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# A Reappraisal of the Effects of Adenosine 3′:5′-Cyclic Monophosphate on the Function and Morphology of the Rat Prostate Gland

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1. A comparison was made of the binding of 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and cyclic AMP in the rat prostate gland. Distinct binding mechanisms exist for these compounds, and cyclic AMP cannot serve as a competitor for the 5α-dihydrotestosterone-binding sites and vice versa. In contrast with the results obtained with 5α-dihydrotestosterone, very small amounts of cyclic AMP are retained in nuclear chromatin and the overall binding of this cyclic nucleotide is not markedly affected by castration. 2. Androgenic stimulation does not lead to major increases in the adenylate cyclase activities associated with any subcellular fraction of the prostate gland. Accordingly, changes in the concentration of cyclic AMP in the prostate gland after hormonal treatment are likely to be small, but these were not measured directly. 3. When administered to whole animals *in vivo*, small amounts of non-degraded cyclic AMP are found in the prostate gland but sufficient to promote an activation of certain carbohydrate-metabolizing enzymes in the cell supernatant fraction. The stimulatory effects of cyclic AMP were not evident with cytoplasmic enzymes engaged in polyamine synthesis or nuclear RNA polymerases. These latter enzymes were stimulated solely by the administration of testosterone. 4. By making use of antiandrogens, a distinction can be drawn between the biochemical responses attributable to the binding of 5α-dihydrotestosterone but not of cyclic AMP. Evidence is presented to suggest that the stimulation of RNA polymerase, ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase is a consequence of the selective binding of 5α-dihydrotestosterone. Only the stimulation of glucose 6-phosphate dehydrogenase can be attributed to cyclic AMP or other metabolites of testosterone. 5. Overall, this study indicates that the formation of cyclic AMP is not a major feature of the androgenic response and affects only a restricted number of biochemical processes. Certainly, cyclic AMP cannot be considered as interchangeable with testosterone and its metabolites in the control of the function of the prostate gland. This difference is additionally emphasized by the failure of cyclic AMP to restore the morphology of the prostate gland in castrated animals; morphological restoration only follows the administration of androgens.

Cyclic AMP has been widely reported to act as an intracellular mediator or 'second messenger' in the mechanism of action of several polypeptide hormones and biogenic amines. An authoritative account of the current knowledge of this important area of investigation may be found in the review by Robison & Sutherland (1972). Recent studies have implicated cyclic AMP in the mechanism of action of steroid sex hormones on the accessory sexual organs in adult male and female rats. Androgens, for example, have been shown to induce the activity of many enzymes involved in carbohydrate metabolism in the seminal vesicle and ventral prostate gland (Singhal & Valadares, 1968; Singhal *et al.*, 1968; Santti & Villee, 1971) and these enzymes are similarly stimulated in uterus by the administration of oestrogens (Barker & Warren, 1966; Eckstein & Villee, 1966; Hilf *et al.*, 1972). Since R. L. Singhal and his colleagues (Singhal *et al.*, 1970, 1971) have shown that cyclic AMP is also capable of directly inducing the activity of many carbohydrate-metabolizing enzymes in the prostate gland and seminal vesicle, the distinct possibility exists that cyclic AMP is implicated in the overall mechanism of action of the steroid sex hormones in cells on which they exert profound biochemical effects. Not all of the currently available evidence necessarily supports such a close connexion between the formation of cyclic AMP and the trophic and biochemical effects of steroid hormones. Studies conducted by Rosenfeld & O'Malley (1970) failed to demonstrate a stimulation of adenylate cyclase activity in the prostate gland after androgenic stimulation, and Liao *et al.* (1971) were similarly unable to detect any significant alteration in adenylate cyclase activity in prostatic nuclei after

either castration or the administration of testosterone *in vivo*. Kissel *et al.* (1970) also reported that the rate of formation of cyclic AMP in chick oviduct was unaffected by injection of diethylstilboestrol, a hormonal treatment that promotes cellular differentiation and the synthesis of ovalbumin in this organ (O'Malley *et al.*, 1967). These authors did report, however, that subsequent administration of progesterone stimulated the synthesis of another protein, avidin, from a different cell type, together with a delayed increase in adenylate cyclase activity (O'Malley *et al.*, 1967). Owing to the delay in the response of this enzymic activity, O'Malley *et al.* (1967) concluded that the formation of cyclic AMP was not an early and integral event in the accelerated synthesis of avidin promoted by progesterone. In direct opposition to these findings, other reports have indicated that a rise in intracellular concentrations of cyclic AMP follows the administration of steroid sex hormones *in vivo*. Singhal *et al.* (1971) have reported that testosterone increases the cyclic AMP content of the accessory sexual glands of castrated male rats after a prolonged latent period of some 8h; conversely, Szego & Davis (1967) reported a remarkably rapid increase in the concentration of this cyclic nucleotide in the uterus of ovariectomized rats after the administration of oestrogens. Significant changes were reported within 15s of hormonal stimulation. On the basis of these somewhat conflicting findings, the implication of cyclic AMP in the overall mechanism of action of steroid sex hormones can only be described at best as equivocal.

In a critical appraisal of the similarities between the effects of cyclic AMP and testosterone on the prostate gland, it is essential to bear in mind that hormonal stimulation of this accessory sexual gland involves profound changes in enzymes other than those engaged in carbohydrate metabolism. A particularly rapid increase in the activity of the $Mg^{2+}$-activated $\alpha$-amanitin-insensitive RNA polymerase after androgenic stimulation has been widely documented (Liao *et al.*, 1965; Mainwaring *et al.*, 1971) and slower but profound increases in the activities of enzymes responsible for the formation of the nitrogenous bases spermine and spermidine are also stimulated by the administration of androgens (Pegg *et al.*, 1970). Further, androgens also control the morphology of the prostate gland, particularly the size and function of the nucleolus (Cavazos & Mellampy, 1954), the height and secretory activity of the luminal epithelium (Brandes & Groth, 1963) and the degree of cytoplasmic basophilia, equatable with the ribosomal RNA content of the gland (Seamen & Studen, 1960; Brandes, 1963). It is essential therefore that such a morphological study should feature in any investigation of the overall influence of cyclic AMP on the prostate gland. Perhaps the most pertinent insight into the similarities between the effects of cyclic AMP and testosterone on male accessory sexual glands can be gained by the use of antiandrogenic steroids, known to compete selectively for the androgen-binding sites. There is abundant evidence that testosterone is extensively metabolized to 5$\alpha$-dihydrotestosterone (17$\beta$-hydroxy-5$\alpha$-androstan-3-one) in the prostate gland (Bruchovsky & Wilson, 1968) and that this metabolite is specifically bound to androgen-binding proteins or 'receptors' within androgen-dependent cells (Anderson & Liao, 1968; Mainwaring, 1969*a*,*b*). The ultimate destination of the 5$\alpha$-dihydrotestosterone–receptor complex is nuclear chromatin and the implication of the receptors in the overall hormonal response is based on the finding that antiandrogens such as cyproterone acetate (6$\alpha$-chloro-17$\alpha$-hydroxy-1,2$\alpha$-methylene-4,6-pregnadiene-3,20-dione 17-acetate) and 6$\alpha$-bromo-17$\beta$-hydroxy-17$\alpha$-methyl-4-oxa-5$\alpha$-androstan-3-one decrease the high-affinity binding of 5$\alpha$-dihydrotestosterone (Belham & Neal, 1971; Mangan & Mainwaring, 1972) and also stop the activities of such indicators of the androgenic response as RNA polymerase (Mangan & Mainwaring, 1972) and acid phosphatase (Geller *et al.*, 1969). By the use of both biochemical and morphological criteria, the principal objective of the present study was to assess critically the importance of cyclic AMP in the androgenic response in the rat ventral prostate gland.

