The Androgenic Regulation of the Activities of Enzymes Engaged in the Synthesis of Deoxyribonucleic Acid in Rat Ventral Prostate Gland

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1. The restoration of mitosis and growth of the prostate gland of castrated animals by androgens provides a favourable experimental system for studying the hormonal regulation of enzymes engaged in DNA replication. 2. Many DNA polymerase activities were identified in the prostate gland, but only a 9S form with a particular preference for denatured DNA as template was conspicuously enhanced by androgenic stimulation. 3. Thymidine kinase also provided a sensitive indicator of the hormonal regulation of DNA replication, and on electrophoretic criteria, one discrete form of the enzyme appeared precisely with the onset of mitosis. 4. Evidence is presented to support the view that DNA ligase activity is intimately associated in the process of DNA replication in the prostate gland. 5. A spectrum of deoxyribonuclease activities is present in the prostate gland, but only one form (pI 7.0) can safely be said to be implicated in the process of DNA replication. 6. Androgenic stimulation of the prostate gland leads to the appearance of a component capable of denaturing or unwinding prostate DNA. This component is seemingly distinct from RNA or DNA polymerase activities on the basis of several distinct physico-chemical characteristics. 7. The conspicuous feature of all the changes in enzyme activities evoked by androgens in the prostate gland is their acute tissue- and steroid-specificity. Such changes could not be mimicked in liver or spleen and the regulatory role of androgens could not be simulated by other classes of steroid hormones. Particularly on the basis of studies with the anti-androgen cyproterone acetate, it is concluded that the changes are initially mediated by the androgen-receptor system and the high-affinity binding of 5 α -dihydrotestosterone in the prostate gland. 8. The results are discussed in the context of the mechanism of action of androgens.

The induction of growth and proliferation in the male accessory sexual glands by androgens has long been recognized as a characteristic property of this class of trophic steroid hormones [see the collected papers in Brandes (1974) for the historical background]. The enhancement of prostatic mitosis by androgens was originally monitored by histological procedures (Burkhart, 1942; Allen, 1958), but the more refined technique of mitotic labelling indices, based on the incorporation of [³H]thymidine into DNA, has been adopted in most contemporary investigations (for example, Tuohimaa & Niemi, 1968; Morley *et al.*, 1973). Apart from the innovative studies conducted by H. G. Williams-Ashman and his collaborators, however, on prostate gland (Coffey *et al.*, 1968) and coagulating gland (Brasel *et al.*, 1969) there is a paucity of information on the enzymes engaged in DNA synthesis in male accessory sexual glands. To a certain extent, this can be attributed to the currently confusing status of DNA replication in eukaryotes and of the enzymes mandatorily associated with mitosis. On presently available evidence, a diversity of enzymes are impli-

cated in DNA synthesis in mammalian cells and their characterization is the centre of considerable debate [see the excellent review by Keir & Craig (1973)]. The DNA polymerases (EC 2.7.7.7), catalysing the polymerization of 2-deoxyribonucleoside 5'-triphosphates according to the nucleotide sequence of the DNA template, exist in several structurally distinct forms, but principally as a cytoplasmic 6–8S form and a nuclear 3–4S species (Haines *et al.*, 1971). Of these, only the activity of the 6–8S form appears to be stimulated by the onset of cell division (Chang & Bollum, 1972; Chang *et al.*, 1973). The situation is additionally complicated by the possibility that these forms of DNA polymerase are freely interconvertible (Lazarus & Kitron, 1973; Hecht, 1973) and may represent fundamental enzyme subunits that are assembled and disassembled in response to the demands of cell replication (Keir & Craig, 1973). Another class of enzymes, the terminal nucleotidyl-transferases, promote the random addition of a single deoxyribonucleotide 5'-triphosphate to the 3'-terminal hydroxyl group of oligodeoxyribonucleotides (Chang & Bollum, 1971). McCaffery *et al.* (1973)

have advocated the adoption of a standard nomenclature for DNA polymerases, including the terminal-addition enzymes. However, such a suggestion is not entirely satisfactory, because the connexion between the terminal nucleotidyltransferases and DNA replication remains in conjecture (Tatò *et al.*, 1974). In addition, such DNA polymerases are essentially restricted to the thymus gland (Chang, 1971; Bekkering-Kuylaars & Campagnari, 1974) and perhaps a few other cell types of lymphoid origin (Penit *et al.*, 1974). Another distinct group of enzymes, the DNA ligases (EC 6.5.1.1), are capable of joining short fragments of DNA together (Lindahl & Edelman, 1968) and they also have been implicated in the process of DNA synthesis in eukaryotes (Hayton *et al.*, 1973). Although their involvement remains to be established unequivocally, yet another class of enzymes, the deoxyribonucleases, also appears to be necessary for the process of DNA repair and replication in higher animals (Lehman, 1967). Until the recent advent of analytical methods of high resolution, however, the part played by deoxyribonucleases in DNA synthesis has been difficult to envisage, since they also exist in a complex spectrum of molecular forms (Keir & Craig, 1973). As a final feature of DNA synthesis in eukaryotic cells, intracellular pools of 2-deoxyribonucleotides are known to fluctuate during the various phases of cell division (Grav, 1967; Nordenskjöld *et al.*, 1970) and the enzyme responsible for the synthesis of dTMP, thymidine kinase (EC 2.7.1.75), is a particularly sensitive marker for the onset of DNA synthesis (Bollum & Potter, 1959; McAuslan & Joklik, 1962).

The male accessory sexual glands provide excellent experimental systems for a critical appraisal of the enzymes engaged in DNA replication, since mitosis is totally suppressed in the castrated animal and the restoration of cell division is stringently dependent on androgenic stimulation. Further, the rat ventral prostate gland essentially responds to prolonged androgenic stimulation in a single dramatic burst of mitotic activity (Tuohimaa & Niemi, 1968) and this greatly facilitates experimental interpretation, since changes in the properties of enzymes may be unequivocally associated with the onset of cell division.

Materials and Methods

Chemicals

[8-³H]dGTP (15 Ci/mmol), [5-³H]dTTP (13.1 Ci/mmol), [methyl-³H]thymidine (19.2 Ci/mmol) and [γ -³²P]ATP (16.2 Ci/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Tris base (99.5% pure), deoxyribonucleotide 5'-triphosphates (sodium salts), highly polymerized calf thymus DNA, dithioerythritol, alkaline phosphatase from *Escherichia coli* and ATP (sodium salt)

were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. *E. coli* cells (strain MRE 600) were obtained from the Microbiological Research Establishment, Porton Down, Wilts., U.K. Denatured thymus DNA was immobilized on cellulose (Macherey-Nagel, type 2200ff; Camlab Ltd., Cambridge, U.K.) as previously described by Mainwaring & Irving (1973). Pancreas DNAase* I and spleen DNAase II were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. The synthetic polymers poly(dA-dT) and poly(dG-dC) were supplied by Miles-Seravac, Maidenhead, Berks., U.K. The anti-androgen, cyproterone acetate (6 α -chloro-17 α -acetoxy-1,2 α -methylene-4,6-pregnadiene-3,20-dione) was generously given by Schering Pharmaceuticals, Burgess Hill, Sussex, U.K. All other chemicals were of the highest available purity, and glass-distilled water was used in making up all solutions.

