Japanese and Caucasian men in the different age groups. Urinary 17-ketosteroid excretion was lower in the former, although the plasma concentrations of testosterone and cortisol were similar in the two populations. In a more recent study, Okamoto and colleagues suggested that there was a unique pattern of testosterone metabolism in the Japanese male.

Detailed, careful epidemiological studies must now be of great potential value in assessing those biological factors that may be concerned with the etiology of prostatic cancer. The high mortality rate from this disease in the American Negro compared to the Negro from Africa directs attention to the environment. The positive correlation between prostatic cancer and carcinoma of the breast (Figure 2) suggests a common type of etiological background for these hormone-related cancers, and, obviously, intensive investigation into the endocrinology of the normal and diseased prostate gland could eventually provide a valuable guide to possible cause. The following sections outline some of these investigations.

II. HORMONES AND AGING

Clinical data indicate that carcinoma of the prostate in man is, to some extent, func-
tionally dependent upon androgenic stimulation, yet the role of the various C₁₇-steroids circulating in the blood and formed within the prostate, in the etiology of the disease, remains obscure. Considerable interest has been directed to the changes in plasma steroids that are associated with aging in man and to the relative activity of the endocrine glands that play a part in the regulation of prostatic growth and function.

Testosterone must be considered to be the most important plasma androgen and the concentration, of its free, nonprotein-bound form, is generally accepted as a reasonable indicator of androgenic status. This status does not depend solely upon plasma testosterone, however, but also relates to other C₁₇-steroids, many of which can achieve relatively high levels in blood, steroids such as androstenediol (androst-5-ene-3β,17β-diol), androstenedione, DHA (dehydroepiandrosterone), and DHA sulfate. The interrelationship between these various plasma steroids and their glands of origin must be considered in any discussion concerned with the endocrinology of prostatic disease and the influence of aging on androgen balance. The recent development of radioimmunoassay for hormone analysis has allowed the sensitive and precise measurement of various steroids and protein hormones present in plasma and has contributed greatly to our understanding of the problem.

A. Endogeneous Steroid Hormone Levels in Plasma

It was interesting that, although prostatic disease occurs at a time when it is generally accepted that testicular function is declining, Kent and Acone reported⁴⁴ that the plasma concentration of testosterone in the human male is maintained at a relatively constant level from the age of 20 to the ninth decade. This resulted, however, from a decrease in the metabolic clearance rate of the hormone rather than a sustained ability of the testis to continue with its active synthesis and secretion. In contrast, Vermeulen and colleagues⁴⁵ observed that the testosterone concentration decreased after the sixth decade, although individual differences were large (Table 1).

In this respect, Vermeulen also showed that sex hormone binding globulin (SHBG) retained a constant binding capacity until about the fifth decade, when a gradual increase occurred until, at the age of 80, its value was approximately twice that found in young men. The free, nonprotein-bound, testosterone fraction, which represents about 2% of the total testosterone concentration in the younger man, decreases to 1.75% in the fifth decade and to 1.25% in the eighth. Therefore, although the total testosterone concentration in plasma at the age of 80 is one third of that at 25, the “active” free fraction represents only one sixth. This is referred to again in a later section.

Androgen levels in plasma may, therefore, decrease with aging in certain individuals, particularly after the sixth decade, and data from other groups tend to support this concept;⁴⁴-⁴⁷ although in most of these investigations the significance of the differences found between the various age groups studied has not been sufficiently convincing to indicate that this is a general phenomenon. For most studies, control subjects can only be men without clinically manifest prostatic disease, and it would seem that more detailed studies of individual subjects will be necessary. On the other hand, plasma estradiol-17β concentration was found to increase with age in the healthy, apparently normal, adult male⁴⁴-⁴⁸ (Table 1), thereby supporting the concept that changes in the androgen-estrogen balance in elderly men may be implicated the etiology of prostatic disease.

Little is known about the physiological importance of estrogens in the male. It has been established that the testis secretes estradiol-17β and estrone,⁴⁹-⁵¹ although its contribution to the plasma estrogen levels is small. Any involvement of the adrenal cortex is, however, negligible and the major proportion of plasma estrogen in the male origi-
must, therefore, so nent that the such low keto-function appear necessary referred ulin high affinity tan-3-one), the their activity. many that
Steroid Hormone B.
to exogenous activity with fluoxymesterone. of the testis response difficult to whether this increases of group older concentration of HCG group, There was 100% rise in tant resulted in elderly subjects and cortex could, therefore, which reported,55 that increased been or data quantitative 53 the former testosterone,52 from the nates Arone4J 70—80 6.50 60—70 5.90 5.60 4.70 30—40 5.60 5.34 4.70 4.62 4.45 30—40 5.60 4.70 6.40 5.62 810 50—60 5.90 6.35 6.16 5.90 6.40 5.54 50—60 5.90 6.34 5.54 6.6 5.77 6.40 6.16 5.77 25.6 28.7 24.2 5.77 6.50 5.43 4.45 6.34 5.54 25.6 28.7 24.2 640 640 640 640 600 * Accumulated data from the Tenovus Institute for Cancer Research.

nates from the peripheral aromatization of such steroids as androstenedione and testosterone,52,53 the former being synthesized and secreted by the adrenal cortex.54 Little quantitative data are available on estrogen production from C19-steroids in relation to aging or in patients with carcinoma or hypertrophy of the prostate; although it has been reported,55 that increased peripheral aromatization occurs in the elderly man, which could, therefore, account for the increased plasma estrogen/androgen ratio.

Kley and colleagues56 assessed the effect of age on the responsiveness of the adrenal cortex and testis to trophic stimulation. In 12 younger men (19 to 40 years old) and 12 elderly subjects (60 to 86 years old), adrenocorticotropic hormone (ACTH) administration resulted in a 250% rise in plasma cortisol in both age groups, with a concomitant 100% rise in plasma estrone in the former and a 75% rise in the older group. There was little or no effect of ACTH on plasma estradiol-17β levels. In the younger group, HCG (human chorionic gonadotropin) administration increased plasma concentration of estrone (122%) and also that of estradiol-17β (250%), whereas in the older group it was considered there was a relative, decreased response to HCG, with increases of plasma estrone and estradiol-17β of 103% and 150%, respectively. Again, whether this response reflects direct secretion of estrogen or that of a prehormone is difficult to determine from such experiments. Doerr and Pirke57 showed a similar response of the testis to HCG and also studied the suppression of testicular secretory activity with fluoxymesterone. Both studies show, however, that the ability to respond to exogenous gonadotropin is preserved despite aging and loss of libido.

B. Steroid Hormone Binding Proteins

In assessing the androgenicity of the plasma steroids, it is important to recognize that many of them are bound to plasma proteins58 which would, therefore, modulate their activity. Testosterone, 5α-dihydrotestosterone (5α-DHT, 17β-hydroxy-5α-androstan-3-one), the 5α-androstenediols, androstenediol, and, also, estradiol-17β, bind with high affinity but low capacity—and with some degree of stereospecificity—to a β-globulin referred to as the sex hormone binding globulin. A 17β-hydroxyl group and a 3-keto-function appear necessary for binding.51 Albumin will also bind steroids but with such low affinity that it has little physiological significance. There is also good evidence that the free, nonprotein-bound hormone represents the biologically active component52 so the distribution of the various plasma steroids between free and bound states must, therefore, be taken into account when considering potential androgenicity and
TABLE 2

Sex Hormone Binding Globulin in Man

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Sex hormone binding globulin (SHBG) binding capacity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20—50</td>
<td>5.2 x 10^-3 M</td>
<td>63</td>
</tr>
<tr>
<td>70—85</td>
<td>8.9 x 10^-3 M</td>
<td></td>
</tr>
<tr>
<td>20—40</td>
<td>2.28 x 10^-3 M</td>
<td>65</td>
</tr>
<tr>
<td>50—85</td>
<td>3.62 x 10^-3 M</td>
<td></td>
</tr>
<tr>
<td>22—44</td>
<td>2.85 x 10^-3 M</td>
<td>64</td>
</tr>
<tr>
<td>45—64</td>
<td>4.66 x 10^-3 M</td>
<td></td>
</tr>
<tr>
<td>20—50</td>
<td>4.3 x 10^-3 M</td>
<td>66</td>
</tr>
<tr>
<td>50—90</td>
<td>7.8 x 10^-3 M</td>
<td></td>
</tr>
<tr>
<td>Prostatic cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54—80</td>
<td>3.89 x 10^-3 M</td>
<td>64</td>
</tr>
<tr>
<td>50—85</td>
<td>4.00 x 10^-3 M</td>
<td>65</td>
</tr>
<tr>
<td>Prostatic hypertrophy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62—97</td>
<td>4.07 x 10^-3 M</td>
<td>64</td>
</tr>
<tr>
<td>Estrogen-treated carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.0 x 10^-3 M</td>
<td>64</td>
</tr>
</tbody>
</table>

also the concentration of SHBG. In this respect, therefore, the observed increase in SHBG with advancing age44,63-66 (Table 2) together with the consequent decrease in plasma free testosterone levels43,67 must be relevant when considering the effect of this androgen on the aging prostate. The increase in SHBG would seem to have a lesser effect on the plasma concentration of free estradiol. In the case of this hormone, there was an approximate 70% decrease in the level of the free steroid with advancing age, which emphasizes again the change in the "biologically active" estrogen and androgen balance in the elderly male (Table 3). It is generally assumed that the increase in the concentration of SHBG results from the increase in plasma estrogens.

Little is known about the biochemical processes concerned with the transfer of plasma steroid into the target cells, but obviously the relationship between plasma-bound and free concentrations must play a role. Farnsworth considered this relationship as a possible mechanism for controlling the intracellular accumulation of steroid within the prostate,68 and it has been generally accepted that only free steroid is available to androgen-dependent tissues.69 Lasnitzki and Franklin70 showed with explants of rat prostate in culture that serum containing SHBG inhibited the entry of testosterone into the cells. Similar results from human prostatic tissue have also been obtained.71 A more intensive investigation into the factors which control steroid entry would be valuable, and it would seem reasonable that intracellular receptor concentration, steroid metabolism in the prostate, and possibly certain protein hormones such as prolactin may be concerned. There has also been some speculation that SHBG, with steroid attached, may bind to the plasma membrane to mediate steroid transfer into the cell or, indeed, that SHBG could pass through the membrane. There are many studies to develop on this aspect of prostatic endocrinology, some of which may be of particular relevance to chemotherapy.

C. Steroid Metabolism in the Prostate: 5α-Dihydrotestosterone and the 5α-Androstane-diol: Biological Effects

In normal men, more than 90% of the testosterone produced each day, which is
approximately 5 to 10 mg, is secreted by the testes with a relatively small contribution from the adrenal cortex, although, as discussed later, this is significant in the estrogen-treated or orchidectomized man. Furthermore, there is now considerable evidence to indicate that 5α-dihydrotestosterone, rather than testosterone, is the active, intracellular hormone in the prostate gland and other accessory sex organs. The formation in vitro of 5α-dihydrotestosterone from testosterone by tissue preparations from human benign hypertrophic prostate was originally demonstrated in the now classical experiments of Farnsworth and Brown. Further studies, with human and animal tissues, undertaken by Farnsworth and a number of other groups including our own, confirmed and expanded these original findings. Many other metabolites of testosterone are formed in vitro, especially 5α-androstan-3β,17β-diol and 5α-androstane-3β,17β-diol, and it was of interest when [7α-3H]testosterone was infused as a bolus into the cephalic vein of human subjects undergoing Millin retropubic prostatectomy for benign prostatic hypertrophy that the radioactive steroids isolated from the prostatic adenoma, removed about 30 min later, showed a similar pattern of testosterone metabolism to that observed in vitro with 5α-dihydrotestosterone appearing as the major radioactive product (Table 4). Androstanediols were also formed, together with androsterone, epiandrosterone, 5α-androstanedione, androstenedione, and, in some of the experiments in vivo, epitestosterone (17α-hydroxyandrost-4-en-3-one) and 5α-androstan-3β,17α-diol (Figure 3). It is possible of course that radiometabolites formed by systemic interconversion may be localized in the prostate and this has been discussed by Voigt and colleagues who have performed similar studies in vivo.

It would also appear that metabolites of testosterone other than 5α-dihydrotestosterone may have specific roles to play within the target tissue. Baulieu, Lasnitzki and Robel showed that certain 5α-androstanediols, as well as 5α-dihydrotestosterone, stimulated cell division and induced epithelial hyperplasia and secretory activity in cultured explants of rat prostate. The investigations of Farnsworth suggest that 5α-androstan-3α,17β-diol may influence the steroid-sensitive, cation-dependent ATP-ase in human prostatic tissue directing attention to effects of steroids on prostatic biochemistry other than intranuclear processes. Our own studies have indicated that certain of the 5α-androstanediols stimulate a semipurified prostatic DNA polymerase (DNA nucleotidyltransferase, E.C. 2.7.7.7) and DNA-dependent RNA polymerase (nucleoside triphosphate–RNA nucleotidyltransferase E.C. 2.7.7.6). It seems then that testosterone and certain of its metabolites are concerned in the control of prostatic growth and function, and interconversion of these steroids within the gland may provide a delicate regulatory mechanism for the various glandular processes. Imbalance of this mechanism could be considered a possible factor which might contribute to prostatic dysfunction. The part played by other hormones such as prolactin, growth

### TABLE 3

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Plasma-free steroid (% Total)</th>
<th>Conc-free steroid in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testosterone</td>
<td>5α-DHT</td>
</tr>
<tr>
<td>22—61</td>
<td>2.24</td>
<td>1.17</td>
</tr>
<tr>
<td>67—93</td>
<td>1.65</td>
<td>0.83</td>
</tr>
<tr>
<td>20—50</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td>50—70</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td>70—90</td>
<td>1.36</td>
<td></td>
</tr>
</tbody>
</table>
Radioactive Steroids Isolated in Human Prostatic Tissue after In Vivo Infusion of 50μCi \([7\alpha^3H]\)Testosterone, \([7\alpha^3H]\)Androstenedione, or \([7\alpha^3H]\)Dehydroepiandrosterone (DHA) Sulfate

<table>
<thead>
<tr>
<th></th>
<th>Testosterone infusion</th>
<th>Androstenedione infusion</th>
<th>DHA sulfate infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient no.</td>
<td>Patient no.</td>
<td>Patient no.</td>
</tr>
<tr>
<td></td>
<td>31 32 33 34 35</td>
<td>23 24 38 39</td>
<td>36 37 40</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5\alpha)-Dihydo...</td>
<td>9.2 12.8 7.4 3.2 3.1</td>
<td>6.6 18.1 4.7 2.0</td>
<td>2.2 2.0 0.2</td>
</tr>
<tr>
<td>Androsterone</td>
<td>8.7 5.9 11.1 6.2 14.3</td>
<td>8.8 9.2 24.3 24.6</td>
<td>3.2 7.5 0.3</td>
</tr>
<tr>
<td>Epiandrostro...</td>
<td>5.5 2.3 1.9 3.4 6.2</td>
<td>39.7 47.3 29.4 17.7</td>
<td>2.2 6.6 0.3</td>
</tr>
<tr>
<td>(5\beta)-Androstano...</td>
<td>1.5 1.2 1.3 4.5 5.2</td>
<td>0 13.8 1.9</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>6.8 0.9 0.4 5.6 2.8</td>
<td>35.2 0 10.9 14.2</td>
<td>1.6 3.1 0.3</td>
</tr>
<tr>
<td>(5\alpha)-Androstan-...</td>
<td>8.9 2.7 2.4 5.6 5.5</td>
<td>3.1 10.6 2.8 9.2</td>
<td>0 0 0</td>
</tr>
<tr>
<td>(5\alpha)-Androstan-...</td>
<td>5.7 — 2.9 5.1 7.4</td>
<td>3.8 8.3 2.6 13.8</td>
<td>0 0 0</td>
</tr>
<tr>
<td>DHA</td>
<td>— — — — —</td>
<td>— — — — —</td>
<td>2.2 2.6 8.8</td>
</tr>
<tr>
<td>DHA sulfate</td>
<td>— — — — —</td>
<td>— — — — —</td>
<td>89.4 72.9 89.8</td>
</tr>
</tbody>
</table>

**Notes:** Dashes indicate steroid was not investigated in these experiments.

hormone, insulin, and estrogens in influencing this metabolic pattern is not yet understood. It must be accepted that at present the androgenic role of the androstane diols is not particularly clear. Possibly, knowledge of their concentrations in plasma could well provide an index of prostatic activity with regard to testosterone metabolism. Furthermore, 5α-androstane-3α,17β-diol may have a function in inhibiting FSH and LH secretion by the pituitary, but whether it has any androgenic function per se must be doubtful. Although this diol is apparently an effective androgen in that its administration to castrated rats increased the ventral prostate weight to the same extent as that achieved with 5α-dihydrotestosterone (Table 5), the elegant, extensive investigations of Voigt and colleagues effectively demonstrated that the major product in hypertrophic prostates of man, injected with [3H]5α-androstane-3α,17β-diol, was 5α-dihydrotestosterone. Less 5α-dihydrotestosterone was found after administration of [3H]5α-androstane-3β-17β-diol. It is difficult, therefore, to distinguish the andro-

FIGURE 3. Testosterone metabolism by the prostate.
TABLE 5

Weight of the Ventral Prostate After Steroid Administration to Castrated, Mature Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ventral prostate wt (mg/100 g body wt) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesame oil</td>
<td>16.5 ± 6.4</td>
</tr>
<tr>
<td>Testosterone</td>
<td>50.4 ± 2.8</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>22.3 ± 3.6</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>67.5 ± 1.8</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>66.4 ± 10.2</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>20.1 ± 3.3</td>
</tr>
<tr>
<td>5β-Androstane-3α,17β-diol</td>
<td>22.1 ± 10.4</td>
</tr>
</tbody>
</table>

*Rats were treated daily with 100 μg steroid/100 g body weight, s.c., for 7 days, starting on the day of orchidectomy. Steroids were given in sesame oil.


...genic effects of these diols from those of 5α-dihydrotestosterone to which they are both metabolized. In the rat, however, if conversion of the 5α-androstane-3β,17β-diol to 5α-dihydrotestosterone does not occur, then the biological effects observed by Robel et al. and Schmidt et al. must be attributed to that diol for which there may exist an equally specific receptor.

Early studies from the Tenovus Institute provided evidence that 5α-androstane-3α,17α-diol may play a role in controlling RNA synthesis in the canine prostate. Evidence for a specific receptor for this diol has been found by Evans and Pierrepoint as well as for 5α-dihydrotestosterone, although only the former steroid was able to maintain the histological integrity of the tissue in organ culture. Epitestosterone and 5α-dihydroepitestosterone were only partially successful in maintaining epithelial height, while secretory activity was not preserved. Testosterone and 5α-dihydrotestosterone were entirely unsuccessful in this respect and induced a marked stromal reaction. Obviously, a greater insight is needed into the effects of the various metabolites of testosterone of prostatic biochemistry.

D. Metabolic Activity of the Prostate Gland

Although an assessment of prostatic activity could be made from enzyme studies on tissue preparations in vitro, it has always been believed that the measurement of the various prostatic metabolites of testosterone in biological fluids could ultimately provide the best index of prostatic function, or dysfunction, in vivo. Specific radioimmunoassay for the different 5α-androstanediols are only now being developed, however, and at present, little is known about the levels of these steroids in plasma.

Vermeulen and colleagues studied the effect of advancing age on testosterone metabolism along the lines previously followed by Mauvais-Jarvis and colleagues when investigating androgen metabolism in normal women. Decreased formation of the 5α-androstanediols in the older man was reported, with an increase in the 5β/5α-metabolite ratio, a metabolic pattern observed in men on estrogen therapy and in hypogonadism. Since estrogen treatment decreases the concentration of plasma 5α-androstane-3α,17β-diol, which is related to the level of the free plasma testosterone
fraction, Vermeulen et al.\textsuperscript{43} suggested that these metabolic changes observed with advancing age were consistent with a decreased metabolism of testosterone in target tissues such as the prostate.

It was established by Ito and Horton\textsuperscript{104} that in the normal male plasma 5α-dihydrotestosterone is derived principally from testosterone. Its blood-production rate is approximately 400 μg/day,\textsuperscript{108} half of which originates from peripheral conversion of plasma testosterone, 20% from androstenedione,\textsuperscript{106,107} and a smaller amount is synthesized and secreted by the testes.\textsuperscript{108} Although it is unlikely that metabolism of androgens within the prostate makes a significant contribution to the levels of 5α-dihydrotestosterone in the circulation, the observations by Mahoudeau et al.\textsuperscript{44} are of interest, for they found that blood taken from the prostatic capsular veins of nine men undergoing open prostatectomy for benign hyperplasia contained slightly higher levels of this steroid than systemic venous blood. It is, however, difficult to accept that prostatic capsular venous blood is likely to reflect accurately the character of blood leaving the true prostate, for there is a complex plexus in this region communicating with the dorsal vein of the penis, the base of the bladder, and, possibly, the epididymis and testicular venous drainage through the deferential vein. Mahoudeau and colleagues recognized this limitation. They also found that there was no difference in the ratio of 5α-dihydrotestosterone to testosterone in the plasma of 29 men with benign prostatic hyperplasia when compared with age-matched controls whose prostates were observed to be normal at operation for other disorders. Furthermore, no significant reduction of this ratio was found in peripheral venous blood after removal of a prostatic adenoma. This contrasts with the findings of Berberia and his colleagues\textsuperscript{109} who reported an increased ratio of 5α-dihydrotestosterone to testosterone in the plasma of men with benign prostatic hypertrophy and, after resection of the prostatic tissue, the level of 5α-dihydrotestosterone decreased. These data, therefore, suggest that a prostatic adenoma can contribute substantially to plasma 5α-dihydrotestosterone levels, and the impressive superfusion studies of Giorgi et al.\textsuperscript{110} certainly indicated that 5α-dihydrotestosterone was released from prostatic tissue. Whether the normal prostate contributes in a similar manner remains to be demonstrated. Further study is also required to consider the effect of prostatectomy on pituitary hormone secretion, since it would seem from experimental evidence, to be discussed later, that certain protein hormones such as prolactin may influence prostatic activity.\textsuperscript{111,112}

It is also important to establish whether 5α-dihydrotestosterone secreted by the normal prostate gland reflects the loss of an excess of metabolite formed, but not associated with androgen receptor,\textsuperscript{113} or the release of 5α-dihydrotestosterone previously bound to nuclear chromatin after translocation of steroid-receptor complex from cytoplasm to nucleus. The biochemical mechanisms which regulate prostatic metabolism of testosterone and the processes which govern 5α-dihydrotestosterone action and release are, as yet, poorly understood, and, consequently, the value of measuring plasma 5α-dihydrotestosterone levels for the assessment of prostatic activity requires careful consideration. In contrast to testosterone, the plasma concentration of total 5α-dihydrotestosterone was shown not to decrease with advancing age when Pirke and Doerr\textsuperscript{114} analyzed plasma from men between the ages of 22 to 61 and 69 to 93. Mahoudeau et al.\textsuperscript{45} also failed to show a significant decrease in 5α-dihydrotestosterone levels with increasing age (Table 1), although conflicting data from the laboratory of Chisholm\textsuperscript{49} did indicate a lower concentration in elderly men.

A significant 26% decrease in free, nonprotein-bound 5α-dihydrotestosterone in plasma was reported\textsuperscript{114} however, although this was still smaller than the 46% decrease in free testosterone levels found between the different age groups (Table 3), and the close relationship between the concentration of the two steroids—with a 5α-dihyro-
testosterone to testosterone ratio of approximately 0.1—suggests that plasma 5α-dihydrotestosterone concentration is essentially a function of plasma testosterone levels.\textsuperscript{116}

It is difficult to know whether plasma levels of 5α-dihydrotestosterone accurately reflect 5α-reductase activity in that, although the prostate may secrete this steroid, further intracellular metabolism to the 5α-androstanediols obviously affects the amount released. It was shown in the rat, however, by Shimazaki et al.\textsuperscript{117} that castration decreased 5α-reductase activity and, furthermore, by Moore and Wilson,\textsuperscript{118} that administration of testosterone increased the activity of the enzyme. It is noteworthy that opposite effects of these endocrine manipulations have been reported for the 5α-reductase of the adrenal cortex\textsuperscript{119} and pituitary glands.\textsuperscript{120}

Animal model systems can often be used to investigate human disease and Eik-Nes\textsuperscript{91} has recently considered the relevance of the prostatic secretion of 5α-dihydrotestosterone in the isolated organ from the dog when substances that affect the metabolic activity of the prostate enter the gland through the arteries under controlled blood flow\textsuperscript{121} and then are removed, together with their metabolites, in the venous drainage. The effect of end-product inhibition of enzymatic activity is, therefore, avoided. The perfused canine prostate concentrates androgens, and perfused testosterone is rapidly metabolized to 5α-dihydrotestosterone and other metabolites.\textsuperscript{122} Furthermore, analytical data on affluent and effluent blood indicated that the prostate secreted 5α-dihydrotestosterone. It is important, however, when studying the endocrinology of the canine prostate, that extrapolation to man should be undertaken with considerable care. The tendency of the dog, like man, to develop an age-related hypertrophy of the prostate\textsuperscript{123,124} and, less commonly, neoplasia,\textsuperscript{125,126} recommends this animal for special consideration in its own right. From such a study in the Tenovus Institute, the presence of a specific receptor protein for 5α-androstan-3α,17α-diol,\textsuperscript{99} which stimulated RNA polymerase activity in an isolated in vitro system,\textsuperscript{127,128} suggested that there are probably basic underlying differences in the endocrine control of prostatic function between the two species. More intensive research on the dog may make more evident the endocrine changes that initiate or accompany prostatic dysfunction in this species.

III. HORMONAL STATUS OF PATIENTS WITH PROSTATIC DYSFUNCTION

It seems reasonable that in the search for the biochemical or endocrine abnormality that could be concerned in the etiology of prostatic cancer, a detailed study of the hormone levels in plasma and tissue from patients with this disease could provide valuable information. The generally accepted experimental approach has been that a comprehensive knowledge of hormone action and steroid metabolism in diseased prostatic tissue and in tissue from normal elderly men, together with information on the hormonal status of patients from whom tissue was removed, could provide the necessary background that would lead to a greater understanding and possibly control of prostatic disease.

It has been realized, however, that there is yet little evidence to indicate that plasma hormones are actively concerned in prostatic carcinogenesis, although few would deny that the differentiated carcinoma is usually androgen dependent. Certainly, conventional therapy has involved reducing plasma testosterone or possibly inhibiting its action within the prostatic cell. It is possible, therefore, that activation of latent carcinoma and promotion of its early growth involve hormone intervention, although, as described earlier, opinions tend to differ as to whether a reduced androgenic status with associated prostatic atrophy\textsuperscript{8,20} or prostatic hyperplasia\textsuperscript{19,22} precedes neoplasia.

In relation to this, hormone profiles in patients with benign prostatic hypertrophy
have also been determined, again in comparison with the asymptomatic "normal" male, and it is not yet understood whether any endocrine disturbances associated with hypertrophy similarly influence to any extent the development of neoplasia.

A. Plasma Hormones and Prostatic Disease

It has been accepted for many years that estrogens may play an important role in male reproductive physiology and there has been long speculation that changes in the androgen-estrogen balance may be implicated in the etiology of prostatic disease. Prostatic hyperplasia and the synergism occurring between androgens and estrogens in promoting the growth of the prostate and seminal vesicles have been discussed often.

Since the plasma concentration of estradiol-17β has been shown to increase with age in the apparently normal, healthy adult male, a report from Brandes et al. on ultrastructural studies of prostatic adenomas, indicating androgen stimulation with an overriding estrogenic effect, would, therefore, seem reasonable; others have confirmed this observation. Studies with experimental animals also indicate that estrogens can induce prostatic enlargement, although the relevance of the effect in relation to the pathogenesis of prostatic hypertrophy in man must be viewed with some degree of caution. Spontaneous prostatic adenocarcinoma has been described in rodents, but benign hyperplasia can only be induced by hormone manipulation. For example, Burrows and Kennaway produced urinary obstruction by the application of estrogenic material to the skin of mice and Fingerhut and Veenema reported growth of the periurethral glands and the prostate in mice after prolonged treatment with diethylstilbestrol. Although these classical studies are interesting, experimental prostatic enlargement does not have the histological fibrotic characteristics typical of the human benign tumor, and use of rodents as a model system may be limited.

Studies from our own laboratories have been concerned with the plasma levels of testosterone, androstenedione, estradiol-17β, FSH, LH, and prolactin in patients with prostatic cancer, with benign prostatic hyperplasia, and in controlled hospitalized patients without symptomatic or clinical prostatic disease (Table 6). With the exception of prolactin, no significant difference was noted in the concentration of these hormones between any of these groups. The mean plasma prolactin concentration was significantly lower in the patients with benign hyperplasia than in those with malignant disease. Asano earlier, using a bioassay, claimed an increased urinary excretion of prolactin in patients with prostatic cancer; and Bartsch et al. have also reported an elevated prolactin level in 15% of such patients with carcinoma.

The data on plasma C19-steroid levels agree well with results of Moon and Flocks. Previously, Isurugi had found no evidence of an elevated testosterone production rate in patients with prostatic carcinoma. Although Gandy and Peterson described an elevated peripheral concentration of androstenedione in six patients with prostatic carcinoma, implicating the adrenal cortex, in our study of 23 patients (Table 6), no such elevation was detected. Bartsch et al. also confirmed that plasma levels of testosterone, 5α-dihydrotestosterone, estrone, and estradiol-17β, in patients with prostatic cancer, did not differ from those of a control group of similar age. Despite the implication, therefore, that estradiol-17β may be concerned in the pathogenesis of prostatic hyperplasia, these investigations indicated that all the patients studied had similar levels of plasma estradiol-17β. This agreed with earlier reports of Marmoronon et al. on estrogen measurements in urine from a similar group of patients.