## Materials and Methods

### *Animals and materials*

*Animals.* Adult male Sprague–Dawley rats, 8–10 weeks old (210–240g), were used throughout this study. Castration was performed by the scrotal route under fluothane anaesthesia. When administered, testosterone phenylpropionate (2.5mg) was given subcutaneously into the flank in arachis oil (0.25ml). Cyclic AMP (2.5mg) and theophylline (2.5mg) were administered together intraperitoneally as a single injection in 0.25ml of physiological saline (0.9% NaCl). Cycloheximide (100$\mu$g), actinomycin D (70$\mu$g) and cyclic [$^3$H]AMP (10$\mu$Ci) plus theophylline (2.5mg) were injected intraperitoneally in 0.25ml of 0.9% NaCl. Cyproterone acetate (10mg) was given subcutaneously in 0.5ml of propane-1,2-diol. Depending on the nature of the experiment, control animals received an equivalent volume of injection vehicle only.

*Chemicals.* [U-$^{14}$C]ATP (ammonium salt; sp. radioactivity 550mCi/mmol), [8-$^3$H]GTP (ammonium salt; sp. radioactivity 13.3Ci/mmol), cyclic [8-$^3$H]AMP (ammonium salt; sp. radioactivity 25Ci/mmol) and [4-$^{14}$C]testosterone (sp. radioactivity 61mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 5$\alpha$-[4,5-$^3$H]Dihydrotestosterone (30Ci/mmol) was synthesized by Dr. M. M. Coombs of this Institute and

stored in oxygen-free benzene at 8–10°C. [$^{14}$C]-Testosterone (5$\mu$Ci) was converted into 5$\alpha$-[$^{14}$C]-dihydrotestosterone by incubation with a prostate microsomal preparation (4mg of protein) at 37°C for 1h in the presence of an NADPH-generating system, composed of glucose 6-phosphate (1.4mg), $NADP^+$ (2.0mg) and 150$\mu$g of yeast glucose 6-phosphate dehydrogenase. The $^{14}$C-labelled steroids were extracted and separated by t.l.c. as described in detail by Mainwaring (1969*b*). The overall conversion into 5$\alpha$-[$^{14}$C]dihydrotestosterone was 57% and the labelled metabolite was purified to a radiochemical purity of 95% by repeated chromatography. Dithiothreitol, ATP, CTP, UTP, GTP (sodium salts), Tris base (99.5% pure), protamine sulphate (grade II), phenazine methosulphate, theophylline and pyridoxal phosphate were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Actinomycin D was obtained from Merck, Sharp and Dohme, Hoddesdon, Herts., U.K. Cyproterone acetate was a gift from Schering Chemicals, Burgess Hill, Sussex, U.K. Cycloheximide was supplied by Ralph N. Emanuel Ltd., Alperton, Middx., U.K. $NAD^+$, $NADP^+$, NADH, yeast glucose 6-phosphate dehydrogenase and Nitro Blue Tetrazolium were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Glucose 6-phosphate (disodium salt) and yeast pyruvate kinase were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Camlabs (Cambridge, U.K.) supplied Macherey–Nagel cellulose MN 300 polyethyleneimine-impregnated chromatography sheets (20cm×20cm; 0.1mm thickness; code no. Polygram CEL 300 PEI). The plates were washed with deionized water and air-dried just before use.

*Fractionation procedures*

*Isolation of subcellular fractions.* All preparative procedures were conducted in ice within a cold laboratory maintained at 4°C.

*Incubation of prostate minces in vitro and the subsequent isolation of subcellular fractions.* Prostate glands pooled from five rats were coarsely minced with scissors and placed in 5ml of Eagle's minimal medium containing various radioactive compounds as indicated in the text. After incubation in air at 30°C for 45min, the tissue was drained, washed with several changes of ice-cold Eagle's medium and finally homogenized in 10ml of 0.25M-sucrose–1mM-EDTA with a motor-driven Potter–Elvehjem homogenizer. The homogenate was filtered through a 100-gauge wire mesh and centrifuged at 700*g* for 10min. Nuclei were purified from the sediment by further centrifugation in 2.2M-sucrose–1mM-$MgCl_2$ as described by Mainwaring *et al.* (1971). The 700*g* supernatant fraction was centrifuged at 10000*g* for 20min to provide a crude mitochondrial preparation. The supernatant from the centrifugation at 10000*g* was centrifuged at 100000*g* for 60min to provide a microsomal preparation and a cell supernatant fraction. The particulate fractions were resuspended in 1.0ml of 1M-NaOH and solubilized by standing overnight at 30°C. Samples of cell supernatant fraction (0.5ml) were treated with 50$\mu$l of 2% (w/v) protamine sulphate and left for 5min in ice. The sediment was then removed by centrifugation and dissolved in 1.0ml of 1M-NaOH. This treatment has been shown to precipitate the specific 5$\alpha$-dihydrotestosterone–receptor complex in the cell supernatant fraction of accessory sexual glands (Mainwaring, 1969*b*). Samples of all subcellular fractions were assayed for protein content and counted for radioactivity.

*Isolation of nuclei and cell supernatant after labelling or hormonal treatments in vivo.* Prostate glands (five per experimental group) were removed and chilled in ice-cold 0.25M-sucrose–1mM-$MgCl_2$. The tissue was homogenized in 5ml of 0.25M-sucrose–1mM-$MgCl_2$ and the homogenate was centrifuged at 700*g* for 10min. Purified nuclei were then isolated from the sediment (Mainwaring *et al.*, 1971) and the 700*g* supernatant was centrifuged at 100000*g* for 60min to yield a cell supernatant fraction.

*Enzyme assays*

*Assay of RNA polymerase.* RNA polymerase activity was determined in purified nuclei essentially as described in detail by Mainwaring *et al.* (1971). The standard assay system contained 50$\mu$mol of Tris–HCl buffer, pH8.1, 0.2$\mu$mol of dithiothreitol, 3.5$\mu$mol of KCl, 0.3$\mu$mol each of ATP, CTP and UTP (all adjusted to pH8.0 with 50mM-Tris base), 0.625$\mu$Ci of [$^3$H]GTP and 12.5nmol of GTP, nuclei equivalent to 100$\mu$g of DNA and the final volume was adjusted to 0.5ml by the addition of 50mM-Tris–HCl, pH8.1. The bivalent cations included in the assay medium were either 2.5$\mu$mol of $MgCl_2$ alone or 2.5$\mu$mol of $MnCl_2$ plus 150$\mu$mol of $(NH_4)_2SO_4$. Full details of the conditions of incubation and the determination of the incorporation of [$^3$H]GTP are given by Mainwaring *et al.* (1971). The incorporation of [$^3$H]GTP was calculated in terms of acid-insoluble d.p.m./mg of DNA after correction for background and zero-time controls. Under these conditions of assay, 186d.p.m. was equivalent to the incorporation of 1pmol of GMP into RNA.