Animals

Adult male Sprague-Dawley rats, 270–300 g body wt., were castrated by the scrotal route under fluothane anaesthesia. Beginning 7 days after castration, animals were injected with steroids subcutaneously into the flank region in 0.25 ml of arachis oil; castrated control animals received injections of oil only. Details of the dosage of steroids and the duration of individual experiments are given in the legends to Tables. Where administered, actinomycin D and cycloheximide were given intraperitoneally (40 μ g/100 g body wt.) in 0.1 ml of 0.9% NaCl.

Determination of mitotic indices

At 2 h before death, animals were injected subcutaneously with colchicine (100 μ g/100 g body wt.) in 0.25 ml of 0.9% NaCl to arrest dividing cells in metaphase. The prostate glands were fixed in Carnoy's medium (ethanol-chloroform-acetic acid, 6:3:1, by vol.) for 2 h and embedded in paraffin wax, and sections (2–4 nm thick) were cut in the standard histological procedure. After staining with Haematoxylin-Eosin Y dye mixture, metaphase plates were counted under the microscope. At least 1000 cells were scanned and the mitotic index was calculated as the number of dividing cells per 1000 cells observed; a minimum of six slides was analysed for each sample of prostate tissue.

Preparation of labelled substrates

All preparations were stored frozen at –20°C in small lots (volume 0.5 ml).

(a) ³H-labelled prostate DNA. As documented in detail in the Results section, mitosis is undetectable in the prostate gland of normal (non-castrated) animals. Significant incorporation of [³H]thymidine into prostate DNA can only be accomplished on incubation of glands taken from castrated animals

* Abbreviation: DNAase, deoxyribonuclease.

that had subsequently received 4 consecutive days of androgenic stimulation. Pooled glands from eight animals were minced finely with scissors and placed in 20 ml of Eagle's medium in a Grant bath at 37°C. On shaking at 120 cycles/min, temperature equilibration was attained in 5 min. [^3H]Thymidine (200 μCi in 0.2 ml of Eagle's medium) was added and labelling allowed to proceed for 1 h. The tissue was drained, rinsed three times with 20 ml of ^3H -free Eagle's medium and the ^3H -labelled DNA extracted by the procedure developed by Colter *et al.* (1962). To ensure complete extraction of the prostate DNA in an undegraded form it was essential that the labelled tissue was gently homogenized directly in extraction medium (Colter *et al.*, 1962) and promptly treated with an equal volume of water-saturated phenol. The final preparation contained less than 2% of protein and had a specific radioactivity of 4400 c.p.m./10 μg of DNA. From sedimentation runs in the Spinco model E analytical centrifuge, the ^3H -labelled prostate DNA had a sedimentation coefficient of 60S.

(b) ^3H -labelled *E. coli* DNA. Approx. 500 mg wet wt. of *E. coli* cells were suspended in 400 ml of sterile culture medium containing 3 g of NaCl, 5 g of glucose and 4 g of Difco yeast extract and allowed to grow for 2 h at 33°C in a micro-fermentor (Bio Tec Instruments, Croydon, Surrey, U.K.; model FE 007) under conditions of vigorous aeration and stirring. [^3H]Thymidine (200 μCi in 0.5 ml of water) was introduced into the apparatus and the culture incubated for a further 16 h. Highly polymerized ^3H -labelled DNA was extracted by the method developed by Miura (1967). The amount of radioactivity in the final preparation was 4.9×10^6 c.p.m./100 μg of DNA.

(c) ^{32}P -labelled calf thymus DNA. The assay of DNA ligase activity requires partially digested DNA with ^{32}P -labelled phosphate groups from [γ - ^{32}P]ATP introduced at the 5'-hydroxyl ends by polynucleotide kinase. Satisfactory preparations of ^{32}P -labelled DNA were obtained by using the procedure advocated by Ichimura & Tsukada (1971) and the polynucleotide kinase of rat liver nuclei. Our sole departure from the published procedure (Ichimura & Tsukada, 1971) was that the DNA was not precipitated with ethanol at any stage. Removal of proteins was accomplished by extraction with water-saturated phenol and the resultant aqueous phase, containing the DNA, was repeatedly extracted with ether. Residual ether was removed under a gentle stream of N_2 . The final preparation was exhaustively dialysed against water and contained 5800 c.p.m./10 μg of DNA. Over 90% of the ^{32}P was released on incubation with *E. coli* alkaline phosphatase.

Preparation of subcellular fractions

Except where specified, isolations were performed at temperatures as close to 0°C as possible.

(a) *Soluble extracts (cell 105000g supernatant fraction)*. Tissues were thoroughly cooled on ice, minced finely with scissors and homogenized in medium A (50 mM-Tris-HCl buffer, pH 7.5 at 25°C, containing 1 mM-EDTA and 1 mM-dithioerythritol) in the proportion 4 ml of medium A/g wet wt. of tissue, by using a motor-driven Potter-Elvehjem homogenizer. After centrifugation at 105000g for 1 h, the soluble extract was carefully aspirated, avoiding the sediment of particulate elements and the floating layer of lipid. Over 90% of the tissue DNA was recovered in the 105000g sediments and these were qualitatively assayed for DNA to provide a reference for enzyme activities measured in the soluble extracts.

(b) *Proteins with a high affinity for DNA*. Tissues were homogenized in medium B (20 mM-Tris-HCl buffer, pH 8.0 at 25°C, containing 1 mM-EDTA and 5 mM-2-mercaptoethanol) in the proportion of 5 ml of medium B/g wet wt. of tissue. Solid NaCl was added to an overall concentration of 2M and the viscous extracts were stirred rapidly for 30 min. After three cycles of rapid freezing and thawing by using an acetone-solid CO_2 freezing mixture and a water bath at 45°C, 0.5 vol. of medium B containing 2M-NaCl and 42% (w/v) polyethylene glycol 6000 was added dropwise, and stirring continued for 30 min. It should be emphasized that the freezing and thawing procedure is essential for the efficient release and solubilization of this class of proteins in tissue homogenates. A heavy precipitate was sedimented by centrifugation at 20000g for 20 min and the clear supernatant fraction was dialysed overnight against 5 litres of medium B. Up to 8 ml of dialysed extract was applied to columns (1.5 cm $\times$ 4.0 cm) of DNA-cellulose, previously equilibrated with medium B, and washing in medium B continued until non-absorbed proteins were removed. Proteins with a pronounced affinity for DNA were selectively eluted in medium B containing 1M-NaCl and peak protein fractions were pooled and dialysed against medium B for 3 h.

(c) *Nuclei*. These were purified as described by Mainwaring (1969).

Enzyme activity assays

The objective of all the assays was to synthesize a labelled product which could be selectively adsorbed on DEAE-cellulose discs (Whatman DE 81; 2.5 cm diam.) and washed free of radioactive precursor. Assays were conducted in triplicate and at the completion of incubation, 100 μl samples were pipetted on to DE 81 discs and thoroughly dried under an i.r. lamp. To facilitate the performance of assays in large numbers it was found most convenient to store incubation mixtures at -20°C in small lots, to which enzyme extracts could be added directly for assay. All enzyme activity assays were conducted under

conditions where the rates of reaction were proportional to the time of incubation.