It may well be, however, that the relevant time to study such estrogen changes is many years earlier, long before the disease is manifest. Alternatively, differences may exist between concentrations of unbound estradiol-17β in the various groups. It is also
TABLE 6

Plasma Hormone Concentrations in Patients With and Without Prostatic Disease*

<table>
<thead>
<tr>
<th></th>
<th>Testosterone (ng/ml)</th>
<th>Androstenedione (ng/ml)</th>
<th>Estradiol (pg/ml)</th>
<th>LH (mIU/ml)</th>
<th>FSH (mIU/ml)</th>
<th>Prolactin (mamp/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control groups of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>patients without</td>
<td>Mean value</td>
<td>6.4</td>
<td>0.56</td>
<td>40</td>
<td>7.0</td>
<td>14.0</td>
</tr>
<tr>
<td>prostatic disease</td>
<td>SEM</td>
<td>0.5</td>
<td>0.06</td>
<td>1.2</td>
<td>1.4</td>
<td>4.2</td>
</tr>
<tr>
<td>n</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>Patients with</td>
<td>Mean value</td>
<td>5.9</td>
<td>0.59</td>
<td>41.7</td>
<td>11.1</td>
<td>6.6</td>
</tr>
<tr>
<td>benign prostatic</td>
<td>SEM</td>
<td>0.4</td>
<td>0.06</td>
<td>1.6</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>hypertrophy</td>
<td>n</td>
<td>41</td>
<td>27</td>
<td>41</td>
<td>41</td>
<td>37</td>
</tr>
<tr>
<td>Patients with</td>
<td>Mean value</td>
<td>5.45</td>
<td>0.70</td>
<td>38.5</td>
<td>8.2</td>
<td>6.9</td>
</tr>
<tr>
<td>prostatic carcinoma</td>
<td>SEM</td>
<td>0.46</td>
<td>0.11</td>
<td>1.6</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>n</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.5</td>
</tr>
</tbody>
</table>

* Benign v. prostatic carcinoma P>0.01, n = number of patients, SEM = standard error of the mean.
possible that biochemical changes within the cell, resulting in an increased sensitivity and responsiveness to the normal hormone levels, could lead to abnormal growth.

Despite experimental work in animals that suggested that pituitary hormones may influence prostatic growth and function, there have been few studies on plasma protein hormones and prostatic cancer. Hypophysectomy, for example, in dog and rat produces a more marked prostatic atrophy than does castration. Administration of growth hormone (GH) and ACTH to hypophysectomized-castrated rats stimulated prostatic growth, whereas LH, FSH, and prolactin were without effect. Synergism of hormonal effects was evident, for simultaneous administration of prolactin and ACTH to castrated rats produced greater prostatic growth than ACTH alone. A more marked increase in fructose and citric acid content of the prostate of hypophysectomized-castrated rats was found after prolactin and testosterone administration than after testosterone alone, the effect being mainly on the dorsolateral lobe of the gland. GH was generally found to complement the effects of prolactin and testosterone on prostatic weight.

Although plasma hormones may not be actively concerned in prostatic carcinogenesis, a permissive role should not be overlooked and a particular endocrine milieu could influence tumor dissemination. Similarly, tumor spread may affect the secretion of stress-related hormones such as ACTH, cortisol, GH, and prolactin. A study was established in the U.K. to examine in considerable detail possible interrelationships between these plasma hormones and a variety of clinical parameters. The investigation was established under the auspices of the British Prostate Study Group consisting of urologists from various clinics in the country in association with the Tenovus Institute for Cancer Research. Patients who presented for treatment in different clinics were selected, assessed, and classified in a standardized manner, according to their primary tumor grade and metastatic status, and a profile of plasma hormone levels was determined prior to and during therapy. Possible differences in hormone concentrations between these particular groups were investigated by using a multivariate-statistical-analysis technique developed at the Institute by Wilson and Tan. Canonical variate analysis was used to determine if group separation could be achieved and to establish the contribution of the various hormones in discriminating between the various patient populations. Particular attention was given to ensure consistent assay performance over a prolonged period, and special quality control schemes were devised for use in the Institute's hormone assay laboratories.

Concentrations of testosterone, estradiol-17β, FSH, LH, prolactin, and GH in the plasma of patients classified according to their primary tumor staging (modified UICC classification) are given in Table 7. Mean values, together with standard deviations, are shown and the wide variation between individuals in each group is evident. No significant differences were found between these groups using the standard Student's 't' test. Table 8 shows the mean values for plasma hormones of patients with (M1) and without (M0) evidence of metastases. Again, no significant differences were found. Use of canonical variate analysis led to discrimination between various groups. Figure 4 illustrates the separation of the variate-group means when patients with T3- and T4-stage tumors were analyzed against those with T0, T1, and T2 tumors, the circles representing the 95% confidence regions for these means. The principal components resulting in the separation of the groups were estradiol-17β and GH, which contributed 21% and 27% of the variance, respectively. GH levels were lower in patients with more advanced tumors, which is interesting since this hormone is released in response to stress, a response that might be expected in this group of patients. When plasma hormone concentrations of patients classified into two groups, based on the presence and absence of clinically evident metastases, were subjected to multivariate analysis, separation of the variate means was obtained (Figure 5) with age (62%) and
TABLE 7

Plasma Hormone Concentrations of Patients with Prostatic Carcinoma Separated According to UICC Classification of Their Primary Tumors

<table>
<thead>
<tr>
<th>Classification group (No. of patients)</th>
<th>Age (years)</th>
<th>T ng/ml</th>
<th>E₂ pg/ml</th>
<th>LH mIU/ml</th>
<th>FSH mIU/ml</th>
<th>GH mIU/ml</th>
<th>Prolactin µ/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀ category (n = 24)</td>
<td>mean 71</td>
<td>4.5</td>
<td>29.5</td>
<td>6.4</td>
<td>15.5</td>
<td>3.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD 9.04</td>
<td>1.48</td>
<td>17.65</td>
<td>5.33</td>
<td>13.05</td>
<td>3.72</td>
<td>0.13</td>
</tr>
<tr>
<td>T₁ category (n = 14)</td>
<td>mean 68</td>
<td>4.6</td>
<td>27.0</td>
<td>6.7</td>
<td>16.9</td>
<td>1.6</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD 7.07</td>
<td>2.03</td>
<td>9.33</td>
<td>5.28</td>
<td>16.07</td>
<td>2.44</td>
<td>0.09</td>
</tr>
<tr>
<td>T₂ category (n = 31)</td>
<td>mean 71</td>
<td>4.4</td>
<td>29.4</td>
<td>5.4</td>
<td>15.1</td>
<td>3.8</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD 9.23</td>
<td>1.60</td>
<td>11.06</td>
<td>4.13</td>
<td>14.03</td>
<td>7.06</td>
<td>0.1</td>
</tr>
<tr>
<td>T₃ category (n = 73)</td>
<td>mean 70</td>
<td>4.1</td>
<td>26.2</td>
<td>8.2</td>
<td>16.8</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD 7.86</td>
<td>1.53</td>
<td>12.11</td>
<td>8.09</td>
<td>17.27</td>
<td>3.49</td>
<td>0.11</td>
</tr>
<tr>
<td>T₄ category (n = 21)</td>
<td>mean 68</td>
<td>3.7</td>
<td>24.5</td>
<td>6.5</td>
<td>14.6</td>
<td>3.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD 7.43</td>
<td>1.31</td>
<td>7.53</td>
<td>7.15</td>
<td>22.46</td>
<td>4.85</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Note: T refers to testosterone, E₂ to estradiol-17β, S.D. to standard deviation and n to the number of patients.

GH (31%) is the principal discriminating component. GH means were higher in the group with metastases, and the biochemistry of somatomedin in relation to metastatic spread of tumor to bone may well be of interest. FSH levels were found to be significantly different in patients with benign and malignant disease when using this form of analysis (Figure 6; Table 7). Whether concentrations of these various hormones are in any way related to the histological grading of the tumor in individual patients is currently being studied by the Prostate Study Group. This may be relevant when assessing the possible feedback control by tumor metabolites. Androgen metabolism of the more differentiated prostatic tumors probably differs from those which are anaplastic, and other studies from the Institute in the field of carcinoma of the breast indicated that the more highly differentiated primary breast tumor rarely lacks estradiol-17β receptors.

It is obvious, however, that further studies along these lines, on the interrelationship of hormone levels and various clinical parameters, can do much to elucidate the role of the endocrine factors in the pathogenesis of prostatic cancer.

The relationship of prolactin to prostatic disease requires detailed investigation. The effect on plasma prolactin levels of the increasing estradiol to testosterone ratio in the elderly male has not been fully documented and is under review by the British Prostate Study Group, but the well-established stimulatory effect of estrogen on prolactin release may be concerned in disturbing prostatic biochemistry. Preliminary studies have produced evidence that prolactin in vitro, may increase the effectiveness of testosterone action on the prostate by increasing the uptake of the steroid by the gland, but the precise mechanism by which prolactin affects the prostate remains uncertain. Results from this Institute clearly indicate that administration of CB154 (2-bromo-α-ergocryptine, Sandoz Ltd.), an inhibitor of prolactin secretion to male rats for periods up to 30 days, failed to affect the weights of the accessory sex glands despite the decrease in plasma prolactin concentration to an undetectable level.

B. Endogenous Steroid Levels in Prostatic Tissue

It has been suggested that changes in steroid metabolic activity of the prostate may
<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>T (ng/ml)</th>
<th>E (pg/ml)</th>
<th>LH (mIU/ml)</th>
<th>FSH (mIU/ml)</th>
<th>GH (μU/ml)</th>
<th>Prolactin (μU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients without metastases MO</td>
<td>mean 70</td>
<td>4.3</td>
<td>25.9</td>
<td>7.8</td>
<td>18.5</td>
<td>2.4</td>
<td>0.10</td>
</tr>
<tr>
<td>(n = 78)</td>
<td>SD 8.15</td>
<td>1.38</td>
<td>9.46</td>
<td>7.80</td>
<td>19.70</td>
<td>4.71</td>
<td>0.11</td>
</tr>
<tr>
<td>Patients with metastases M1</td>
<td>mean 70</td>
<td>4.1</td>
<td>27.1</td>
<td>6.0</td>
<td>13.0</td>
<td>3.4</td>
<td>0.10</td>
</tr>
<tr>
<td>(n = 74)</td>
<td>SD 8.28</td>
<td>1.74</td>
<td>13.34</td>
<td>5.05</td>
<td>13.00</td>
<td>4.28</td>
<td>0.12</td>
</tr>
<tr>
<td>Benign prostatic hyperplasia</td>
<td>mean 68</td>
<td>4.3</td>
<td>30.9</td>
<td>7.3</td>
<td>7.7</td>
<td>2.6</td>
<td>0.14</td>
</tr>
<tr>
<td>(n = 72)</td>
<td>SD 8.3</td>
<td>1.9</td>
<td>12.0</td>
<td>5.6</td>
<td>7.7</td>
<td>2.2</td>
<td>0.08</td>
</tr>
<tr>
<td>Significant differences</td>
<td>MO V Benign</td>
<td>NS</td>
<td>0.01</td>
<td>NS</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>M1 V Benign</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Note: T refers to testosterone; E, to estradiol-17β; n to numbers of patients; SD to standard deviation and NS to not significant.
Growth Hormone (27%)

FIGURE 4. Separation of variate group means by canonical variate analysis. The circles represent the confidence regions for each of the tumor categories; the centres of the circle represent the variate group means. GH contributes 27% of the variance in the separation between group T0, T1, T2 and T3, T4.

Age (62%)
Growth Hormone (31%)

FIGURE 5. Separation of variate groups means by canonical variate analysis. As with Figure 4, group separation was achieved and GH and age contributed to the discrimination of the M1 and M0 groups.
be responsible for the pathogenesis of the diseased state. Whether prolactin affects metabolism or uptake of testosterone by benign or malignant tumor cells remains to be elucidated. Interesting in this respect, however, was the observation of Siiteri and Wilson\textsuperscript{167} that the endogenous concentration of 5a-dihydrotestosterone in benign prostatic adenomas was five times greater (Table 9) than in normal post-mortem prostatic tissue and, furthermore, that there was a higher level of the "active androgen" in the periurethral region of the prostate compared with the other zones.

The known effect of 5a-dihydrotestosterone on prostatic growth,\textsuperscript{99} its mitogenic effect on explants of rat ventral prostate in culture,\textsuperscript{72,73} the marked rise in its concentration in prostatic tissue from men over 60 years of age, and the correlation between canine prostatic size and 5a-dihydrotestosterone content\textsuperscript{166} prompted Siiteri and Wilson\textsuperscript{167} to suggest that increased production and accumulation of this steroid in the periurethral area of the prostate results in abnormal tissue growth.

From our own work on the development of high-resolution, selected ion-monitoring procedures for the analysis of steroid levels in biological tissues,\textsuperscript{166} endogenous concentrations of testosterone, 5a-dihydrotestosterone, 5a-androstane-3α,17β-diol, and 5a-androstane-3β,17β-diol were also determined\textsuperscript{170} in a limited number of benign prostatic adenomas (Table 9). The levels of testosterone and 5a-dihydrotestosterone were similar to those reported by Siiteri and Wilson, and the 5a-androstanediol concentrations to results recently obtained, by Geller et al.\textsuperscript{171} for "total androstanediol" levels. The data of Geller et al. suggest that the increased level of 5a-dihydrotestosterone results from a decreased 3-hydroxysteroid oxidoreductase activity which is responsible for converting 5a-dihydrotestosterone to the diols. Certainly it appears that there is no difference in the 5α-reductase activity of normal and abnormal tissue.\textsuperscript{167} The levels of endogenous steroids in prostatic carcinoma will be awaited with particular interest.

It has been mentioned that the canine prostate is also prone to hyperplastic change with age. Early reports from Ofner\textsuperscript{79} and ourselves\textsuperscript{78} recorded the formation, in the
TABLE 9
Endogenous Steroid Concentrations in Prostatic Tissue

<table>
<thead>
<tr>
<th>Endogenous steroid concentrations (ng/g tissue)</th>
<th>Investigation (method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>Testosterone</td>
</tr>
<tr>
<td>Normal prostate</td>
<td>2.1 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>(0.78—2.9)</td>
</tr>
<tr>
<td></td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>12.7 ± 7.1</td>
</tr>
<tr>
<td>Hypertrophic prostate</td>
<td>5.6 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>(3.1—9.2)</td>
</tr>
<tr>
<td></td>
<td>6.0 ± 1.0</td>
</tr>
</tbody>
</table>

Note: RIA refers to radioimmunoassay and GC-MS to gas chromatography - mass spectrometry.

Canine prostate, of 5α-dihydrotestosterone and the various androstanediols, and it was recognized that the formation of the latter group of steroids may regulate the intracellular concentration of 5α-dihydrotestosterone. An interesting experiment of Jacobi and Wilson172 involved the incubation of 5α-dihydrotestosterone with microsomal preparations from homogenates of 31 dog prostates, from 1 to 15 g weight. 5α-Androstane-3α,17β-diol formation correlated with the size of the gland, which is also known to relate to age, and it was suggested that androstanediol biosynthesis may be a limiting factor in controlling prostatic growth in the dog with excessive synthesis inducing prostatic hyperplasia. The 3α,17β-diol does increase prostatic size in the dog118 although it was originally considered to act only after conversion to 5α-dihydrotestosterone.173 As previously stated, the androstanediols could have a unique role in mediating androgen action133,174 and may be worthy of further study.

IV. ESTROGENS, PROLACTIN, AND THE TESTICULAR-PROSTATIC AXIS

Obviously, androgen uptake by the prostate is influenced by the nature of the steroid in the blood and the capacity of the cell to retain and metabolize it. The potential effects, both direct and indirect, of the higher estrogen to androgen ratio in the older man on androgen action and prostatic metabolism, require more careful consideration.

The well-established elevation of plasma prolactin after estrogen administration has been discussed. The effects in the elderly man of increasing plasma SHBG levels, probably-induced by estrogen, have been considered. SHBG levels control the plasma concentration of free C19-steroid and estradiol-17β and, therefore, steroid availability to the hormone-dependent tissues. Voigt and his co-workers19 recently confirmed the age dependency of SHBG binding capacity and produced data suggesting that a lower
SHBG binding capacity occurred in benign and malignant disease of the prostate. However, the increased level of SHBG determined in these patients after subsequent estrogen therapy clearly did not indicate any disturbance in SHBG regulation, and subsequent studies failed to confirm this interesting observation.

The increased concentration of plasma free estradiol-17β in the elderly man could exert a more direct action on the prostate and the observed effect of estradiol-17β, in increasing the uptake of testosterone by the lateral lobe of the rat prostate is noteworthy. Superfusion studies of Giorgi et al. also indicate that estrogens increased the entry of testosterone into the prostate. On the other hand, in other experimental systems there was a decreased formation of 5α-dihydrotestosterone in the presence of estrogen. Shimazaki and colleagues had also shown previously that estrogen reduced 5α-reductase activity, but had no effect on the reduction in vitro of 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol.

Circulating estradiol-17β as well as prolactin could, therefore, influence the endogenous C19-steroid content of the aging and, also, dysfunctional prostate, although the experimental data available as yet suggest that there is no concomitant, increased formation of 5α-dihydrotestosterone. Indeed, treatment of dogs with estradiol-17β for 30 days produced a marked change in the C19-steroid metabolic pattern of the prostate, with an apparent stimulus to the "oxidative pathway" (Figure 3), resulting in the formation of the less androgenic androstenedione and 5α-androstane-3α,17β-dione at the expense of testosterone and 5α-dihydrotestosterone. Our own experiments also produced evidence that estrogen administration increased the rate at which testosterone was metabolized by canine prostatic tissue, an effect subsequently confirmed in rat prostatic tissue. These latter studies (Figure 7) demonstrated that daily administration of estradiol-17β or diethylstilbestrol in vivo for 10 days markedly af-

![Figure 7](image-url)
fected testosterone metabolism in vitro by prostatic tissue, with a decreased formation of 5α-dihydrotestosterone and a corresponding increase in the synthesis of the 5α-androstanediols and 5α-androstane-dione.

A. Estrogen and the Pituitary-Testicular Axis

Although there is evidence that estrogens directly influence the prostate gland, it has generally been considered that the principal antiandrogenic effect of estrogen therapy in the treatment of prostatic carcinoma is exercised, indirectly, on prostatic tissue, via the pituitary, reducing testosterone secretion by suppression of LH release. Such an effect has been observed in patients with prostatic cancer and Figure 8 illustrates the marked changes in gonadotropin levels in treated patients from our own studies. There is little evidence that the changing estrogen to androgen balance can influence circulating FSH or LH, or that changes in gonadotropin levels relate to prostatic dysfunction, except as mentioned earlier; accumulated analytical data indicated a differ-

ence in the levels of FSH between patients with benign and malignant tumors (Figure 6). Geller et al.183 have reported a decreased LH reserve in elderly men with benign disease, although no age-matched controls were used in this study. Such studies to assess the relationship of circulating hormone levels to prostatic disease have generally involved only single plasma determinations and must, therefore, be considered with caution. Frequent sampling during a 24-hr period would be more effective, as would an investigation in depth into the chronobiology of the endocrine aspects of prostatic disease. This is illustrated by the observed change in LH secretion with age. Detailed studies of plasma LH levels during the complete 24-hr sleep-wake cycle of males up to the age of 45 clearly demonstrated an augmented LH secretion during the sleep period in late prepubertal and early pubertal males184,185 which contrasted with the low LH secretion before puberty. The elevated LH secretory activity during sleep marks the onset of clinical puberty,186 a process in which secretion of androgens by the adrenal cortex may be concerned.187,188 Advancing sexual maturity is characterized by episodic secretion of LH during the wake periods until the mean LH levels during these times are the same as those found in sleep.189

Similar detailed studies of LH secretion with advancing age are not yet available, although there are investigations which indicate that plasma LH levels from single analyses increase each decade from the age of 40,190 but data on prostatic disease are limited. Urinary-protein-hormone analysis with sampling through a 24-hr period to assess secretion in relation to prostatic cancer may be worth considering.

An increase in plasma LH with advancing age190 may well reflect a decreasing capacity of the testis to synthesize testosterone,49 although this cannot be considered hypogonadism, since in both young and elderly men, HCG administration has been shown to increase both plasma testosterone57,191-194 and estrogens.36,194 The absolute response was a little less in the elderly, but the ability to respond was preserved. Others195,196 were unable to show a change in basal levels of FSH and LH with advancing age, although the capacity of the pituitary to respond to administered LH-RH tended to decrease with age. Similar decreased FSH and LH "responses" to LH-RH stimulation have been described198,197 in relation to male senescence, although in every study marked pituitary stimulation was observed. The relative "unresponsiveness" may well result from the effect of the increasing plasma estradiol-17β in older men on LH-RH action. Our own studies,198-200 which illustrate the effect of estradiol-17β on pituitary responsiveness to LH-RH in dog and man, are shown in Figure 9.

In considering the changing testicular-prostatic axis with age, the suggested direct inhibitory action of estrogen on the testis199,201 should also be considered. Estrogen administration in vivo was subsequently shown to have affected the testicular synthesis of testosterone in vitro198,199,201 in particular by decreasing the activity of both the 17β-hydroxysteroid dehydrogenase and 17α-pregnen-C-17,20-stereoid lyase.

The concept of a direct inhibitory action of estrogens on testicular synthesis of testosterone has long been controversial,205 but experimental data tend to support both an immediate direct action together with a delayed, less sensitive effect at the pituitary level. It is clear, however, that advancing age is accompanied by changes in testicular function reflected by decreased spermatogenesis, although whether this results from reduced steroid synthesis remains to be elucidated. Possibly the number of Leydig cells decreases with age,206 or they are relatively less sensitive to gonadotropins, but the interrelationship of these testicular changes to prostatic disease is not clear. Little is also known about the effect of aging on the Sertoli cells and their function.

B. Prolactin and the Testicular-Prostatic Axis

Prolactin secretion by the pituitary is controlled by prolactin inhibiting factor re-
FIGURE 9. Gonadotropin responses to luteinizing hormone-releasing hormone (LH-RH): (a) in man, with no pretreatment and after injection (i.m.) of either estradiol-17β (E₂) or testosterone (T) 2 hr. previously. FHS (O) and LH (•) concentrations in plasma were normalized by expressing as a fraction of the average within an experiment for each man. Mean values (± S.E.M., n = 5 men) were calculated and are presented as percentages of the initial, basal sample. (Data from Cole, E. N., Llewelyn, H., Link, J., and Boyns, A. R., J. Endocrinol., 63, 251, 1974. With permission.) (b) in male beagle dogs (6) given 50 pg E₂ in 0.15 M NaCl or vehicle alone. Experiments were over 4 weeks and were randomized. Blood was collected before and after administration of 5 µg LH-RH 15, 60, or 165 min after E₂ injection. Vertical lines indicate ± S.E.M. (Data from Jones, G. E. and Boyns, A. R.; J. Endocrinol., 61, 123, 1974, and 68, 475, 1976. With permission.)

leased by the hypothalamus and, currently, considered by some to be dopamine.²⁰⁷ There is at present little information on the effect of aging on prolactin secretion, although there was a comparable prolactin response when TRH was administered to groups of males of different ages,²⁰⁸ an observation subsequently confirmed by Yamaji et al.,²⁰⁹ who also stated that aging per se did not appear to be associated with altera-
tions in the basal prolactin secretion or the pituitary reserve of the hormone. Data are needed, however, on the episodic secretion of prolactin in the older man and the changes in the circadian rhythms of prolactin output with advancing years. In view of this episodic secretion of prolactin, only regular and frequent sampling over a more extended time period will provide the necessary reliable assessment of the physiological role of prolactin in the elderly man.

Evidence is now accumulating that prolactin plays a physiological role, probably synergistically with LH, in regulating testicular activity. Specific binding of $^{125}$I-ovine prolactin to rat testicular membrane preparations has been reported, and the work of Hafiez and colleagues suggests that prolactin and LH are required to maintain normal plasma levels of testosterone in hypophysectomized rats. Although in some species, there is a parallel fluctuation in the plasma levels of testosterone and LH, indicating that testosterone production is predominantly under LH control: such a relationship was not found in man. Studies from our own laboratories on the interrelationship between gonadotropins, testosterone, and prolactin in six normal young men over a 24-hr period showed a correlation between prolactin and testosterone in three subjects. Rubin et al. also reported a correlation between peripheral levels of prolactin and testosterone and have previously observed from nocturnal episodic-hormone-secretion studies that the testosterone peak was preceded by an elevation in prolactin.

Administration to normal men of sulpiride, a dopaminergic antagonist promoting prolactin synthesis, followed by HCG-stimulation of testicular activity, also resulted in an enhanced testosterone output in the presence of hyperprolactinemia. In contrast, however, hyperprolactinemia in the female has an antigonadotropic effect and furthermore, transplantation of prolactin-producing tumors to male rats led to testicular atrophy. Our own investigation of infertility in the human male indicated that hypoprolactemia was also related to infertility, the general interpretation probably being that physiological prolactin levels are required for normal testicular function.

A role for prolactin in the endocrine control of the testis seems clear, therefore, although the mechanism by which this regulation is effected still requires clarification. Possible underlying abnormalities in prolactin secretion that could lead to prostatic dysfunction in the elderly man demand further investigation and the physiological control by the testicular-prostatic axis of prolactin synthesis and release from the pituitary, in males of all ages, warrants more intensive study. The heterogeneity of prolactin in serum may also vary with age and disease, and it is probable that not all immunoreactive prolactin is biologically active. Estradiol-17$\beta$ certainly promotes prolactin release although testosterone has little, if any, effect on pituitary prolactin content. Early bioassay studies of Asano suggested that pituitary prolactin content increased after prostatectomy. The possible feedback control of the 5$\alpha$-androstenediols on prolactin output has been mentioned earlier in this chapter.

There is a considerable body of evidence which would indicate that prolactin directly governs certain aspects of prostatic biochemistry, and some of the reports relating to this have been previously discussed. It would seem that prolactin can accentuate the effects of androgens in stimulating prostatic growth and function, and it is interesting that the presence of specific binding sites for the hormone on prostatic membranes has recently been demonstrated. The steroid-prolactin interrelationship that influences the putative effect of prolactin on prostatic tissue requires consideration. Friesen and colleagues have described prolactin receptors with high specificity and affinity in female rat liver. Estrogen treatment induced the receptor in male rat liver, but not after hypophysectomy. Removal of the pituitary in the female rat resulted in the loss
of the prolactin receptor, and it was suggested, therefore, that plasma prolactin concentration probably regulates the prolactin receptor level.\textsuperscript{224, 255} Similar mechanisms are reported to control prolactin binding sites of the rat prostate,\textsuperscript{226} except that estrogens inhibit the specific binding of \textsuperscript{125}I-ovine prolactin to the membrane preparation. Furthermore, active protein synthesis was found necessary for the maintenance of the receptors,\textsuperscript{226} and, obviously, with such a rapid turnover, regulation of their concentration would play a major role in controlling prolactin action and might well be influenced by aging.

At present, prolactin cannot be said to have a trophic effect on the prostate and it is difficult to ascribe a functional role for the prolactin receptor. Although Asano et al.\textsuperscript{227} showed that administration of antiserum to prolactin decreased the weight of the prostate gland of the rabbit, our own studies\textsuperscript{165} indicated that CB154 administration to male rats for 30 days failed to affect prostatic weight. Moreover, equivocal data were obtained on the effect of prolactin on adenyl cyclase, the activity of which was stimulated only in certain of the homogenates prepared from the different lobes. At the same time, in these studies prolactin did not appear to markedly influence androgen metabolism by the rat prostate, although other experiments (Table 10) and work from other groups provided evidence to the contrary.\textsuperscript{228, 229} It may be, however, that since experimental evidence indicates that prolactin tends to act synergistically with other hormones,\textsuperscript{112, 148, 211, 230} it is possible that the inconsistent effects caused by prolactin\textsuperscript{165} could mean that the ideal system in vitro had not been realized.

Our ultrastructural studies with related electron microscope microanalysis, using EMMA-4 (Associated Electrical Industries, Manchester), did, however, indicate changes in the lateral lobe after CB154 treatment,\textsuperscript{165} with a marked decrease in the zinc concentrations in most regions of the cell. Aragona and Friesen have subsequently reported\textsuperscript{226} that prolactin receptors are localized in the dorsolateral lobes of the rat prostate.

C. A Direct Control of the Prostate

Currently available evidence indicated that the determination of circulating, plasma hormone concentrations has, as yet, failed to clearly define any real abnormality in the endocrine status of an individual that can be readily seen to be implicated in the etiology of prostatic disease. In our own research program, consideration was given to alternative routes which might allow for a testicular influence on the prostate and, thereby, explain the age-related changes that occur. Attention was directed to the anatomical link between the testis and prostate by the excurrent duct system, the seminiferous tubules, rete testis, vasa efferentia, epididymal ducts, and the vasa deferentia, which could provide a connection by which androgens reach the target organs without entering the general circulation. Skinner and Rowson\textsuperscript{231, 232} have shown previously that severing the vasa deferentia caused a reduction in weight and in the fructose and citric acid content of the ampullae, which could be restored by the infusion of testosterone along the vasa.

Preliminary studies were established\textsuperscript{233} to test the hypothesis that a direct hormonal influence could similarly be imposed on the prostate. The results indicated that vasoligation reduced the activity of the DNA dependent-RNA polymerase in the ventral lobes of the rat prostate. Furthermore, \textsuperscript{[3H]}testosterone, infused along the vasa, was preferentially taken up by the prostate and seminal vesicles, implying, therefore, the feasibility of normal passage of androgen from the testis via the vas deferentia.

Unilateral castration of the fetus\textsuperscript{234, 235} restricts the development of the ipsilateral genital structures, suggesting that testicular secretion is not distributed to the general circulation, but produces a local effect via the genital tract.
<table>
<thead>
<tr>
<th>Precursor steroid</th>
<th>Hormone added (5 I.U.)</th>
<th>Tissue protein in cultured tissue (mg)</th>
<th>Specific Radioactivity (dpm/nmol/mg protein)</th>
<th>Total 5α-reduced steroids formed</th>
<th>Total 5α-reduced steroid/precursor steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>A</td>
<td>5α-DHT</td>
<td>5αA</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>Control</td>
<td>1.45</td>
<td>-</td>
<td>84.9</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>Ovine prolactin</td>
<td>1.86</td>
<td>-</td>
<td>99.5</td>
<td>41.2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Control</td>
<td>1.73</td>
<td>203.1</td>
<td>12.4</td>
<td>163.6</td>
</tr>
<tr>
<td></td>
<td>Ovine prolactin</td>
<td>1.67</td>
<td>265.4</td>
<td>6.5</td>
<td>111.1</td>
</tr>
</tbody>
</table>

Note: T = testosterone; A = androstenedione; 5α-DHT = 5α-dihydrotestosterone; 5αA = 5α-androstane-3,17-dione and An = androsterone

* Mean specific radioactivity of steroids isolated from human prostatic explants (benign prostatic hyperplasia) cultured with either [7α-'H]testosterone or [1,2-'H]androstenedione in the presence of ovine prolactin. Steroids were characterized after routine isotope dilution procedures.

Further studies of Pierrepont et al.\textsuperscript{246} provided added support for the hypothesis that the epididymis exercises a direct and unilateral androgenic control over the prostate. It was shown that unilateral castration and vasectomy reduced the activity of the DNA-dependent RNA polymerase of the ipsilateral lobes of the prostate. This was not observed after unilateral orchidectomy when the epididymis was not removed. Also the epididymides, in the absence of the testis, but maintained by exogenous testosterone, sustained the prostate gland at a level not achieved in their absence. Furthermore, the epididymis in the absence of ipsilateral gonad, but maintained by the contralateral testis, provided a greater sustaining influence on the prostate than did the testis and epididymis separated from the target organs by vasoligation.