*Assay of enzyme activities in cell supernatant fractions.* (a) Glucose 6-phosphate dehydrogenase. To a 3.0ml silica cuvette was added the following: 2.5ml of 50mM-Tris–HCl buffer, pH7.6, 0.20ml of 0.1M-$MgCl_2$, 0.1ml of glucose 6-phosphate (sodium salt; 10mg/ml) and 0.1ml of $NADP^+$, and the enzymic reaction was initiated by the addition of 0.1ml of cell supernatant fraction (approx. 1.5mg of protein).

The increase in $E$ at 360nm was followed in a thermostatically controlled cuvette-holder at 25°C in a Gilford recording spectrophotometer.

(b) Putrescine-dependent *S*-adenosyl-L-methionine decarboxylase. For the preparation of a cell supernatant fraction suitable for this assay, prostate glands were homogenized in 0.25M-sucrose–1mM-$MgCl_2$–5mM-dithiothreitol; the inclusion of the thiol-protecting agent follows the recommendation of Jänne & Williams-Ashman (1970). Essentially, the measurement of enzymic activity is based on the determination of the release of $^{14}CO_2$ from *S*-adenosyl-L-[*carboxy*-$^{14}$C]methionine (Pegg & Williams-Ashman, 1969). The assay medium (final volume 1.0ml) contained 2$\mu$mol of putrescine, 100$\mu$mol of $K_2HPO_4$–$KH_2PO_4$ buffer, pH7.0, 0.2$\mu$mol of *S*-adenosyl-L-[*carboxy*-$^{14}$C]methionine and 0.4ml of 100000*g* supernatant. Incubation was performed for 30min at 37°C, and full details of the trapping of $^{14}CO_2$ released are given by Pegg & Williams-Ashman (1969).

(c) Ornithine decarboxylase. Ornithine decarboxylase activity was assayed in the cell supernatant fraction prepared in the manner described for the assay of *S*-adenosyl-L-methionine decarboxylase. The activity was measured by the release of $^{14}CO_2$ from [1-$^{14}$C]ornithine, as described by Pegg *et al.* (1970). The assay mixture (final volume 1.0ml) contained 0.2$\mu$mol of L-[1-$^{14}$C]ornithine, 0.1$\mu$mol of pyridoxal phosphate, 50$\mu$mol of Tris–HCl buffer, pH7.5, and 0.1ml of cell supernatant fraction. The reaction was incubated for 30min at 37°C and terminated as described elsewhere (Pegg *et al.*, 1970).

*Assay of adenylate cyclase activity in subcellular fractions.* Adenylate cyclase activity was measured in subcellular fractions with [U-$^{14}$C]ATP as substrate. The reaction mixture contained the following, in a total volume of 0.05ml: 2$\mu$mol of Tris–HCl, pH7.5, 0.25$\mu$mol of $MgCl_2$, 0.6$\mu$mol of NaF, 0.05$\mu$mol of phosphoenolpyruvate, 0.05$\mu$mol of cyclic AMP, 0.05$\mu$mol of theophylline, 1$\mu$g of pyruvate kinase and 0.2$\mu$mol of ATP containing 0.2$\mu$Ci of [U-$^{14}$C]ATP. The reaction was initiated by the addition of 0.02ml of prostatic subcellular fraction suspended in 0.25M-sucrose (approx. 100$\mu$g of protein). The reaction was carried out at 37°C for 15min and terminated by heating in a boiling-water bath for 3min. The tubes were then cooled in ice, 0.05ml of ethanol was added and the contents were centrifuged. The supernatant was collected and a portion (usually 0.02 or 0.01ml) was applied to polyethyleneimine-impregnated cellulose t.l.c. sheets, pre-washed in deionized water. The plates were developed in 0.25M-LiCl for 3h as described by Bär & Hechter (1969). Standards corresponding to cyclic AMP ($R_F$ 0.69), 5′-AMP ($R_F$ 0.38), 5′-ADP ($R_F$ 0.20) and 5′-ATP ($R_F$ 0.05) were located in guide strips under u.v. light. The plates were dried in air, cut into 1cm squares and the radioactivity was counted in 10ml of phosphor composed of 5g of 2,5-diphenyloxazole/litre of toluene. The percentage recovery of radioactivity from the plates was between 75% and 80% of a pre-counted sample. Counts corresponding to the cyclic AMP marker were determined and the total conversion of [U-$^{14}$C]ATP into cyclic [$^{14}$C]-AMP was calculated in terms of nmol of cyclic [$^{14}$C]AMP formed/15min per mg of protein at 37°C.

*Detection of isoenzymes of glucose 6-phosphate dehydrogenase*

Discontinuous electrophoresis of prostate cell supernatant fraction (0.2ml) was conducted by the procedure of Ornstein (1964) in 7.5% (w/v) polyacrylamide gels. Electrophoresis was performed for 3h at a current of 5mA/gel, in 100mM-Tris–glycine, pH8.3, as electrode buffer. At the completion of the separation, the gels were promptly removed from the running tube and the isoenzymes detected by incubation, for 4h in the dark at room temperature, in a solution containing 1.0ml of 20mM-glucose 6-phosphate, 1.0ml of $NADP^+$ (2.5mg/ml), 2.0ml of 20mM-$MgCl_2$ in 100mM-Tris–HCl buffer, pH7.5, 2.5ml of Nitro Blue Tetrazolium (1mg/ml) and 0.25ml of phenazine methosulphate (1mg/ml). The gels were stored in 7% (v/v) acetic acid and scanned at 540nm in a Gilford spectrophotometer, by using the model 34B gel-holder.

*Release of enzymes from microsomal preparation and polyribosomes*

A total microsomal preparation and purified polyribosomes were prepared from the prostate gland by a method based essentially on that of Tata & Williams-Ashman (1967). Samples of the preparation (1.5mg of protein) were suspended in 50mM-Tris–HCl buffer, pH7.8, containing 1mM-$MgCl_2$ alone or in medium supplemented with cyclic AMP, 5$\alpha$-dihydrotestosterone or 5$\alpha$-androstane-3$\beta$,17$\beta$-diol. After standing for 1h at 4°C, the particulate elements were sedimented by centrifugation at 100000*g* for 1h (microsomal preparation) or 3h (polyribosomes) and the supernatant fraction was assayed for glucose 6-phosphate dehydrogenase activity.

*General techniques*

*Staining of sections of the prostate gland.* Prostate glands were fixed for 18h in Carnoy's medium and sections were stained by the standard Haemotoxylin–Eosin method.

*Isoelectric focusing.* This was performed in small columns, volume 12.5ml, made to the design recommended by Osterman (1970). Samples for analysis (maximum load 2.0mg of protein) were first desalted

by passage through columns of Sephadex G-25, equilibrated with water only, and evenly distributed throughout a 12.5ml linear 0–60% (w/v) sucrose gradient containing narrow-range ampholytes (LKB, South Croydon, Surrey, U.K.) suitable for analyses in the range pH5–8. After thorough cooling in a cold-room and the circulation of ice-cold water through the cooling jacket, an initial potential of 300V (2mA) was applied for 30min, after which the potential was adjusted to 800V. Focusing was conducted overnight for a total of 16h. Samples of prostate extract were supplemented with 50$\mu$g of marker proteins ferritin, catalase and haemoglobin just before analysis; these coloured proteins, of known pI, enabled the satisfactory performance of focusing to be observed throughout.