(a) *DNA polymerases*. When required, denatured DNA was prepared just before use by heating the DNA solution (in water) in a boiling-water bath for 15 min followed by rapid cooling to 0°C. Each assay, total volume 0.30 ml, contained 100 µl of enzyme extract, 30 µmol of Tris-HCl buffer, pH 8.0, 0.6 µmol of MgCl₂, 0.3 µmol of dithioerythritol, 50 nmol each of dATP, dCTP and dTTP, 0.1 µCi of [³H]dGTP and 5 nmol of non-radioactive dGTP, 50 µg of native or denatured DNA as template and glycerol to 10% (v/v). Other investigators, including Coffey *et al.* (1968), performed assays for DNA polymerase in the presence of a nucleoside 5'-triphosphate-regenerating system. In our experience, the inclusion of ATP and phosphoenolpyruvate had only a marginal influence on the rate of DNA synthesis and were therefore not included in our standard assay system. In assays for terminal nucleotidyltransferase activity, complementary deoxyribonucleotide 5'-triphosphates were omitted and only [³H]dTTP was incubated with 10 µg of poly(dG-dC) or [³H]dGTP with 10 µg of poly(dA-dT). Incubations were for 30 min at 37°C. Under these conditions of assay, the incorporation of 10 pmol of dGTP into DNA is equivalent to the conversion of 440 d.p.m. of radioactivity into a high-molecular-weight form.

(b) *DNA ligase*. This enzyme is monitored by the radioactivity in ³²P-labelled DNA, which can be converted into a form resistant to alkaline phosphatase. Each assay, total volume 0.20 ml, contained 100 µl of enzyme, 10 µmol of Tris-HCl buffer, pH 8.0, 0.2 µmol of MgCl₂, 0.4 µmol of dithioerythritol, 0.2 µmol of ATP and 10 µg (5800 c.p.m.) of ³²P-labelled thymus DNA. Incubation was for 30 min at 37°C and the tubes were then transferred to a bath at 65°C for 5 min to inactivate the DNA ligase. After cooling to room temperature, 20 µl (20 µg) of *E. coli* alkaline phosphatase was added to each tube and incubation at 65°C continued for 30 min. This procedure is simpler and less time-consuming than other published methods.

(c) *Thymidine kinase*. Each assay, total volume 0.10 ml, contained 50 µl of enzyme, 5 µmol of Tris-HCl buffer, pH 8.0, 1 µmol of MgCl₂, 0.2 µmol of dithioerythritol, 1 µmol of ATP, 0.1 µCi of [³H]-thymidine and 10 nmol of non-radioactive thymidine and 0.25 µmol of NaF. Incubation was for 30 min at 37°C and the samples were diluted with 100 µl of water. This was necessary to decrease the protein content, which otherwise competed with the reaction product, [³H]dTTP, for the anion-binding sites on DEAE-cellulose. From analyses conducted by t.l.c. on polyethyleneimine-impregnated cellulose sheets with 0.25 M-NaCl as developing medium (Randerath & Randerath, 1964), over 95% of the ³H in the products of the thymidine kinase assay was [³H]dTTP,

the remainder being traces of [³H]dTTP. The conversion of 10 pmol of [³H]thymidine into [³H]dTTP is equivalent to the selective retention of 1100 d.p.m. of radioactivity under the conditions of assay used.

(d) *Washing of DE 81 discs*. The 100 µl taken from all assay mixtures was just sufficient to soak a DE 81 disc completely. After thorough drying, up to 30 discs from a given assay were combined in 150 ml beakers with holes in both the base and sides. Each beaker was mounted on a hollow Perspex cylinder (2 cm high; 7 cm diam.), containing a magnetic stirring bar, within a beaker of 800 ml capacity. Then 750 ml of washing solution was introduced and thorough stirring continued for 10 min. The discs were then drained completely and placed in fresh washing solution. The washing cycle consisted of three washes with 5% (w/v) Na₂HPO₄ and one wash in 50% (v/v) ethanol. Specifically in the assay of thymidine kinase only, the discs were washed three times with 1 mM-ammonium acetate in 5 mM-Tris-HCl buffer, pH 8.0, and once with ethanol. After drying under an i.r. lamp, the discs were counted for radioactivity. This recommended procedure was a vast improvement in terms of time and effort over conventional methods based on precipitation and washing with cold acids. Our procedure was prompted by the innovative work of Litman (1968) and Berk & Clayton (1973).

Separation of enzyme activities

Many of the enzymes investigated during this study exist as distinct physicochemical entities, and methods of high resolution are required for separating these polymeric enzymes.

(a) *Sucrose gradients*. Linear 5–20% (w/v) sucrose gradients, volume 4.8 ml, were prepared in medium A. After centrifugation at 100000 g_{av} in the SW 52 titanium rotor of a Christ Omega II ultracentrifuge for 16 h at 4°C, the gradients were subdivided into approx. 25 fractions (0.2 ml each) and analysed for enzymic activity. The sedimentation marker ox heart catalase (11.4 S) was run in a separate gradient.

(b) *Polyacrylamide gels*. The various forms of thymidine kinase have similar sedimentation coefficients, but they may be satisfactorily resolved by electrophoresis in polyacrylamide gels (see, e.g. Kit *et al.*, 1974). Samples of soluble extracts were separated in 7% (w/v) polyacrylamide gels under the conditions of discontinuous electrophoresis described by Ornstein & Davis (1964). As originally reported by Kit *et al.* (1974) it was found necessary to include ATP at a concentration of 2 mM in the upper (cathode) buffer medium in order to preserve prostate thymidine kinase during electrophoresis. Separations were conducted at 4°C and 4 mA/gel until the tracking dye, Bromophenol Blue, had migrated 7 cm in the direction of the anode. The gel was cut in approx. 30 equal

slices (2.2 mm-thick), each of which was suspended in 0.1 ml of medium A and 0.1 ml of thymidine kinase assay mixture (for details, see above); incubation was for 1 h at 37°C. The product, [^3H]dTMP, was again selectively retained on DE 81 discs. An identical approach was used for analysing DNA polymerases in polyacrylamide gels, the sole difference being the substitution of the DNA polymerase assay medium for the incubation of the gel slices.

(c) *Isoelectric focusing in polyacrylamide gels.* Zöllner *et al.* (1974) have proposed a novel and sensitive assay for DNAases in which high-molecular-weight ^3H -labelled DNA is incorporated within polyacrylamide gels. After electrophoretic separation of enzyme extracts in the gels, DNAase activity is located by incubating individual gel slices and measuring the release of ^3H . In our experience, the original procedure advocated by Zöllner *et al.* (1974) was eminently satisfactory for the analysis of commercial samples of DNAases I and II. However, certain prostate DNAases barely penetrated into the analytical gel during electrophoresis at pH 8.3 (Ornstein & Davis, 1964), presumably because this pH is exceedingly close to their isoelectric points. To ensure that we were detecting all the potential DNAase activities in prostate extracts recourse was made to isoelectric focusing in polyacrylamide gels in a pH gradient of wide range. Each gel (volume 3.0 ml) was prepared by mixing the following components; 1.0 ml of 15% (w/v) Cyanogum 41 (BDH, Poole, Dorset, U.K.), 0.1 ml of ^3H -labelled *E. coli* DNA (50 μg ; 2.4×10^6 c.p.m.), 50 μl each of 40% (w/v) ampholytes (LKB, Croydon, Surrey, U.K.) of pH ranges 5–8 and 8–9.5, 0.9 ml of water, 0.9 ml of 0.14% (w/v) $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and 20 μl of *NNN'*-tetramethylethylenediamine. The mixture was poured into a glass tube (0.8 cm diam.), overlaid with water to promote the formation of a flat upper gel surface and left for 15 min for polymerization to reach completion. Up to eight gels were mounted in the standard Ornstein & Davis (1964) electrophoresis apparatus; the lower (anode) reservoir contained 0.02 M- H_2SO_4 and the upper reservoir 0.02 M- NaOH . A brief period of pre-electrophoresis at 100 V for 15 min was performed to remove residual $(\text{NH}_4)_2\text{S}_2\text{O}_8$ from the gels. A 0.2 ml layer of 10% (w/v) sucrose was placed on each gel and the enzyme extract underlaid beneath this first layer. The extract for analysis (0.2 ml) was mixed with 25 μl of 40% (w/v) ampholytes (LKB), pH range 3–10, and 0.1 ml of 60% (w/v) sucrose just before analysis. One gel was loaded with a 0.2 ml mixture of coloured proteins of known isoelectric point (25 μg each of catalase, haemoglobin and cytochrome c). A potential difference of 15 V/gel was applied at 4°C for approx. 5 h; at this time the marker proteins had reached stable positions in the gels and focusing was deemed complete. Two principal classes of DNAase activity were investigated after cutting the gels into approx. 30 equal slices (2.2 mm-thick). For DNAase I