The evidence, therefore, suggests that the epididymides modulate prostatic activity through an intact vas deferens. Subsequently,\textsuperscript{237} it was shown that the concentrations of testosterone in the deferential and testicular veins of the dog were comparable and higher than in peripheral blood, further suggesting that the deferential vein serves as a direct transporting system for androgens from the epididymis to the prostatic complex. The venous drainage of the cauda epididymidis is solely through the deferential vein, which is generally severed at vasectomy. Ligation of this vein reduces the DNA and RNA content of the ventral lobe of the prostate and also the RNA polymerase activity. Fluorescent and radio-opaque material can be transferred from the deferential vein directly to the prostate,\textsuperscript{238,239} and a study of the venous drainage of the canine prostate\textsuperscript{240} supported the concept that a small retrograde flow of blood would take androgen-rich blood from the deferential vein into the prostate gland.

Therefore, man and dog, virtually alone in the animal kingdom in their susceptibility to prostatic hypertrophy, are species in which the vasa deferentia pass through the prostate to reach the urethra (Figure 10). This direct-control mechanism of the prostate should receive further investigation with regard to its possible role in inducing prostatic dysfunction, perhaps by a retrospective study of the incidence and type of prostatic dysfunction in men who have had vasectomies earlier in life.

V. PROLACTIN, THE ADRENAL GLAND, AND THE PROSTATE

There are a number of reports indicating a relationship between the adrenal gland and the prostate,\textsuperscript{152,241-244} and it would seem that adrenocortical steroids, progesterone, and the adrenal androgens may all influence prostatic growth and function. Tisell\textsuperscript{242} noted that cortisone induced growth and secretory activity of prostatic epithelium, an effect accentuated by estradiol-17β. Adrenal hyperplasia and urinary retention was observed\textsuperscript{241} in mice with estrogen-induced prostatic hyperplasia. Subsequent adrenalectomy induced urinary flow; the prostate then appeared histologically normal; and elevated adrenal activity, resulting from estrogen-promoted release of ACTH, was considered a potential factor in the pathogenesis of, at least, prostatic hyperplasia. Certainly, estrogen-promoted ACTH release results in adrenal hyperplasia,\textsuperscript{203,245} and estrogens increased the adrenal responsiveness to ACTH.\textsuperscript{246} Progesterone administration also stimulated the growth of the ventral prostate of castrated rats and prevented involution of the gland in castrated, hypophysectomized, adrenalectomized animals.\textsuperscript{247}

Although at present, there is little evidence that the adrenal gland is related in any way to prostatic disease, experiments of Farnsworth and from our laboratories\textsuperscript{60,248} indicated that the adrenal C\textsubscript{19} steroids, DHA sulfate, DHA, and androstenedione were metabolized in vivo and in vitro by benign hypertrophic tissue (Table 4). DHA sulfate, a major secretory product of the human adrenal cortex and present in large quantities in plasma, offers a potentially large source of precursor for androgenic hormone formation by the prostate.
The influence of these adrenal C₁₉-steroids on prostatic tissue may be more significant, however, in patients with prostatic cancer treated by castration or estrogen therapy. Normally, administration of ACTH suppresses plasma testosterone levels in men, and investigations of our own group on untreated patients with prostatic cancer showed that Synacthen® administration produced a similar suppression. Androstenedione levels in plasma increased after Synacthen® treatment. A similar Synacthen® test, undertaken after at least 6 months of estrogen therapy when plasma testosterone concentrations had been markedly decreased to below 70 ng/100 ml, resulted in an elevation in the concentrations of both androstenedione and testosterone, the latter showing a two- to ninefold increase. Plasma androstenedione levels in untreated patients did not differ significantly from those in elderly, control, hospitalized men (10 to 125 ng/100 ml), neither were they elevated after prolonged estrogen ther-
therapy. The plasma testosterone response to Synacthen® during the estrogen treatment may have been a result of increased DHA sulfate or DHA secretion, followed by peripheral conversion to testosterone. There is evidence, however, of a small direct secretion of testosterone by the adrenal gland, and the adrenal synthesis of testosterone and its stimulation by Synacthen® has been demonstrated by human adrenal explants in organ culture. These and other studies from the Institute provided some of the first evidence that prolactin may influence the synthesis of C₁₆-steroids from the adrenal gland, and recent investigations of Vermeulen et al. revealed that three males with prolactinomas and patients on psychotropic drugs with elevated plasma prolactin levels had significantly raised plasma concentrations of DHA and its sulfate. Furthermore, the findings of an increased urinary DHA excretion in patients with hyperprolactinemic amenorrhea and hyperprolactinemia associated with bilateral adrenal hyperplasia suggest a trophic effect of prolactin on the human adrenal gland. Specific binding sites for prolactin have also been reported in adrenal tissue, and, in male rats and mice, ectopic pituitary isografts produced a significant elevation of prolactin levels with concomitant adrenal stimulation. The relationship between DHA levels in the plasma with increasing age has been investigated by De Peretti and Forest, and the changes in DHA concentration at early puberty associated with a possible “adrenarche” were discussed. Prolactin also must be considered a factor influencing sexual maturation during prepuberty and puberty.

It would seem, therefore, that the adrenal cortex of the patient receiving endocrine therapy for carcinoma of the prostate is responsive to ACTH, supporting earlier observations that a secondary rise in plasma androgen concentration during therapy in some of these patients may be of adrenal origin. Dexamethasone treatment decreased plasma testosterone from 50 to 100 ng/100 ml to below the limits of detection. Whether this secondary rise in plasma testosterone relates to a resumption of tumor growth, dissemination of the disease, and subsequent relapse is at present under study, but initial observations would indicate that it may not be the endocrine parameters but factors such as polyamines, carcinoembryonic antigen, α-fetoprotein, or other tumor markers that will provide the means of assessing recurrence and metastatic spread.

VI. ANDROGEN ACTION, HORMONE RECEPTORS, AND THE PROSTATE

A considerable weight of evidence supports the assumption that benign and malignant prostatic growth is androgen mediated. Plasma hormones may well have a permissive role in influencing this abnormal growth of prostatic tissue, but it would seem reasonable that attention should be directed to abnormalities within the target tissue itself. Indeed, there has been an underlying, fundamental assumption in many laboratories over the past decade that information on the endocrine disturbance inherent in prostatic disease will accrue from precise biochemical investigations into the cellular processes by which androgens control the growth and function of the gland. Such investigations, particularly with the ventral lobes of the rat prostate, clearly indicate that in the prostate, as in other steroid-responsive cells of other target tissues, steroid hormones function through a well-defined, integrated series of intracellular events, the most important of which is probably the association of the steroid with a cytoplasmic “receptor” protein, through which the hormone elicits the initial transcriptional processes at specific nuclear “receptor” sites. The selective binding of a particular steroid, followed by translocation to the nucleus and the specific binding of the steroid-receptor complex to these nuclear acceptor sites, thereby regulates gene expression, as
PLASMA PROTEIN

FIGURE 11. Schematic representation of the effects of androgens within the prostatic cell, depicting the essential characteristics of the mechanism of steroid action. T, testosterone; DHT, 5α-dihydrotestosterone; Adiols, the various androstaneadiols; PP, plasma proteins responsible for the transport of testosterone (SHBG in man); R, specific intracellular receptor; DHT-R, steroid-receptor complex; DHT-R*, the active complex; mRNA and rRNA, messenger and ribosomal ribonucleic acid respectively. Question marks refer to processes not completely elucidated; thick arrows relate to processes of growth and function, dotted arrows to theoretical relationships, and thin arrows to the development of processes within the cell.

illustrated in Figure 11. It is interesting that, with other target tissues, circulating hormones such as estradiol-17β or aldosterone bind to receptor and are then translocated to the nucleus, whereas in the prostate, testosterone is converted to the “intracellular hormone”, 5α-dihydrotestosterone, before translocation to the nucleus. Detailed information on the mechanism of action of androgens has recently been discussed, but essentially the steroid-receptor complex regulates transcription, gene activation, and the integrated processes related to growth.

The precise mechanism by which testosterone enters the prostatic cell is, as yet, poorly defined, but some degree of selectivity is probably involved, and plasma SHBG will influence steroid entry. Current evidence suggests that the nonprotein-bound testosterone crosses the plasma membrane and is metabolized to a variety of 5α-reduced steroids (Figure 3), although high levels of SHBG have been found associated with the stroma of the hypertrophied prostate. As stated earlier, 5α-androstanediols and unmetabolized testosterone may have roles in physiological processes independent of receptor or transcription, but 5α-dihydrotestosterone is retained by the receptor proteins of selective high affinity; the steroid-receptor complex then attains a conformation with increased affinity for nuclear components and is then translocated to the chromatin where it is retained to initiate and promote the cellular mechanisms leading to tissue growth. After some time, the 5α-dihydrotestosterone leaves the nucleus by some, as yet, ill-defined mechanism and may well be secreted directly from the prostate or further metabolized. In effect, therefore, the process is concerned in maintaining an effective intracellular concentration of active 5α-dihydrotestosterone-receptor complex to control normal prostatic function.

It is reasonable, therefore, that since prostatic dysfunction may originate from an age-dependent imbalance of C17,20-steroid metabolism, resulting in localized androgenic stimulation, the intracellular formation of 5α-dihydrotestosterone has received consid-
erable attention. The reported age-dependent accumulation of this steroid in the prostate gland of elderly men without concomitant change in testosterone concentration, particularly in the periurethral area,\textsuperscript{167} may result in localized stimulation. Enzymatic imbalance, facilitated steroid entry, or inhibited release of androgen may, therefore, result in prostatic dysfunction. Of interest in relation to the high concentration of 5α-dihydrotestosterone in hypertrophy are the reports of a lower conversion of testosterone to 5α-dihydrotestosterone in neoplastic tissue.\textsuperscript{268} 269 Since testosterone also binds to the androgen-receptor protein,\textsuperscript{270} the resultant testosterone-receptor complex may well elicit cellular responses which, in the absence of the normal 5α-dihydrotestosterone regulation, could induce prostatic dysfunction. A higher conversion of 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol has recently been implicated in prostatic hypertrophy\textsuperscript{271} with the diol exerting some effect on cell growth in a situation corresponding to the canine prostate, but in other studies\textsuperscript{271,272} this conversion has been reported to be minimal. Moreover, the evidence would indicate that 5α-androstane-3α,17β-diol usually exerts its action through conversion to 5α-dihydrotestosterone\textsuperscript{273} and normally does not bind significantly to the androgen receptor.\textsuperscript{274} Furthermore, although it can eventually induce benign growth of the prostate of castrated dogs,\textsuperscript{133} it may not be the active androgen or androstanediol of this tissue.\textsuperscript{96,275} Extranuclear, receptor-independent effects of the androstanediols on other sites in the cell cannot be excluded from a consideration of etiology. Therefore, although an imbalance in the C19-steroid metabolic pattern of the prostate cell may be functionally concerned in promoting benign or malignant growth, the presentation of an increased concentration of intracellular androgen might be dependent on an elevated level of effective receptor protein. Formation of the steroid-receptor complex is a prerequisite for steroid hormone action, and abnormal prostatic growth may result from an overloading of the receptor-acceptor-gene system with consequent lack of control of the regulatory unit (Figure 11).

A. Receptors in Human Prostatic Tissue

Considerable effort has been directed to the characterization and quantitation of steroid-binding components of the human prostate. This work has recently been the subject of a review by Davies,\textsuperscript{276} who considered the clinical possibilities and limitations of receptor analysis in relation to its inherent, technological difficulties, one of the principal being tissue contamination with SHBG, considered by some\textsuperscript{166} a potential reservoir of androgen within prostatic cells. Also discussed\textsuperscript{276} was the procedure used in the Tenovus Institute for the measurement in human tissue of total receptor site concentration in which receptor is first stabilized by protamine sulfate precipitation.\textsuperscript{277,278}

Figure 12 illustrates results that have been obtained from our own studies with sucrose density gradient centrifugation, Figures 12a, b, and c showing that labeling of cytosol preparations from normal, carcinomatous, and hypertrophic prostate, respectively, with [3H]5α-dihydrotestosterone yields two peaks of radioactivity with sedimentation coefficients in the ranges 3 to 4S and 7 to 8S. Evidence for the specificity and low capacity of the 7 to 8S peak, the cytoplasmic receptor, has been previously described.\textsuperscript{279,280} 5α-Dihydrotestosterone localization within the nucleus\textsuperscript{281} due to translocation of the cytoplasmic steroid-receptor complex has been reported,\textsuperscript{279,280} together with the marked stimulation by the complex of chromatin transcription by RNA polymerase.\textsuperscript{279,282} 283 Figure 12d shows a sedimentation profile of [3H]5α-dihydrotestosterone-receptor complex derived from salt extraction of a hypertrophied prostate nuclear preparation previously incubated with prelabeled cytosol. The characteristic single peak of protein-bound radioactivity corresponds to the nuclear 5α-dihydrotestosterone-receptor complex with sedimentation coefficient of approximately 4S.
FIGURE 12. Sedimentation analysis of [3H]5α-dihydrotestosterone binding components in human prostate subcellular fractions. Samples of normal, hypertrophied or neoplastic prostate were homogenized and subcellular fractions prepared as previously described. Aliquots of cytosols (100,000 x g supernatant) were incubated (1 hr at 0 to 4°C) with [3H]DHT (4 nmol/l) in the absence (○) and presence of (●) of unlabeled DHT (400 nmol/l). Portions (400 μl) of [3H]-labeled cytosols were layered on linear 5 ml sucrose gradients (5 to 20% w/v) and centrifuged at 100,000 x g for 16 hr at 0 to 4°C in a Beckman L2-65B or L5-65B ultracentrifuge using a SW50.1 (6 x 5 ml) swinging bucket rotor (r, 8.35 cm). Gradients were fractionated by upward displacement with 40 to 50% (w/v) sucrose and approximately 30 fractions were collected in scintillation vials. Attempts were made to label nuclear receptors by the incubation of [3H]-labeled cytosol with equal volumes of prostate nuclei for 15 min at 37°C. Nuclei were sedimented, washed several times with buffer to remove free [3H]steroid and extranuclear [3H]steroid-receptor complex, and then extracted with KCl (0.4 mol/l). Nuclear debris was sedimented from the viscous extract by centrifugation at 100,000 g for 10 to 30 min and samples of salt-extract examined on sucrose gradients containing an uniform concentration of KCl (0.5 mol/l) as described for cytosol. Profiles obtained from such studies on cytosol from human normal prostate (a), human hypertrophied prostate (b), and human neoplastic prostate (c), and on nuclei from human hypertrophied prostate (d). Also shown is an example of human hypertrophied prostate exhibiting no low-capacity binding components (e). Throughout, the direction of centrifugation was from left to right and the sedimentation marker (arrow) was bovine serum albumin (S20w, 4.6S).
FIGURE 13. Calculation of androgen-receptor concentration and affinity of androgen-receptor interaction. Values for specific androgen binding were subjected to Scatchard-type analysis so that the concentration of receptor ([R]) and the dissociation constants for androgen-receptor interaction (K_a) could be calculated. Representative results are shown in this figure. (a) Androgen receptor from human normal prostate cytosol: [R] = 52 fmol/mg protein, K_a = 1.6 nmol/l. (b) Androgen receptor from human hypertrophied prostate cytosol: [R] = 56.6 fmol/mg protein, K_a = 1.48 nmol/l. (d) Androgen receptor from human prostate cancer cytosol: [R] = 63 fmol/mg protein, K_a = 0.69 nmol/l.

B. Levels of Androgen Receptors in Human Prostatic Tissue

The determination in our laboratories of androgen receptor proteins in normal, hypertrophic, and carcinomatous prostate tissue by saturation analysis, using the protamine sulfate procedure, indicated in all cases the presence of one class of high-affinity binding sites (Figure 13). Specific [3H]5α-dihydrotestosterone-binding components were present in nuclear and cytoplasmic preparations of all samples of prostatic tissue.
analyzed, and values for receptor-site concentration for occupied plus unoccupied sites, obtained by incubation at 15°C for 16 hr, are given in Table 11.276 The unoccupied sites contributed between 2 and 30% of the total, but were usually less than 10%, in line with a previous report.284

The amount of data is limited, but assessment of the results available does suggest a higher cellular content of androgen receptors in hypertrophic and malignant prostatic tissue, with a less efficient translocation process in the neoplastic state implicated by the cytoplasmic receptor to nuclear receptor ratio. Furthermore, the apparent dissociation constants imply a higher affinity of ligand-receptor interaction in the malignant tissue. It will be important to determine whether there are different receptor characteristics in the various cells and areas of the prostate which might relate to the occurrence of hypertrophy or carcinoma, since molecular rearrangement may affect affinity for 5α-dihydrotestosterone, for another metabolite of testosterone or possibly for estradiol-17β. Further data will accumulate, and it will be most interesting to find whether these preliminary differences are maintained.

It has always been hoped that the analysis of androgen receptors in carcinoma of the prostate may be valuable in determining the potential value of endocrine therapy for the treatment of the disease in a manner similar to the situation found in studies of breast cancer. Estradiol-17β receptor analysis of breast tumor tissue has been found of value in predicting the response to hormone therapy.285 Since certain chemotherapeutic agents are considered to act through an interaction with the receptor,286,287 then the presence of the receptor protein would be indicative of the potential effect of such antiandrogens in inducing tumor response. Treatment of prostatic cancer is, however, the subject of another chapter of this volume. It is noteworthy at this stage, however, to emphasize the relatively unpleasant side effects of diethylstilbestrol generally used in the treatment of prostatic carcinoma. Cardiovascular problems, gynecomastia, and loss of libido are relatively common, and, yet, the steroidal compounds with supposed antiandrogenic properties are comparatively ineffective.287 It must be hoped that the extensive knowledge gained during the past decade into the mechanism by which androgenic hormones regulate prostatic function may eventually be of value in the development of a more effective therapy, possibly with nonsteroidal compounds such as flutamide.288,290

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TABLE 11

Androgen Receptors in Human Prostate Subcellular Fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tissue</th>
<th>Receptor concentration</th>
<th>Cytoplasmic Nuclear</th>
<th>Dissociation constant (nmol/1)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>fmol</td>
<td>Molecules/cell</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>Normal</td>
<td>Central</td>
<td>44—52</td>
<td>1218—1231</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peripheral</td>
<td>53—57</td>
<td>1041—1354</td>
</tr>
<tr>
<td></td>
<td>Hypertrophy</td>
<td>Cancer</td>
<td>38—92</td>
<td>2423—5461</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63</td>
<td>3363</td>
<td>2.20</td>
</tr>
<tr>
<td>Nuclei</td>
<td>Normal</td>
<td>Central</td>
<td>196—223</td>
<td>758—860</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peripheral</td>
<td>221—248</td>
<td>850—955</td>
</tr>
<tr>
<td></td>
<td>Hypertrophy</td>
<td>Cancer</td>
<td>412—868</td>
<td>1387—3922</td>
</tr>
<tr>
<td></td>
<td></td>
<td>546</td>
<td>1529</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Note: Concentrations of cytoplasmic receptor are expressed as fmol/mg protein and those of nuclear receptor as fmol/mg DNA. Values shown are the range of concentrations obtained in this series of experiments.
C. Other Receptor Proteins in Prostatic Tissue

Over the past few years evidence has been found for the presence in prostatic tissue of specific estrogen receptors. High affinity binding in the absence of 5α-dihydrotestosterone binding was reported in benign prostatic adenomas, whereas Wagner and colleagues found receptors for both 5α-dihydrotestosterone and estradiol-17β in normal and hypertrophic tissue. None of the prostatic tissue in our laboratory investigations was found to have estradiol-17β receptors when studied with standard dextran-charcoal adsorption techniques and competition with unlabeled diethylstilbestrol. Endogenous estrogen was not replaced, however. It has already been stated earlier in this chapter that a more direct role has often been assumed for estradiol-17β in the pathogenesis of prostatic dysfunction, and the presence of these estradiol-17β receptor proteins tends to suggest the presence of an estrogen regulatory mechanism in the prostatic cell, although its physiological significance must await further investigation. It may well be that such a process functions merely to remove estrogens from the androgen dominated cellular processes.

Furthermore, an investigation to determine whether the estradiol-17β receptor is localized in stromal or epithelial tissue should be interesting, since stromal and epithelial tissues respond differently to hormones and may well have a different receptor status. Progesterone receptor-like binding in stromal tissue, rather than epithelial tissue, has already been described by Cowan et al. Although stromal tissue possesses a 5α-reductase, metabolizes C₃₉-steroids and responds in organ culture to androgens, the presence of estradiol-17β and progesterone receptors certainly suggests a different hormonal control mechanism, further elucidation of which may be of interest in relation to prostatic disease. Recent unpublished preliminary studies of Pierrepoint and Chaisiri have indicated that most of the estrogen receptor of the dog prostate appears to be located in the stromal tissue.

VII. ENVIRONMENTAL ASPECTS OF PROSTATIC CARCINOMA

In the foregoing discussions attention was drawn to McNeal’s hypothesis that prostatic carcinoma originates in active epithelial tissue rather than from regions of atrophy. The hypothesis does not, however, assign the role of prostatic carcinogen to the androgens. Indeed, the evidence tends to indicate that the androgenic hormones have more of a permissive role than one of induction in the promotion of prostatic neoplasia. A change in the general hormonal status or an increased sensitivity to either androgen or estrogen may predispose the prostate gland to respond to some exogenous stimulus. Lasnitzki, for example, demonstrated that estrogens enhanced the hyperplastic effect resulting from the incubation of rat prostatic explants with the carcinogen methylcholanthrene.

There is now some evidence that viruses are, at least, associated with human tumors and there are many who now argue strongly for an association of RNA oncogenic viruses with prostatic cancer. Ultrastructural studies have identified viruslike particles in prostatic carcinoma, and there is convincing evidence for an etiological role for viruses in certain other animal malignancies. The possibility, therefore, that a viral agent may be implicated in prostatic carcinoma in man should be borne in mind, although a recent epidemiological study indicated that there was no evidence to support any relationship between a venereally transmissible oncogenic agent and the disease, despite the suggested association between prostatic cancer and excessive sexual activity.

Rotkin’s report, however, directed attention to the possible risk to man from an environmental or industrial source, and it is interesting in this respect, that a number of investigations have described an increased mortality from prostatic cancer among
workers exposed to the heavy metal cadmium. It was suggested that replacement of intracellular prostatic zinc with cadmium may affect cellular function and promote neoplasia. The clinical effects of cadmium on the male reproductive tract are well established, and it is known that parenteral administration of zinc prior to cadmium to experimental animals prevents testicular damage. These, and other studies, over the past 20 years have been concerned with understanding the mechanism by which cadmium affects the testis, and evidence suggested that its primary target was the vascular supply of this gland. The relationship between zinc and cadmium in the reproductive system of the rat was investigated by Gunn et al., who also concluded that the principal effect of cadmium was on the testes and the subsequent decrease in plasma androgen levels resulted in a marked fall in prostatic weight. Testicular synthesis of testosterone in vitro was impaired after cadmium administration, and subsequent studies suggested that there was no direct effect of cadmium on the prostate gland.

The implication that cadmium could be a potential carcinogen in animals and in man stimulated, in our laboratories, an interest in the effect of the metal on the lateral prostate of the rat, which is known to contain high concentrations of zinc. Its uptake is influenced by various hormones and is also affected by cadmium. Although cadmium administration in vivo resulted in a decreased plasma concentration of testosterone and an elevated level of LH, ultrastructural studies clearly suggested that cadmium exerts a direct effect on the lateral lobe of the prostate. Electron microscopy microanalysis using EMMA-4 indicated that there were large reductions in the levels of zinc in all subcellular regions of the epithelial cells of the lateral lobe soon after cadmium administration. Cadmium was found localized in the nucleoli and lysosomes of the epithelium. An interesting feature of the study was the reaction of the basal cells which appeared not to be adversely affected by the presence of cadmium. On the contrary, apparent stimulation of these cells was observed, and there was an increased pinocytotic activity at the basement membrane. There were no involutionary changes as was seen in the epithelial cell, and it is interesting that, in a separate investigation, basal cells also responded differently to hormonal changes, being seen to proliferate after castration. Incubation of cadmium in vitro with explants of lateral prostate of rat produced similar results, with the metal entering the cell, causing local necrosis and subsequent proliferation of basal cells. Basal cell growth also has been observed after administration of 20-methylcholanthrene directly into the prostate, and it would seem that their potential for rapid growth is in relation to a hormonal or physiological environment which does not promote the normal function and maintenance of the prostatic epithelial cell.

These observations serve to emphasize that the various cell types of the prostate gland, the epithelial cells, muscle fibers, basal cells, fibroblasts, etc. do not respond in a similar manner to changes in the hormonal environment. The basis of the major advances in recent years in our understanding of the mechanism of hormone action at the molecular level has been primarily centered on the epithelial cell as the principal target. There have been comparatively few biochemical studies which were directly concerned with the interrelationship between the epithelial and stromal elements of the prostate gland. It is noteworthy, however, that in benign prostatic hypertrophy the normal, coordinated growth pattern of the epithelial and stromal cells in the periurethral tissue is disturbed to produce adenomata, which are not of one-cell type, but contain varying proportions of the various cellular components, although one particular cell type may well predominate. The extent to which hormones control the growth and development of the stromal elements is at present not very clear, and more fundamental work is required on the coordinated growth processes that would seem to control the interrelationship between epithelial and stromal elements in the prostate gland. Equally important are more definitive studies of the cells of the various zones of the
prostate gland, investigations which may provide more insight into the reasons why the epithelial tissue of the peripheral zone of the human prostate is predominantly the site of origin of carcinoma. Moreover, the differences between this type of androgen-dependent, prostatic, epithelial cell and those of other male, sex-accessory glands which rarely develop cancer remain to be elucidated. Any changes in circulating hormones with increasing age must affect these other glands of the male urogenital system, and, again, intensive studies into the effects of aging on fundamental biochemical processes within these various cell types would seem warranted.

There is evidence from cell culture that elevated intracellular levels of cAMP may be concerned in contact inhibition, whereby cultured cells cease dividing when contact is made with other cells. Goldberg et al. have also implicated cGMP in these control mechanisms and suggest that concentrations of these two cyclic nucleotides are inversely related in regulating biochemical processes concerned with cell growth. The effect of various protein hormones, prolactin, GH, FSH, etc. which normally act through membrane-receptors, with consequent stimulation of adenyl cyclase and elevation of cAMP concentrations, on such a regulatory system within the various cells of the prostate could well be interesting.

Other aspects of prostatic intracellular biochemistry should also be considered in relation to neoplasia. The high concentration of aliphatic polyamines in prostatic tissue and in seminal fluid has been well documented. Although one of these studies clearly demonstrated a relationship between the levels of spermine in seminal plasma and sperm count and motility, the role of the polyamines in human reproduction is still uncertain. It is well established, however, that polyamines can influence intracellular macromolecular synthesis in prostatic preparations. They have a marked effect on RNA transcription and translocation stabilization of the membranes of the endoplasmic reticulum; they interact with polyribosomes and inhibit nucleolytic enzymes. The reported increase in the spermidine to spermine ratio in rapidly proliferating neoplastic tissue and associated elevation in the activity of ornithine-decarboxylase, an enzyme concerned in polyamine biosynthesis, also directs attention to the relationship between polyamines and DNA replication. Whether the use of specific and sensitive analysis for the measurement of polyamine levels in plasma and seminal fluid will reflect abnormal growth patterns within prostatic tissue remains to be determined, but recent investigations by Russell offer considerable encouragement. Also important in relation to etiology is the suggestion of Williams-Ashman et al. that the oxidation of spermine or spermidine by certain amine oxidases present in seminal plasma and, possibly, in microbial enzymes present in the prostate to aldehydic oxidation products may be deleterious to the prostatic cells. Such oxidation products are cytotoxic and their potential carcinogenic effect requires further consideration.

It was stated at the beginning of this review that prostatic disease, benign hyperplasia or malignant neoplasia, represents a major clinical problem affecting a large proportion of elderly men. Of all species, it appears that only the aging dog is similarly afflicted. Whether this species susceptibility is a function of changes in the pattern of circulating hormones with age and/or alterations in the sensitivity and interaction of the various cellular elements of the prostate in association with environmental factors shared by these two animals remains to be determined.

It has long been accepted in man that, after the early period of prostatic growth during adolescence, the size of the gland remains relatively stable until the fifth decade when hypertrophy and sometimes carcinoma can then be found. Prostatic disease is related to the aging process. Relevant, however, are the studies of MacMahon et al. which drew attention to the decreased risk from breast cancer in women who had an early pregnancy, suggesting hormonal changes in the breast tissue during this adoles-
cent period of life may "trigger" certain biochemical events, which, later in life, become manifest as neoplastic growth or, conversely, protects against it. Endocrinologists will no doubt be directing their attention to this early phase in the life of women in an attempt to understand these "risk or protective factors" and equally, in the male, prostatic disease may result in the elderly man as a consequence of endocrine changes established in the adolescent youth. In this respect, the recent report from Rotkin* is most interesting. A detailed study of patients with prostatic cancer suggested that a delayed onset of sexuality in the late adolescent period with an early suppression of sexual activity were characteristics prevalent in those with the disease. A shorter span of sexual activity may then relate to prostatic cancer, with interest centering on the late pubertal phase of the male life as a period of cancer initiation, when the potential cancer patient may be endocrinologically different from the "normal" male. Whatever the outcome, however, the importance of the epidemiological approach to our understanding of the etiology of prostatic carcinoma is becoming steadily more evident.

VIII. ACKNOWLEDGMENTS

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ANDROGENS AND PROSTATIC CANCER

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Although it has long been recognised that the growth and function of the normal prostate is dependent upon testosterone secretion by the testes, the fact that prostatic cancer retained some degree of hormone dependence was only established a few decades ago from the classical studies of Huggins and his colleagues in the early 1940's. Antiandrogen therapy by administration of the synthetic oestrogen diethylstilboestrol or by orchidectomy has since been the generally accepted, conventional form of treatment for early prostatic cancer, although their relative merits have never been clearly defined possibly because of the inexact procedures for diagnosing, staging and assessing the progress of the disease. It must now be accepted that the well-differentiated cancer will respond to a change in hormonal status of the patient. The duration of the clinical response is unpredictable and often brief, and many patients relapse, presumably due to the progression of the autonomous cancer cells. Furthermore the clinical management of the secondary phase of the disease is ill-defined and the endocrinology relating to aetiology is little understood, although there is general acceptance of a hormonal role in the pathogenesis of prostatic cancer (Griffiths et al., 1979).

A detailed investigation into the relationship between plasma hormones and various clinical parameters, together with a study of the changes in hormone levels during treatment, was undertaken by the British Prostate Study Group, at present consisting of 20 urologists from various clinics in the United Kingdom in association with the Tenovus Institute. Patients
without previous therapy were classified according to primary tumour grade and metastatic status. Wide variations in hormone levels were found. Prolactin, FSH and LH appeared to play a minimal role in the discrimination of the primary tumour groups. There was also no systematic change in group means of the concentrations of growth hormone (GH), testosterone and oestradiol -17β, (Table 1), associated with the progression of the disease from the To to the T4 stage, in patients without metastatic disease (Mo) (British Prostate Study Group, 1979).