*Chemical analyses.* DNA was determined by the procedure of Burton (1956) with calf thymus DNA as standard. The method of Lowry *et al.* (1951) was employed for the determination of protein with bovine serum albumin as standard.

## Results

### *Binding of cyclic AMP and 5α-dihydrotestosterone in the subcellular fractions of the prostate gland*

*Particulate elements.* In initial experiments, a comparison was made of the intracellular binding sites for cyclic [$^3$H]AMP and 5α-[$^3$H]dihydrotestosterone, the principal metabolite formed from testosterone in the prostate gland. Particular use was made of the effect of castration on the binding of the tracer compounds; orchidectomy results in a pronounced rise in the number of available binding sites for 5α-[$^3$H]dihydrotestosterone (Mangan *et al.*, 1968; Mainwaring, 1971) and, if cyclic [$^3$H]AMP is bound to identical sites, then a similar enhancement of binding of this cyclic nucleotide should result from castration. On the basis of the results presented in Table 1, the intracellular binding sites for 5α-dihydrotestosterone and cyclic AMP are totally different. Two criteria in particular distinguish the binding sites. First, the specific or high-affinity binding of 5α-[$^3$H]dihydrotestosterone was predominantly in the nucleus; binding to these sites was augmented by castration, yet significantly decreased by the antiandrogen cyproterone acetate. Binding of 5α-[$^3$H]dihydrotestosterone to prostate microsomal preparations and mitochondria was essentially of low affinity and non-specific, since neither castration nor the presence of antiandrogen effected a significant change in the degree of binding. Secondly, the nuclear binding of cyclic [$^3$H]AMP was low, and, further, the binding of this cyclic nucleotide to all particulate fractions of the prostate gland was largely unchanged by castration or the presence of cyproterone acetate. Throughout this series of experiments, cyproterone acetate served as a competitor only for the 5α-dihydrotestosterone-binding sites; it had negligible effects on the binding of cyclic AMP. The displacement of the binding of 5α-[$^3$H]dihydrotestosterone was revealed particularly in studies conducted on tissues from androgen-depleted animals. The results on the binding of the $^3$H-labelled androgen are entirely consistent with earlier experimental

Table 1. *Comparison of the binding of 5α-[$^3$H]dihydrotestosterone and cyclic [$^3$H]AMP in the particulate subcellular fractions of the rat prostate gland*

Minces of prostate tissue, pooled from five animals, were incubated *in vitro* with $1.8\times10^6$c.p.m. (60pmol) of either 5α-[$^3$H]dihydrotestosterone or cyclic [$^3$H]AMP; both tracer compounds had a specific radioactivity of 25Ci/mmol. Theophylline (2.5mg) was included in all incubations with cyclic [$^3$H]AMP; where indicated, a 1000-fold excess of cyproterone acetate (60nmol) was also present in some incubations. Full details of the experimental procedures are given in the Materials and Methods section. The results are the means±S.D. of determinations in triplicate.

| | Binding (d.p.m./mg of protein) | | | |
|---|---|---|---|---|
| | Normal non-castrated animals | | Animals 24h after castration | |
| Tracer | Cyproterone acetate absent | Cyproterone acetate present | Cyproterone acetate absent | Cyproterone acetate present |
| 5α-[$^3$H]Dihydrotestosterone | | | | |
| Nuclei | 1280±113 | 1010±47 | 12130±392 | 4320±376 |
| Mitochondria | 16440±172 | 15020±136 | 18530±904 | 16940±876 |
| Microsomes | 10690±121 | 8420±171 | 18840±1200 | 14290±916 |
| Cyclic [$^3$H]AMP | | | | |
| Nuclei | 480±60 | 460±37 | 656±140 | 599±46 |
| Mitochondria | 2920±204 | 2820±224 | 2840±272 | 2760±299 |
| Microsomes | 1820±162 | 1760±142 | 2210±241 | 2140±196 |

findings (Mainwaring, 1971). In additional experiments, not shown in detail here, where [$^3$H]testosterone replaced 5α-[$^3$H]dihydrotestosterone, an identical subcellular distribution of radioactivity was found after incubation of prostate tissue. The sole difference in experiments with [$^3$H]testosterone is that the system must be supplemented with an NADPH-generating system.

*Cell supernatant fraction.* Highly selective methods must be employed for the determination of the specific high-affinity androgen receptors in the cell supernatant fraction of the prostate gland, since extensive but non-specific binding of labelled androgens has been widely documented by many investigators (Mainwaring, 1969*b*; Unhjem *et al.*, 1969; Baulieu & Jung, 1970). One simple means for specifically measuring the androgen-receptor–steroid complexes in the presence of other steroid-binding proteins is precipitation with protamine sulphate (Mainwaring, 1969*b*). From the evidence summarized in Table 2, the binding sites for 5α-[$^3$H]dihydrotestosterone and cyclic [$^3$H]AMP in the prostate cell supernatant fraction are different. In general terms, protamine sulphate failed to precipitate significant amounts of radioactivity from soluble preparations labelled with cyclic [$^3$H]AMP, even with preparations derived from castrated animals. The difference in the binding of the two tracer compounds under comparison was forcibly demonstrated in the competitive effects of non-radioactive compounds. Cyproterone acetate, 5α-androstane-3β,17β-diol (another metabolite formed from testosterone in the prostate gland) and, most notably, 5α-dihydrotestosterone significantly decreased the binding of 5α-[$^3$H]dihydrotestosterone; such marked competition was not found in the experiments conducted with cyclic [$^3$H]AMP. Of outstanding importance, however, was the observation that non-radioactive cyclic AMP did not decrease the binding of 5α-[$^3$H]dihydrotestosterone.

To confirm the difference in the binding components for cyclic AMP and 5α-dihydrotestosterone in the cell supernatant fraction, isoelectric focusing was conducted on a mixed sample of 100000*g* supernatant fractions, initially labelled with either 5α-[$^{14}$C]dihydrotestosterone or cyclic [$^3$H]AMP (Fig. 1). This experiment provided confirmation of the unique nature of the binding components for these labelled compounds. A complex of bound 5α-[$^{14}$C]dihydrotestosterone with a protein of pI 5.8 was formed. Binding of cyclic [$^3$H]AMP to several proteins was observed, but there was negligible binding in the area of the gradient of pH range 5.6–6.0 where androgen binding was maximal. The presence of considerable amounts of $^3$H at the bottom of the pH gradient indicated significant dissociation of the highly charged cyclic nucleotide during the course of isoelectric focusing. On the basis of these experiments, both cyclic AMP and 5α-dihydrotestosterone are retained in the prostate gland, but the cellular components responsible for this binding are different. The low binding of cyclic [$^3$H]AMP to the soluble androgen receptor is particularly important, since it provides substantial corroboration of the low nuclear binding of cyclic [$^3$H]AMP on incubation

Table 2. *Relative precipitation of radioactivity from the cell supernatant fraction by protamine sulphate after labelling with either 5α-[$^3$H]dihydrotestosterone or cyclic [$^3$H]AMP*

Minces of prostate tissue, pooled from three animals, were labelled as described in the legend to Table 1. After dilution to a uniform concentration of proteins, samples of 105000*g* supernatant were precipitated with protamine sulphate and the radioactivity in the precipitate was determined. Where indicated, an excess of non-radioactive compounds was included in the incubation medium: androstanediol and 5α-dihydrotestosterone, 20-fold excess; cyproterone acetate and cyclic AMP, 100-fold excess. The results are the means ± S.D. of triplicate determinations.