activity, slices were incubated at 37°C for 2 h in 0.20 ml of 0.2 M-Tris-HCl buffer, pH 8.0, containing 5 mM- MgCl_2 ; incubation was performed in 0.20 ml of 0.2 M-sodium acetate-acetic acid buffer, pH 5.0, containing 2 mM-EDTA, for DNAase II activity. The supernatant was carefully withdrawn, avoiding the slice of gel, and counted for radioactivity. Despite the high specific radioactivity of the ^3H -labelled DNA, the background in the absence of DNAase activity was exceedingly low (of the order 20 c.p.m./slice). This procedure ensured sharper and more reproducible resolution of protein extracts than alternative methods where the proteins are initially distributed throughout the gel (Wrigley, 1968).

Assay of the unwinding (or denaturation) of DNA

Native (highly polymerized) DNA is not retained by nitrocellulose filters, but strand cleavage, by either physical means or attachment of certain proteins, converts the DNA into a form which cannot permeate such membranes. In this assay, 10 μl of ^3H -labelled prostate DNA (10 μg ; 4400 c.p.m.) was mixed with 50 μl of protein extracts (20–50 μg of protein) and 50 μl of medium C (50 mM-Tris-HCl buffer, pH 8.0, containing 5 mM-EDTA and 0.5 mM-dithioerythritol). After incubation at 37°C for 2 min, the samples were diluted with 0.60 ml of medium C at room temperature and drawn into disposable plastic hypodermic syringes (1.0 ml capacity). Millipore membranes (0.45 μm pore size; code HAWP 01300), pre-soaked in medium C, were mounted in Millipore holders (code SX 0001300) and after filling the assemblies with medium C, the syringes were attached. Up to 12 syringes can be mounted together in a Perspex rack and the contents were expressed through the filters with a total emptying time of 20 s. The membranes were dried thoroughly under an i.r. lamp and counted for radioactivity.

Analytical procedures

Protein was determined by the Folin-Ciocalteu method (Lowry *et al.*, 1951), with bovine serum albumin as the standard. The diphenylamine-acetaldehyde procedure of Giles & Myers (1965) was used for the assay of DNA, with calf thymus DNA as the reference.

Counting of radioactivity

Labelled compounds retained on Millipore membrane or Whatman DE 81 discs were counted in 10 ml of a toluene-based phosphor [15 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/l] in

a Nuclear-Chicago mark II liquid-scintillation spectrometer. Counting efficiency for ^3H under these conditions was 54%. Aqueous samples containing radioactivity (up to 0.40 ml) were counted in 10 ml of a water-miscible phosphor [15 g of 5-(4-biphenyl) 2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole dissolved in 1770 ml of toluene, 110 ml of water and 625 ml of Triton X-100]. Counting efficiency for ^3H in this scintillation phosphor was 40%.

Results

Calculation of enzyme activities

Throughout the present work, enzyme activities are expressed with reference to a unit amount of protein. In all experiments, however, enzyme activities were also calculated with reference to DNA, and without exception, identical trends in the changes of enzyme activities were recorded. For simplicity in the presentation of our experimental findings, however, all data are quoted solely with respect to the protein concentration of the enzyme extracts.

Mitotic activity evoked in the prostate gland by androgens

The time-course of the induction of growth and mitosis evoked by androgens in the prostate gland of castrated rats is presented in Fig. 1. Over a protracted period of androgenic stimulation, some 10 days in all, the weight of the prostate progressively increased until it exceeded that in normal (non-castrated) animals. Precisely as documented by Coffey *et al.* (1968), there was a latent period of approx. 48 h in the androgen-mediated stimulation of mitosis in the prostate, after which the number of dividing cells suddenly increased to a maximum and then slowly declined despite the continued presence of androgenic hormones. Also concordant with former investigations (Coffey *et al.*, 1968), the stimulation of DNA polymerase activity followed precisely this surge and subsequent decline in mitotic activity. On the basis of these experimental findings, the biochemical activities mandatorily implicated in the process of DNA replication in the prostate gland should be barely detectable in the resting state of the castrated animal, yet demonstrate a dramatic stimulation after 72–96 h of hormonal stimulation.

The only difficulty encountered during the calculation of the data summarized in Fig. 1 was the accurate assessment of the baseline or reference mitotic activity in the prostate of castrated (control) animals. In essence, we never observed a dividing cell in histological sections of prostate gland derived from animals depleted in androgens, but solely to provide a basis for comparison the mitotic index in castrated animals was arbitrarily set at 1 dividing cell per 10000.

Fig. 1. Time-course of the changes in mitotic activity and growth of the ventral prostate gland of castrated rats promoted by androgens

Beginning 7 days after castration, rats were given daily injections of 2.5 mg of testosterone phenylpropionate subcutaneously in oil; six animals served as castrated controls and were killed directly, without receiving androgenic stimulation. At timed intervals, four animals were killed and three measurements were conducted on the prostate glands to delineate the onset of growth and mitosis; ▲, mitotic index; △, DNA polymerase activity measured in the cell 105000g supernatant fraction with double-stranded DNA as template; ■, weight of the prostate gland. All data are quoted as percentage changes relative to baseline determinations made on prostate glands from castrated controls. The reference values were as follows: mitotic index, 1 dividing cell/10000 cells; DNA polymerase, 16 pmol of [^3H]dGTP incorporated into DNA/30 min per mg of protein at 37°C; wet wt. of gland, 94 mg. The values enclosed in squares at the extreme right of the abscissa are from normal (non-castrated) animals. The range of experimental findings was $\pm 10\%$ for all parameters studied; the data are plotted as mean values from determinations made in triplicate.

Stimulation of thymidine kinase activity promoted by androgens

As documented in Table 1, prostate thymidine kinase activity was regulated by androgens in a highly steroid- and tissue-specific manner. Consistent with findings in other experimental systems (Bollum & Potter, 1959; McAuslan & Joklik, 1962), this enzyme provides a sensitive indicator of DNA replication, for the pronounced enhancement of prostate thymidine kinase activity was concomitant with the onset of mitosis. These dramatic changes in enzyme activity could not be evoked by other classes of steroid hormones and were essentially restricted to the prostate gland. The only anomaly in the experimental results presented in Table 1 was the modest increase in spleen thymidine kinase activity after androgenic stimulation. This finding remains unexplained, particularly since a specific androgen-binding mechanism of the type present in accessory sexual glands has not been detected in spleen (Mainwaring & Peterken, 1971; Mangan & Mainwaring, 1972). It is possible, however, that the androgen treatment enhanced the

Table 1. *Tissue- and steroid-specificity in the stimulation of prostate thymidine kinase activity by androgens*

Two experiments are summarized here. (a) Tissue specificity. Beginning 7 days after castration, rats were injected subcutaneously with 2.5 mg of testosterone phenylpropionate in oil every 24 h; six animals served as controls and were injected with oil only. At timed intervals, groups of animals were killed and tissue pooled from four animals was used to prepare cell 105000g supernatant fraction. Samples were assayed in triplicate for thymidine kinase activity; results are quoted as means $\pm$ s.e.m. Enzyme activities in tissues from normal (non-castrated) animals are included for comparison. (b) Steroid specificity. At 7 days after castration, groups of six rats were administered daily injections of 2.5 mg of steroids subcutaneously in oil; six animals (controls) received injections of oil only. After 3 days of treatment, cell 105000g supernatant fractions were prepared from the prostate glands and analysed for thymidine kinase activity.