Although there were few consistent relationships between the hormone levels and the various clinical parameters, of interest was the application of multivariate analysis to the data which showed that the results allowed some degree of separation of the different groups of patients (Fig. 1) and indeed, that the growth hormone concentration was a major contributory factor in the discrimination between those patients with (M1) and those without (Mo) metastases, with GH values significantly larger ($p < 0.02$) in the former. The study illustrates the value of such an analysis in evaluating the complex endocrine status of patients with prostatic cancer assessed in a standard-

![First Canonical Variate](image)

**Fig. 1.** Multivariate analysis of data on hormone conc. and patient's age showing group separation based on stage (T) and metastatic status (M).
Table 1.

Plasma hormone concentrations of patients with prostatic carcinoma separated according to (a) UICC classification of primary tumours (b) metastatic spread

<table>
<thead>
<tr>
<th>(a) Primary tumour category</th>
<th>Age (years)</th>
<th>Testosterone (ng/ml)</th>
<th>Oestradiol (pg/ml)</th>
<th>LH (mIU/ml)</th>
<th>FSH (mIU/ml)</th>
<th>GH (ng/ml)</th>
<th>Prolactin (ng/ml)</th>
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<tr>
<td>To category (n=24)</td>
<td>mean 71</td>
<td>4.5</td>
<td>29.5</td>
<td>6.4</td>
<td>15.5</td>
<td>3.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD  9.04</td>
<td>1.48</td>
<td>17.65</td>
<td>5.33</td>
<td>13.05</td>
<td>3.72</td>
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<tr>
<td>T1 category (n=14)</td>
<td>mean 68</td>
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<td></td>
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<td>5.28</td>
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<tr>
<td></td>
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<td>T4 category (n=21)</td>
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<td>14.6</td>
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</tr>
<tr>
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<td>SD  7.43</td>
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<td>7.15</td>
<td>22.46</td>
<td>4.85</td>
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(b) Metastatic Spread

<table>
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<tr>
<th>Patients without metastases MO (n=78)</th>
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<th>4.3</th>
<th>25.9</th>
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<tr>
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<td>7.80</td>
<td>19.70</td>
<td>4.71</td>
</tr>
<tr>
<td>Patients with metastases ML (n=72)</td>
<td>mean 70</td>
<td>4.1</td>
<td>27.1</td>
<td>6.0</td>
<td>13.0</td>
<td>3.4</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>8.28</td>
<td>1.74</td>
<td>13.24</td>
<td>5.05</td>
<td>13.00</td>
<td>4.28</td>
</tr>
</tbody>
</table>

T refers to testosterone; E, to oestradiol-17β; n to numbers of patients; SD to standard deviation and NS to not significant.

ised manner in a multicentre project.

Of particular interest was the monitoring of hormone concentrations during oestrogen treatment of the patients. Although it is generally considered that the principal effect of diethylstilboestrol on prostatic cancer is exercised indirectly via the pituitary by suppressing LH output and thereby decreasing testosterone secretion by the testis, there is also evidence, some from our own group (Danutra et al., 1973) that oestrogens directly inhibit testicular activity. Patient monitoring however clearly indicates that within 7 days of diethylstilboestrol treatment (1 mg t.d.s.), levels of FSH and LH have fallen to undetectable levels (Harper et al., 1976), whereas in some patients, prolactin and GH concentrations rise. The plasma testosterone concentration falls to a value of approximately 60 ng/100ml. Although prolactin would appear to play a role in the regulation of the testis and the prostate and possibly may exercise some control of androgen production by the adrenal (Griffiths et al., 1979), no evidence has yet been produced to indicate that any subsequent rise in the levels of plasma testosterone is a consequence of an effect of the hormone on the adrenal or indeed that any rise in plasma
testosterone is related to recurrence of the disease. The basal level of plasma testosterone appears of adrenal origin however (Fig. 2) when the patient is receiving oestrogen treatment and it is therefore difficult to understand the further patient response to subsequent orchidectomy being testosterone mediated.

Indeed, it is tempting to suggest that an operation such as subcapsular orchidectomy in association with the administration of the adrenal inhibitor aminoglutethimide might offer a form of primary treatment worth considering. Evidence from the

Fig. 2. Changes in hormone conc. in patients on oestrogen therapy and during Synacthen stimulation and dexamethasone suppression tests.

246
Table 2. Plasma hormone levels in patients after orchidectomy, before and 24 hr after, HCG (3000 i.u., i.m.) administered at least 3 months post-op.

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Testosterone (ng/100ml) Before HCG</th>
<th>Testosterone (ng/100ml) After HCG</th>
<th>Oestradiol-17ß (pg/ml) Before HCG</th>
<th>Oestradiol-17ß (pg/ml) After HCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total orchidectomy</td>
<td>12 (n=6)</td>
<td>37</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>36</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>18</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>16</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>36</td>
<td>34</td>
<td>14</td>
</tr>
<tr>
<td>Subcapsular orchidectomy</td>
<td>45 (n=7)</td>
<td>24</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>14</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>30</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>49</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>22</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Non-castrated elderly</td>
<td>291 (n=5)</td>
<td>644</td>
<td>34</td>
<td>92</td>
</tr>
<tr>
<td>Male controls</td>
<td>286</td>
<td>518</td>
<td>27</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>312</td>
<td>492</td>
<td>33</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>329</td>
<td>412</td>
<td>38</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>439</td>
<td>803</td>
<td>45</td>
<td>150</td>
</tr>
</tbody>
</table>

Institute studies would indicate the value of subcapsular orchidectomy (Table 2), considered by some however to be ineffective because of residual Leydig cells remaining in the tunica albuginea.

A new approach to the study of the endocrinology of prostatic cancer is now possible from the development in the Institute by Dr. Fahmy of new sensitive radioimmunoassays for salivary steroid determination. The advantages are obvious: easy non-invasive, stress-free collection, serial sampling possible and probably most important, the fact that the salivary steroid concentration reflects the level of non-protein bound, free biologically active plasma steroid. For testosterone, the sensitivity of the assay is down to 0.5 pg/tube (Walker et al., 1979). Shown in Fig. 3 are the levels of salivary testosterone during the day and the application of cosinor analysis indicates that the concentration peaks at 07.00 hr, (Fig. 4). Fig. 5 shows the salivary and plasma testosterone levels in the assessment of testicular function after HCG (5000 i.u./d) administration. Furthermore, parotid fluid levels of steroid are identical to those in whole saliva (Fig. 5a), the concentration does not fall with increased flow rate if the sample collected is small and there is excellent correlation between plasma and salivary steroid levels in various endocrine situations as illustrated by Fig. 5b. The potential of these assays to study the chronoendocrinology of prostatic disease is enormous and data is already being
Fig. 3. Circadian rhythm in salivary testosterone. Nos. refer to subjects.

Fig. 4. Cosinor analysis showing a 07.00 hr peak for salivary testosterone.

Fig. 5. HCG (5000 i.u./d) stimulation test. Effect on plasma and salivary testosterone.
Fig. 6(a) Correlation between levels of testosterone in parotid fluid and saliva.

(b) Correlation between testosterone conc. in saliva (○—○), parotid fluid (●—●) and plasma (○---○) in patients with congenital adrenal hyperplasia.

accumulated to attempt to understand the aetiology of prostatic cancer.

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ANALYSIS OF TESTOSTERONE AND DEHYDROEPIANDROSTERONE IN SALIVA BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Cardiff, CF4 4XX, U.K.

Received 4-15-80

ABSTRACT

Testosterone and 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone) have been identified in human parotid fluid and saliva by gas chromatography-mass spectrometry/selected ion monitoring analyses of the t-butyldimethylsilyl ether and methyl oxime, t-butyldimethylsilyl ether derivatives. High specificity of analysis has been achieved by the use of high mass spectrometric resolution or by the monitoring of metastable peaks. Quantitative analyses indicate concentrations of both unconjugated testosterone and unconjugated dehydroepiandrosterone in the range 200-800 pmol/l in the saliva and parotid fluid of the normal males examined. These represent 1.5-7.5% of the concentrations of the steroids in blood plasma taken from the same subjects.

INTRODUCTION

The current interest in the assay of salivary steroids is attributable in part to the advantages of a non-invasive, and hence stress-free, sampling technique (1,2). Furthermore, the available data suggest that the well-substantiated correlation of salivary concentrations of many drugs with the concentrations of the non-protein bound forms in blood plasma (3,4) may also be applicable to steroids (5). Thus, salivary steroid assays may provide information of greater diagnostic value than analyses of plasma, where significant changes in the concentrations of a physiologically active steroid may be obscured by relatively high concentrations of its protein-bound form.
The low concentrations of steroids in mixed and parotid saliva severely restrict the number of applicable analytical methods. Immunoassay techniques have been applied (1,2,5,6,7) and will doubtless continue to be the methods of choice for routine use. The unequivocal identification of steroids in saliva, however, requires the application of alternative highly selective techniques which may also be used to validate quantitative data obtained by the routine methods. Analytical procedures incorporating gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM) are now widely accepted as reference methods for the determination of steroids in blood (8,9,10). The refinements of high mass spectrometric resolution (11, 12) or of monitoring metastable peaks (13) further enhance analytical specificity whilst retaining high sensitivity. This paper reports the application of these techniques to the analysis of unconjugated testosterone and 3β-hydroxy-androstan-5-en-17-one (dehydroepiandrosterone; DHA) in saliva. Preliminary aspects of the work have been reported elsewhere (12).

METHODS

Collection of samples
Blood samples were collected by venepuncture. Parotid fluid was obtained by fitting a Curby cup over the duct of the parotid gland; secretion was stimulated by administration of citric acid on the tongue, as described elsewhere (1). Saliva was obtained after subjects had rinsed the mouth with water and a minimum of 15 min had elapsed. All samples were collected in glass tubes and stored at -20°C until use. Saliva samples were centrifuged prior to extraction; solid material was discarded.

Extraction and fractionation of extracts
Saliva or parotid fluid (5-7.5 ml) and plasma (1-2 ml) samples were twice extracted with diethyl ether (15 ml). Where applicable, total extracts of aliquots of a single fluid sample were combined. Solvent was removed under a stream of nitrogen and the residue dissolved in 200 μl of methanol:water:chloroform (9:1:2, by vol.; solvent A) and applied to a column (4 cm x 0.5 cm) of Lipidex 5000 (Packard Instruments, Downers Grove, IL 60515, U.S.A.)
swollen in solvent A (14). A 0-2 ml eluate fraction was collected. Solvent was removed under nitrogen and the residue dissolved in hexane:ethanol (4:1, by vol.; solvent B). The solution was applied to a column (4 cm x 0.5 cm) of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), swollen in solvent B; a 0-4 ml fraction was collected and solvent again removed under nitrogen. Recoveries from each gel chromatographic separation exceeded 92%, as judged by model experiments with radiolabelled steroids. For quantitative analyses, samples of blood plasma (1-2 ml) or saliva (10-20 ml) were supplemented with a solution of 17α-hydroxy-androst-4-en-3-one (17-epitestosterone; 5 ng) in 10 μl ethanol. After mixing and equilibration overnight, extraction and extract purification were performed as described above.

**Derivatisation**

t-Butyldimethylsilyl (TBDMS) ethers were prepared by dissolving the steroid (or fraction of an extract of a biological sample) in t-butyldimethylchlorosilane : imidazole : dimethylformamide (1:1:6, by vol.; Applied Science Laboratories, State College, PA 16801, U.S.A.) and allowing to stand overnight at room temperature. Excess reagent was removed by passage through a column (2 cm x 0.5 cm) of Sephadex LH-20, swollen in hexane : chloroform : methanol (10:10:1, by vol.) (15). Methyl oximes were prepared, before or after TBDMS ether formation, by dissolving the steroid in 20 μl of a solution of methoxyamine hydrochloride (Eastman Kodak, Rochester, NY 14650, U.S.A.) in pyridine (15 mg ml⁻¹) and heating at 60⁰ for 1 h. Pyridine was removed under a stream of nitrogen. All samples were dissolved in ethyl acetate for GC-MS analysis.

**Gas chromatography-mass spectrometry (GC-MS)**

GC-MS analyses were performed using a Varian 2700 gas chromatograph coupled to a Varian MAT 731 double-focusing mass spectrometer via a two-stage Watson-Biemann separator. Separations were achieved on glass columns (2 m or 3 m x 3.5 mm, i.d.) of 1% OV-1, 1% OV-17, or 3% Poly-S 179 on Gas Chrom Q (100 - 120 mesh). The electron energy was 70 eV and the ion source temperature was 200⁰. For selected ion monitoring at high mass spectrometric resolution (m/Δm 8500-12000, 10% valley definition), ions of chosen exact mass were focused, using the peak matching unit, by reference to ions derived from perfluorokerosene (Pierce Chemical Co., Rockford, IL 61105, U.S.A.), which was independently introduced into the ion source. For metastable peak monitoring analyses of methyl oxime t-butyldimethylsilyl ether derivatives (16), daughter ions of m/z 374 were focused at an accelerating voltage of 8 KV. The accelerating voltage was subsequently increased, with constant electric sector voltage, to locate the metastable peak corresponding to the transition, m/z 431 + m/z 374, occurring in the first field-free region. The
mass spectrometric resolution was 1000.

RESULTS AND DISCUSSION

Table 1 records salient features of the mass spectra (electron impact; 70 eV) of the t-butyldimethylsilyl (TBDMS) ethers and methyl oxime (MO) TBDMS ethers of testosterone, DHA and isomeric steroids. In all cases, $|\text{M-C}_4\text{H}_9|^+$ ions constitute the base peaks and comprise a high proportion of the total ion current. Selected ion monitoring (SIM) of these ions therefore affords analyses of high sensitivity; detection limits of 20-50 pg are obtained during GC-high resolution MS/SIM of standard compounds. Molecular ions of TBDMS derivatives of steroids are not generally observed at significant intensities (15, 17) but the MO, TBDMS derivatives of testosterone and 17-epitestosterone are exceptions (Table 1). In these instances, molecular ions are presumably stabilised by the 4-ene, 3-methyl oxime system. Intense metastable peaks are observed corresponding to the fragmentation, $\text{M}^+ \rightarrow |\text{M-C}_4\text{H}_9|^+$.

Table 1. Mass spectrometric data for derivatives of testosterone, dehydroepiandrosterone (DHA) and isomeric steroids.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Mass Spectrum (relative intensities)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M^+$</td>
</tr>
<tr>
<td><strong>TBDMS ethers</strong></td>
<td></td>
</tr>
<tr>
<td>testosterone</td>
<td>m/z 402</td>
</tr>
<tr>
<td>17-epitestosterone</td>
<td>100</td>
</tr>
<tr>
<td>DHA</td>
<td>100</td>
</tr>
<tr>
<td>3α-hydroxyandrost-5-en-17-one</td>
<td>100</td>
</tr>
<tr>
<td><strong>MO, TBDMS ethers</strong></td>
<td></td>
</tr>
<tr>
<td>testosterone</td>
<td>m/z 431</td>
</tr>
<tr>
<td>17-epitestosterone</td>
<td>52</td>
</tr>
<tr>
<td>DHA</td>
<td>55</td>
</tr>
<tr>
<td>3α-hydroxyandrost-5-en-17-one</td>
<td>100</td>
</tr>
</tbody>
</table>
occurring in the first field-free region of the double-focusing mass spectrometer (16). GC-MS with metastable peak monitoring of MO, TBDMS derivatives of testosterone and 17-epitestosterone yields detection limits of ca. 30 pg. In this mode of analysis, detection of DHA and 3α-hydroxyandrost-5-en-17-one is, as expected (Table 1), less sensitive by at least a factor of 10. Loss of the elements of dimethylsilenol from Δ-2H₄ ions represents the only other significant fragmentation pathway observed for these derivatives (Table 1).

An extract of pooled mixed saliva from a normal male was purified by gel chromatography and converted to the TBDMS derivative. Fig. 1A shows the trace obtained by GC-high resolution MS with SIM of m/z 345.2250 (Δ-2H₄ for testosterone TBDMS and isomeric steroids). Intense peaks were observed at retention times characteristic of testosterone and DHA derivatives. The remaining sample was converted to the MO, TBDMS ether and analysed by GC-high resolution MS with SIM of m/z 374.2515 (Δ-2H₄ for testosterone MO, TBDMS and isomeric steroids). Two peaks were again observed at retention times corresponding to testosterone and DHA (Fig. 1B). Analyses of the same samples using GC columns of differing selectivity (OV-1, OV-17 and Poly S-179) in each case afforded peaks at the appropriate retention times.

An extract of saliva taken from the same subject on a different occasion was analysed as the MO, TBDMS derivative with SIM at high mass spectrometric resolution (Fig. 2A) and by metastable peak monitoring (Fig. 2B). In the latter analysis, a single peak was observed at a retention time corresponding to testosterone MO, TBDMS; as expected, DHA MO, TBDMS was not detected during metastable peak monitoring.

Further evidence for the identification of testosterone and DHA in saliva was obtained following addition of 17-epitestosterone as internal standard. Purified extracts
Fig. 1: GC-high resolution MS/SIM analyses of aliquots of a purified extract of mixed saliva (40 ml). A. TBDMS ether; m/z 345.2250. GC conditions: 1% OV-1, 245°. Analysis of 10% aliquot. B. MO, TBDMS ether; m/z 374.2515. GC conditions: 1% OV-1, 255°. Analysis of 15% aliquot.
Fig. 2: GC-MS analyses of aliquots of the MO, TBDMS derivative of a purified extract of mixed saliva (40 ml). A. SIM of m/z 374.2515. B. metastable peak monitoring of m/z 431 → m/z 374. GC conditions: 1% OV-1, 255°.
were analysed as TBDMS ethers with SIM at mass spectrometric resolutions of 8,500 and 12,000. The ratios of the intensities of the peaks attributable to testosterone and DHA to the intensity of the 17-epitestosterone peak were unchanged at the higher resolution, confirming that the ions detected were indeed of the exact mass monitored (rather than of a closely similar, and incompletely separated, mass).

The addition of 17-epitestosterone internal standard also enabled quantitative estimation of testosterone and DHA by GC-MS/SIM of TBDMS derivatives. Table 2 records concentrations of the two steroids in matched samples of blood plasma and mixed or parotid saliva from male subjects (25-30 years). Salivary concentrations of testosterone are in accordance with data obtained by enzyme- and radioimmunoassays (5,6,7), substantiating the specificity of the routine immunoassay procedures. Walker et al., for example, reported radioimmunoassay data which indicated morning concentrations of testosterone of $0.37 \pm 0.17$ nmol/l and evening concentrations of $0.21 \pm 0.13$ nmol/l in the saliva of normal males (7). The analysis of DHA in saliva has not been reported previously. For both steroids, the salivary concentrations represent 1.5-7.5% of the concentration in blood plasma. This is consistent with the hypothesis that salivary steroid concentrations correspond to the concentrations of the non-protein bound form in blood plasma (5). The present limited data, however, do not permit an assessment of the correlation of plasma and saliva concentrations.

**CONCLUSION**

Highly specific techniques of GC-MS have been applied to the identification of testosterone and dehydroepiandrosterone in mixed and parotid saliva. Quantitative determinations by GC-MS are in accordance with recently reported immunoassay data. The application of GC-high resolution MS/SIM to the rigorous validation of routine immunoassay procedures is in progress and will be reported elsewhere.
Table 2. Concentrations of testosterone and dehydroepiandrosterone in blood plasma, mixed saliva and parotid saliva of male subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Testosterone (nmol/l)*</th>
<th>DHA (nmol/l)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Mixed Saliva</td>
</tr>
<tr>
<td>A1**</td>
<td>20.7(19.3)</td>
<td>0.364</td>
</tr>
<tr>
<td>A2**</td>
<td>21.8</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>13.2(11.5)</td>
<td>0.243</td>
</tr>
<tr>
<td>C</td>
<td>9.20</td>
<td>0.271</td>
</tr>
<tr>
<td>D</td>
<td>34.2</td>
<td>-</td>
</tr>
</tbody>
</table>

* Values in parentheses are from duplicate determinations.
** Samples from the same subject taken on different occasions.
ACKNOWLEDGEMENTS

The financial support of the Tenovus Organisation is gratefully acknowledged.

REFERENCES

PLASMA SPERmidine CONCENTRATIONS IN PATIENTS WITH TUMOURS OF THE BREAST OR PROSTATE OR TESTIS

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(Received October 15th, 1979)

Summary

Plasma spermidine concentrations were measured by radioimmunoassay in normal subjects and in patients with various tumours of the breast, prostate or the testis. The sensitivity of the method was 0.45 pmol spermidine/20 μl plasma and the cross reactivity was 13% with putrescine and 2% with spermine. Plasma spermidine concentrations were raised in 25% of the patients with prostatic cancer (mean concentration 316.7 ± 240.69 nmol/l) and in 8% of the patients with benign prostatic hyperplasia (mean concentration 198.9 ± 169.92 nmol/l). No correlation was found between elevated plasma levels of spermidine in the prostatic cancer patients and tumour stage or metastatic status of the patients. No correlation of plasma spermidine concentrations and age was found in 61 normal male subjects (mean concentration 200.3 ± 137.71 nmol/l plasma). Only 29% of the patients with breast carcinoma had elevated levels of spermidine compared to normal female subjects. Plasma spermidine concentrations did not correlate with clinical stage or oestrogen receptor status in these patients. Patients with testicular tumours had elevated mean concentrations of plasma spermidine. One out of five patients with seminoma of the testis and six out of 16 patients with teratoma of the testis had significantly elevated concentrations.

Introduction

Polyamine concentrations have been found to increase during proliferation, accumulation being observed in embryonic development [1], in liver after

* To whom correspondence should be addressed.
partial hepatectomy [2,3] and during neoplastic growth [4—7]. Raised concentrations of polyamines have been found in tumour tissues and in the blood and urine of patients with malignant tumours [8—10]. Most of the data on polyamine levels in relation to malignancy have been obtained on urinary analysis of these compounds as the methods available were not sensitive enough for plasma determination. In order to assess the value of plasma polyamine analysis in the diagnosis of early cancer and for monitoring the response of a tumour to therapy, it was considered necessary to develop rapid and sensitive radio-immunoassays for their measurement [11,12]. Measurement of plasma spermine concentrations by radioimmunoassay in patients with either breast or prostatic tumours has been reported earlier [13]. Previous urinary analysis of these patients have indicated that spermidine was not a useful marker diagnostically but its possible use in monitoring chemotherapy in such patients would necessitate a preliminary analysis of the plasma values obtained in clinically staged patients [14].

This report now describes the development of a radioimmunoassay for spermidine and the measurement of this polyamine in the plasma of patients with benign and malignant tumours and in normal subjects, in order to initially assess its value in detection and staging of these tumours.

Materials and methods

Spermine, spermidine, cadaverine, putrescine and ornithine hydrochloride, bovine serum albumin, 1-ethyl-3-(3-dimethyl-amino propyl) carbodiimide hydrochloride (CDI) and activated charcoal were obtained from Sigma Chemical Co. (Terminal methylene-3H(N)) spermidine trihydrochloride (41 Ci/mmol) was obtained from New England Nuclear, Boston, U.S.A., and the radiochemical purity was checked at least every two months by thin layer chromatography on cellulose plates in the solvent system 2-methoxyethanol/propionic acid/H_2O (saturated with NaCl) (70 : 10 : 20, v/v/v). Human normal immunoglobulin and human albumin were gifts from Blood Products Laboratory, Lister Institute, Elstree, Herts., U.K.; Dextran T-150 was obtained from Pharmacia, Uppsala, Sweden.

Preparation of immunogen

The spermidine/bovine serum albumin (BSA) conjugate was prepared by a modification of the techniques described by Skowsky et al. [15] and Goodfriend et al. [16] using the carbodiimide reaction. The reaction mixture containing the reagents (spermidine : BSA : CDI) in the molar ratio of 440 : 1 : 50 was at neutral pH. After 18 h, the reaction mixture was applied to a 2.2 X 30 cm Sephadex G-75 column to separate the conjugated and free spermidine. The conjugate fractions were collected, dialysed and freeze-dried. A spermidine/BSA conjugate, with an incorporation ratio of approximately 46 : 1 was obtained.

Immunization

Adult Dunkin Hartley guinea pigs were immunized by the multiple-site injection technique. The guinea pigs were boosted at bi-weekly intervals with 1 mg
of conjugate, dissolved in 1 ml of normal saline solution and emulsified with an equal volume of Freund's complete adjuvant until a sufficiently high titre of antibody was produced. The antibody titre in sera increased, reaching a plateau after six weeks. Antisera collected between 6–8 weeks after first immunization were pooled and used for the assay. The association constant, \( K_a \), for the spermidine antibody was \( 0.34 \times 10^9 \) litres/mol.

**Specificity of antiserum**

The specificity of the spermidine antiserum is shown in Table I. Cross reactivity, expressed as a percentage, is given as the mass of each competitive compound required to displace 50% of bound radiolabelled spermidine from its antibody [17].

**Radioimmunoassay**

All assays were carried out in duplicate on ice. Plasma samples (20 \( \mu l \)) without dilution, or diluted up to five times dependent on plasma spermidine concentrations, were added to each tube together with protein solution (80 \( \mu l \)). [\(^3\)H]Spermidine (100 \( \mu l \); 0.302 pmol; 27 500 dpm) was then added to each tube, followed by antiserum (100 \( \mu l \)), diluted in borate buffer (0.05 mol/l; pH 8.0) to a concentration at which 50–60% of the radiolabelled spermidine was bound, (1:1000 final dilution). To prepare a standard curve, a series of tubes containing 100 \( \mu l \) solution of spermidine in borate buffer, in concentrations ranging between 0.172 and 44.06 pmol together with protein solution (100 \( \mu l \)), prepared from human albumin and human normal globulin were set up. The blank and “total-count” tubes were included in each assay. Sufficient borate buffer was added to all tubes to make a total volume of 1.0 ml. All tubes were vortexed and allowed to equilibrate at 4°C for 4 h. Antibody-bound and free spermidine were then separated after addition of 100 \( \mu l \) of dextran-coated charcoal suspension [5% (w/v) charcoal, 0.1% (w/v) Dextran-150 in assay buffer], mixing, standing on ice for 10 min and centrifuging at 1000 X g for 15 min at 4°C. The supernatant was decanted as a 0.5-ml aliquot directly into scintillation vials containing 6 ml of scintillation fluid (10 g, 2,5-diphenyloxazole, 1 l Triton X-100, 2 l toluene). Radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation spectrometer.

**Sensitivity, precision and accuracy**

The sensitivity of the assay was determined by establishing the 95% confi-

<table>
<thead>
<tr>
<th>Compound investigated</th>
<th>% Cross reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermidine</td>
<td>100</td>
</tr>
<tr>
<td>Putrescine</td>
<td>12.7</td>
</tr>
<tr>
<td>Spermine</td>
<td>1.77</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>0.23</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0</td>
</tr>
</tbody>
</table>
dence limits of the zero standard using 10 replicate standard curves and was defined as the amount of standard which is equal to the value read off the standard curve at mean \(-2\) S.D. of the percentage bound of the 10 zero tubes [18]. The sensitivity for this assay was 0.45 pmol of spermidine per assay tube.

Determination of the precision of the assay was accomplished using plasma pools of high, medium and low spermidine concentrations. Twenty replicates of the same plasma sample were measured in a single assay and the mean and standard deviation of the spermidine concentrations obtained were used to calculate the intra-assay variance. The intra-assay coefficient of variation was 6.9% for a mean value of 294 nmol/l, 7.5% for a mean value of 841 nmol/l and 11.2% for a mean value of 50 nmol/l. The inter-assay coefficient of variation was determined by including high, medium and low titre plasma pools in subsequent assays \((n = 17)\). The coefficient of variation was 9.5% for a mean value of 892 nmol/l, 12.7% for a mean value of 238 nmol/l, 17.7% for a mean value of 43 nmol/l of plasma. The accuracy of the spermidine assay was reflected in the analytical recovery of spermidine added to a plasma sample. There was a linear relationship between amount added and amount found (correlation coefficient = 0.922) as shown in Table II. Accuracy was also checked by parallelism studies [19]. Serial dilutions of a plasma with a high spermidine concentration were assayed and the dose response curve produced was compared with that of a diluted spermidine standard. Regression analysis was used to fit the best straight line to the data for both plasma and standard samples. It was found that at 95% confidence limits, the gradients of the two best fit curves were not significantly different and therefore taken to be parallel.

Normal subjects

Plasma was collected from 61 male volunteers, 19–79 years of age, mean 37.8, and from a group of 98 female volunteers 17–74 years of age, mean 35.6, all apparently in good health and free of any known disease. No effect of age was evident in the two normal populations; when analysed, the correlation coefficients were for male subjects \(r = 0.330\) and for the female subjects \(r = 0.250\). Plasma was also collected from 15 pregnant female subjects. Plasma samples were stored at \(-20^\circ\)C until analysed.

Patients bearing benign or malignant tumours

Samples were collected from patients with cancer before the initiation of

<table>
<thead>
<tr>
<th>Added (pmol)</th>
<th>Measured (pmol)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.71</td>
<td>—</td>
</tr>
<tr>
<td>0.69</td>
<td>4.48</td>
<td>100</td>
</tr>
<tr>
<td>1.38</td>
<td>5.51</td>
<td>106</td>
</tr>
<tr>
<td>2.75</td>
<td>6.82</td>
<td>104</td>
</tr>
<tr>
<td>5.50</td>
<td>8.81</td>
<td>94.8</td>
</tr>
<tr>
<td>11.01</td>
<td>14.11</td>
<td>95.3</td>
</tr>
</tbody>
</table>
therapy. Only histologically proven carcinoma of the prostate or testis was taken as an indication of a malignant tumour. The clinical staging of prostatic cancer patients into T and M categories was assessed according to UICC classification [20]. Separation of the prostatic cancer patients into those with no metastases, M0, and those with metastases, M1, was accomplished by evidence from X-ray analysis, isotopic bone scanning and plasma prostatic acid phosphatase measurements.

Results

Patients with prostatic or testicular tumours

Samples of plasma from 52 patients with prostatic carcinoma and 40 patients with benign prostatic hyperplasia (BPH) were assayed for spermidine. Table II shows the mean ± standard deviation and ranges of spermidine concentrations in the plasma of these patients, in 21 patients with testicular tumours and 61 normal male subjects. Mean plasma spermidine concentrations in patients with prostatic cancer were significantly higher than in normal males but only 13 of the 52 patients had values 2 S.D. above the normal. Three of the 40 patients with BPH had elevated concentrations of spermidine but the mean plasma concentration for this group was not significantly different from the normal. The primary prostatic tumours were classified into T0, T1, T2, T3 and T4 categories and the metastatic status as M0 and M1, using the UICC system. Table IV shows that plasma spermidine concentrations did not correlate with primary tumour staging nor with the metastatic status of the patient.