| | | Precipitated radioactivity (d.p.m.) | |
|---|---|---|---|
| Tracer | Non-radioactive compound | Normal non-castrated animals | Animals 24h after castration |
| 5α-[$^3$H]Dihydrotestosterone | None | 4370 ± 203 | 8650 ± 411 |
| | Cyproterone acetate | — | 4240 ± 376 |
| | Androstanediol | — | 6110 ± 450 |
| | 5α-Dihydrotestosterone | — | 764 ± 112 |
| | Cyclic AMP | — | 7990 ± 342 |
| Cyclic [$^3$H]AMP | None | 940 ± 22 | 1140 ± 144 |
| | Cyproterone acetate | — | 1070 ± 70 |
| | Androstanediol | — | 1250 ± 146 |
| | 5α-Dihydrotestosterone | — | 1020 ± 81 |
| | Cyclic AMP | — | 704 ± 62 |

of minces of whole prostate gland *in vitro* described in Table 1. There is considerable evidence to support the view that steroid–receptor complexes are initially formed in the cell supernatant or cytoplasmic fraction of rat prostate gland (Mainwaring & Peterken, 1971; Steggles *et al.*, 1971), followed by the transfer of the entire protein–steroid complex into the nuclear chromatin.

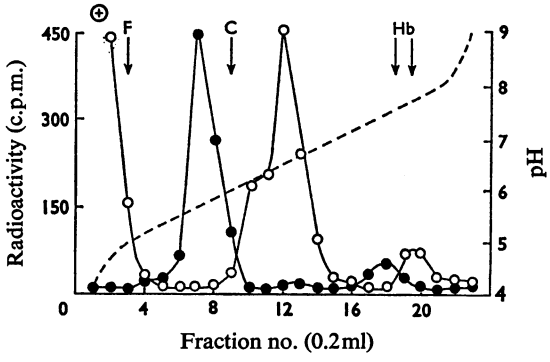



Fig. 1. *Separation of the binding components for 5α-[$^{14}C$]dihydrotestosterone and cyclic [$^{3}H$]AMP in the prostate cell supernatant fraction*

Samples of 100000*g* supernatant fraction were labelled separately with $1 \times 10^6$ c.p.m. of each of the labelled compounds for 1 h at 0°C. Samples containing $^{14}C$ and $^{3}H$ were mixed, desalted in columns of Sephadex G-25 and analysed by isoelectric focusing in the range pH 5–8. ●, $^{14}C$; ○, $^{3}H$; ---, pH. F, C and Hb denote the positions of marker proteins ferritin, catalase and haemoglobin (two bands) of pI 5.0, 6.0, 7.2 and 7.6 respectively.

*Relative uptake of cyclic [$^{3}H$]AMP into the prostate gland*

On the basis of the amounts of the radioactivity associated with various organs after a single injection of cyclic [$^{3}$H]AMP *in vivo*, none of the androgen-dependent organs has a selective propensity for the binding of this cyclic nucleotide (Table 3). Particularly large amounts of radioactivity were detected in the kidney and probably represent excretion of degradation products of the labelled cyclic AMP. When samples of extracted radioactivity were separated on t.l.c. plates of polyethyleneimine-impregnated cellulose, the amount of radioactivity chromatographing like cyclic [$^{3}$H]AMP was not particularly great in any tissue examined, being some 9–14% in seminal vesicle, 14–16% in prostate gland and 19–20% in the adrenal glands.

*Effect of the administration of androgens on the adenylate cyclase activity in the prostate gland*

Before an investigation was undertaken of the possible hormonal stimulation of adenylate cyclase activity, the subcellular distribution of the enzyme was established (Table 4). Adenylate cyclase activity was essentially recovered in only the particulate fractions of the prostate gland, and, in harmony with the results of Liao *et al.* (1971), some activity was associated with the nuclear fraction. Owing to the very high activities in the microsomal and mitochondrial fractions, however, a valid assessment of the nuclear-associated adenylate cyclase activity can only be made after extensive purification of the nuclei, such as further sedimentation in 2.2 M-sucrose.

Previous investigations on the possible androgenic stimulation of adenylate cyclase activity were conducted in unfractionated tissue homogenates (Rosenfeld & O'Malley, 1970), but in the present study

Table 3. *Retention of radioactivity in various organs after the administration of cyclic [$^{3}H$]AMP in vivo*

Cyclic [$^{3}$H]AMP (10 μCi) plus 2.5 mg of theophylline was administered to either normal non-castrated animals or to animals castrated 24 h previously. Tissues were removed 1 h later and radioactivity was determined in pooled tissue derived from five animals. The results are the means of determinations performed in triplicate.

| | Radioactivity (d.p.m./g of tissue) | |
|---|---|---|
| Tissue | Animals 24 h after castration | Normal non-castrated animals |
| Kidney | 741000 | 1441000 |
| Liver | 65060 | 109200 |
| Adrenal gland | 25200 | 31600 |
| Seminal vesicle | 10300 | 17000 |
| Prostate gland | 11200 | 14200 |
| Skeletal muscle | 6100 | 15900 |

enzymic activity was determined in discrete subcellular fractions (Table 5). Even with this more elaborate experimental approach, however, the effects of hormonal stimulation on the adenylate cyclase activity in the prostate gland are difficult to interpret. Overall, they are generally in agreement with the conclusion of Rosenfeld & O'Malley (1970), namely that the changes, if any, are small and even equivocal. The enzymic activity associated with the mitochondria was partially depressed by castration, but in the early period of hormonal stimulation restoration of activity to that in non-castrated animals did not occur. The nuclear adenylate cyclase activity was decreased by castration, yet restored by the administration of androgens. The low specific activity of the nuclear-associated activity, however, places severe doubt on the overall importance of this subcellular site of cyclic AMP synthesis. Some stimulation of the adenylate cyclase associated with the microsomal preparation was elicited by the administration of testosterone, but this finding is difficult to interpret on the grounds that little decrease in enzymic activity resulted from castration. The determination of the concentrations of cyclic AMP directly seemingly offered an alternative means of elucidating the effects of androgens on adenylate cyclase activity. This was not undertaken extensively in the present study because it is likely that significant dissociation of cyclic AMP occurred during the isolation and washing of the subcellular fractions. The results were exceedingly variable and did little to aid our appraisal of the effects of androgens on the rate of formation of cyclic AMP in the prostate gland, or its intracellular distribution.