Hormonal treatment	Thymidine kinase activity (pmol of dTMP formed/30 min per mg of protein at 37°C)		
	Liver	Prostate gland	Spleen
(a) Tissue specificity			
None (castrated controls)	8.4 $\pm$ 0.4	10.2 $\pm$ 0.2	85.2 $\pm$ 6.2
Testosterone, 48 h	8.0 $\pm$ 0.8	6.6 $\pm$ 0.4	88.2 $\pm$ 8.2
Testosterone, 72 h	6.2 $\pm$ 0.8	112.6 $\pm$ 5.6	101.4 $\pm$ 5.4
Testosterone, 96 h	9.4 $\pm$ 0.6	155.4 $\pm$ 10.2	100.6 $\pm$ 3.4
Normal (non-castrated animals)	9.6 $\pm$ 0.4	14.4 $\pm$ 0.4	96.0 $\pm$ 6.0
(b) Steroid specificity		Prostate gland only	
None (castrated controls)		14.0 $\pm$ 0.2	
Testosterone, 72 h		133.2 $\pm$ 8.8	
Oestradiol-17 β , 72 h		18.2 $\pm$ 0.4	
Corticosterone, 72 h		16.6 $\pm$ 0.8	

release of erythropoietin from the kidney into the general circulation, thereby stimulating spleen thymidine kinase.

Androgenic stimulation of the prostate gland was also accompanied by the appearance of a novel species of thymidine kinase (Fig. 2). Although the resting (non-proliferating) prostate gland of castrated animals contains a single form of thymidine kinase activity of relatively low electrophoretic mobility, the induction of mitosis is accompanied not only by a pronounced enhancement of this enzyme form but also by the appearance of a thymidine kinase activity typified by a more rapid migration in the direction of the anode. Although not shown in Fig. 2, the appearance of this form of thymidine kinase is a characteristic feature of the prostate gland; these changes in electrophoretic pattern could not be stimulated in spleen after hormonal stimulation.

Stimulation of DNA polymerase activities by androgens

A multiplicity of DNA polymerase activities exist in the prostate gland, but we primarily restricted our attention to three types of enzymic activity, each being characterized by a pronounced preference for either native or denatured DNA as template and the ability to conduct polymerization in the absence of complementary 2-deoxyribonucleoside 5'-triphosphates. These results are summarized in Table 2. The conspicuous feature of these findings was that although

certain DNA polymerase activities were remarkably enhanced by androgens, other members of this class of enzymes were more resistant to androgenic stimulation. In the prostate preparations from castrated control animals, native DNA was the preferred template and this DNA polymerase activity was enhanced by androgens. The activity which is capable of supporting polymerization in the absence of complementary 2-deoxyribonucleoside 5'-triphosphates was present in only small amounts and was stimulated to a lesser degree by hormonal stimulation. The most impressive enhancement of prostate DNA polymerase activity evoked by androgens was indubitably shown by the enzyme utilizing denatured DNA as template. From an exceedingly low activity in castrated control animals, this particular DNA polymerase was stimulated by almost two orders of magnitude after 4 days of testosterone treatment. This experimental observation is totally harmonious with the original findings of Coffey *et al.* (1968). The stimulation of the activities of all the prostate DNA polymerases was acutely dependent on the restoration of the circulating concentrations of androgens; other steroid hormones did not share this ability. The extreme tissue specificity of these changes was amply illustrated by concomitant determinations of DNA polymerase activities in liver and spleen, for although the activities in spleen were particularly high, they were totally insensitive to hormonal manipulation *in vivo*.

From an inspection of the results summarized in Table 2, particularly from a comparison of the three

Fig. 2. Analysis of prostate thymidine kinase in polyacrylamide gels

Samples of prostate cell 105000g supernatant fractions (350 μ g of protein) were separated in 7% (w/v) polyacrylamide gels. ATP was present in the upper (cathode) reservoir at a concentration of 2mM and electrophoresis was conducted until the tracking dye, Bromophenol Blue, had migrated 7.0cm in the direction of the anode. After sectioning, individual slices of gel were analysed for thymidine kinase activity. For full details, see the Materials and Methods section. The direction of the anode is to the right; B, Bromophenol Blue marker at the ion front; H, internal marker of haemoglobin present in the extracts. Prostate extracts from (a) animals 7 days after castration; (b) animals 7 days after castration, then 3 days of treatment with testosterone phenylpropionate (2.5mg/animal); and (c) normal (non-castrated) animals. Enzyme recovery from the gels was 65–70% in all experimental groups.

enzyme activities and their modulation by androgens, it would be predicted that each activity was conducted exclusively by a unique DNA polymerase enzyme. To corroborate this conclusion, prostate

DNA polymerase activities were separated by centrifugation in sucrose density gradients (Fig. 3). These experimental results unequivocally support the contention that the three DNA polymerase activities were conducted by discrete, separable enzyme moieties. The polymerization reaction in the absence of complementary 2-deoxyribonucleoside 5'-triphosphates was conducted by an enzyme present in a broad peak of mean sedimentation coefficient approx. 4S, and this was only marginally stimulated after the administration of androgens. On the other hand, the enzyme with a preference for native DNA as template was similarly recovered in the 4S region of the gradients but was markedly enhanced in activity by androgenic stimulation. The most striking outcome of the restoration of the androgenic status of castrated animals, however, was the dramatic appearance of a 9S DNA polymerase which supported polymerization with denatured DNA as template. This experimental finding is entirely consistent with studies conducted on other experimental systems (Chang & Bollum, 1972; Chang *et al.*, 1973) and which indicate that the 6–8S DNA polymerase is the form most deeply implicated in the process of DNA replication. Enzyme activities in gradient fractions were not influenced by the presence of sucrose. This was validated in additional experiments.

In addition to the three enzyme activities investigated in Table 1, the prostate gland also contains detectable terminal-addition DNA polymerase activities which utilize the synthetic templates poly(dG-dC) and poly(dA-dT). However, since these activities were present in variable but always low amounts and were not particularly influenced by androgenic stimulation, they were not investigated in great depth. It appears therefore that these terminal nucleotidyltransferases are not an invariant feature of eukaryotic cells, and their general association with mitosis remains in question.