Patients with testicular tumours had a significantly raised mean plasma spermidine concentration, 1/5 of the patients with seminoma and 6/16 of the patients with teratoma of the testis having elevated plasma levels (Table III).

Patients with breast tumours

Plasma spermidine concentrations were measured from a group of 69 patients with breast carcinoma, 23 patients with benign breast tumours, 98 normal females and 15 pregnant females. The breast carcinoma patients were

---

**Table III**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age (yr)</th>
<th>No. of subjects</th>
<th>Spermidine (nmol/l)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mean ± S.D. range</td>
<td></td>
</tr>
<tr>
<td>Patients with BPH</td>
<td>52–85</td>
<td>49</td>
<td>198 ± 169.9 36–705</td>
<td>3</td>
</tr>
<tr>
<td>Patients with prostatic cancer</td>
<td>49–93</td>
<td>52</td>
<td>316 ± 240.6 * 36–1015</td>
<td>13</td>
</tr>
<tr>
<td>Patients with testicular tumours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) seminoma</td>
<td>26–64</td>
<td>5</td>
<td>354 ± 460.0 68–1170</td>
<td>1</td>
</tr>
<tr>
<td>(b) teratoma</td>
<td>20–42</td>
<td>16</td>
<td>563 ± 612.1 68–2478</td>
<td>6</td>
</tr>
<tr>
<td>Normal males</td>
<td>19–79</td>
<td>61</td>
<td>200 ± 137.7 48–636</td>
<td></td>
</tr>
</tbody>
</table>

An elevated level is defined as a concentration greater than the mean plus two standard deviations of normal males.

* Difference from normal males is statistically significant, $2 p = 0.001$. 
PLASMA SPERMIDINE CONCENTRATIONS IN PATIENTS WITH PROSTATIC CANCER CLINICALLY STAGED INTO THE VARIOUS T AND M-CATEGORIES (UICC)

<table>
<thead>
<tr>
<th>Patient categories</th>
<th>No. of patients</th>
<th>Spermidine (nmol/l)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean ± S.D.</td>
<td>range</td>
</tr>
<tr>
<td>Primary tumour staging</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>14</td>
<td>305 ± 228.7</td>
<td>99— 912</td>
</tr>
<tr>
<td>T1</td>
<td>9</td>
<td>305 ± 237.1</td>
<td>43— 826</td>
</tr>
<tr>
<td>T2</td>
<td>13</td>
<td>374 ± 294.8</td>
<td>36—1015</td>
</tr>
<tr>
<td>T3</td>
<td>8</td>
<td>225 ± 105.5</td>
<td>108—437</td>
</tr>
<tr>
<td>T4</td>
<td>5</td>
<td>400 ± 280.7</td>
<td>159—826</td>
</tr>
<tr>
<td>Metastatic status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>21</td>
<td>285 ± 202</td>
<td>36— 826</td>
</tr>
<tr>
<td>M1</td>
<td>15</td>
<td>304 ± 192.8</td>
<td>117—791</td>
</tr>
</tbody>
</table>

An elevated level is defined as a concentration greater than the mean plus two standard deviations of the normal males.

divided into three groups depending on their menopausal status. Peri-menopausal refers to those patients who have experienced some recession in menstrual bleeding but neither they nor their clinicians are certain they are actually menopausal. Patients with benign tumours had a significantly raised mean value of plasma spermidine as did both pre- and post-menopausal with breast carcinoma when compared to normal females as shown in Table V. The number of patients with elevated levels was, however, low, 2 out of 23 (8%) of the benign group, 4 out of 15 (26%) of the pre-menopausal and 16 out of 50 (32%) of the post-menopausal breast carcinoma groups. Table VI shows the spermidine concentration obtained in breast cancer patients staged as described by Blamey et al. [21]. The number of patients in stage C was too small to show any significant difference when compared to stage A and B patients. The mean plasma spermidine concentrations were similar in both stage A and stage B patients. Furthermore, no difference between spermidine concentrations of

PLASMA SPERMIDINE CONCENTRATIONS IN NORMAL SUBJECTS AND PATIENTS WITH BREAST TUMOURS

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age (yr)</th>
<th>No. of subjects</th>
<th>Spermidine (nmol/l)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mean ± S.D.</td>
<td>range</td>
</tr>
<tr>
<td>Benign</td>
<td>17—72</td>
<td>23</td>
<td>287 ± 194.9 a</td>
<td>79—963</td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre-menopausal</td>
<td>32—50</td>
<td>15</td>
<td>371 ± 246.4 b</td>
<td>134—912</td>
</tr>
<tr>
<td>peri-menopausal</td>
<td>48—56</td>
<td>4</td>
<td>165 ± 69.2</td>
<td>75—244</td>
</tr>
<tr>
<td>post-menopausal</td>
<td>40—82</td>
<td>50</td>
<td>374 ± 234.7 c</td>
<td>61—1087</td>
</tr>
<tr>
<td>Normal females</td>
<td>17—74</td>
<td>98</td>
<td>201 ± 116.8</td>
<td>55—550</td>
</tr>
<tr>
<td>Normal pregnancy</td>
<td>18—34</td>
<td>15</td>
<td>93 ± 99.6</td>
<td>34—43.3</td>
</tr>
</tbody>
</table>

An elevated level is defined as greater than the mean plus two standard deviations of normal females, the 2p values of 0.007, 0.000, 0.000 were obtained by the Student's t-test for the groups suffixed a,b,c, respectively when compared with the normal individuals.
TABLE VI
PLASMA SPERMIDINE CONCENTRATIONS IN PATIENTS WITH CARCINOMA OF THE BREAST

<table>
<thead>
<tr>
<th>Breast cancer patient groups</th>
<th>No. of patients</th>
<th>Spermidine (nmol/l)</th>
<th>No. with elevated levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean ± S.D.</td>
<td>range</td>
</tr>
<tr>
<td>Primary</td>
<td>61</td>
<td>371 ± 238</td>
<td>61—1087</td>
</tr>
<tr>
<td>Advanced</td>
<td>41</td>
<td>317 ± 214.2</td>
<td>33—1101</td>
</tr>
<tr>
<td>Oestradiol-17β receptor content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>21</td>
<td>352 ± 233.5</td>
<td>144—912</td>
</tr>
<tr>
<td>Positive</td>
<td>40</td>
<td>373 ± 235.1</td>
<td>61—1087</td>
</tr>
<tr>
<td>Clinical stage of tumour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>17</td>
<td>353 ± 243.7</td>
<td>86—912</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>418 ± 188.2</td>
<td>192—688</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>399 ± 31.2</td>
<td>285—791</td>
</tr>
</tbody>
</table>

An elevated level is defined as greater than the mean plus two standard deviations of normal females.

patients with primary and advanced breast carcinoma was observed, nor did the concentrations relate to the presence or absence of cytoplasmic oestradiol-17β receptor (Table VI).

Discussion

Spermidine antiserum used in this study provided a sensitive assay for the determination of this compound in plasma, although it does cross react with putrescine. Using this assay, the normal range of plasma spermidine in 61 male subjects was found to be 48—637 nmol/l (mean 200 nmol/l) and in 98 female subjects, 55—551 nmol/l (mean 201 nmol/l). The mean values are therefore in agreement with those obtained by Bartos et al. [12] for five normal subjects, but slightly lower than those obtained for hydrolysed sera by Nishioka and Romsdahl [22,23]. It has been suggested that spermidine is present in the plasma predominantly in the conjugated form [24], and as this radioimmunoassay is capable of measuring only free spermidine, the lower concentrations would therefore be expected.

The mean value of plasma spermidine in patients with prostatic cancer was significantly higher than that found in normal males, although only 25% of the patients had concentrations higher than 2 S.D. of the mean of normal subjects. The increased plasma spermidine concentration in prostatic cancer patients is partly in agreement with the results of Sanford et al. [25], Fair et al. [26] and Lipton et al. [10], which indicated a relationship between urinary polyamine concentrations and patients with prostatic adenocarcinoma. These investigators, however, reported high levels in 70% of their patients in contrast to the present results. The difference may be due to the smaller number of subjects studied by these investigators. Also their quoted elevations in polyamine concentration refers to any amount greater than a chosen value and not to those higher than 2 S.D. of their mean concentration for normal subjects. Alternatively the difference may be due to the measurement by the radioimmunoassay of free spermidine in the plasma, whereas the total spermidine concentra-
tion is determined in the urine. There was no significant difference in plasma spermidine concentrations in patients with localized malignancy of the prostate compared to those with widespread disease. This is in agreement with the results of Lipton et al. [10] but at variance with those of Fair et al. [26]. The low incidence of raised plasma spermidine concentrations in prostatic cancer patients would suggest that this parameter is of little value in the detection of this malignancy but may be of some value in monitoring response to therapy in certain patients and this will receive further investigation.

In patients with breast tumours, both pre- and post-menopausal groups of patients exhibited higher mean plasma spermidine concentrations than normal females, as did the patients with benign breast disease. Approximately 29% of the 69 breast cancer patients had elevated levels. Although direct comparison is difficult, this figure is higher than that quoted by Tormey et al. [9], who found 11% of their patients with breast cancer had elevated urinary spermidine concentrations. The plasma data show no difference between plasma spermidine concentrations in patients with primary breast cancer and advanced disease, nor any relation with the clinical staging of the tumour, or oestradiol-17β receptor status. It would appear that plasma spermidine is of little value diagnostically in breast cancer, although again it may prove useful in monitoring response to cytotoxic therapy in certain patients [27].

Acknowledgements

This work has been supported by the Tenovus Institute for Cancer Research and a grant from the Welsh Office. We are grateful to Dr. P.V. Maynard, Tenovus Institute for Cancer Research, who was responsible for the oestradiol-17β receptor assays. We would also like to thank Dr. P. Doyle and Dr. H. Bishop, City Hospital, Nottingham and Dr. K. Queen, St. Woolos' Hospital, Newport for their assistance.

References

Hormonal relationships of prostatic cancer

Clinical evidence suggests that prostatic cancer is, in some patients, functionally dependent on androgenic stimulation. Fig. 1 provides a relatively simple illustration of the various endocrine factors that are thought to be concerned with the regulation of prostatic growth and function, and

**Figure 1**
Schematic representation of certain aspects of the endocrinology relating to control of the prostate gland. DHA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; SHBG, sex hormone binding globulin; R, specific intracellular receptor; DHT-R, steroid receptor complex; mRNA and rRNA, messenger and ribosomal ribonucleic acid respectively. Question marks refer to processes not completely elucidated.
which must be considered in relation to the development of pathological change and subsequent hormone therapy.

Testosterone is considered to be the most important plasma androgen, although it must be remembered that the androgenic status of the male normally involves high concentrations of other C₁₉-steroids, such as androstenedione and dehydroepiandrosterone (DHA) and its sulphate, which are secreted by the adrenal gland. The latter steroids are present in relatively high concentrations in blood and are thus available for metabolism by the prostate to more potent androgens.

The development of sensitive radioimmunoassays for plasma hormone analysis has been of particular benefit in understanding those endocrine changes associated with ageing that might relate to prostatic cancer. The investigations of Vermeulen and his colleagues, supported by others, have indicated that the concentration of plasma testosterone tends to decrease after the sixth decade (Table 1), although variations between individuals are large. This decrease was less marked than might be expected from the declining ability of the testis to secrete testosterone, presumably because of a concomitant reduction in the metabolic clearance of the hormone. The ability of both testis and adrenal to respond to exogenously administered trophic hormones appears, however, to be retained despite ageing and loss of libido.

Other studies have found that the plasma oestradiol-17β concentration increased with age in the clinically normal adult male (Table 1). The physiological importance of oestradiol-17β in the male is not well understood, and, although the testis secretes both oestradiol-17β and oestrone, large proportion of plasma oestrogen originates from the peripheral aromatization of androstenedione secreted by the adrenal gland. It has been reported that there is an increased peripheral aromatization in the elderly male, which may account for this increased oestrogen:androgen ratio in later life. Although comparative data on the conversion of C₁₉-steroids to oestrogens in normal men and in those with prostatic cancer are not yet available, the effect of a change in the oestrogen:androgen balance on prostatic tissue could be considerable.

When assessing the androgenicity of plasma C₁₉-steroids, it has to be recognized that many are bound to sex hormone binding globulin (SHBG) in plasma. Only 2% of the testosterone in younger men is non-protein-bound and it is this fraction which is considered to represent the biologically active hormone capable of being transferred through the plasma membrane into the target cell. It would therefore seem reasonable that the observed increase (Table 1) in SHBG levels with age (believed to be promoted by the higher concentration of plasma oestrogens, and resulting in a decreased plasma-free testosterone concentration) may also influence prostatic function in the elderly male and accentuate the effect of the changing oestrogen-androgen balance.

There are also data to suggest that prolactin

Table 1
Plasma levels of testosterone, oestradiol-17β and SHBG binding capacity in relation to ageing

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Testosterone* (ng/ml)</th>
<th>Oestradiol-17β** (pg/ml)</th>
<th>SHBG binding*** capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 - 30</td>
<td>6-16</td>
<td>16-6</td>
<td>5-2 x 10⁻⁶M</td>
</tr>
<tr>
<td>30 - 40</td>
<td>6-34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 - 50</td>
<td>6-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 - 60</td>
<td>5-82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 - 70</td>
<td>4-62</td>
<td>25-6</td>
<td>8-9 x 10⁻⁶M</td>
</tr>
<tr>
<td>70 - 80</td>
<td>3-73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>2-45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data from Vermeulen et al.
** Data from Pirke & Doerr
*** Data from Vermeulen et al.
may influence the uptake of testosterone by the prostate\textsuperscript{14, 15} and the superfusion studies of Giorgi et al.\textsuperscript{16} indicate that oestrogens increase the entry of testosterone into the gland.

There are few reported studies on the relationship between protein hormones and carcinoma of the prostate, despite experimental work in animals which suggested that these hormones may influence prostatic growth.\textsuperscript{17, 18} For example, growth of the prostate was stimulated by both ACTH and growth hormone (GH) in hypophysectomized-castrated rats.\textsuperscript{19} Hypophysectomy produced a more marked atrophy of the prostate than did castration\textsuperscript{19, 20} and GH generally synergizes with testosterone and prolactin to affect prostatic weight.\textsuperscript{21} However, studies comparing plasma hormone levels of clinically normal, asymptomatic men with those of patients with prostatic carcinoma have generally failed to show any marked differences.\textsuperscript{22-24}

A more detailed study was undertaken in the United Kingdom under the auspices of the British Prostate Study Group to investigate the relationship between plasma hormones and various clinical parameters. Patients with histologically proven carcinoma of the prostate, without previous therapy, were classified according to primary tumour grade and metastatic status by a modification of the UICC classification, recommended by a British sub-committee.\textsuperscript{25} The presence of clinically evident metastases was assessed by X-ray examination and isotopic bone scanning.

Some of the data presented in a recent report of the Group\textsuperscript{26} are given in Table 2. They clearly indicate that, in patients without obvious metastases, there is no difference in the group means of the hormone concentrations associated with the progression of the disease from the T0 to the T4 (see Table 2) stage. Wide variations in plasma hormone concentrations were observed in this extensive study, and testosterone concentrations in elderly men in their 70's and 80's were often comparable to those of younger men.

Mean FSH, LH and prolactin levels did not differ significantly between the stages in the primary tumour group and were unrelated also to the presence of metastatic disease. Multivariate analysis of the data, however, clearly showed that GH concentration was a major discriminating factor between patients with and without metastases, the GH values being significantly higher (\textit{p} < 0.02) in the former. The clinical relevance of such an observation is, as yet, not clear.

This co-operative study illustrates both the value of a multicentre investigation to generate data from a large number of patients who have been assessed clinically in a standardized manner, and also the potential of multivariate statistical analysis in the evaluation of the complex endocrine status of patients with prostatic carcinoma.

It is possible, of course, that a more appropriate time to study such hormone profiles is earlier in life, before the disease is manifest. Comparatively little has been done to consider the various social, sexual and hormonal influences on the earlier life of the male that might "prime" the prostate, such that pre-cancerous changes become established in later life. On the other hand, it may well be that biochemical changes within the prostatic cell itself could provide an increased responsiveness or sensitivity to normal hormone concentrations.

**Metabolism of androgen by prostate: androgenic effects**

It is now well accepted that testosterone is actively metabolized by prostatic tissue. The classical experiments of Farnsworth and Brown\textsuperscript{27} demonstrated the formation of a reduced metabolite of testosterone, 5α-dihydrotestosterone, by human prostatic tissue and there is now a considerable body of evidence to indicate that it is this metabolite which is the active androgen within the cell.\textsuperscript{28-32} There is also evidence that other metabolites of testosterone synthesized in the prostate, particularly the various 5α-androstanediols, may have a specific biochemical role to play\textsuperscript{33-35} in the control of prostatic growth and function. The pattern of testosterone metabolism in the prostate may well provide a regulatory influence, and imbalance may lead to dysfunction of the gland.

It is established that 5α-dihydrotestosterone regulates prostatic growth through a well-defined process involving, firstly, a specific association with a cytoplasmic "receptor" protein (Fig. 1) followed by translocation of this steroid-receptor complex to the nucleus. The specific binding of the complex to nuclear acceptor sites elicits transcriptional processes and mRNA synthesis, thereby regulating genetic expression and the integrated cellular processes concerned with tissue growth.
Table 2
Plasma hormone concentrations of patients with prostatic carcinoma separated according to UICC classification of (a) primary tumours (b) metastatic spread

<table>
<thead>
<tr>
<th>(a) Primary tumour</th>
<th>Age (years)</th>
<th>T ng/ml</th>
<th>E₂ pg/ml</th>
<th>LH mU/ml</th>
<th>FSH mU/ml</th>
<th>GH μU/ml</th>
<th>Prolactin μU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0 category (n = 24)</td>
<td>mean 71</td>
<td>4·5</td>
<td>29·5</td>
<td>6·4</td>
<td>15·5</td>
<td>3·1</td>
<td>0·1</td>
</tr>
<tr>
<td></td>
<td>SD 9·04</td>
<td>1·48</td>
<td>17·65</td>
<td>5·33</td>
<td>13·05</td>
<td>3·72</td>
<td>0·13</td>
</tr>
<tr>
<td>T1 category (n = 14)</td>
<td>mean 68</td>
<td>4·6</td>
<td>27·0</td>
<td>6·7</td>
<td>16·9</td>
<td>1·6</td>
<td>0·1</td>
</tr>
<tr>
<td></td>
<td>SD 7·07</td>
<td>2·03</td>
<td>9·33</td>
<td>5·28</td>
<td>16·07</td>
<td>2·44</td>
<td>0·09</td>
</tr>
<tr>
<td>T2 category (n = 31)</td>
<td>mean 71</td>
<td>4·4</td>
<td>29·4</td>
<td>5·4</td>
<td>15·1</td>
<td>3·8</td>
<td>0·1</td>
</tr>
<tr>
<td></td>
<td>SD 9·23</td>
<td>1·60</td>
<td>11·06</td>
<td>4·13</td>
<td>14·03</td>
<td>7·06</td>
<td>0·10</td>
</tr>
<tr>
<td>T3 category (n = 73)</td>
<td>mean 70</td>
<td>4·1</td>
<td>26·2</td>
<td>8·2</td>
<td>16·8</td>
<td>2·5</td>
<td>0·1</td>
</tr>
<tr>
<td></td>
<td>SD 7·86</td>
<td>1·53</td>
<td>12·11</td>
<td>8·09</td>
<td>17·27</td>
<td>3·49</td>
<td>0·11</td>
</tr>
<tr>
<td>T4 category (n = 21)</td>
<td>mean 68</td>
<td>3·7</td>
<td>24·5</td>
<td>6·5</td>
<td>14·6</td>
<td>3·7</td>
<td>0·1</td>
</tr>
<tr>
<td></td>
<td>SD 7·43</td>
<td>1·31</td>
<td>7·53</td>
<td>7·15</td>
<td>22·46</td>
<td>4·85</td>
<td>0·11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Metastatic spread</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients without</td>
<td>mean 70</td>
<td>4·3</td>
<td>25·9</td>
<td>7·8</td>
<td>18·5</td>
<td>2·4</td>
<td>0·10</td>
</tr>
<tr>
<td>metastases M0 (n = 78)</td>
<td>SD 8·15</td>
<td>1·38</td>
<td>9·46</td>
<td>7·80</td>
<td>19·70</td>
<td>4·71</td>
<td>0·11</td>
</tr>
<tr>
<td>Patients with</td>
<td>mean 70</td>
<td>4·1</td>
<td>27·1</td>
<td>6·0</td>
<td>13·0</td>
<td>3·4</td>
<td>0·10</td>
</tr>
<tr>
<td>metastases M1 (n = 72)</td>
<td>SD 8·28</td>
<td>1·74</td>
<td>13·24</td>
<td>5·05</td>
<td>13·00</td>
<td>4·28</td>
<td>0·12</td>
</tr>
</tbody>
</table>

Key: T = testosterone; E₂ = oestradiol-17β; n = numbers of patients; SD = standard deviation;

After association of the receptor complex with the chromatin, the precise mechanism by which 5α-dihydrotestosterone leaves the nucleus and then the cell, is poorly defined. The steroid may be directly secreted from the prostate or possibly metabolized to an androstanediol, but, obviously, to control normal prostatic function, it is necessary to maintain an effective, intracellular concentration of the 5α-dihydrotestosterone-receptor complex. It is interesting that a concentration of endogenous 5α-dihydrotestosterone 5-fold higher than the normal prostate, has been demonstrated in benign hypertrophic prostatic tissue. Clearly, however, increased testosterone entry into the cell, imbalance of C₂₅-steroid metabolizing enzymes or inhibited release could each result in androgen accumulation and promotion of prostatic growth. Increased concentrations of receptor protein could also produce this accumulation of effective androgen and, currently, a considerable amount of research is directed to the analysis and characterization of steroid receptor protein in both normal and abnormal human prostatic tissue.

At present the available data are limited, but preliminary results from our own laboratory suggest that there may be a higher concentration of androgen receptor in hypertrophic and malignant human prostatic tissue than in the normal gland, and also a higher affinity of steroid-receptor interaction in the neoplastic tissue. It is important to determine whether receptor characteristics in diseased prostatic tissue differ from those in normal tissue, for this could
influence the affinity of the protein for either 5α-dihydrotestosterone or other metabolites of testosterone, or possibly even for oestradiol-17β.

The analysis of breast tumour tissue for oestradiol-17β receptor protein has proved of value in predicting a likelihood of response to endocrine therapy in patients with mammary carcinoma, and it is possible that analysis of the androgen receptor content of prostatic carcinoma may prove equally valuable in determining which patients will respond to anti-androgen therapy.

The nature of the available tumour tissue for such analysis is obviously crucial and the use of a cold-punch to collect biopsy samples, rather than the ‘hot-loop’, would facilitate analysis of this labile receptor material.

Receptors for oestradiol have also been found in human prostatic tissue and recent studies from this laboratory have shown such receptors in the stromal tissue of the canine prostate. The physiological significance of the prostatic oestrogen receptor is as yet unknown, although oestradiol-17β is suspected of being implicated in the pathogenesis of prostatic disease, particularly benign prostatic hypertrophy.

A direct control mechanism for the prostate

The evidence currently available has not defined any real abnormality of the endocrine status of the ageing male that can be related to the aetiology of prostatic cancer. By accepted convention, changes in hormone concentration are sought in the systemic circulation. Recently, however, investigations in our laboratories have drawn attention to the anatomical link between the testis and prostate through the ejaculatory duct system, the seminal vesicles, vasa efferentia, epididymal duct and the vas deferens, which might provide a path for androgens to reach the prostate without entering the general circulation.

Experimental evidence now exists to support the hypothesis that the androgen-maintained epididymis exercises unilateral control over the prostate via an intact vas deferens. Unilateral castration or vasectomy decreased RNA polymerase activity of the ipsilateral lobes of the rat prostate, an effect not observed after unilateral orchidectomy when the epididymis was not simultaneously removed with the gonad. The concentration of testosterone in the deferential vein of the dog was very much higher than in peripheral blood, and of the same order as that of the spermatid veins. The venous drainage of the cauda epididymis is through the deferential vein, which could therefore directly transport androgen from the epididymis to the prostatic complex and which, at vasectomy, is often severed. It has been shown that radio-opaque material can be carried to the prostate directly from the deferential vein and a retrograde flow of blood from the vessel would convey androgen-rich blood into the prostate gland.

A study of prostatic dysfunction and disease in men who have had vasectomies early in life is currently being conducted. In addition, however, this direct control of prostatic tissue requires investigation in relation to hormone therapy for prostatic cancer.

References
40 Chaisiri, N. and Pierrepoint, C. G. (1979) J. Endocrinol., 81, 147P.
Hormonal Relationships, Receptors, and Tumour Markers

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Tenovus Institute for Cancer Research, Welsh National School of Medicine, GB - Cardiff CF4 4XN

1 Introduction

Prostatic cancer is one of the most common causes of death from malignant disease in men. It is unfortunate, however, that this particular form of cancer and benign hyperplasia of the prostate gland are generally considered mere appurtenances of advancing years and relatively little research has been directed to the study of the biological factors that may be concerned with the aetiology of the condition. Furthermore, prostatic cancer is often clinically recognised only when the disease has become systemic and has extended beyond the prostate, yet there is little information on potential risk factors relating to the disease, and limited data on the practical value of various 'tumour markers' for the early detection of the condition.

Although the classic laboratory experiments of Dr. Charles Huggins and his colleagues (Huggins and Clark 1940; Huggins and Hodges 1941; Huggins et al. 1941) clearly established the androgen dependence of the prostatic cancer cell, thereby laying the foundation for treatment of the disease by oestrogen or antiandrogen therapy, the endocrine factors concerned with the aetiology of cancer of the prostate are little understood. There is, as yet, no evidence that hormones can initiate prostatic cancer, although the disease has not been reported in a prepubertally castrated male and it would seem that any role for hormones in the pathogenesis of this condition is essentially permissive.

It is not surprising, however, that investigations into the 'risk factors' concerned with such a hormone-dependent cancer, were directed primarily to a search for an endocrine 'abnormality' of the prostatic cancer patient, although at present there is little evidence that a changed endocrine status is implicated in the promotion of neoplasia (Griffiths et al. 1979). It is interesting, however, that although zones of the true prostate undergo a progressive, but slow atrophy with age (McNeal 1975), certain older men have prostatic tissue showing glandular morphology comparable to that seen in glands from younger men. Furthermore, McNeal (1970, 1972) has described an age-related increase in atypical hyperplasia of the prostate, changes which were considered premalignant and which related closely to prostatic carcinoma. Whether this age-related hyperplasia reflects some degree of hormonal stimulation associated with a change in the endocrine status of the male remains to be determined.

Also relevant in this context is the presence of small foci of latent, asymptomatic, differentiated carcinoma in the prostate of the older man. Although a common finding

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in the peripheral zone of the prostate gland, it is believed that many of these microscopic foci of cancer never become clinically evident (Varkarakis et al. 1970; Correa et al. 1974). The time course of development of such latent carcinoma is not known, although there is evidence that a time lapse of at least 20 years could occur between the appearance of neoplasia and the clinical manifestation of the disease (Hirst and Bergman 1954). The relationship between the changing endocrine status with ageing and these microcarcinoma may be of particular interest. It is also noteworthy that despite the low incidence of carcinoma of the prostate in Japan, latent carcinoma is as prevalent there as in a Caucasian population (Buell and Dunn 1965; Haenszel and Kurihara 1968), and investigations exploring the possibility of a different endocrine status in the ageing Japanese male could well be of immense value in helping us to understand the biological factors concerned in the promotion of prostatic cancer.

2 Hormonal Status

Figure 1 provides some indication of the hormones and glands that could be concerned in controlling the growth and function of the prostate gland. Testosterone, synthesised and secreted by the testis, is considered the principal plasma androgen although other C₁₉-steroids, dehydroepiandrosterone (DHA) and its sulphate, together with androstenedione are secreted in relatively high concentrations from the adrenal gland and contribute to the androgenic status of the male. These adrenal androgens could be metabolised by the prostate to more potent androgens (Pike et al. 1970; Harper et al. 1974).

Studies by Vermeulen et al. (1972) have suggested that the plasma concentration of testosterone tends to fall after the sixth decade, although the testis and adrenal can still respond to trophic hormone stimulation despite ageing (Doerr and Pirke 1974; Kley et al. 1975). The concentration of oestradiol-17β in the plasma increases with age (Pirke and Doerr 1973; Kley et al. 1974). Oestradiol-17β is secreted by the testis (Longcope et al. 1972) and, furthermore, is synthesised from the peripheral metabolism of the adrenal C₁₉-steroid, androstenedione; it is this increased peripheral aromatisation of androstenedione that is believed responsible for the higher levels of oestradiol-17β in the older man (Longcope et al. 1969; MacDonald et al. 1972).

It is equally important to consider the level of sex hormone-binding globulin (SHBG) in plasma. Little of the testosterone in plasma is free and unbound to protein, but it is this free hormone which is considered the biologically active material that can be transferred into the cells of the prostate gland. The level of SHBG also increases with age (Vermeulen et al. 1971), which will therefore influence the amount of hormone taken up by the target tissues. Prolactin, which appears to have a synergistic role with luteinising hormone (LH) in controlling the secretory activity of testis, might also influence not only the uptake of testosterone by the prostate, but the biological effect of other protein hormones, such as growth hormone, on the gland. A recent review (Griffiths et al. 1979) discusses in detail the endocrinology of prostatic disease. It was reasonable, however, that studies directed to understanding the endocrinology of prostatic disease should be concentrated on the determination of plasma hormone levels of patients with the condition. Unfortunately, the accumulated data have tended to be disappointing (Harper et al. 1976; Bartsch et al. 1977; Hammond et al. 1977).
Fig. 1. Schematic representation of certain aspects of the endocrinology relating to control of the prostate gland. DHA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; SHBG, sex hormone-binding globulin; R, specific intracellular receptor; DHT-R, steroid-receptor complex; mRNA and rRNA, messenger and ribosomal ribonucleic acid, respectively. Question marks refer to processes not completely elucidated.