Table 4. *Subcellular distribution of adenylate cyclase activity in the rat prostate gland*

Pooled tissue from six normal animals was homogenized in 0.25M-sucrose–EDTA and separated into various subcellular fractions by differential centrifugation. Where indicated, the crude nuclei sedimented at 700*g* were further purified by resuspension and centrifugation in 2.2M-sucrose. Details of the assay of adenylate cyclase are given in the Materials and Methods section and the results are the means of duplicate determinations.

| | Adenylate cyclase activity | |
|---|---|---|
| | Cyclic AMP formed (nmol/15min per mg of protein at 37°C) | Total ATP formed (μmol/15min per fraction at 37°C) |
| 100000*g* supernatant | 8.7 | 0.37 |
| 100000*g* sediment (microsomal fraction) | 2094 | 5.56 |
| 10000*g* sediment (mitochondria) | 1801 | 2.72 |
| Crude nuclei (initial 700*g* sediment) | 424 | 0.36 |
| Purified nuclei | 53.2 | 0.12 |

Table 5. *Effect of the administration of testosterone to castrated animals on the activity of adenylate cyclase in the subcellular fractions of the prostate gland*

At 72h after castration, testosterone (2.5mg) was given *in vivo* for a period of 1–3h. Homogenates were prepared from tissue pooled from six animals, separated into subcellular fractions as described in the legend to Table 4 and assayed for adenylate cyclase activity. The results are the means of determinations performed in duplicate.

| | Adenylate cyclase activity (nmol of cyclic AMP formed/mg of protein) | | |
|---|---|---|---|
| Animals | Microsomal preparation | Mitochondria | Nuclei |
| Normal non-castrated animals | 2190 | 1830 | 58.9 |
| Castrated animals | 1913 | 1739 | 18.6 |
| Castrated testosterone-treated animals | | | |
| 1h | 1883 | 1630 | 62.9 |
| 2h | 2627 | 1719 | 60.6 |
| 3h | 2352 | 1690 | 26.0 |

*Influence of cyclic AMP and androgens on the activity of cytoplasmic enzymes*

In attempts to elucidate the part played by cyclic AMP in the androgenic response in the prostate gland, a comparison was made between the effects of the administration of cyclic AMP or testosterone *in vivo* on the activity of enzymes in the cell supernatant fraction that are known to be influenced by the androgenic status of the animal. Since testosterone is essentially converted into 5α-dihydrotestosterone in the prostate gland, these studies provide a mechanistic evaluation of the importance of the binding of cyclic AMP and 5α-dihydrotestosterone described in the earlier part of the Results section.

*Glucose 6-phosphate dehydrogenase.* By using the protocol of injections suggested by Singhal *et al.* (1971), the effects of the administration of cyclic AMP and testosterone on the activity of glucose 6-phosphate dehydrogenase were compared. These results are summarized in Table 6. Our experimental findings are in complete agreement with those of Singhal & Valadares (1968), Singhal & Ling (1969) and Singhal *et al.* (1971). Castration results in a significant fall in the activity of glucose 6-phosphate dehydrogenase in the prostate cell supernatant fraction, and enzymic activity may be restored and even stimulated to values in excess of the activities of non-castrated animals by the administration of either testosterone or cyclic AMP *in vivo*. With both of these stimulatory agents, the restoration of enzymic activity is suppressed by the concomitant administration of the metabolic inhibitors cycloheximide or actinomycin D. The inhibitory effects of these compounds suggest a need for continued synthesis of both RNA and protein in the manifestation of enzymic stimulation. By far the most important observation, however, was that simultaneous administration of cyproterone acetate failed to inhibit the stimulation of enzymic activity promoted by either cyclic AMP or testosterone. The full implications of this result will be evident from the suppressive effects of this antiandrogen on all the other biochemical changes induced in the prostate gland by the administration of testosterone.

Work by Singhal and his colleagues (see, e.g., Singhal *et al.*, 1971) has indicated that carbohydrate-metabolizing enzymes in the prostate gland other than glucose 6-phosphate dehydrogenase are similarly stimulated by the administration of both cyclic AMP and testosterone *in vivo*. These additional results are not described in detail, but we confirmed that the activity of glyceraldehyde 3-phosphate dehydrogenase is enhanced by injection of either cyclic AMP or testosterone; this stimulation was removed by simultaneous administration of the metabolic inhibitors cycloheximide and actinomycin D, but not by the presence of cyproterone acetate. In all respects these results are entirely in accord with those obtained with glucose 6-phosphate dehydrogenase.

One possible mechanism to explain the stimulatory effects of cyclic AMP and testosterone on the overall activity of glucose 6-phosphate dehydrogenase is that they promote a change in the complement of isoenzymes, favouring the synthesis or activation of a

Table 6. *Effects of the administration of cyclic AMP or testosterone in vivo on the activity of prostate glucose 6-phosphate dehydrogenase*

Castrated animals (72h after castration) were injected with either testosterone alone (2.5mg) or cyclic AMP plus theophylline (2.5mg each). Other compounds were administered in the doses and routes given in the Materials and Methods section. Pooled tissue from four animals was used to prepare a 100000*g* supernatant fraction, and this was used for the assay of enzymic activity. The numbers of experiments are given in parentheses; in each experiment, the assays were made in duplicate and the overall results are quoted as means±S.D.

| | Treatment | Glucose 6-phosphate dehydrogenase activity (μmol/h per 20mg of protein at 25°C) |
|---|---|---|
| Castrated animals | None (5) | 5.25±0.04 |
| | Testosterone (5) | 10.02±0.06 |
| | Testosterone plus cycloheximide (1) | 5.18±0.07 |
| | Testosterone plus actinomycin D (1) | 2.80±0.03 |
| | Testosterone plus cyproterone acetate (2) | 9.68±0.04 |
| | Cyclic AMP (5) | 7.55±0.05 |
| | Cyclic AMP plus cycloheximide (1) | 5.25±0.04 |
| | Cyclic AMP plus actinomycin D (1) | 3.65±0.07 |
| | Cyclic AMP plus cyproterone acetate (2) | 7.68±0.06 |
| Normal, non-castrated animals | None (5) | 7.05±0.05 |

species of enzyme with a particularly high turnover number. However, detailed comparisons of the glucose 6-phosphate isoenzyme patterns in castrated and androgen-stimulated animals failed to distinguish a significant change in the qualitative or quantitative nature of the enzyme. Studies by Chuah & Oliver (1971) on the activation of tyrosine aminotransferase in foetal liver by cyclic AMP offered an alternative explanation of the influence of testosterone and cyclic AMP on glucose 6-phosphate dehydrogenase in the prostate gland. The studies in foetal liver indicated that cyclic AMP stimulated the release of preformed enzyme bound to membrane-associated polyribosomes. Such a mechanism is not operative for the prostate enzyme under discussion. Not only was the glucose 6-phosphate dehydrogenase activity associated with prostate polyribosomes of barely detectable activity, but incubation of polyribosomes with cyclic AMP, testosterone or its metabolites, including 5α-dihydrotestosterone, did not release the enzyme into a solubilized form.

*Enzymes engaged in the synthesis of polyamines.* As originally reported by Pegg & Williams-Ashman (1969) and Pegg *et al.* (1970), the activities of two enzymes involved in the synthesis of polyamines in the prostate gland, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, are significantly impaired after castration (Table 7). When cyclic AMP and testosterone were administered to castrated animals *in vivo* in an effort to restore these enzymic activities to normal values, the experimental findings were in marked contrast to those obtained with glucose 6-phosphate dehydrogenase. Stimulation of these enzymes was only effected by injection of testosterone; cyclic AMP had no effect on the activity of enzymes engaged in polyamine synthesis. The restoration of these enzymic activities by testosterone was impaired by concomitant administration of actinomycin D or cyproterone acetate. The latter finding provides a particularly important distinction in the behaviour of carbohydrate-metabolizing enzymes and other enzymes in the cell supernatant fraction to androgenic stimulation.