DNA ligase and DNA replication in the prostate gland

Perhaps owing to other interfering enzyme activities, we were unable to conduct reproducible analyses for DNA ligase activity in cell 105000g supernatant fractions of the prostate gland directly. This contrasted sharply with the experience of other investigators using similar preparations from liver, for example Tsukada (1974). To circumvent this difficulty, samples of cell 105000g supernatant fraction were separated in sucrose density gradients and individual fractions were analysed for DNA ligase activity (Fig. 4). This enzyme has a sedimentation coefficient of approx. 11S and during ultracentrifugation it is presumably sedimented free from interfering enzyme activities. Androgenic stimulation of castrated animals led to a pronounced increase in prostate DNA ligase activity to a value exceeding that in normal

Table 2. Tissue- and steroid-specificity in the stimulation of prostate DNA polymerases by androgens

The regimen of steroid injections is that described in the legend to Table 1. (a) Tissue specificity. With six animals per experimental group, tissues were removed after various periods of androgenic stimulation and used for the preparation of cell 105 000 g supernatant fractions. (b) Steroid specificity. Groups of six animals were treated for 3 consecutive days with various steroids and cell 105 000 g supernatant fractions prepared from the prostate glands. Three different polymerase activities were measured by the incorporation of [³H]dGTP into a polymerized form (see the Materials and Methods section for complete details): (1) with native DNA as template; (2) with thermally denatured DNA as template; (3) with native DNA as template but with the omission of complementary deoxyribonucleoside 5'-triphosphates. Assays were conducted in triplicate and the results are expressed as means ± s.e.m. All data were corrected for the incorporation of [³H]dGTP in the absence of added DNA; for the purpose of comparison, certain assays were performed on enzyme extracts from normal (non-castrated) animals.

Hormonal treatment	DNA polymerase activity (pmol of [³ H]dGTP incorporated/30 min per mg of protein at 37°C)									
	Liver			Prostate gland			Spleen			
	(1) Native DNA template	(2) Denatured DNA template	(3) Minus deoxyribonucleotides	(1) Native DNA template	(2) Denatured DNA template	(3) Minus deoxyribonucleotides	(1) Native DNA template	(2) Denatured DNA template	(3) Minus deoxyribonucleotides	
(a) Tissue specificity										
None (castrated controls)	40.4 ± 4.1	26.6 ± 4.1	13.1 ± 4.1	35.2 ± 6.2	14.1 ± 2.1	10.0 ± 1.7	451.6 ± 40.7	534.7 ± 29.0	70.7 ± 11.1	
Testosterone, 48 h	32.6 ± 2.4	29.9 ± 5.1	17.0 ± 3.1	72.5 ± 7.1	33.6 ± 5.2	4.2 ± 1.1	447.4 ± 41.4	512.2 ± 40.0	74.1 ± 13.6	
Testosterone, 96 h	44.7 ± 6.8	23.2 ± 1.9	14.9 ± 3.0	132.8 ± 20.2	220.6 ± 20.1	21.4 ± 4.4	487.2 ± 20.6	550.7 ± 36.2	81.2 ± 17.7	
Normal (non-castrated animals)	—	—	—	88.0 ± 14.1	64.6 ± 7.6	5.9 ± 1.1	—	—	—	
				Prostate gland only						
(b) Steroid specificity										
None (castrated controls)				33.1 ± 4.0	12.1 ± 1.2	7.6 ± 0.7				
Testosterone, 72 h				136.6 ± 17.0	264.0 ± 21.0	24.1 ± 3.7				
Oestradiol-17β, 72 h				27.1 ± 2.7	9.4 ± 1.7	9.0 ± 0.9				
Corticosterone, 72 h				39.1 ± 4.0	8.7 ± 1.8	8.1 ± 1.1

Fig. 3. Analysis of prostate DNA polymerases in sucrose gradients

Samples of prostate 105000g supernatant fraction (500 µg of protein) were centrifuged at 100000g_{av} for 18 h in linear 5–20% (w/v) sucrose gradients, volume 4.8 ml, prepared in medium A. The direction of centrifugation is from right to left; C, sedimentation marker, catalase (11.4S); H, sedimentation marker, haemoglobin (4.6S) present in the enzyme extracts. Individual gradient fractions were analysed for three separate DNA polymerase activities; for complete details, see the Materials and Methods section. Prostate extracts from (a) animals 7 days after castration; (b) castrated animals and 3 days of treatment with testosterone phenylpropionate (2.5 mg/animal); (c) normal (non-castrated) animals. DNA polymerase activities: ●, with denatured DNA as template; ○, with native DNA as template; ▲, with native DNA as template in the presence of [³H]dGTP only. Enzyme recovery from the gradients was 90–95% in all experimental groups.

(non-castrated) animals. This change was essentially tissue-specific because it could not be mimicked in spleen extracts, and hence the androgenic regulation of DNA ligase activity appears to be a characteristic feature of DNA replication in the accessory sexual glands only.

Deoxyribonucleases and DNA replication in the prostate gland

Attempts were initially made to investigate the androgenic control of DNAase activity in the prostate gland by the application of spectrophotometric and radiochemical methods of enzyme assay to prostate cell 105000g supernatant fractions. These studies were somewhat inconclusive, and one plausible explanation was that the changes in DNAase activities were too subtle to be analysed by such a relatively unsophisticated approach. Under rigorously controlled conditions, the procedure of Zöllner *et al.* (1974) is capable of providing a qualitative and quantitative analysis of DNAases and this procedure was applied to prostate extracts derived from animals of varying androgenic status. Our sole departure from the original method (Zöllner *et al.*, 1974) was that the separations were performed by isoelectric focusing in polyacrylamide gels rather than the standard electrophoretic separation (Ornstein & Davis, 1964). The authenticity of the modified procedure was confirmed by analysis of proteins of known pI; under our conditions, a linear relationship was established between the pI of the standard proteins and their final position in the isoelectric-focusing gels.

Androgens provoked significant changes in the DNAase activity in the prostate gland (Fig. 5) and their complexity was such that they would not be detected except by methods embodying both sensitivity and high resolution. Two types of DNAase activity were investigated; DNAase I, proceeding optimally at pH 8.0 in the obligatory presence of MgCl₂, and DNAase II, pH optimum 5.0 and not requiring bivalent cations. The pI of the enzymes was estimated by comparison with marker proteins run in parallel. In the prostate of castrated animals, little DNAase I activity was present, the major enzyme being a DNAase II activity, pI 7.0. After androgenic stimulation, DNAase II activity was represented equally by forms of pI 7.0 and 8.8, but the principal enzyme was now a DNAase I, pI 7.5, that had been barely detectable in extracts derived from castrated (control) animals. In normal (non-castrated) animals, DNAase II activity was predominantly present in the form of pI 8.8 and the DNAase I activity, pI 7.5, was significantly decreased, being replaced by a form of pI 5.1. It is evident that the DNAase activity in the prostate gland is represented by a wide spectrum of discrete enzyme moieties whose relative presence is stringently regulated by androgens. Of all these forms of prostate DNAase, only the DNAase I form, pI 7.0, can safely be said to be associated with the process of DNA replication, because its appearance after hormonal stimulation is correlated with the induction of mitosis. Further, like the process of mitosis itself (see Fig. 1), this form of DNAase I is not maintained during protracted androgenic stimulation such as exists in the normal (non-castrated) animal.

Fig. 4. Analysis of prostate DNA ligase in sucrose gradients

Samples of prostate 105000g supernatant fraction (500 μ g of protein) were centrifuged at 100000 g_{av} for 18 h in linear 5–20% (w/v) sucrose gradients, volume 4.8 ml, prepared in medium A. The direction of centrifugation is from right to left; C, sedimentation marker, catalase (11.4S); H, haemoglobin marker, haemoglobin (4.6S) present in the enzyme extracts. Samples of 50 μ l of each gradient fraction were incubated with 10 μ g of 32 P-labelled DNA (5800 c.p.m.) and the radioactivity converted into a form resistant to *E. coli* alkaline phosphatase was determined. A background of 600 c.p.m. of 32 P resistant to phosphatase without incubation with DNA ligase was subtracted from all determinations. Extracts from (a) animals 7 days after castration; (b) castrated animals and 3 days of testosterone phenylpropionate treatment (2.5 mg/animal); (c) normal (non-castrated) animals.