A detailed study in the United Kingdom, undertaken by the British Prostate Study Group (1979), was focussed on the relationship between hormone concentrations in plasma of patients with proven prostatic cancer and various clinical parameters. There were no differences between hormone levels of patients with cancer and the controls. Moreover, there was no systematic change in the group means of the hormone concentrations related to the progression of the disease from stage T0 to stage T4 (Table 1). Wide variations in hormone concentrations were found, levels in older men
Table 1. Plasma hormone concentrations of patients with prostatic carcinoma grouped according to (a) UICC classification of primary tumours and (b) metastatic spread

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Plasma hormone concentrations</th>
<th>LH mIU/ml</th>
<th>FSH mIU/ml</th>
<th>GH μU/ml</th>
<th>Prolactin mIU/ml</th>
<th>T ng/ml</th>
<th>E2 pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Primary tumour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0 category</td>
<td>Mean</td>
<td>71</td>
<td>6.4</td>
<td>15.5</td>
<td>3.1</td>
<td>0.1</td>
<td>4.5</td>
</tr>
<tr>
<td>(n = 24)</td>
<td>SD</td>
<td>9.04</td>
<td>5.33</td>
<td>14.05</td>
<td>3.72</td>
<td>0.13</td>
<td>1.48</td>
</tr>
<tr>
<td>T1 category</td>
<td>Mean</td>
<td>68</td>
<td>6.7</td>
<td>16.9</td>
<td>1.6</td>
<td>0.1</td>
<td>4.6</td>
</tr>
<tr>
<td>(n = 14)</td>
<td>SD</td>
<td>7.07</td>
<td>5.28</td>
<td>16.07</td>
<td>2.44</td>
<td>0.09</td>
<td>2.03</td>
</tr>
<tr>
<td>T2 category</td>
<td>Mean</td>
<td>71</td>
<td>5.4</td>
<td>15.1</td>
<td>3.8</td>
<td>0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>(n = 31)</td>
<td>SD</td>
<td>9.23</td>
<td>4.13</td>
<td>14.03</td>
<td>7.06</td>
<td>0.10</td>
<td>1.60</td>
</tr>
<tr>
<td>T3 category</td>
<td>Mean</td>
<td>70</td>
<td>8.2</td>
<td>16.8</td>
<td>2.5</td>
<td>0.1</td>
<td>4.1</td>
</tr>
<tr>
<td>(n = 73)</td>
<td>SD</td>
<td>7.86</td>
<td>8.09</td>
<td>17.27</td>
<td>3.49</td>
<td>0.11</td>
<td>1.53</td>
</tr>
<tr>
<td>T4 category</td>
<td>Mean</td>
<td>68</td>
<td>6.5</td>
<td>14.6</td>
<td>3.7</td>
<td>0.1</td>
<td>3.7</td>
</tr>
<tr>
<td>(n = 21)</td>
<td>SD</td>
<td>7.43</td>
<td>7.15</td>
<td>22.46</td>
<td>4.85</td>
<td>0.11</td>
<td>1.31</td>
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<td>(b) Metastatic spread</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients without metastases M0</td>
<td>Mean</td>
<td>70</td>
<td>7.8</td>
<td>18.5</td>
<td>2.4</td>
<td>0.10</td>
<td>4.3</td>
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<tr>
<td>(n = 78)</td>
<td>SD</td>
<td>8.15</td>
<td>7.80</td>
<td>19.70</td>
<td>2.71</td>
<td>0.11</td>
<td>1.38</td>
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<tr>
<td>Patients with metastases M1</td>
<td>Mean</td>
<td>70</td>
<td>6.0</td>
<td>13.0</td>
<td>3.4</td>
<td>0.10</td>
<td>4.1</td>
</tr>
<tr>
<td>(n = 72)</td>
<td>SD</td>
<td>8.28</td>
<td>5.05</td>
<td>13.00</td>
<td>4.28</td>
<td>0.12</td>
<td>1.74</td>
</tr>
</tbody>
</table>

T, testosterone; E2, oestradiol-17β; n, numbers of patients; SD, standard deviation; NS, not significant
in their seventies being similar to those of young men. Generally the hormone concentrations appeared unrelated to the presence of metastatic disease, although when the data were subjected to multivariate analysis it was seen that growth hormone concentration was a major contributory factor in the discrimination between patients with (M1) and without (M0) metastases. The study emphasised the potential value of multivariate analysis in assessment of the complex endocrine status of the ageing male in relation to disease. Analysis of the preliminary hormone and clinical data clearly indicated that group differences existed when the computer print-outs were considered (Fig. 2), although the clinical relevance of the higher growth hormone levels in patients with metastatic disease is obviously not clear. Promotion of prostatic neoplasia may well be dependent on some particular, abnormal endocrine status of certain individuals, but current evidence indicates therefore that it will be very difficult to identify a high-risk group from simple plasma analysis of hormones.

It is possible, however, that the recent development by the Tenovus Institute of radio-immunoassays for salivary steroids (Walker et al. 1978a, 1979; Turkes et al. 1979; Fahmy et al. 1980) might allow a more detailed study of the chrono-endocrinology relating to ageing and prostatic cancer. The non-invasive nature of saliva collection makes sequential sampling very easy and the fact that the salivary steroid concentration reflects the level of non-protein-bound, free, biologically active steroid in plasma renders the assays of immense value. Recent studies of testosterone levels in saliva (Walker et al. 1980) from a group of males clearly demonstrate their potential and illustrate the ease with which the circadian rhythm of testosterone production can be shown (Fig. 3).

![Fig. 2. Multivariate analysis of data on hormone concentrations and patients age, showing group separation based on stage (T) and metastatic status (M)](image-url)
Fig. 3a, b. Circadian rhythm in salivary testosterone from one individual (a) and a group of males (b). Numbers refer to subjects.
It may be, however, that the most appropriate time to study hormone profiles is earlier in life, before the disease is clinically manifest. It was always hoped that factors that might reflect the hormonal status of younger men prior to cancer development — demographic features such as marital status, sex drive and other social and hormonal influences in the early life of men who present with prostatic cancer — might help to differentiate a high-risk group, but such studies have generally been disappointing (King et al. 1963; Steele et al. 1971; Greenwald et al. 1974). The close correlation between the incidence of prostatic cancer and carcinoma of the breast in various countries around the world (Wynder et al. 1971), and the established relationship between early first-term birth and risk of developing breast cancer (MacMahon et al. 1970) possibly suggest that various biological changes in adolescence may 'trigger' biochemical events, which in later life are manifest in neoplastic growth. In relation to this, a recent investigation (Rotkin 1977) suggested that delayed onset of sexuality in the late adolescent period, with an early suppression of sexual activity, were features prevalent in those with prostatic cancer.

2.1 Role of Testosterone in Androgen Receptor Assays

Consideration must also be given to the possibility that biochemical changes within the prostatic cells might be responsible for an increased responsiveness to normal hormone levels in plasma. Man and dog are virtually alone in the animal kingdom in their susceptibility to prostatic hyperplasia, and the epithelial tissue of the peripheral zone of the human prostate is the predominant site of origin of carcinoma. The difference between this type of androgen-dependent prostatic epithelial tissue and those of other male accessory glands such as the seminal vesicles, which rarely develop cancer, remains to be determined. Particular attention is presently being devoted to the cellular processes concerned with the mode of action of testosterone (Fig. 1). It is now well established that testosterone is metabolised within the prostatic cell to 5α-dihydrotestosterone, which is the active androgen within the cell (see Griffiths et al. 1979), although other metabolites, the 5α-androstanediols, may also have specific biochemical roles in the control of prostatic function. Indeed the pattern of testosterone metabolism may well provide an intracellular, regulatory process. The 5α-dihydrotestosterone regulates prostatic growth through a mechanism involving its specific association with a cytoplasmic receptor protein (Fig. 1), followed by translocation of the steroid-receptor complex to the nucleus. Binding of the complex to nuclear acceptor sites on the chromatin elicits transcription, mRNA synthesis, and tissue growth. It is important to determine whether receptor characteristics in diseased prostatic tissue differ from those of normal tissue (Davies 1978) in such a way that the affinity of the protein for 5α-dihydrotestosterone, or possibly other metabolites of testosterone or oestradiol-17β, could influence prostatic growth. Furthermore, since it has been shown that the analysis of oestradiol-17β receptor protein content in breast tumour tissue can be used to predict the response to endocrine therapy of patients with advanced cancer (McGuire et al. 1975), it has been hoped that 5α-dihydrotestosterone receptor levels in prostatic carcinoma could also be of value in determining the most appropriate form of therapy. It is well accepted now that breast tumours devoid of oestradiol receptors are rarely influenced by endocrine therapy. The value of androgen receptor assays in human prostatic carcinoma as a predictor of endocrine response has not yet been established, however. Although the methodology
is now available to study these relatively labile proteins (Davies et al. 1977; Shain and Boesel 1978), obviously the nature of the tumour tissue available for analysis is important in determining the real value of the receptor status of the tissue in relation to response to therapy. The material obtained by electroresection is clearly unacceptable. Analysis of metastatic deposits could well be the most useful, although such tissue is difficult to obtain. A recent report by Ekman et al. (1979), who analysed tissue removed by perineal punch needle biopsy of primary tumour (50–200 mg tissue), indicated that reliable receptor data could be obtained which related well to short-term response to endocrine therapy. Although support for this work has been provided by the work of Mobbs et al. (1978), who measured androgen receptor content by means of a protamine sulphate precipitation assay, de Voogt and Dingjan (1978), with an agar gel electrophoretic procedure, were unable to relate receptor levels to response. Clearly further studies are necessary in this important field to establish the clinical value of the oestrogen receptor concentration in prostatic cancer.

3 Some Aspects of Tumour Marker Analysis

3.1 Polyamines

The high concentration of aliphatic polyamines in prostatic tissue and in seminal plasma is well documented (Williams-Ashman et al. 1972a, 1975), although their role in human reproduction is uncertain. Polyamines, however, influence micromolecular synthesis in prostatic tissue preparations (Williams-Ashman et al. 1972a), effect RNA transcription, and give rise to the increased spermidine levels in proliferating neoplastic tissue (Snyder and Russell 1970); the related activity of ornithine-decarboxylase, an enzyme concerned with polyamine synthesis, indicates an association with DNA replication (Williams-Ashman et al. 1972b). It was reasonable to expect that patients with malignant tumours might have elevated levels of polyamines in urine and plasma, and high levels have been reported in such patients (Russell 1978; Marton et al. 1973). The possibility existed, therefore, that measurement of polyamine levels in plasma might reflect normal growth patterns within prostatic tissue. The studies of Russell et al. (1978) provided considerable support for this concept, and it seemed that polyamine analysis might offer a valuable tumour marker for the early detection of prostatic carcinoma. It has generally been accepted, however, that methods for the measurement of polyamines in biological fluids are tedious, and/or lack sensitivity. To assess the value of polyamine analysis, both in the diagnosis of early cancer and in the assessment of response to therapy, it was necessary to develop sensitive and relatively rapid radio-immunoassays for the specific analysis of spermine and spermidine. These assays have been previously described (Chaisiri et al. 1979, 1980) and some of the preliminary data from patients with prostatic disease are shown in the following section.

Table 2 shows the mean ± standard deviation and ranges of spermine concentration in the plasma of patients with benign prostatic hyperplasia and prostatic carcinoma compared with normal male subjects. There were no significant differences between the groups, and of the 54 patients with carcinoma only three had spermine levels higher than the normal range. When the primary prostatic tumours were classified into
Table 2. Levels of plasma spermine in patients with prostatic tumours and in normal subjects

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>No. of subjects</th>
<th>Spermine (pmol/ml)</th>
<th>No. with elevated levels&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td><strong>(A)</strong> Benign</td>
<td>52–82</td>
<td>20</td>
<td>210 ± 140</td>
<td>50–540</td>
</tr>
<tr>
<td>Malignant</td>
<td>48–89</td>
<td>54</td>
<td>210 ± 110</td>
<td>60–640</td>
</tr>
<tr>
<td>Normal males</td>
<td>18–79</td>
<td>66</td>
<td>200 ± 100</td>
<td>50–320</td>
</tr>
<tr>
<td><strong>(B)</strong> Patients with malignant tumour staged by UICC T categories</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td></td>
<td>8</td>
<td>200 ± 90</td>
<td>70–380</td>
</tr>
<tr>
<td>T1</td>
<td></td>
<td>6</td>
<td>200 ± 60</td>
<td>120–270</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td>12</td>
<td>210 ± 80</td>
<td>100–390</td>
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<tr>
<td>T3</td>
<td></td>
<td>18</td>
<td>210 ± 120</td>
<td>130–640</td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td>9</td>
<td>240 ± 160</td>
<td>60–520</td>
</tr>
<tr>
<td><strong>(C)</strong> Patients with and without metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td></td>
<td>24</td>
<td>230 ± 80</td>
<td>130–520</td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td>19</td>
<td>230 ± 150</td>
<td>20–640</td>
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</table>

<sup>a</sup> Elevated level is defined as a higher level than the mean ± 2 SD for normal males
<table>
<thead>
<tr>
<th>Category</th>
<th>Age (years)</th>
<th>No. of subjects</th>
<th>Spermidine (pmol/ml)</th>
<th>No. with elevated levels&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Benign</td>
<td>52–85</td>
<td>40</td>
<td>198 ± 169</td>
<td>3</td>
</tr>
<tr>
<td>Malignant</td>
<td>49–93</td>
<td>52</td>
<td>316 ± 240</td>
<td>13</td>
</tr>
<tr>
<td>Normal males</td>
<td>19–79</td>
<td>61</td>
<td>200 ± 137</td>
<td>13</td>
</tr>
<tr>
<td>(B) Patients with malignant tumour staged by UICC T categories</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>14</td>
<td>305 ± 228</td>
<td>99–912</td>
<td>3</td>
</tr>
<tr>
<td>T1</td>
<td>9</td>
<td>305 ± 237</td>
<td>43–826</td>
<td>2</td>
</tr>
<tr>
<td>T2</td>
<td>13</td>
<td>374 ± 294</td>
<td>36–1,015&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>T3</td>
<td>8</td>
<td>225 ± 105</td>
<td>108–437</td>
<td>0</td>
</tr>
<tr>
<td>T4</td>
<td>5</td>
<td>400 ± 280</td>
<td>159–826</td>
<td>2</td>
</tr>
<tr>
<td>(C) Patients with and without metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>21</td>
<td>285 ± 202</td>
<td>36–826</td>
<td>4</td>
</tr>
<tr>
<td>M1</td>
<td>15</td>
<td>304 ± 192</td>
<td>117–791</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Elevated level is defined as a higher level than the mean + 2 SD for normal males

<sup>b</sup> Difference from normal males in statistically significant (2 <i>p</i> = 0.001)
T categories according to the UICC system (Wallace et al. 1975) and the metastatic status was noted as M0 and M1, again, there was no correlation between spermine concentration and clinical staging or between those with localised cancer and those with widespread metastatic disease. Spermine concentration in plasma therefore appears to be of little value, either for detection or in monitoring response to therapy.

Table 3 shows similar data for spermidine concentrations in the plasma of patients with prostatic disease. Although the mean spermidine concentration in the plasma of patients with prostatic cancer was significantly higher than that of normal males, only 13 of the 52 patients had values 2SD above the normal. Three of the 40 patients with benign prostatic hyperplasia had elevated levels. Again, spermidine levels did not relate to primary tumour staging or to metastatic status of the patient. It should be noted however that it has been suggested that spermidine is present in plasma predominantly in the conjugated form (Rosenblum et al. 1978), and the radio-immunoassay used in the Institute measures only free spermidine.

The higher levels of spermidine in certain samples of plasma from patients with prostate cancer are to some extent in agreement with previous results (Sanford et al. 1975; Fair et al. 1975; Lipton et al. 1976), which indicated that urinary polyamine concentrations were elevated in 70% of such patients. The number of patients was smaller in these studies, however, and the quoted elevations were not 2SD higher than

---

**Fig. 4.** Plasma acid phosphatase levels in BPH, non-metastatic prostate cancer, and metastatic prostate cancer, compared with those in a control group of normal males
the mean concentration for normal males. Total spermidine concentration is also measured by the urinary assay.

3.2 Acid Phosphatase

One of the first tumour markers to be measured in serum was the activity of the enzyme acid phosphatase. This enzyme is secreted by prostatic epithelium and although its physiological role is not clear, it has been established for some time (Gutman and Gutman 1938; Huggins and Hodges 1941) that serum acid phosphatase activity is elevated in patients with prostatic carcinoma, 65%–90% of patients with bone metastases, and 5%–10% of those with clinically demonstrable metastatic cancer (Yam 1974). Serum acid phosphatase activity can, however, be attributed to many tissues other than the prostate. The tissue specificity of this marker is therefore to some extent limited unless the ‘prostatic proportion’ of the activity is defined. The isolation of tissue isoenzymes and the use of particular enzymatic incubation conditions and specific inhibitors, together with the incorporation of certain enzyme substrates, was found to be unsatisfactory; the realisation that the enzyme activity was

![Graph showing plasma acid phosphatase levels in non-metastatic and metastatic prostate cancer in relation to stage classification](image-url)

**Fig. 5.** Plasma acid phosphatase levels in non-metastatic and metastatic prostate cancer in relation to stage classification
to some extent irrelevant to the role of the enzyme as a tumour marker stimulated the development of an immunoassay for the measurement of the enzyme protein. Initial reports of studies with antisera raised to purified prostatic acid phosphatase were encouraging, with an increased sensitivity being found for the detection of elevated levels of the protein in earlier stages of prostatic cancer (Foti et al. 1977; Chu et al. 1977/1978; Romas et al. 1979). The involvement of the Institute in the British Prostate Study Group's hormonal survey of patients presenting with cancer stimulated the search for effective tumour markers and a radio-immunoassay for prostatic acid phosphatase had been established, together with those for carcinoembryonic antigen (CEA) and \( \beta \)-hCG. Since samples of plasma from various clinics were frozen and sent to the Institute for analysis, obviously the immunoassay for the protein offered more stability than an assay determining enzyme activity.

Very briefly, the enzyme was purified from benign hyperplastic tissue by ammonium sulphate precipitation, followed by DEAE cellulose and Sephadex G 150 column chromatography. The enzyme was homogeneous in polyacrylamide gel electrophoresis, and antisera were raised to the protein in New Zealand white rabbits. A double-antibody assay with \( ^{125}\text{I} \)-labelled antigen was established. Although certain investigators (Vihko et al. 1978) have been able to show a complete separation of the cancer group with elevated serum enzyme levels from the control group, Fig. 4 indicates that in this particular study, now reported, considerable overlap was found, although a larger proportion of patients with metastatic disease had higher levels. Figure 5 illustrates the data in relation to \( T \) classification. Further work is obviously required, but it is clear that progress is being made in the establishment and assessment of new assays for tumour markers.

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13. Prostatic Cancer: Rationale for Hormonal Therapy

K. GRIFFITHS, W. B. PEELING, M. E. HARPER, P. DAVIES AND C. G. PIERREPOINT

Prostatic cancer is the fourth most common cause of death in man from malignant disease, and together with benign hyperplasia of the gland is accepted as a concomitant of advancing years. Unfortunately, the condition is generally recognized only after it has become disseminate and usually, at this stage, some form of systemic therapy is required.

Although it was recognized by John Hunter (1786) that the testes are required for the growth and function of the prostate, orchidectomy was not introduced as therapy for prostatic cancer until the reports of Huggins and his colleagues (Huggins and Clark, 1940; Huggins and Hodges, 1941; Huggins et al., 1941). Their classical experiments showed that castration or the administration of oestrogens caused epithelial degeneration and inhibition of secretory activity in normal prostatic tissue. The same treatment caused regression of human prostatic cancer, thereby showing that it retained some of the hormone dependence of the normal gland. The timely preparation by Dodds and his colleagues (1938) of the first orally active synthetic oestrogen, diethylstilboestrol, provided what was to become a popular alternative to orchidectomy.

The endocrine factors concerned with the aetiology of prostatic cancer are little understood. Nevertheless, because of the clinical response by the established disease to hormonal manipulation, a hormonal role is assumed in the pathogenesis of the condition. Certainly the disease has not been observed in the pre-pubertally castrated male, but as yet there is little direct evidence to implicate hormones in the initiation of prostatic cancer. Rather, they can be considered permissive to the condition.

The rationale for hormone therapy in this disease will be considered under four headings:

- The pathogenesis and aetiology of prostatic cancer;
- Hormonal relationships of prostatic cancer;
- Metabolism of androgen by the prostate: androgenic effects;
- Rationale of anti-androgen therapy.

**The Pathogenesis and Aetiology of Prostatic Cancer**

It needs to be stated that following the controversy over the years concerning the relationship between benign prostatic hypertrophy and carcinoma of the gland (Sommers, 1957; Turner and Belt, 1957; Armenian et al., 1974; Greenwald et al., 1974; Rotkin, 1975), available evidence now indicates that the pathogenesis of these conditions should be considered distinct and independent. They originate in different regions of the prostate and thus prob-
ably do not share a common aetiology. The concept that benign hyperplasia constitutes a premalignant condition developed from reports of a high incidence of nodular hyperplasia associated with prostatic cancer. Since prostatic hypertrophy is a feature of ageing, its association with carcinoma of the gland is to be expected.

McNeal, (1970; 1975), considers the prostate to be made up of three distinct structures within the capsule, with two, the peripheral and central zones, containing well developed glandular tissue, forming the "true prostate" (Fig. 1). The third structure is the perirethral gland, with simple glandular tissue opening into the upper region of the urethra above the upper end of the verumontanum, and enclosed by a tube of muscle tissue (the "preprostatic sphincter"). It is considered to be separate from the "true prostate" and the unique site of origin of benign nodular hyperplasia. This latter concept is in accordance with the views of Franks.

Although the zones of the "true prostate" generally atrophy with age, some older patients show a prostatic morphology consistent with that found in younger men. Nevertheless,
eNeal described an age-related increase in typical hyperplasia, a proliferation of epithelial tissue from "persistently active glandular tissue" and this premalignant histology was associated with a high incidence of prostatic carcinoma. Of 170 small cancers which he identified in the 415 prostates studied, 148 were the peripheral zone, only 6 were in the central zone and none was found in the periurethral ind (McNeal, 1975).

Small foci of latent, asymptomatic, differentiated carcinoma are a common occurrence in the peripheral region of the prostate, although it is known that they rarely become clinically evident. There are data (Hirst and Bergman, 1974) to suggest that there is a lapse of at least 10 years between the early development of hyperplasia and the appearance of symptoms. Nevertheless, little is known about the time-view relating to the development of such latent carcinomas, and extensive growth and spread of the disease outside the prostate is possible long before the clinical signs are manifest.

Although the incidence of prostatic cancer is low in Japan, data from autopsies indicate that latent carcinoma of the gland is as prevalent among Japanese as in Caucasians of similar ages (Seuell and Dunn, 1965; Haenszel and Kurihara, 1968). Promotion of hyperplasia may well be dependent upon an abnormal endocrine situation in certain ageing males and a better understanding of such differences between Japanese and European men could be of particular interest. It is also important to carry out detailed epidemiological studies to identify biological factors in the earlier life of the male that might relate to the appearance of prostatic cancer in later life.

Direct Control Mechanism for the Prostate

The evidence currently available has not defined any real abnormality of the endocrine status of the ageing male that can be related to the aetiology of prostatic cancer (Griffiths et al., 1979). By accepted convention, changes in hormone concentration are sought in the systemic circulation. Recently however, investigations in our laboratories (Pierrepoint & Davies, 1973; Pierrepoint et al., 1974) have drawn attention to the anatomical link between the testis and prostate through the excurrent duct system, the seminaliferous tubules, vasa efferentia, epididymal ducts and the vasa deferentia which might provide a path for androgens to reach the prostate without entering the general circulation.

Experimental evidence now exists (Pierrepoint, 1975) to support the hypothesis that the androgen-maintained epididymis exercises unilateral control over the prostate via an intact venous drainage. Unilateral castration or vasectomy decreased RNA polymerase activity of the ipsilateral lobes of the rat prostate, an effect not observed after unilateral orchidectomy when the epididymis was not simultaneously removed with the gonad. The concentration of testosterone in the deferential vein of the dog was very much higher than in peripheral blood, and of the same order as that of the spermatic vein (Pierrepoint et al., 1975). The venous drainage of the cauda epididymidis is through the deferential vein, which could therefore directly transport androgen from the epididymis to the prostatic complex and which, at vasectomy, is often severed. It has been shown that radio-opaque material can be carried to the prostate directly from the deferential vein (Dhabuwala et al., 1978) and a retrograde flow of blood from the vessel would convey androgen-rich blood into the prostate gland (Dhabuwala and Pierrepoint, 1977).

A study of prostatic dysfunction and disease in men who have had vasectomies early in life, would provide an interesting study and is currently being conducted. In addition, however, this direct control of prostatic tissue requires investigation in relation to hormone therapy for prostatic cancer.

Hormonal Relationships of Prostatic Cancer

Clinical evidence suggests that prostatic cancer is in some patients functionally dependent on androgenic stimulation. Fig. 2 provides a relatively simple illustration of the various endocrine factors that are thought to be concerned with the regulation of prostatic growth and function, and which must be considered in relation to the development of pathological change and subsequent hormone therapy. Testosterone is considered to be the most important plasma androgen, although it must be remembered that the androgenic status of
the male normally involves high concentrations of other C19 steroids, such as androstenedione and dehydroepiandrosterone (DHA) and its sulphate, which are secreted by the adrenal gland. The latter steroids are present in relatively high concentrations in blood and are thus available for metabolism by the prostate to more potent androgens.

The development of sensitive radioimmunoassays for plasma hormone analysis has been
of particular benefit in understanding those endocrine changes associated with ageing that might relate to prostatic cancer. The investigations of Vermeulen and his colleagues (1972), supported by others (Pirke and Doerr, 1973; Nieschlag et al., 1973), have indicated that the concentration of plasma testosterone tends to decrease after the sixth decade (Table 1), although variations between individuals are large. This decrease was less marked than might be expected from the declining ability of the testis to secrete testosterone presumably because of a concomitant reduction in the metabolic clearance of the hormone (Kent and Acoc. 1966). The ability of both testis and adrenal to respond to exogenously administered androgenic hormones appears however to be retained despite ageing and loss of libido (Doerr and Pirke, 1974; Kley et al., 1975).

Other studies (Pirke and Doerr, 1973; Kley et al., 1974) have found that the plasma oestradiol-17β concentration increased with age in the clinically normal adult male (Table 1). The physiological importance of oestradiol-17β in the male is not well understood, and although the testis secretes both oestradiol-17β and oestrogen (Longcope et al., 1972; Weinstein et al., 1974), a large proportion of plasma oestrogen originates from the peripheral aromatization of androstenedione secreted by the adrenal gland (Longcope et al., 1969; MacDonald et al., 1972).

It has been reported (Hemseh et al., 1974) that there is an increased peripheral aromatization in the elderly male, which may account for this increased oestrogen : androgen ratio in later life. Although comparative data on the conversion of C18 steroids to oestrogens in normal men and in those with prostatic cancer are not yet available, the effect of a change in the oestro-

### Table 13.1. Plasma levels of testosterone, oestradiol-17β and SHBG binding capacity in relation to ageing

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Testosterone* (ng/ml)</th>
<th>Oestradiol-17β** (pg/ml)</th>
<th>SHBG Binding*** Capacity</th>
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<tr>
<td>20-30</td>
<td>6.16</td>
<td>16-6</td>
<td>5.2x10^-6M</td>
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<td>30-40</td>
<td>6.34</td>
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<tr>
<td>40-50</td>
<td>6.40</td>
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<td>50-60</td>
<td>5.82</td>
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<td></td>
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<td>60-70</td>
<td>4.62</td>
<td>25-6</td>
<td>8.9x10^-8M</td>
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<td>70-80</td>
<td>3.73</td>
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</tr>
<tr>
<td>80+</td>
<td>2.45</td>
<td></td>
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</table>

* Data from Vermeulen et al. (1972). ** Data from Pirke and Doerr (1973). *** Data from Vermeulen et al. (1971)
testosterone and prolactin to affect prostatic weight (Chase et al., 1957). However, studies comparing plasma hormone levels of clinically normal, asymptomatic men with those of patients with prostatic carcinoma have generally failed to show any marked differences (Harper et al., 1976; Bartsch et al., 1977; Hammond et al., 1977).

A more detailed study was undertaken in the United Kingdom under the auspices of the British Prostate Study Group to investigate the relationship between plasma hormones and various clinical parameters. Patients with histologically proven carcinoma of the prostate, without previous therapy, were classified according to primary tumour grade and metastatic status by a modification of the UICC classification, recommended by a British sub-committee (Wallace et al., 1975). The presence of clinically evident metastases was assessed by X-ray examination and isotopic bone scanning.

Some of the data presented in a recent report of the Group (British Prostate Study Group, 1979) are given in Table 2. They clearly indicate that in patients without obvious metastases there is no difference in the group means of the hormone concentrations, associated with the progression of the disease from the T0 to the T4 stage. Wide variations in plasma hormone concentrations were observed in this extensive study, and testosterone concentrations of elderly men in their 70s and 80s were often comparable with those of younger men.

Mean FSH, LH and prolactin levels did not differ significantly between the stages of the primary tumour group and were unrelated also to the presence of metastatic disease. However, multivariate analysis of the data clearly showed that growth hormone concentration was a major discriminating factor between patients with and without metastases, the GH values being significantly larger ($P<0.02$) in the former. The clinical relevance of such an observation is, as yet, not clear.

This co-operative study illustrates both the value of a multicentre investigation to generate data from a large number of patients who have been assessed clinically in a standardized manner, and also the potential of multivariate statistical analysis in the evaluation of the

<table>
<thead>
<tr>
<th>Table 13.2</th>
<th>Plasma hormone concentrations of patients with prostatic carcinoma, separated according to (a) UICC classification of primary tumours, (b) metastatic spread</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Plasma hormone concentrations</strong></td>
</tr>
<tr>
<td></td>
<td><strong>(a) Primary tumour category</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Age (years)</strong></td>
</tr>
<tr>
<td>T0 category</td>
<td>mean</td>
</tr>
<tr>
<td>(n = 24)</td>
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<tr>
<td>T1 category</td>
<td>mean</td>
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<td>(n = 14)</td>
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<td>(n = 31)</td>
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<td>T3 category</td>
<td>mean</td>
</tr>
<tr>
<td>(n = 73)</td>
<td>SD</td>
</tr>
<tr>
<td>T4 category</td>
<td>mean</td>
</tr>
<tr>
<td>(n = 21)</td>
<td>SD</td>
</tr>
<tr>
<td>(b) Metastatic spread</td>
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</tr>
<tr>
<td>Patients with metastases M1</td>
<td>mean</td>
</tr>
<tr>
<td>(n = 72)</td>
<td>SD</td>
</tr>
</tbody>
</table>

T refers to testosterone; E$_2$ to oestradiol-17β; n to numbers of patients; SD to standard deviation.
complex endocrine status of patients with prostatic carcinoma.

It is possible, of course, that a more appropriate time to study such hormone profiles is earlier in life, before the disease is manifest. Comparatively little has been done to consider the various social, sexual and hormonal influences on the earlier life of the male, that might "prime" the prostate, such that precancerous changes become established in later life. On the other hand, it may well be that biochemical changes within the prostatic cell itself could provide an increased responsiveness or sensitivity to normal hormone concentrations.