*Influence of cyclic AMP and testosterone on the activity of a nuclear enzyme, RNA polymerase*

At least two RNA polymerase enzymes are present in nuclei of eukaryotes: one activated by $Mg^{2+}$ in media of low ionic strength, the other activated by $Mn^{2+}$ at high ionic strength. Preliminary experiments showed that the incorporation of [$^3$H]GTP into RNA in purified prostate nuclei was dependent on the additional presence of ATP, CTP and UTP, and that the incorporation was totally suppressed by the inclusion of actinomycin D, ribonuclease or deoxyribonuclease in the incubation media. Further, the $Mn^{2+}$-activated enzyme was selectively inhibited by the toxin from *Amanita phalloides*, α-amanitin. As documented by many investigators (Liao *et al.*, 1965;

Table 7. *Effects of the administration of cyclic AMP or testosterone in vivo on the activities of two enzymes involved in polyamine synthesis in the prostate gland*

At 72h after castration, animals were injected with either testosterone alone (2.5mg) or cyclic AMP plus theophylline (2.5mg each), and again 12h later. After a further 12h, animals were killed. In certain experiments other compounds were administered concomitantly. Pooled tissue from four animals was homogenized and the activities of enzymes were measured in the resultant 100000*g* supernatant fraction. The results are the means ±S.D. of assays performed in triplicate.

| | | Enzyme activities (nmol of $^{14}CO_2$ formed/30min per mg of protein at 37°C) | |
|---|---|---|---|
| | Treatment | Ornithine decarboxylase | *S*-Adenosylmethionine decarboxylase |
| Castrated animals | None | 0.055±0.06 | 0.185±0.09 |
| | Testosterone | 0.805±0.12 | 1.230±0.18 |
| | Testosterone plus actinomycin D | 0.590±0.10 | 0.770±0.12 |
| | Testosterone plus cyproterone acetate | 0.408±0.03 | 0.846±0.26 |
| | Cyclic AMP | 0.062±0.04 | 0.180±0.04 |
| | Cyclic AMP plus actinomycin D | 0.065±0.06 | 0.185±0.06 |
| | Cyclic AMP plus cyproterone acetate | 0.062±0.04 | 0.178±0.07 |
| Normal non-castrated animals | None | 1.506±0.17 | 3.140±0.28 |

Liao & Lin, 1967; Mainwaring *et al.*, 1971) castration results in a marked decrease in the activity of the $Mg^{2+}$-activated RNA polymerase (Table 8). This decrease in $Mg^{2+}$-activated RNA polymerase activity was effectively countered by the administration of testosterone *in vivo*. The enzymic activity was not restored with cyclic AMP. The $Mn^{2+}$-activated RNA polymerase activity was also decreased by castration and responded similarly to the administration of testosterone; again, in contrast, cyclic AMP was unable to restore this enzymic activity in castrated animals. Despite the different activities of the RNA polymerase in the various experimental groups, the sensitivity of the enzymes to α-amanitin was remarkably uniform, the $Mg^{2+}$-activated enzyme being inhibited only 5–10% but 56–70% for the $Mn^{2+}$-activated enzyme. A similar differential sensitivity in the RNA polymerases of liver nuclei to α-amanitin has been reported by Tata *et al.* (1972).

To emphasize further the fundamental differences in the response of the $Mg^{2+}$-activated RNA polymerase activity in prostate nuclei to the administration of testosterone and cyclic AMP *in vivo*, a final series of experiments was conducted in which metabolic inhibitors and an antiandrogen were injected simultaneously with the compounds under critical evaluation (Table 9). The stimulation of this nuclear enzymic activity by testosterone was impaired by concomitant administration of actinomycin D and cycloheximide, implicating the requirement for both exposed sites on the DNA template and continued protein synthesis for the hormonal response. Cyproterone acetate also suppressed the androgenic response, suggesting that the high-affinity binding of

Table 8. *Comparison of the ability of testosterone and cyclic AMP to restore the RNA polymerase activities in prostate nuclei prepared from castrated animals*

Rats were castrated and 72h later testosterone or cyclic AMP plus theophylline were administered *in vivo* in the manner described in the legend to Table 7. Nuclei were prepared from prostate glands pooled from five animals and assayed for RNA polymerase activities in the presence of either $Mg^{2+}$ alone or $Mn^{2+}$ plus $(NH_4)_2SO_4$. The total numbers of experiments are given in parentheses; results are the means±S.D. of assays conducted in duplicate for each experiment.

| | | RNA polymerase activity (pmol of GTP incorporated/15min per mg of DNA at 37°C) | |
|---|---|---|---|
| | Treatment | $Mg^{2+}$-activated enzyme | $Mn^{2+}$-activated enzyme |
| Castrated animals | None (5) | 118±17 | 134±16 |
| | Testosterone (5) | 238±22 | 267±27 |
| | Cyclic AMP (5) | 123±16 | 150± 9 |
| Normal non-castrated animals (2) | None | 228±21 | 312±28 |

Table 9. *Effect of metabolic inhibitors and cyproterone acetate on the testosterone-mediated restoration of the $Mg^{2+}$-activated RNA polymerase activity in prostate nuclei isolated from castrated animals*

Rats were castrated and 72h later testosterone or cyclic AMP plus theophylline were administered alone or in combination with metabolic inhibitors and an antiandrogen. Details of the injection schedule are given in the legend to Table 7. The $Mg^{2+}$-activated RNA polymerase was determined in nuclei prepared from tissue pooled from five animals. The results are the means of experiments performed in triplicate.

| Treatment | $Mg^{2+}$-activated RNA polymerase activity (pmol of GTP incorporated/15min per mg of DNA at 37°C) |
|---|---|
| None | 128 |
| Testosterone | 252 |
| Testosterone plus cycloheximide | 126 |
| Testosterone plus actinomycin D | 124 |
| Testosterone plus cyproterone acetate | 168 |
| Cyclic AMP | 120 |
| Cyclic AMP plus cycloheximide | 107 |
| Cyclic AMP plus actinomycin D | 111 |
| Cyclic AMP plus cyproterone acetate | 126 |

5α-dihydrotestosterone is intimately associated with the control of transcription in the prostate gland. None of these compounds produced any significant alteration in the activity of this form of RNA polymerase when administered with cyclic AMP. This cyclic nucleotide is unable to promote any change in RNA polymerase activity and the effect of metabolic inhibitors was simply to depress the low enzymic activity in castrated animals even further. This is illustrated in the studies with cycloheximide. On the basis of these experimental findings, the similar response of the carbohydrate-metabolizing enzymes to testosterone and cyclic AMP must be considered an exception to the general response of the biochemical processes in the prostate gland; the majority of other enzymic processes may be stimulated only by the administration of testosterone.