These changes in prostate DNAase activity may reasonably be considered as a characteristic feature of accessory sexual glands because the pattern of spleen DNAases was not influenced by androgens.

Unwinding (or denaturation) of prostate DNA

Since the principal DNA polymerase present in the prostate gland after androgenic stimulation had a marked propensity for utilizing denatured DNA as template (see Fig. 3), we investigated the possible existence of a prostate protein whose function was to promote the formation of this favoured form of template at a time consistent with the induction of mitosis. Evidence supporting the presence of a DNA-unwinding protein in the prostate gland is presented in Table 3. Considerable DNA-unwinding activity was identified in spleen, but it was not influenced by the concentrations of circulating androgens. Conversely, DNA-unwinding activity in the prostate gland was regulated by androgens in a tissue- and steroid-specific manner. An important feature of the appearance of the DNA-unwinding protein in the prostate gland after androgenic stimulation was that it reached a maximum before the pronounced rise in mitotic activity (see Fig. 1) or indeed the enhancement of the activities of the enzymes thymidine kinase and DNA polymerase (see Tables 1 and 2).

Many proteins in eukaryotes can combine avidly with purified DNA and promote local regions of

denaturation or strand separation in helical poly-deoxyribonucleotides; a particularly good example is form B (or II) RNA polymerase (Meilhac *et al.*, 1972). Proteins sharing this ability often require DNA as an obligatory component in the expression of their enzymic function, and binding to DNA may be profitably exploited in their purification, for example RNA polymerase (Dezelee *et al.*, 1972) and DNA polymerase (Haines *et al.*, 1972). As a logical continuation of the studies on the DNA-unwinding activity it was necessary to eliminate the possibility that this function was attributable to either RNA polymerase or DNA polymerase, for the activity of both enzymes is stringently subject to control (Coffey *et al.*, 1968; Brasel *et al.*, 1969; Mainwaring *et al.*, 1971), as indeed is the DNA-unwinding activity (see Table 3). Prostate cell 105000g supernatant fractions were fractionated by either ultracentrifugation in sucrose gradients (Fig. 6a) or electrophoresis in polyacrylamide gels (Fig. 6b) and analysed for DNA-unwinding activity. In the studies reported above in Table 3, the DNA-unwinding activity was isolated by DNA-cellulose chromatography. Recourse to cell 105000g supernatant fractions was made on two principal grounds; first, practical expedience, and second, certain DNA polymerases were only partially retained on columns containing immobilized DNA (W. I. P. Mainwaring, unpublished work). These extracts were prepared from androgen-stimulated animals so that the activities of the prostate DNA polymerases were ex-

Fig. 5. Analysis of prostate DNAases by isoelectric focusing in polyacrylamide gels

Prostate cell 105000g supernatant extracts (500 μ g of protein) were separated by isoelectric focusing in 5% (w/v) polyacrylamide gels within the pH range 3–10; see the Materials and Methods section for complete details. Each gel contained 50 μ g of 3 H-labelled *E. coli* DNA (2.4×10^6 c.p.m.). At the completion of isoelectric focusing, the gels were cut into 30 equal slices (2.5 mm-thick) and incubated under optimum conditions for either DNAase I activity (pH 8.0; presence of 5 mM-MgCl₂) or DNAase II activity (pH 5.0; absence of added cations). Enzyme activity was located by the release of 3 H into a soluble form. Prostate 105000g supernatant fractions from (a) animals 7 days after castration; (b) castrated animals receiving 3 days of injections of testosterone phenylpropionate (2.5 mg/animal); (c) normal (non-castrated) animals. DNAase I, ●, DNAase II, ○. The anode is to the right. Marker proteins, used in the calculation of the pI of DNAase activities, were as follows: Cyt, cytochrome c (pI 9.2); Hb, haemoglobin present in the extracts (pI 7.2 and 7.6) and Cat, catalase (pI 6.0).

pressed at their maxima. In sucrose gradients, DNA-unwinding activity was present in two areas of the gradient profile corresponding to components of sedimentation coefficients 6.7S and 4.2S (Fig. 6a). This

essentially eliminates the implication of two important enzymes in the partial denaturation of prostate DNA, the 9S DNA polymerase (see Fig. 3) and RNA polymerase, sedimentation coefficient 22S under conditions of low ionic strength (Mainwaring *et al.*, 1971). However, the 4S DNA polymerases still remained potential candidates for promoting the unwinding of DNA, at least in part. From the experimental evidence presented in Fig. 6(b), the DNA polymerase with a preference for native DNA as template is not responsible for the DNA-unwinding activity in prostate extracts. The low activity of the DNA polymerase catalysing the polymerization of [3 H]dGTP alone with native DNA as template prevented its unequivocal location in polyacrylamide gels. However, the difference in the period of androgenic stimulation required to raise this DNA polymerase and the DNA-unwinding activity to their respective maxima (compare Tables 2 and 3) tends to obviate their common identity. Overall, the partial denaturation of DNA appears to be conducted by discrete protein entities other than RNA or DNA polymerases.

Anti-androgens and the induction of enzymes engaged in DNA replication

An impressive body of evidence has now established that many critical biochemical events in the prostate gland are set in train by the specific high-affinity binding of 5 α -dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one) to the androgen receptor proteins [see review by King & Mainwaring (1974)]. In support of this hypothesis, certain synthetic steroids counter both the high-affinity binding of 5 α -dihydrotestosterone and the subsequent manifestation of the androgenic response; a particularly well studied anti-androgen is cyproterone acetate (Geller *et al.*, 1968; Fang & Liao, 1969; Mangan *et al.*, 1973). It was decided to establish whether the enhancement of the prostate enzymes engaged in DNA replication was similarly triggered by the androgen-receptor system. To conform with other aspects of this study, testosterone was again used as the androgenic stimulus *in vivo*; however, there is no evidence to suggest that cyproterone acetate impairs the metabolism of testosterone to 5 α -dihydrotestosterone (Belham & Neal, 1971; Giorgi *et al.*, 1973) and its negation of androgen action is essentially expressed by means of the androgen-receptor system. Cyproterone acetate is not a potent competitor for the binding sites of 5 α -dihydrotestosterone (Fang & Liao, 1969; Liao & Fang, 1969) and large doses are needed to ablate the androgenic response. This necessitated a minor change in the hormonal regimen where this anti-androgen was used, but the concomitant administration of cyproterone acetate with testosterone *in vivo* severely curtailed the androgen-mediated enhancement of enzymes engaged in DNA replication (Table

Table 3. Hormonal regulation of DNA-unwinding activity in the prostate gland

On the basis of the protocol of steroid injections given in Table 1, the hormonal regulation of DNA-unwinding activity in the prostate gland and spleen was investigated. Tissues were pooled from four animals per experimental group and proteins with a high affinity for denatured DNA-cellulose were isolated (see the Materials and Methods section for details). Samples containing 20–50 µg of protein were incubated in triplicate with native ³H-labelled prostate DNA (10 µg; 4400 c.p.m.) and radioactivity retained on nitrocellulose filters was determined. Without unwinding (or denaturation), only 20 c.p.m. of radioactivity was retained, and all values are corrected for this background. The results are expressed as means ± S.E.M.