**Metabolism of Androgen by Prostate: Androgenic Effects**

It is now well accepted that testosterone is actively metabolized by prostatic tissue. The classical experiments of Farnsworth and Brown (1963) demonstrated the formation of a reduced metabolite of testosterone, 5α-dihydrotestosterone (Fig. 3), by human prostatic tissue and there is now a considerable body of evidence to indicate that it is this metabolite which is the active androgen within the cell (Bruchovsky and Wilson, 1968a,b; Anderson and Liao, 1968; Mainwaring, 1975; Griffiths et al., 1979). There is also evidence that other metabolites of testosterone, synthesized in the prostate (Fig. 3), particularly the various 5α-androstanediols may have specific biochemical roles to play (Baulieu et al., 1968a,b; Farnsworth, 1972) in the control of prostatic growth and function. The pattern of testosterone metabolism in the prostate may well provide a regulatory influence, and imbalance may lead to dysfunction of the gland.

It is established that 5α-dihydrotestosterone regulates prostatic growth through a well-defined process, involving firstly a specific association with a cytoplasmic 'receptor' protein (Fig. 2) followed by translocation of this steroid-receptor complex to the nucleus. The specific binding of the complex to nuclear acceptor sites elicits transcriptional processes and mRNA synthesis, thereby regulating genetic expression and the integrated cellular processes concerned with tissue growth.

After association of the receptor complex with the chromatin, the precise mechanism by which 5α-dihydrotestosterone leaves the nucleus and then the cell, is poorly defined. The steroid may be directly secreted from the prostate or possibly metabolized to an androstanediol, but obviously to control normal prostatic function, it is necessary to maintain an effective, intracellular concentration of the 5α-dihydrotestosterone-receptor complex. It is interesting that a 5-fold higher concentration of endogenous 5α-dihydrotestosterone, compared to the normal prostate, has been demonstrated in benign hypertrophic prostatic tissue (Siiteri and Wilson, 1970). Obviously, however, increased testosterone entry into the cell, imbalance of C19-steroid-metabolizing enzymes or inhibited release could each result in androgen accumulation and promotion of prostatic growth. Increased concentrations of receptor protein could also produce this accumulation of effective androgen and currently, a considerable amount of research is directed to the analysis and characterization of steroid receptor protein in both normal and abnormal human prostatic tissue.

At present the available data are limited, but preliminary results from our own laboratory (Davies, 1978; Griffiths et al., 1979) suggest that there may be a higher concentration of androgen receptor in hypertrophic and malignant human prostatic tissue than in the normal gland, and also a higher affinity of steroid-receptor interaction in the neoplastic tissue. It is important to determine whether receptor characteristics in diseased prostatic tissue differ from those in normal tissue, for this could influence the affinity of the protein for either 5α-dihydrotestosterone or other metabolites of testosterone, or possibly even for oestradiol-17β.

The analysis of breast-tumour tissue for oestradiol-17β receptor protein has proved of value in predicting a likelihood of response to endocrine therapy in patients with mammary carcinoma (McGuire et al., 1975), and it is possible that analysis of the androgen receptor content of prostatic carcinoma may prove equally valuable in determining which patients will respond to anti-androgen therapy. The nature of the available tumour tissue for such analysis is obviously crucial and the use of a cold-punch to collect biopsy samples, rather than the 'hot loop', would facilitate analysis of this labile receptor material.

Receptors for oestradiol have also been found in human prostatic tissue (Wagner et al., 1975; Hawkins et al., 1975; Bashirelahi and Arm-
strong, 1975) and recent studies from this laboratory (Chaisiri and Pierrepoint, 1979) have shown such receptors in the stromal tissue of the canine prostate. The physiological significance of the prostatic oestrogen receptor is as yet unknown, although oestradiol-17β is suspected of being implicated in the pathogenesis of prostatic disease, particularly benign prostatic hypertrophy.

Rationale of Anti-androgen Therapy

We have discussed above the evidence that malignant prostatic growth may be dependent upon androgen stimulation. The removal of approximately 90 per cent of plasma testosterone (Baird et al., 1969) by castration offers a rationale for this form of treatment. However, the mechanism by which administered oestro-
prostatic cancer: rationale for hormonal therapy

It is probable that the major anti-androgenic effect of diethylstilboestrol on the prostatic carcinoma is exercised indirectly via the pituitary (Fig. 2), by suppressing LH secretion and thus decreasing testicular synthesis and secretion of testosterone. Figure 4 illustrates the changes in plasma gonadotrophin concentrations observed in our own studies of patients with prostatic carcinoma treated with either diethylstilboestrol (1 mg t.d.s.) or Honvan, di-ethyldiethylstilboestrol diphasate (100 mg b.d.). The concentrations of both FSH and LH decreased to undetectable levels after seven days of treatment with either regime.

Corresponding changes in plasma testosterone levels were seen, with the concentration falling to below 100 ng/100 ml plasma within seven days (Fig. 5) again with no difference between the high and low dose of oestrogen. It seems reasonable, that if reduction of plasma testosterone levels is the principal mode of action of oestrogen treatment, then the lower, daily dose of 3 mg diethylstilboestrol should be as effective in clinical practice as the higher dose of the diphasate. A dose of 1 mg per day has been shown not to be so effective. Concentration of oestradiol-17β in plasma fell to 50 per cent of pre-treatment with either dosage level (Fig. 5) and was maintained as such for at least 3 months after treatment.

Plasma prolactin levels increased significantly (Fig. 5) after treatment with either diethylstilboestrol or its diphasate, and growth hormone concentrations increased in some patients but not in others. Honvan administration pro-

![Graph](image-url)

Figure 13.4. Changes in plasma FSH and LH in patients with prostatic carcinoma before and after treatment with diethylstilboestrol (○) 1 mg t.d.s., or Honvan (●) 100 mg b.d.)

duced the greater effect on prolactin. The physiological significance of the change is as yet uncertain, but evidence is accumulating to indicate that prolactin acts synergistically with LH in regulating testicular activity (see review by Griffiths et al., 1979). There are also data to suggest that prolactin may exercise some control of certain biochemical processes within the prostate, for it accentuates certain androgenic effects on prostatic function, and recently specific binding sites for prolactin on cellular membranes were demonstrated (Aragona and Friesen, 1975; Kledzik et al., 1976).

It has been suggested (Harper et al., 1976)
Figure 13.5. Changes in plasma testosterone, oestradiol-17β, prolactin and growth hormone in patients with prostatic carcinoma before and after treatment with diethylstilboestrol (O) 1 mg t.d.s., and Honvan (●) 100 mg b.d.
that elevated prolactin levels might be implicated in the eventual relapse of the patient by promoting androgen production by the adrenal. There are data to indicate that prolactin can stimulate androgen production (Boyns et al., 1972; Millington et al., 1976; Vermeulen et al., 1977), but in our studies there is generally no relative increase in plasma testosterone associated with subsequent relapse of the patient under oestrogen therapy. Monitoring patients' plasma testosterone concentrations during adequate, extended oestrogen therapy indicates an undulating pattern with a mean value of the order of 60 ng/100 ml.

Adrenal stimulation or dexamethasone suppression tests on such patients show that the source of residual plasma testosterone (or more specifically of its precursor, androstenedione) is the adrenal gland (Robinson and Thomas, 1971; Cowley et al., 1976). Monitoring the plasma testosterone concentration therefore provides a good guide to patient compliance with oestrogen therapy but since relapse can occur with gonadal function completely suppressed, it would seem that the analysis of other tumour markers, possibly polyamines, hydroxyproline or CEA, will offer a more effective means of predicting the progression of the disease and of metastatic spread.

Despite considerable controversy over the years, there is now evidence to indicate that at least part of the anti-androgenic effect of oestrogen therapy is promoted by a direct inhibition of testicular production of testosterone (Samuels et al., 1964; Oshima et al., 1967; Danutra et al., 1973a), in particular by decreasing the activity of both the 17β-hydroxysteroid dehydrogenase and 17α-pregnen-C17,20-lyase (Slaunwhite et al., 1962; Danutra et al., 1973b) (Fig. 6). It is interesting that chlorotrianisene, which has been used for the treatment of carcinoma of the prostate, has been reported to exert its effect not at the pituitary level, but by a direct action on the testis (Baker et al., 1973).

There is additional evidence, although still equivocal, to suggest that oestrogens may have a direct effect on prostatic tissue at the cellular level, although the precise mechanism is not understood. The experiments of Goodwin and his colleagues (Goodwin et al., 1961) demonstrated a direct inhibitory effect of oestrogen on the androgen-maintained prostatic secretion of hypophysectomized dogs and later studies showed that oestrogen administration markedly influenced C19-steroid metabolism by the prostate, with a reduced formation of 5α-dihydrotestosterone from testosterone (Shimazaki et al., 1965; Farnsworth, 1969; Groom et al., 1971) and a corresponding increased synthesis of the less androgenic steroids, androstenedione and 5α-androstanedione (Fig. 3) (Leav et al., 1971; Danutra et al., 1973b).

The rationale behind the administration of large doses of diethylstilboestrol diphasphate (Honvan) is that the free and active form of the hormone would be released at the required site in the gland by prostatic phosphatases. To date, there is little evidence of such a concentration of administered oestrogens within prostatic tissue.

Secondary Endocrine Therapy

While loss of control of tumour growth after initial response to oestrogen therapy may reflect

![Figure 13.6. Pathway for the synthesis of testosterone from progesterone by rat testicular tissue. 17β-OHSD = 17β-hydroxy-steroid dehydrogenase.](image)

the tumour's progress to autonomy, the endocrinology of the situation does not confirm this. It is often reported that castration, subsequent to a relapse after oestrogen therapy, is followed by a further clinical response, and this has become standard treatment in many centres. It is however difficult to accept that, with the low levels of LH and FSH resulting from the administration of adequate oestrogen, the testes are the source of the residual plasma testosterone (Fig. 5).

In fact ACTH stimulation, or dexamethasone
suppression, of adrenal activity result respectively in elevation or complete inhibition of plasma testosterone levels (Griffiths et al., 1976) and evidence from detailed studies of many such patients with prostate carcinoma suggests that the adrenal cortex is the source of the residual circulating testosterone. The observation that the clinical results of adrenalectomy or ablation of the pituitary are not very encouraging may be ascribed to the fact that the patients are generally very debilitated by the time they come to such major surgery. Possibly the treatment of choice to eliminate plasma testosterone completely would be subcapsular orchidectomy together with aminoglutethimide to inhibit adrenal production of androgen.

**Subcapsular Orchidectomy**

Although subcapsular orchidectomy is a more cosmetically acceptable operation, doubt has often been expressed about its effectiveness in removing all tissue capable of producing testosterone (McDonald and Calams, 1959). Data shown in Table 3 (from a study made by the Tenovus Institute and Mr. J. C. Gingell, Southmead Hospital, Bristol) clearly indicate

<table>
<thead>
<tr>
<th>Table 13.3. Plasma hormone concentrations in patients with prostatic cancer</th>
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<tr>
<td><strong>(a) In patients before treatment and in those after orchidectomy</strong></td>
</tr>
<tr>
<td><strong>Testosterone (ng/ml)</strong></td>
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<td>Pre-treatment group</td>
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<td></td>
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<td>Post-subcapsular orchidectomy</td>
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<td>Post-total orchidectomy</td>
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<th><strong>(b) In patients after orchidectomy before and 24 hr after HCG (3000 i.u., i.m.) admin.</strong></th>
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<tr>
<td><strong>Patient No.</strong></td>
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<td><strong>Before HCG</strong></td>
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<td>A Total orchidectomy group</td>
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<td>B Subcapsular orchidectomy group</td>
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<td>C Non-castrated elderly men</td>
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Statistical analysis using Wilcoxon-matched pairs signed-rank test give \( P < .04 \) for testosterone and oestradiol levels before and after HCG in group C. \( n = \) number of patients, S.D. = standard deviation.
the endocrinological effectiveness of subcapsular orchidectomy. Plasma hormone concentrations and their response to human chorionic gonadotrophin (HCG) stimulation were determined in patients who had undergone either total or subcapsular orchidectomy. The concentration of testosterone, oestradiol-17β, FSH and LH, after castration by either procedure, were significantly different from pre-treatment values. The fall in testosterone and the elevation of gonadotrophin levels after surgery suggest that the two operations were equally effective.

Testosterone and oestradiol-17β concentrations were measured before and 24 h after i.m. injection of 3000 i.u. of HCG to 13 patients who had been orchidectomized at least three months previously, and compared with results obtained from similar stimulation tests on five elderly men (64–94 years) with no clinical evidence of prostatic disease. There was no significant change in steroid concentrations after HCG administration to either group of orchidectomized patients (Table 3), clearly indicating that after that period of time there is no androgen formation by the residual tunica albuginea after subcapsular orchidectomy.

Alternative Anti-androgenic Hormonal Therapy

While the administration of 1 mg diethylstilboestrol three times daily will effectively suppress pituitary activity for a time, the injectable, long-acting polyoestradiol phosphate (Estradiurin) only weakly inhibits gonadotrophin secretion (Jönsson et al., 1975) and the residual levels of testosterone in the plasma are relatively high. Another alternative to diethylstilboestrol is Premarin, a mixture of conjugated equine oestrogens with half the oestrogenic activity of diethylstilboestrol (Israel, 1967). It has been used in prostatic cancer at a dose level of 15 mg/day (Boyns et al., 1974). The use of the synthetic and potent ethinyl oestradiol was found to effectively lower testosterone levels in plasma at a dose of 50 µg, twice daily (Shearer et al., 1973).

Systemic administration of progestational steroids has been known for many years to produce anti-androgenic effects and certain of these compounds, hydroxyprogesterone cap-

roate (Delalutin), cyproterone acetate (Androcur) and medroxyprogesterone acetate (Provera) have been used for the treatment of carcinoma of the prostate. As with oestrogens, at least part of the anti-androgenic effect of a progestagen is through its action on the pituitary, inhibiting LH release and subsequently lowering the plasma concentration of testosterone.

In addition however, it has been shown (Southren et al., 1977) that the administration of the potent progestagen, medroxyprogesterone acetate, increases the metabolic clearance rate of testosterone, presumably affecting its peripheral metabolism and resulting in a decreased availability of the androgen to the target tissues. This may partly explain the observation of Dorfman (1965) that progestagens antagonize the target-organ effects of exogenously administered testosterone in castrated animals, although it appears that progestagens can also interfere to some extent, with the binding of androgens (Fig. 2) to intracellular receptors of the prostate (Mowszowicz et al., 1974).

Cyproterone acetate is also an effective competitor for 5α-dihydrotestosterone-receptor sites (Fang et al., 1969; Mangan and Mainwaring, 1972) and its administration to rats results in prostatic atrophy. Furthermore, by similarly affecting pituitary androgen-receptors, but acting as an agonist with weak androgenic properties, it also decreases the release of LH.

Our understanding of the biochemistry of androgen action within the target cell may ultimately allow the development of an effective anti-androgen with a selective action against prostatic cancer and with no other endocrine effects. Interest and excitement were considerable, when flutamide (4'-nitro-3'-trifluormethylbutyrylanilide) was reported by Neri et al. (1972) to have potent anti-androgenic effects in the orchidectomized, androgen-treated rat, and was devoid of adverse side-effects. This work was confirmed by Liao et al. (1974) and Mainwaring et al. (1974), the latter indicating furthermore, that a hydroxylated metabolite of flutamide was probably effective at the receptor level. The work with flutamide emphasizes the potential of such development and highlights the way of further research.
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Measurement of Diethylstilbestrol in Plasma from Patients with Cancer of the Prostate

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ABSTRACT

A specific radioimmunoassay has been developed for diethylstilbestrol (DES), using an antiserum raised against DES monooxymethyl ether and a tritium-labeled radioligand. Prior to radiomunnoassay, a fraction enriched in DES is obtained from a dichloroethane extract of plasma using Sephadex LH-20. The specificity of the assay is good, and the sensitivity (130 pg/ml) is adequate for accurate determination of DES in plasma from prostatic cancer patients treated with the drug. The precision is satisfactory, with an interassay coefficient of variation of approximately 10% at concentrations of approximately 1 ng/ml, and the blank values are negligible. Excellent agreement (r = 0.96) is observed between data obtained by radioimmunoassay and those obtained by a procedure using gas chromatography-high-resolution mass spectrometry. DES concentrations in the plasma of six treated (1 mg DES three times daily) patients were in the range 0.15 to 6.0 ng/ml. Increases in plasma concentration were observed within 2 hr of administration, with secondary rises occurring 5 to 6 hr later. Plasma testosterone concentrations were low in four of the patients; in a single subject, relatively high levels of testosterone were further elevated following administration of luteinizing hormone-releasing hormone.

INTRODUCTION

Although there is still controversy as to the most effective form of therapy for early prostatic cancer and on the relative merits of surgery and radiotherapy (16), it has long been accepted that endocrine therapy, generally in the form of DES treatment, is the most effective for the management of the advanced progressive disease. There is still discussion concerning the precise mode of action of DES (10), though it is generally accepted that its principal effect is indirect, decreasing testicular secretion and secretion of testosterone by inhibiting luteinizing hormone secretion from the pituitary. There is, however, evidence for a direct action of DES on the testis and on the prostate gland (6), and it is possibly this latter effect which prompted the use of large doses (>200 mg/day) of DES phosphate (Honvan) for the treatment of prostatic cancer. Endocrine studies of the type undertaken by this Institute (11), however, have clearly indicated that a dose of 1 mg DES tds effectively decreases plasma testosterone levels and does not result in concomitant increases in the concentrations of prolactin and growth hormone. Such observations, and the concern over side effects (3), have tended to direct clinicians towards treatment schedules using low-dosage DES.

Despite its extensive use, there is no information on the levels of DES in patients being successfully treated or in those who apparently fail to respond to such therapy. Initially, 70% of treated patients respond clinically to DES therapy, but the majority eventually relapse. It remains to be determined whether this is related to a change in the hormone sensitivity of the tumor resulting from dedifferentiation or to changes in the pharmacokinetics of DES in certain patients. RIA's with the potential to measure the plasma concentrations of DES have been described (2), but no data on the levels in patients have been reported. Here we report a sensitive RIA for DES and illustrate its application to the determination of concentrations in the plasma of patients receiving 1 mg tds.

MATERIALS AND METHODS

Materials. [monoethyl-3H]DES (50 to 100 Ci/mmole) was purchased from the Radiolabeled Chemical Centre, Amersham, United Kingdom, and stored in ethanol and toluene solution (50 µCi/ml) at –20°C. For use in the assay, an aliquot was taken, the solvent was evaporated under nitrogen, and the residue was dissolved in assay buffer (see below) containing antiserum. The working solution contained 35 to 40 pg/100 µl (about 0.125 µCi/ml). Nonradioactive DES, hexestrol, and various steroids were purchased from Sigma London Chemical Company, Poole, Dorset, United Kingdom; the sodium salt of DES diphasphate (Honvan) was obtained from Ward Blenkinsop & Co., Ltd., London, United Kingdom; -hydroxydienestrol, stored as the triacetate, and -dienestrol were a gift from Dr. M. Metzler, Institut fur Pharmakologie und Toxikologie der Universität Würzburg, Würzburg, West Germany; DES monogluconide and DES digluconide were kindly provided by Dr. K. Krohn, Institut für Organische Chemie und Biochemie, Hamburg, West Germany. Solutions of these compounds were stored in ethanol (1 mg/10 ml) at 4°C. Dichloroethane, purchased from Koch-Light Laboratories, Ltd., Colnbrook, Buckinghamshire, United Kingdom, and dichloromethane from BDH Chemicals, Poole, Dorset, United Kingdom, were used without further purification. Ethanol and methanol were obtained from James Burroughs, Ltd., London. A solution of 0.01 M phosphate buffer (pH 7.4) containing 0.9% sodium chloride and 0.1% sodium azide was stored at 4°C; the assay buffer additionally included gelatin (0.1%). Dextran-coated charcoal was prepared by dissolving dextran T-70 (0.06%, Pharmacia Ltd., London) in assay buffer and then adding HCI-washed activated charcoal (No. C-4336, 0.6%; Sigma London Chemical Company). The suspension was stirred at 4°C for 4 hr before use. Sephadex LH-20 from Pharmacia,
**Patients.** Blood was collected from 6 patients with historically proven carcinoma of the prostate using an indwelling catheter over periods of approximately 8 hr (5 patients) or 16 hr (one patient). Five of the patients had received DES, 1 mg tds, for at least 3 months prior to the study. The sixth patient was investigated 6 days, 3 months, and 6 months after commencement of therapy. They were hospitalized for 48 hr and ate, slept, and drank as appropriate. DES was administered at 6:30 a.m., 12:30 p.m., and 6 p.m. Samples of blood (3 ml) were collected from 5 patients at 30-min intervals from 7:30 a.m. until 4 p.m. except between 9 and 10 a.m. when more frequent sampling occurred. Blood was similarly collected on the second day between 7:30 a.m. and 12 noon, and LH-RH (100 µg i.v.) was administered at 9 a.m. The sixth patient had blood collected at 30-min intervals from 8 p.m. to 12 noon the following day. Samples were centrifuged, and plasma was stored at −20° until assayed for testosterone (12) and DES.

**Synthesis of Antigenic Conjugate.** The O-carboxymethyl ether of DES was prepared according to the general procedure of Rao and Moore (17). Pure DES monocarboxymethyl ether was recrystallized from toluene:hexane (m.p. 157°). The product was characterized by field desorption mass spectrometry using a Varian MAT 731 instrument (MH+ = m/z 327) and by IR spectroscopy. A conjugate, produced by coupling the hapten to bovine serum albumin by the mixed anhydride reaction (7), had a molar incorporation ratio of 12:1 (DES:bovine serum albumin).

**Antiserum.** Rabbits were immunized with the conjugate (15), and one antiserum with a titer of 1/8000 was selected for use in the assay. The antiserum was stored at −20° and diluted to 1/100 with assay buffer.

**Extraction from Plasma.** Dichloroethane (3 ml) was added to plasma (0.5 ml). The tubes were shaken for 12 min and centrifuged (2.5 x 10^3 rpm) for 2 min, and 2 ml of the lower dichloroethane layer were removed to a clean tube. The solvent was evaporated under nitrogen.

**Sephadex LH-20 Chromatography.** Each extract was dissolved in the chromatography solvent (dichloromethane:methanol, 94.6: v/v; 2 x 0.2 ml) and applied to a column (4 x 0.6 cm) of Sephadex LH-20, swollen in the same solvent. The first 3-ml eluate was discarded, and the DES fraction was collected in the next 3.0 ml. The fraction was dried and dissolved in methanol (500 µl). The reproducibility of the chromatographic separation was improved by the addition of a layer (1 x 2 mm) of sand to the top of each Sephadex LH-20 column. Duplicate experiments using [3H]DES indicated excellent reproducibility: mean recovery was 96.3 ± 2.9% (n = 6). Eluate (30 ml) was collected from a large column (30 x 1.3 cm) containing swollen Sephadex LH-20. This eluate was used in the preparation of the standard curve to correct for column blank effects. Aliquots (3 ml) were measured into tubes, one for each point on the standard curve, and dried, and methanol (500 µl) was added.

**RIA.** Triplicate aliquots (100 µl) of the methanolic solution were dried under N2. Antiserum, radioligand solution (200 µl) was added, and the mixture was incubated (37°, 2 hr). For the standards, solutions of 20 to 200 pg DES per 10 µl were prepared in ethanol. Triplicate aliquots (10 µl) were mixed with aliquots (100 µl) of column solvent residue in methanol, dried, and treated as the samples. After equilibration, the free and bound fractions were separated by addition of a suspension of dextran-coated charcoal (1 ml) to all tubes except those used for determination of total counts, to which assay buffer (1 ml) only was added. The solutions were mixed, allowed to stand for 10 min, and centrifuged (12 min; 2.5 x 10^3 rpm). The supernatant was decanted into vials, scintillant (6.5 ml) was added, and the radioactivity was measured for 2 min; the tubes used for determination of total counts contained approximately 10,000 cpm. Plasma concentrations were calculated using a Hewlett-Packard 9810 A programmable desk-top calculator. The program used a rectangular hyperbola fit to the standard curve. Recoveries were monitored in preliminary assays by adding an internal standard of radioactive DES to the plasma aliquot before extraction. Recovery of DES (after allowance for the use of aliquots in the assay) was calculated to be 87.7% ± 6.8%; this was considered adequate, and addition of the internal standard was discontinued. Correction for recovery was not included in the calculation.

**Analyses by GC-MS.** A solution of DMS in drug-free plasma was prepared by addition of 50 µl of an ethanolic solution (10 µg/ml) of DMS to 10 ml of plasma. After mixing and standing overnight, 50 µl were added as internal standard to each aliquot (0.25 to 1.0 ml) of plasma to be analyzed. The plasma was extracted with diethyl ether (8 ml), the aqueous layer was frozen, and the extract was removed and dried under a stream of nitrogen. The residue was dissolved in 3 x 100 µl methanol:water:chloroform (80:30:15, v/v; Solvent A) and applied to a column (2 x 0.6 cm) of triethylaminoxypropyl-Lipidex 5000 (4), swollen in Solvent A. Polar constituents were removed by elution with 2 ml of Solvent A. Nonpolar constituents were subsequently eluted with 2 ml of methanol:chloroform (80:15, v/v; Solvent B). Finally, DES and DMS, together with naturally occurring weakly acidic components, were recovered by elution with 2 ml of Solvent B, acidified by the addition of a small quantity of solid carbon dioxide. The final column eluate was dried under nitrogen, and the DES and DMS were converted to the TBDMS derivatives by reaction with tert-butyldimethylchlorosilane:imidazole:dimethylformamide (1:1:6, v/v; Applied Science Laboratories, State College, Pa.) Excess reagent was removed by filtration through a short column of Sephadex LH-20 (9).

Standard mixtures of DES and DMS for the preparation of the standard curve were obtained in 2 ways. First, aliquots of stock solutions of DES and DMS in ethanol were mixed in appropriate proportions. Second, solutions of DES in plasma were prepared, and appropriate aliquots were diluted to 1 ml with drug-free plasma to give total amounts of DES equal to those in standard mixtures prepared from stock ethanolic solutions. The plasma samples were then treated as for samples of unknown DES concentration.

GC-MS was performed using a Varian MAT 731 double-focusing instrument. Samples were achieved on a glass column (length 3 m, inside diameter 3.5 mm) packed with 1% OV-1 on Gas-Chrom Q (100 to 120 mesh) at 280°. The mass spectrometric resolution was 8000. The ion source temperature was 200°, and the electron energy was 70 eV. Analyses were carried out in the single-ion monitoring mode with sequential detection of ions of m/z 468, 2880 for cis- and trans-DMS bis-TBDMS ether and m/z 496, 3193 for cis- and trans-DES bis-TBDMS ether. Each mass/charge ratio corresponded to the respective DES derivative.
molecular ion.) The ratio (R) of peak areas corresponding to the DES and DMS derivatives were determined using the expression $R = \frac{\text{HT}_{\text{DES}} + \text{HT}_{\text{DMS}}}{\text{HT}_{\text{DES}}}$, where $\text{HT}_{\text{DES}}$ is the product of the height of the peak attributed to cis-DES TBDMS and the gas chromatographic retention time (which is proportional to peak width) of that component. Values for peak area ratios were converted to DES concentrations by reference to the standard curves derived as described. Linear regression of ratio values derived from analyses of "spiked" plasma samples (y) on values derived from analyses of standard mixtures (x) gave the equation $y = 1.067x - 0.038$ ng/ml (correlation coefficient, $r = 0.997$), indicating excellent agreement between the 2 methods and confirming the ability of the assay procedure to determine accurately the concentrations of DES in plasma supplemented with the drug. The standard curve derived from mixtures of aliquots of stock ethanolic solutions of DES and DMS was used for the determination of unknown concentrations.

RESULTs

Characteristics of the RIA. The cross-reactions of the antisera (used at a final dilution of 1:8000) are listed in Table 1. There is a negligible degree of cross-reaction with the naturally occurring steroids and only a small degree of cross-reaction with the major metabolites (13) β-dienestrol and α-hydroxydienestrol, indicating acceptable specificity for the hex-3-ene substructure of DES. The degree of cross-reaction with the glucuronides of DES is high, but, since an extraction step is included in the analytical procedure, no glucuronides are expected in the RIA. The cross-reaction with hexestrol (11%), although relatively high, is clinically irrelevant.

A typical standard curve for the DES assay, over the range of 0 to 200 pg, with a corresponding precision profile, is shown in Chart 1. Addition to the standards of column solvent eluate, in methanol, decreases the binding by approximately 8% and is a necessary part of the procedure. The sensitivity of the assay, calculated using the expression $tS/\sqrt{N}$ (5), gave a curve value of 8.8 pg corresponding to 0.132 ng/ml. The accuracy of the method, determined by repeatedly measuring 3 pools of plasma, spiked with known amounts of DES, was similar for high, medium, and low values (Table 2). Assay precision, determined by calculating within-assay and between-assay coefficients of variation for 3 quality control plasma pools, is shown in Table 3.

Comparison of Analytical Data from RIA and GC-MS. Twelve plasma samples from patients treated with DES, representing a spread of concentrations across the range observed in this study, were analyzed by both RIA and GC-MS procedures (Chart 2). Linear regression of RIA values (y) on GC-MS results (x) gave the equation $y = 0.878x + 0.051$ (correlation coefficient, $r = 0.96$; if single outlier is omitted). Agreement between the methods was thus excellent, particularly since no allowance for recovery was made in the RIA. The single discrepancy when the DES concentration was high may suggest incomplete extraction in the RIA in this example. With
few samples of relatively high concentration, it has not, however, been possible to evaluate this further.

**DES Levels in Treated Patients with Prostatic Cancer.** Plasma samples from 6 patients were assayed for levels of DES and testosterone. Patient F. C. showed an expected marked rise in the concentration of DES approximately 1.5 to 2.0 hr after DES administration (1-mg tablet; 6:30 a.m.) and a less significant rise 6 hr later (Chart 3). The first peak was also observed on Day 2. Similar patterns of DES concentration were observed with Patient J. W. (Chart 4) and 3 others not illustrated.

Plasma testosterone concentrations in Patient F. C. (Chart 3) were higher than expected after DES therapy for several months. The circadian rhythm (18) suggests a substantial adrenal contribution to the plasma androgen level, although in this patient an LH-RH stimulation test also resulted in a significant elevation in the concentration of plasma testosterone (Chart 3), indicating a responsive pituitary-testicular axis. Low levels of testosterone were found in Patient J. W. (Chart 4) and in the 3 other patients studied; there was no response in any of these to the administration of LH-RH.

Plasma DES concentrations in a single patient (A. F.) throughout the night, 6 days, 3 months, and 6 months after commencing therapy are shown in Chart 5. The rise in DES levels after the 6:30 a.m. DES tablet was the same at each period. Rises in DES concentration were in each case observed 4 to 6 hr after administration of the 5 p.m. tablet, presumably corresponding to the secondary rises noted above.

**DISCUSSION**

The development of this routine RIA for DES will permit studies of the relationships between DES concentration in plasma, hormone secretion, and clinical response of treated prostatic cancer patients. The assay described has satisfied accepted performance criteria and has been validated by reference to a highly specific GC-MS procedure. The antiserum displayed high specificity, although inclusion of a Sephadex LH-20 chromatography step in the procedure was essential to remove interfering plasma constituents.

The range of DES concentrations during the day in the 6 patients studied was 0.15 to 5.9 ng/ml. The observed second peak of DES concentration in plasma after the p.o. dose could arise when DES glucuronides are hydrolyzed in, or reabsorbed from, the large intestine approximately 5 hr after the tablet has been taken (14). Administration p.o. of [14C]DES glucuronide results in a rise in plasma radioactivity 3 to 6 hr later (8).

The data from Patient F. C. clearly indicate that, although treatment at the level of 1 mg DES tds is sufficient to suppress testicular activity in most patients (11), higher doses may be required for others. A responsive pituitary-testicular axis was demonstrated in Patient F. C., and furthermore, rhythmic se-
cretion of testosterone was observed. This testosterone profile may be related to the secretion of adrenal androstenedione, the precursor of plasma testosterone. These studies again illustrate the difficulties of analyzing single daily samples for the assessment of the endocrine status of patients with prostatic cancer (19). The development of a specific RIA procedure for the measurement of DES allows the investigation of plasma levels of the drug in relation to changes in the rhythmic secretion of hormones during treatment and will facilitate a study of patient variability in relation to response.

ACKNOWLEDGMENTS

We thank Dr. M. Metzler and Dr. K. Krohn for gifts of various DES metabolites and Dr. B. G. Joyce for the determinations of plasma testosterone.

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Changes in male salivary testosterone concentration with age

By
G. F. Read, M. E. Harper, W. B. Peeling and K. Griffiths

Using a specific and sensitive radioimmunoassay for testosterone, the concentration of the steroid has been determined in samples of saliva taken from men of various ages. The results clearly show that the mean salivary testosterone concentration, which related to the plasma-free, non-protein-bound steroid concentration, falls with advancing age from 236 pmol/l in the 3rd decade to 100 pmol/l in the 8th decade.

Keywords: saliva — testosterone — age — immunoassay.

Although clinical data clearly indicate that carcinoma of the prostate in man is, to some extent, functionally dependent upon androgenic stimulation, the role of the various plasma C-19-steroids in the aetiology of the disease remains to be determined. Testosterone is still considered the most important plasma androgen (Griffiths et al. 1979) and the change in the concentration of the hormone in relation to ageing and prostatic dysfunction has been the subject of many investigations (Kent & Acone 1966; Vermeulen et al. 1972; Baker et al. 1976). It is now generally accepted that plasma testosterone concentration declines after the sixth decade (Vermeulen et al. 1972), although wide individual variations are found. It is also recognised however that the free, non-protein-bound testosterone, considered the biologically active moiety in plasma (Ravenfield 1973) and constituting approxi-
mately 2% of the total (Westphal 1971) would provide the most effective parameter of androgen status. There is some evidence that the plasma-free testosterone level falls with ageing (Vermeulen et al. 1971, 1972; Pirke & Doerr 1975), but current procedures for the determination of the free hormone are relatively difficult and have inherent errors.

The development in the Institute of sensitive assays for the measurement of salivary steroid concentrations (Walker et al. 1978, 1979, 1980; Turkes et al. 1980) which correlate well with the level of non-protein-bound hormone (Smith et al. 1979; Baxendale et al. 1980) now provides the means of developing detailed studies of the endocrine status of individuals, using non-invasive means of sample collection. A preliminary study of the changes in salivary testosterone concentration with age is described in this communication.

Materials and Methods

Mixed whole saliva samples were collected between 08.00 and 10.00 h from male volunteers, by spitting directly into glass tubes (75 × 12 mm), as previously described (Walker et al. 1978). The younger subjects were laboratory staff and their acquaintances. The older subjects were volunteers from men attending a day centre for the elderly. No subjects were currently in hospital. All elderly subjects approached were taking at least one drug, and only those with a history of prostate or urinogenital dysfunction were excluded. Samples were stored at —20°C prior to assay. The concentration of testosterone in the samples of saliva (200 μl) was determined using an assay previously described (Walker et al. 1980). An antiserum, raised in rabbits against a testosterone 3-(O-carboxymethyl)-oxime/bovine serum albumin conjugate was used together with tritium-labelled testosterone. The sensitivity of the radioimmunoassay was 500 fg/assay tube corresponding to a level of 9 pmol/l in saliva. Specificity assessment indicated a negligible cross-reactivity except for 5α-dihydrotestosterone (22%) and the concentrations of testosterone in saliva found in this study were consistent with observations from the Institute (Gaskell & Pike 1979), which showed a range of concentrations of testosterone of 200—400 pmol/l when determined by gas chromatography-mass spectrometry.

Results

The concentration of testosterone determined in samples of saliva collected from men of various ages is shown in Fig. 1. Linear regression analysis of these data gives a good fit to the model ($r^2 = 0.625; T = 305.7 - 2.89A$; where $T =$ salivary testosterone concentration, pmol/l; $A =$ age in years). However this model would
predict zero testosterone at 107y, which is not plausible. The data may also be summarized by a geometric regression ($r_g = 0.623; T = 3117A \uparrow^{-0.7946}$), which predicts a testosterone concentration that continues to fall with age but is always positive. This model appears to make superior predictions, but the fit to the present data is slightly less good.

Data from each decade of life may be aggregated, as shown in Table 1. If the

Table 1.
Mean salivary testosterone concentrations in decades of life in adult men.

<table>
<thead>
<tr>
<th>Age</th>
<th>Testosterone in saliva pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–30</td>
<td>236</td>
</tr>
<tr>
<td>30–40</td>
<td>204</td>
</tr>
<tr>
<td>40–50</td>
<td>167</td>
</tr>
<tr>
<td>50–60</td>
<td>150</td>
</tr>
<tr>
<td>60–70</td>
<td>106</td>
</tr>
<tr>
<td>70–80</td>
<td>100</td>
</tr>
</tbody>
</table>
mean testosterone concentration in each decade is correlated with the median age, similar equations to those from the gross data are derived for both the linear and geometric regressions but the correlations are obviously improved ($r_1 = 0.988; r_g = 0.970$).

**Discussion**

The assay of steroids in saliva provides a most effective non-invasive procedure for the detailed assessment of the endocrine status of a patient. Earlier investigations at the Institute (Walker et al. 1978, 1979b) indicated that steroid levels in saliva are independent of flow rate provided collections are restricted to small volumes, and that the concentrations of steroids in parotid fluid were identical to those in mixed whole saliva.

The reported changes in salivary testosterone concentration with age indicate a wide scatter throughout the decades examined, consistent however with similar data derived from studies of plasma testosterone levels (Kent & Acone 1966; Vermeulen et al. 1972; Baker et al. 1976), and regression analysis showed a decreasing value with advancing age. It is possible that these data may be explained by some factor, such as the filtration efficiency of testosterone from blood to saliva, varying with age. However previous investigations of salivary steroid concentration have clearly indicated a close correlation between plasma and salivary hormone levels and also a close relation between the salivary steroid concentration and the level of the free, non-protein-bound steroid in plasma. The reported data are similar to those described for plasma-free testosterone levels in ageing men (Vermeulen et al. 1971), and others (Smith et al. 1979; Baxendale et al. 1980) have now reported that salivary testosterone concentration is a good index of plasma free testosterone.

Obviously, the determination of the level of biologically active moiety of testosterone in plasma through salivary steroid analysis provides a new analytical dimension in the study of endocrine factors that could be concerned with the aetiology of cancer of the prostate or breast. Furthermore, studies of biological rhythms relating to steroid secretion and metabolism can be more readily established using salivary assays. Investigations along these lines are presently being conducted on men with prostatic dysfunction following the relatively disappointing results previously obtained from plasma analyses (Harper et al. 1976; Bartsch et al. 1977; The British Prostate Study Group 1979).

**Acknowledgments**

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References


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Dr. G. F. Read, Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, CF4 4XX, U.K.
The Pituitary-Testicular Axis in Prostatic Disease: The Potential of Salivary Steroid Assays


Although the biological control of the prostate would seem to be relatively complex (Fig. 1), the basis of most endocrine studies concerning the gland centers around the fact that its growth, maintenance, and functional activity are largely dependent on the androgenic hormones secreted by the testis. Other hormones, particularly of pituitary origin, indirectly affect the prostate, and there is evidence that certain of them may exert a direct effect on the gland. Luteinizing hormone (LH), for example, controls testicular synthesis of testosterone, the principal circulating androgen, approximately 95% of which originates in the testis [1]. Prolactin appears to have a synergistic role with LH in regulating testosterone production by the testis [2-4]. There is also a considerable body of evidence, however, to suggest that prolactin may have a direct influence on prostatic growth and function, and can accentuate the effects of androgens on the gland. Grayhack and Lebowitz [5] also observed an augmented increase in fructose and citric acid content of the prostate of castrated-hypophysectomised rats when testosterone and prolactin were administered together compared with that caused by testosterone alone, and specific binding sites for prolactin have been reported in prostatic tissue membranes [6,7]. Furthermore, Farnsworth has reported [8] that prolactin may, to some extent, increase testosterone uptake by the prostate gland, and others [9,10] suggest that growth hormone (GH) can also increase prostatic weight. Investigations in our laboratories, however, indicated that treatment of rats for 30 days with an inhibitor of prolactin secretion (2-bromo-α-ergo-cryptine; Sandoz Ltd.) failed to affect the weight of the accessory sex glands. Prolactin levels in the plasma were undetectable in these animals. The treatment had no effect on the ventral prostate lobe but did influence the zinc levels and its distribution in the dorsolateral lobes, consistent with earlier
Fig. 1. Schematic representation of certain aspects of the endocrinology relating to control of the prostate gland. DHA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; SHBG, sex hormone binding globulin; R, specific intracellular receptor; DHT-R, steroid receptor complex; mRNA and rRNA, messenger and ribosomal ribonucleic acid respectively. Question marks refer to processes not completely elucidated.
observations [11]. Aragona and Friesen have subsequently reported that prolactin receptors are localized in the dorsolateral lobes of the rat prostate [12].

Follicle stimulating hormone (FSH) or LH may also influence the biochemistry of the prostate gland by controlling oestradiol-17β production by the testis. Receptors for oestradiol-17β have been reported to be present in prostatic tissues, but their precise physiological role has still to be determined. It may be that this role is concerned more with stromal tissue rather than epithelial elements since investigations by ourselves [13] have clearly indicated that the oestradiol-17β receptors of the canine prostate are located mainly in the stroma. ACTH has been reported to stimulate rat prostatic growth [14–17] although its relationship with the prostate must be through the production of adrenal androgens, dehydroepiandrosterone and its sulphate, and androstenedione, which can be metabolized by the prostate [18]. Androstenedione can be peripherally converted to oestrogens [19,20]. The evidence that prolactin is concerned with the production of androgens by the adrenal gland remains equivocal [21]. It must be borne in mind, however, that the adrenal gland is unable to maintain prostatic weight in the castrated man or rat, and adrenal androgens are not sufficient to compensate for the loss of testicular function. The endocrinology of the prostate gland has recently been the subject of at least two major reviews [21,22].

**PITUITARY-TESTICULAR AXIS AND PROSTATIC DISEASE**

An involvement of pituitary hormones in the development of prostatic neoplasia or benign hyperplasia of the gland has still to be decided. There is, however, little evidence to implicate any hormone in the initiation of prostatic cancer [21]. Clinical data would indicate that carcinoma of the prostate in man is, to some extent, functionally dependent upon androgenic stimulation, yet the role of the various hormones circulating in the blood and synthesized within the prostate, in the aetiology of the disease, remains obscure.

The changes in plasma hormone concentrations associated with ageing in man have been of interest. There are reports that although the ability of the testis to respond to exogenous gonadotrophin is preserved despite ageing and decline in libido [23,24], plasma levels of testosterone decrease after the sixth decade [25]. On the other hand, the plasma oestradiol-17β concentration increases with age in the healthy normal adult man [26], an effect which is probably responsible for the reported increase in sex hormone binding globulin (SHBG) levels in the older man [27]. It is quite possible therefore that changes in the androgen-oestrogen balance in the elderly male and in the proportion of the free nonprotein-bound steroid hormones of plasma which are considered the biologically active moiety of the circulating hormone, could be implicated in the aetiology of prostatic disease.

In the search for the endocrine abnormality that may be implicated in the
pathogenesis of prostatic cancer, detailed studies of hormone levels in plasma and tissue from patients with this disease have been undertaken with the view that such data will increase our understanding of its aetiology.

Fundamental to this approach is the possibility that a change in the endocrine status of an individual has activated latent carcinoma or a particular pattern of hormone secretion has promoted early neoplastic growth. Studies from our own laboratories [28] and in association with the British Prostate Study Group [29] have been concerned with the plasma levels of hormones in patients with prostatic disease prior to and during treatment. Patients who presented for treatment in the various urological clinics of the group were assessed and classified in a standardized manner according to their primary tumour grade and metastatic status. Possible differences in the concentration of hormones between the various groups of patients was assessed using a multivariate-statistical analysis technique developed at the Institute [30].

Results obtained from the analysis of plasma obtained from patients with histologically proven prostatic cancer, prior to treatment, were somewhat disappointing, but possibly to be expected from the analysis of a single plasma sample from each patient. When hormone concentrations were classified according to primary tumour staging, using a standard Student t test, no significant differences between the group means were found, with the progression of the disease from stage T0 to T4. Furthermore, there were no significant differences seen in the hormone concentrations of patients with and without metastases. Of interest, however, was the separation found, using canonical variate analysis, between those patients with and without clinically evident metastases. GH levels were higher in those with metastatic disease and together with age, was the principal discriminating component responsible for group separation. High GH levels may be associated with the stress of a more debilitating development of the disease, but such studies indicate that careful assessment of the endocrinology of patients, investigated in depth, many ultimately provide the means to identify factors concerned in the pathogenesis of the disease.

Analysis of these data in relation to patient survival serves to illustrate the point. Patients were separated into groups 1–6 (Table I) depending on the number of years they survived from the time the disease was diagnosed and treatment commenced. Pretreatment hormone results were related to these patient groups (Table II). The analysis clearly indicated that the normality of the pituitary-testicular axis of the patient, on presentation at the clinic, was related to ultimate survival.

The higher the levels of plasma testosterone and oestradiol-17β and the lower those of LH, the longer the patient survived. Those patients with low concentrations of testosterone and oestradiol-17β and high LH levels, died relatively quickly from the disease.
TABLE I. Patient survival groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients who died</td>
<td></td>
</tr>
<tr>
<td>&lt; 1 year after initial diagnosis</td>
<td>43</td>
</tr>
<tr>
<td>1-2 years</td>
<td>32</td>
</tr>
<tr>
<td>2-3 years</td>
<td>30</td>
</tr>
<tr>
<td>3-4 years</td>
<td>15</td>
</tr>
<tr>
<td>3-5 years</td>
<td>41</td>
</tr>
<tr>
<td>Patients alive</td>
<td>76</td>
</tr>
</tbody>
</table>

n, Number of patients.

TABLE II. Relationship of survival to hormone concentrations

<table>
<thead>
<tr>
<th>Survival groups</th>
<th>Plasma hormones (2p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>1 v 2</td>
<td>NS</td>
</tr>
<tr>
<td>1 v 3</td>
<td>NS</td>
</tr>
<tr>
<td>1 v 4</td>
<td>0.01</td>
</tr>
<tr>
<td>1 v 5</td>
<td>0.02</td>
</tr>
<tr>
<td>1 v 6</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Mann Whitney U Statistics. NS, not significant; T, testosterone; E2, oestradiol-17β.

MONITORING HORMONE LEVELS THROUGHOUT TREATMENT

As part of this investigation, plasma hormone levels were measured in patients with prostatic cancer throughout treatment until recurrence and eventual death. The profile of hormone changes seen in Figure 2 was generally found in patients receiving treatment with diethylstilboestrol (DES) diphosphate (Honvan), 100 mg, twice daily. Levels of FSH and LH consistently decreased and subsequently so did the plasma concentration of testosterone. It was interesting that although the testis is generally considered to secrete little oestrogen [20], the levels of oestradiol-17β were always decreased by the order of 50% during treatment (Fig. 3). Prolactin and GH concentration increased under the influence of the high doses of synthetic oestrogen, and in line with the belief that prolactin may exercise some role in either promoting androgen uptake by the prostate or may influence the adrenal in synthesizing androgens, it was obvious that the high dose was contraindicated. Over recent years, a dose of DES of 1 mg t.d.s. has
become generally accepted in the United Kingdom as that which provides adequate endocrine control of testicular activity in the patient.

In Figure 2, the increase in testosterone observed in this patient after a year or more of treatment was seen to relate to disease recurrence (Fig. 4), and this rise in testosterone has usually been considered of adrenal origin. In the investigation of more than 300 patients, only rarely did symptoms of disease recurrence relate to an increase in plasma testosterone. The use of adrenal stimulation tests (Synacthen, IM) or dexamethasone suppression tests clearly indicated that the basal 50 ng/100 ml testosterone, with its episodic and circadian rhythm, was of adrenal and not testicular origin, and the clinical value of orchiectomy for treatment of the patient who has relapsed after oestrogen therapy requires reassessment. Although aminogluthethimide, a drug capable of suppressing steroid synthesis, has been used to treat patients with prostatic cancer who have relapsed
after orchiectomy [31], the possibility that it should be used as part of the primary treatment to suppress all testosterone production from all sources must also be considered. Adrenalectomy and hypophysectomy have never been accepted as offering an effective means of controlling recurrent disease, although it has to be accepted that hypophysectomy is of value for the palliation of pain in patients who have failed on endocrine therapy [32]. The possibility that certain hypothalamic-pituitary factors may influence the biochemistry of prostatic cancer cells must also be accepted. It is noteworthy however, that the profiles of hormone changes during various forms of therapy for prostatic cancer are quite different (Fig. 5). The initial clinical response of the patients to treatment can be identical despite vastly different levels of protein hormones in their plasma.

As part of this study throughout treatment, a method for the measurement of DES in plasma was established [33]. This specific radioimmunoassay, developed
using an antiserum raised against DES-carboxymethyl ether and a tritium-labelled radioligand, has a sensitivity of 8.8 pg, corresponding to a plasma level of 132 pg/ml and is adequate for the accurate determination of DES in the plasma of patients with prostatic carcinoma. When validated against gas chromatography-high resolution mass spectrometry, an excellent correlation ($r = 0.96$) was achieved.

Plasma samples from six patients receiving 1 mg DES t.d.s. were assayed for levels of DES and testosterone. DES was administered at 0630, 1230, and 1700 hours and blood collected via indwelling catheter from 0730-1600 hours on two consecutive days. On the second day, luteinizing hormone-releasing hormone (LH-RH; 100 µg, IV) was administered at 0900 hours.

In Figure 6, the expected marked rise in DES concentration occurred 1.5-2.0 hours after the oral administration of a 1-mg tablet. A less significant rise was observed 4 hours later when DES glucuronides are hydrolyzed in, or reabsorbed from the large intestine. Patients had been on DES for the previous three months.
Fig. 5. General hormone profiles with various endocrine therapies. a) Orchidectomy, b) oestrogen therapy, c) antiandrogen therapy, d) antiestrogen therapy. Arrows indicated start of treatment; P, prolactin; T, testosterone.

![Hormone Profiles Diagram](image)

Fig. 6. Plasma testosterone and diethylstilboestrol (DES) concentrations measured at 30-minute intervals. LH-RH (IV 100 μg) administered at 0900 hours on day 2.

![Testosterone and DES Concentration Diagram](image)
Levels of testosterone were low and failed to rise after LH-RH administration (Fig. 6b).

In one of the six patients, however, levels of testosterone were higher than expected after three months of DES treatment (Fig. 7a) and furthermore, were further elevated after LH-RH treatment (Fig. 7b), indicating a responsive pituitary-testicular axis. Although it would seem therefore that 1 mg DES t.d.s. is sufficient to suppress the pituitary-testicular axis in most patients, it can fail occasionally, and the study emphasizes the value of monitoring testosterone levels during treatment.

**HORMONAL RHYTHMS**

Detailed assessment of the hormone concentrations of patients with prostatic cancer reveals a wide variation in the hormone values found in the differing groups. Concentrations of plasma testosterone in the older patient can be as high as that in 20-year-old men. Such results are probably due to the single sample determination of parameters which exhibit episodic, circadian, and possibly even circatrigintan (monthly) or annual variation. In-depth studies of certain patients with prostatic cancer in whom an indwelling catheter was used to collect blood at 30-minute intervals for periods of 12 or 24 hours illustrated the errors concerned with single sample analysis. This multiple-sampling study clearly indicated the wide differences between patients even within the same age group and disease.

![Fig. 7. Plasma testosterone and diethylstilboestrol (DES) concentrations measured at 30-minute intervals. LH-RH (IV 100 µg) administered at 0900 hours on day 2.](image)
category. Plasma FSH and LH levels throughout the day and night of two patients with benign prostatic hyperplasia are shown in Figure 8.

Figures 9 and 10 show the concentrations of prolactin and GH in plasma of patients with prostatic cancer from whom blood was collected during their sleep periods. The high prolactin during the early hours of the morning would seem normal, and the elevation of GH immediately on falling asleep would again
suggest that any differences between patients and clinically normal males may be difficult to identify.

**SALIVARY STEROID ANALYSIS**

The recognition of the difficulties in assessing the endocrine status of patients by either single sample analysis or use of indwelling catheters stimulated the search for a new approach. The ease with which saliva may be collected and the fact that multiple samples may be taken without detriment to the patient make this an obvious body fluid to revisit; earlier attempts at measuring hormones in this secretion had failed in the fifties and sixties because of the lack of sensitivity of the assays at that time.

Samples of saliva can be collected by patients themselves which is obviously cost effective by saving both clinician-time and the materials required for plasma collection. Equally important is that samples of saliva can be stored at $-20^\circ C$ for 6 months without change in steroid concentrations. The following section serves to illustrate the potential value of salivary steroid analysis in endocrinology.
Steroid concentrations in saliva are low compared to plasma (<10% plasma value), and their determination requires assays which are sensitive as well as specific. Such radioimmunoassays, and certain enzymeimmunoassays, have now been developed and validated in these laboratories [33-41] and are now in routine use for both natural steroid hormones and also certain synthetic compounds. Table III shows the list of these assays, the volume of saliva required, and the sensitivity of the procedure. All assays have been validated against gas chromatography-high resolution mass spectrometric procedures and excellent correlation achieved. Noteworthy, also, is the fact that the concentration of steroid in whole saliva is the same as that in parotid fluid, collected by fitting a modified

**TABLE III. Immunoassays for salivary steroids**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Sensitivity (pg/tube)</th>
<th>Sample volume* (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>DHA</td>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td>17α-OH-progesterone</td>
<td>4</td>
<td>200</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7</td>
<td>400</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.5</td>
<td>200</td>
</tr>
<tr>
<td>Oestriol</td>
<td>12</td>
<td>200 &lt; 36 weeks</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>100 &gt; 36 weeks</td>
</tr>
<tr>
<td>Norethisterone</td>
<td>3</td>
<td>100</td>
</tr>
</tbody>
</table>

*Singlet determination.

Fig. 11. a) Concentrations of 17α-hydroxyprogesterone in matched samples of parotid fluid (○), collected under citric acid stimulation, and of mixed saliva (●) collected with no stimulation, from a patient with congenital adrenal hyperplasia. b) Progesterone concentrations in matched samples of parotid fluid (○) collected under citric acid stimulation, and of mixed whole saliva (●) with no stimulation, from a normal woman.
Fig. 12.  
a) Cortisol concentrations in matched plasma and saliva samples collected from a normal male at 15-minute intervals throughout a 12-hour period.  
b) Concentrations of 17α-hydroxyprogesterone in matched plasma (□) and mixed saliva (■) samples collected from a congenital adrenal hyperplasia patient over a 24-hour period. The arrows indicate the time of cortisol administration.
Carlsen-Crittenden device [42] over the duct of the parotid gland (Fig. 11a). Steroid concentrations in parotid fluid do not change with increasing flow rate, stimulated with citric acid on the tongue. The concentration of 17α-hydroxyprogesterone throughout the day of a patient with congenital adrenal hyperplasia and of progesterone, through the luteal phase of the menstrual cycle, in matched whole-saliva samples and in parotid fluid collected under conditions of maximally stimulated salivary flow rate, showed excellent agreement (Fig. 11).

Concentrations of steroid in whole saliva also adequately reflect the changes occurring in the plasma. Figure 12a illustrates the radioimmunoassay of cortisol in matched samples of saliva and plasma collected at 15-minute intervals throughout the day and Fig. 12b, the concentration of 17α-hydroxyprogesterone in matched saliva and plasma from a patient with congenital adrenal hyperplasia. Figure 13 shows the good correlation for progesterone concentrations between matched samples of blood plasma and saliva taken through one ovulatory cycle and related to basal body temperature. The data indicate the potential value of salivary progesterone in the assessment of ovarian function in cases of subfertility. This is further illustrated in Figure 14 which shows salivary progesterone levels in a woman who was seen at an infertility clinic, prior to (a) and after
Griffiths et al

Fig. 14. Daily salivary progesterone concentrations in a female subject with luteal phase insufficiency before and after clomiphene treatment (50 mg/day).

(b) clomiphene treatment. Once therapy has been initiated the levels of progesterone become elevated and are then seen to be in the normal range [43].

Particularly interesting, however, is the fact that the concentration of steroid in saliva closely relates to the level of free non-protein-bound steroid in plasma. The method thus allows the evaluation of the biologically active principle without recourse to the tedious and difficult procedures of separating free and bound steroids in plasma. Table IV shows the levels of free testosterone in blood reported by Vermeulen and his colleagues [27,44] and the close correlation with our saliva data [45]. The two studies indicate that free testosterone declines with increasing age (Fig. 15). Others have also confirmed the close correlation between the concentration of testosterone in saliva and the free nonprotein-bound fraction in plasma [46,47].

The potential value of such assays is obvious. Salivary testosterone levels rise
TABLE IV. Changes in free plasma testosterone* and salivary testosterone with age

<table>
<thead>
<tr>
<th>Age</th>
<th>TBG × 10⁻⁸M (± SE)</th>
<th>% Free T (± SE)</th>
<th>Free T pmole/l</th>
<th>Range pmole/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–50</td>
<td>5.2 ± 0.07</td>
<td>2.08 ± 0.08</td>
<td>402.7 ± 24.3</td>
<td>191–694.4</td>
</tr>
<tr>
<td>50–70</td>
<td>6.5 ± 0.8</td>
<td>1.72 ± 0.12</td>
<td>298.6 ± 34.7</td>
<td></td>
</tr>
<tr>
<td>70–85</td>
<td>8.9 ± 1.5</td>
<td>1.26 ± 0.14</td>
<td>156.3 ± 27.8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age</th>
<th>Saliva/plasma % (± SD)</th>
<th>T In saliva pmole/l (± SD)</th>
<th>Range pmole/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>18–35</td>
<td>1.6 ± 0.42</td>
<td>368 ± 166 (AM)</td>
<td>104–781</td>
</tr>
<tr>
<td>40–60</td>
<td>158 ± 68 (AM)</td>
<td>212 ± 131 (PM)</td>
<td>35–538</td>
</tr>
<tr>
<td>61–80</td>
<td>103 ± 53 (AM)</td>
<td>158 ± 68 (AM)</td>
<td>51–292</td>
</tr>
</tbody>
</table>


Fig. 15. Salivary testosterone levels in normal men of various ages. The mean (○) and SD for each decade also plotted.
PERIOD FITTED = 24.0 HRS
SAMPLING FREQUENCY = 2 Hours (0700 - 2300)
TIME SPAN = 48 Hrs
No of subjects = 6
UNITS pg/ml

<table>
<thead>
<tr>
<th>VARIABLE AND ELLIPSE IDENT</th>
<th># OF DATA</th>
<th>MESOM</th>
<th>SEM</th>
<th>AMPLITUDE AND (95% LIMITS)</th>
<th>ACROPHASE AND (95% LIMITS)</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A NORMAL YOUNG MEN</td>
<td>92</td>
<td>53.09</td>
<td>2.02</td>
<td>21.79 (15.29 - 28.62)</td>
<td>-103 (-15 - -125)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
in parallel with those in plasma in man after HCG stimulation, indicating the value of the assay for assessment of testicular function [39]. The circadian variation in testosterone levels is illustrated in Figure 16a. Cosinor analysis of the data from the subjects studied indicated that a cosine function can be fitted to the results with an acrophase (peak value) at approximately 0700 hours (Fig. 16b).

Obviously, the increase in testosterone production during the night is controlled [48], and it is interesting to speculate on the role of the elevated night prolactin levels. This steady rise in plasma testosterone throughout the night is shown in Figure 17, which illustrates results obtained from patients with prostatic cancer maintained in bed and in whom an indwelling catheter was used to collect blood. Equally interesting in this respect is the comparative rise in prostatic acid

Fig. 16. a) Salivary testosterone was collected from nine normal males at 2-hour intervals throughout a 16-hour period. Shaded area represents mean and standard deviation. The number of samples analyzed at each time point is indicated. b) Cosinor analysis of salivary testosterone showing a significant rhythm.

Fig. 17. Plasma testosterone concentrations in four patients with prostatic cancer. Blood samples were collected at 30-minute intervals throughout a 16-hour period. The dotted line represents the mean value of the determinations.
Fig. 18. Plasma prostatic acid phosphatase (PAP) in three patients with prostatic cancer prior to therapy, and one patient (WW) after cyproterone acetate treatment 200 mg b.d., was measured by RIA. Acid phosphatase enzyme activity was also measured in split plasma samples of patient (WW).

Fig. 19. a) Cosinor analysis of plasma testosterone concentrations of a prostatic cancer patient. A: pretreatment; B: 6 days post cyproterone acetate therapy 200 mg b.d. A significant rhythm seen with peak levels at 0930 hours. b) Cosinor analysis of the same patient's plasma prostatic acid phosphatase (PAP) also revealed a significant rhythm with peak values between 1300-1400 hours.
phosphatase levels (Fig. 18) which appear to display a circadian acrophase, a few hours behind that for testosterone (Fig. 19a, b).

It is clearly obvious that there is still a great deal to be understood about the endocrinology of the prostate and prostatic disease. The use of salivary steroid analysis, a noninvasive multisampling procedure, which can provide information on rhythmic steroid secretion, will do much to increase this understanding. In relation to the use of chronobiological procedures for data analysis and the added potential of assays for protein hormones in saliva, soon to be reported by the Institute [49], these new procedures offer an exciting development in the study of prostatic cancer.

REFERENCES


35. Walker RF, Read GF, Hughes IA, Riad-Fahmy D: Radioimmunoassay of 17α-hydroxyproges-