Hormonal treatment	³ H-labelled DNA retained (c.p.m./100 µg of protein)	
	Prostate gland	Spleen
(a) Tissue specificity		
None (castrated controls)	160 ± 20	2740 ± 120
Testosterone, 24 h	320 ± 20	2880 ± 200
Testosterone, 48 h	510 ± 30	2910 ± 70
Testosterone, 72 h	390 ± 20	2850 ± 20
Testosterone, 96 h	320 ± 10	2790 ± 40
(b) Steroid specificity		
None (castrated controls)	110 ± 10	
Testosterone, 48 h	480 ± 30	
Oestradiol-17β, 48 h	90 ± 10	
Corticosterone, 48 h	120 ± 20

Fig. 6. Analysis of prostate 105 000 g supernatant fractions for components capable of unwinding native prostate DNA

Cell 105 000 g supernatant fraction was prepared from the prostate glands of animals that had been castrated for 7 days and given three daily injections of testosterone phenylpropionate (2.5 mg/animal). Samples (350 µg of protein) were fractionated by two procedures and analysed for DNA-unwinding activity; in one experiment, additional analyses were conducted for DNA polymerase activity in the presence of native DNA as template. (a) Sucrose gradients: direction of centrifugation right to left; C, sedimentation marker, catalase (11.4S) and H, internal marker of haemoglobin (4.6S) present in the extracts. (b) Polyacrylamide-gel electrophoresis: direction of migration left to right (anode); B, tracking dye (Bromophenol Blue), and H, internal marker of haemoglobin. In both (a) and (b) ●, DNA-unwinding activity; ○, in (b) only, DNA polymerase activity. Slices of gel were incubated at 4°C for 2 h in 0.2 ml of medium A to release proteins before analysis.

4). This effect was specific and could not be simulated by the glucocorticoid corticosterone.

Effect of metabolic inhibitors on the induction of enzymes engaged in DNA replication

It was uncertain whether the enhancement of prostate enzyme activities promoted by androgens was

attributable to their synthesis *de novo* or to the activation of pre-existing enzyme proteins. To answer this question, the metabolic inhibitors actinomycin D and cycloheximide were administered concurrently with testosterone *in vivo* at doses previously shown to suppress over 90% of the RNA and protein synthesis occurring in the prostate gland (Mainwaring & Wilce, 1973). From these experiments, continued RNA and

Table 4. *Suppression of the prostate enzymes engaged in DNA replication by cyproterone acetate*

Beginning 7 days after castration, rats were injected with 0.3 mg of testosterone phenylpropionate subcutaneously in oil twice daily for three consecutive days; control animals received injections of oil only. Other steroids (9.0 mg) were also injected by a similar means. Prostate glands pooled from four animals per experimental group were used to prepare cell 105 000 g supernatant fractions. Analyses for thymidine kinase and DNA polymerase activities (denatured DNA as template) were performed in triplicate and the results are expressed as means $\pm$ S.E.M.

Hormonal treatment	Thymidine kinase activity (pmol of [3 H]dTMP formed/30 min per mg of protein at 37°C)	DNA polymerase activity (pmol of [3 H]dGTP incorporated/ 30 min per mg of protein at 37°C)
None (castrated controls)	7.0 $\pm$ 1.4	16.9 $\pm$ 3.1
Testosterone only	79.1 $\pm$ 7.4	157.4 $\pm$ 14.9
Cyproterone acetate only	6.0 $\pm$ 1.0	11.1 $\pm$ 0.9
Corticosterone only	10.1 $\pm$ 2.0	13.4 $\pm$ 1.9
Testosterone + cyproterone acetate	23.0 $\pm$ 2.2	68.6 $\pm$ 4.9
Testosterone + corticosterone	74.0 $\pm$ 4.4	162.2 $\pm$ 13.0

protein synthesis is necessary for the dramatic stimulation of the enzymes associated with DNA replication in the prostate gland after the administration of androgens.

Discussion

One aspect of the model system used throughout this study (see Fig. 1) warrants further comment, namely the apparent co-ordination of mitosis and DNA replication, represented by the induction of DNA polymerase activity, after androgenic stimulation. On the basis of the classical concept of the cell cycle proposed by Howard & Pelc (1951), DNA replication should occur in the S phase preceding the mitotic event itself, termed the M phase. The similarity in the speed of the androgenic induction of these events in the prostate gland is possibly more apparent than real. With more individual experimental points in our determination of the time-course of events related to DNA replication, distinction between the induction times of DNA polymerase activity and mitosis may have been evident. Alternatively, it could be argued that individual prostate cells are entering the process of cell division at slightly different rates, resulting in the apparent overlap of the otherwise temporally distinct biochemical events proposed by Howard & Pelc (1951). By using a more contemporary model of cell division (Smith & Martin, 1973) it is possible that prostate cells leave the indeterminate A phase into the determinate B phase at different rates. Whichever interpretation is used in no way invalidates the use of the system for the principal purpose of the present study, which was to establish the hormonal regulation of the activities of enzymes engaged in DNA replication.

On the evidence presented, notably with respect to the acute specificity in the changes in enzyme activities and the ablative effect of anti-androgens, it is concluded that the process of DNA replication is ulti-

mately controlled by the high-affinity binding of 5 α -dihydrotestosterone, as indeed are virtually all manifestations of the androgenic response (King & Mainwaring, 1974). The involvement of the receptor system is in no way inconsistent with the protracted latent period in the induction of the various enzyme activities, despite the facts that testosterone is rapidly metabolized and bound within the prostate gland and that the receptor complex does not persist for longer than 12 h (Tveter & Attramadal, 1968; Mainwaring & Peterken, 1971). Our present studies with metabolic inhibitors, together with the findings of other investigators (Sufrin & Coffey, 1973; Coffey, 1974), suggest that protein synthesis *de novo* must occur for the expression of the changes in the activities of enzymes associated with DNA replication. These androgen-induced proteins could include the enzyme moieties themselves and perhaps other regulatory proteins, but in either event, such protein synthesis would require a long time to reach a maximum relative to the rapid saturation of the androgen-receptor system with 5 α -dihydrotestosterone.

On the evidence presented, many enzymes are obligatorily involved in the process of DNA replication in the prostate gland. The nature of these enzymes is essentially harmonious with the overall predictions of Keir & Craig (1973). Perhaps the most novel feature of the present study is the implication of one specific deoxyribonuclease in prostate DNA synthesis and the identification of a DNA-unwinding protein which promotes the denaturation or strand separation of the DNA template required for one particular form of DNA polymerase enzyme. We did not undertake a rigorous check that the enzymes regulated by androgens were predominantly located in the nucleus of prostatic cells. Under the conditions of homogenization used, nuclei and perhaps even mitochondria would be disrupted, and hence our studies probably reflect changes in total enzyme activities (Coffey *et al.*, 1968). In a preliminary study of this type, such a

relatively simplistic approach is eminently justified. However, it should be added that for the enzymes, DNA polymerase and thymidine kinase, we did check that over 85% of the total activities present in prostate homogenates was recovered in the cell 105000g supernatant fraction. This is particularly important for thymidine kinase which is partially located within mitochondria (Berk & Clayton, 1973) and suggests that most intracellular structures were disrupted by our means of homogenization. Characterization of the DNA-unwinding protein is our principal interest. Additional studies (P. S. Rennie & E. K. Symes; unpublished work) have demonstrated an increase in the template activity of native (helical) DNA for the 9S form of DNA polymerase after incubation with the DNA-unwinding protein; this effect is not evident with denatured (single-stranded) DNA or with the 4S form of DNA polymerase.

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