*Morphological changes in the prostate gland after the administration of testosterone*

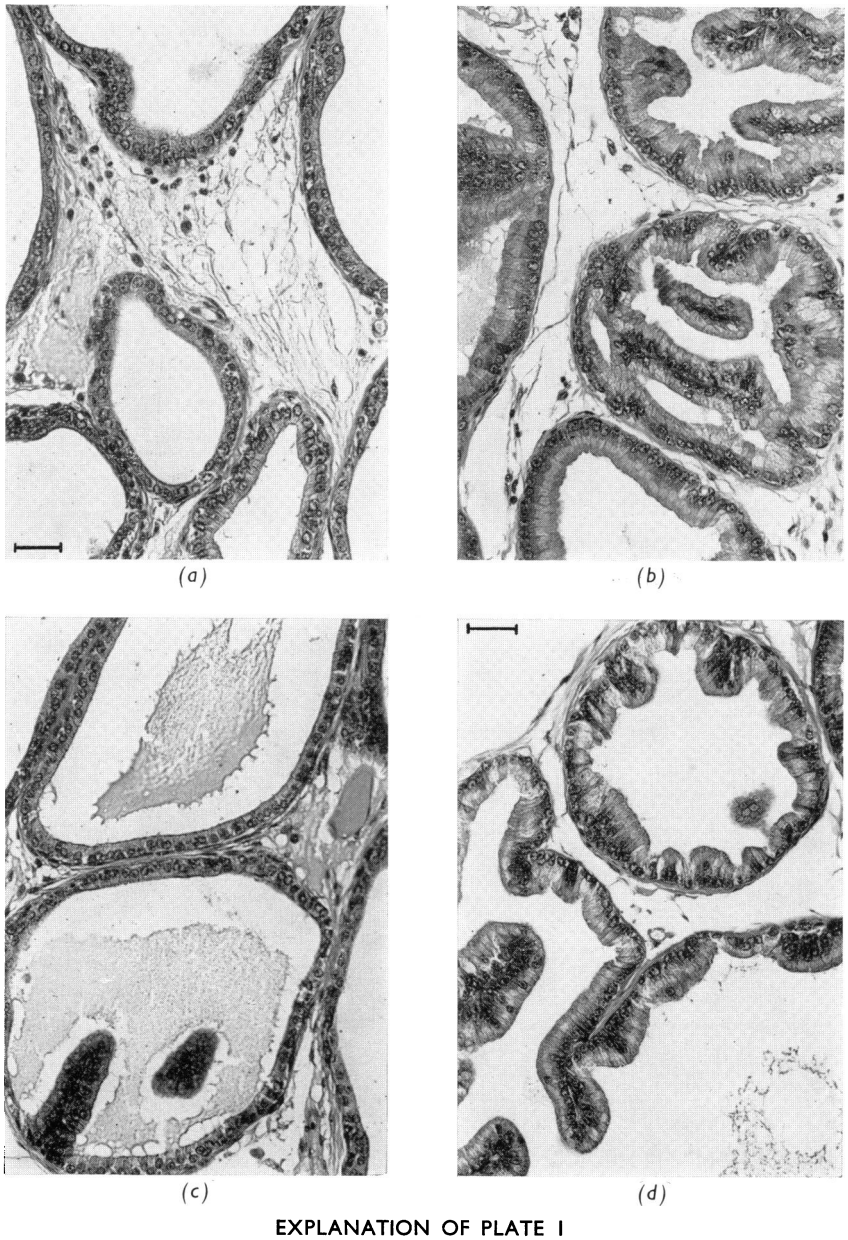
It is well established that testosterone is able to restore the morphological structure of the prostate gland after castration, and it was therefore decided to ascertain whether cyclic AMP could simulate this important aspect of the androgenic response. From the histological evidence presented in Plate 1, it is abundantly clear that, unlike testosterone, cyclic AMP is totally unable to restore the morphological structure of the atrophic prostate gland in the castrated rat. Even from these pictures, taken at relatively low magnification, it is evident that the height and structure of the secretory epithelium is unaltered by the administration of cyclic AMP. The general structure of the prostate gland remains as regressed as in the control sections from castrated animals. Only testosterone restored the compactness and overall structure seen in sections prepared from the prostate gland of normal non-castrated animals. By using additional histochemical techniques (W. I. P. Mainwaring, unpublished work) it was demonstrated that a restoration of the secretion of mucins, revealed by the periodic acid–Schiff-base (fuchsin) reaction and the restoration of cytoplasmic basophilia, detected by the Methyl Green–Pyronin stain, could only be restored by testosterone. The administration of cyclic AMP was totally without effect.

## Discussion

On the strength of the evidence presented, cyclic AMP cannot mimic all of the effects of testosterone, or, more accurately, of its metabolites, on the prostate gland. Rather the reverse is more acceptable; cyclic AMP is possibly implicated in the stimulation of a very restricted number of biochemical processes, and the best example at the present time is provided by the carbohydrate-metabolizing enzymes present in the cell supernatant fraction of tissue homogenates. The failure of cyclic AMP to simulate the androgenic stimulation of the prostate gland is particularly evident from its total inability to stimulate many other enzymic processes both in the cytoplasmic and nuclear subcellular compartments and its lack of effect on the morphology of the gland. It is not surprising that cyclic AMP should fail to stimulate both nuclear RNA polymerase and the soluble enzymes engaged in polyamine synthesis, whereas androgens stimulate both of these processes. A selective stimulation of either of these reactions, independent of effects on the other enzymic process, would be difficult to reconcile with current evidence indicating that RNA and polyamine synthesis are closely coordinated. Possibly the best indication of this functional interdependence is provided by studies on the anucleolate mutant of *Xenopus laevis*, where synthesis of both rRNA and polyamines is virtually absent (Russell, 1971). Our failure to stimulate RNA synthesis with cyclic AMP is at variance with previous reports on other steroid-sensitive systems, such as rat uterus (Sharma & Talwar, 1970; Rao & Talwar, 1972). It should be emphasized, however, that the selection of radioactive precursor (Miller & Baggett, 1972) and large changes in the pools of nucleotides (Oliver & Kellie, 1970; Greenman, 1970, 1971) are known to influence dramatically the incorporation of radioactive precursors into uterine RNA during whole-tissue incubations. Such criticisms cannot be justifiably levelled at the present results, derived entirely from studies on the RNA polymerase activity associated with highly purified nuclei.

The fundamental difference in the response of biochemical processes in the prostate gland to either cyclic AMP or 5α-dihydrotestosterone, the principal metabolite of testosterone formed within the gland, would be predicted from the differences in the binding processes for these compounds. Whereas a specific mechanism exists for the selective retention of 5α-dihydrotestosterone in all accessory sexual glands, we could find no substantial evidence to support the view that cyclic AMP could be similarly retained by this process or that it in any way influenced the high-affinity binding of 5α-dihydrotestosterone. Little cyclic AMP was bound to either the androgen receptor in the cell supernatant fraction or to nuclear chromatin; further, the extent of binding of cyclic AMP was not influenced by castration. In all these respects, the binding process for 5α-dihydrotestosterone is fundamentally different from that responsible for the intracellular retention of cyclic AMP.

Considerations of binding apart, androgenic stimulation of the prostate gland did not elicit a major change in adenylate cyclase activity; this lack of hormonal effect had been reported in the prostate gland (Rosenfeld & O'Malley, 1970) and other steroid-sensitive systems (Kissel *et al.*, 1970; Lang & Edelman, 1972). Direct measurement of the con-




EXPLANATION OF PLATE 1

*Sections of prostate gland, stained with Haemotoxylin and Eosin*

(*a*) From castrated animals, 72h after orchidectomy; (*b*) from normal, non-castrated animals; (*c*) from castrated animals, after 1 day of treatment with 2.5mg each of cyclic AMP and theophylline; (*d*) from castrated animals, after 1 day of treatment with 2.5mg of testosterone. For further details see the text. The bars in (*a*) and (*d*) represent 33$\mu$m.

centrations of cyclic AMP after hormonal stimulation has not been widely reported. Singhal *et al.* (1971), however, reported an increase in this cyclic nucleotide in the prostate gland after the administration of testosterone, but these experiments were conducted in long-term castrated animals, when increases in most biochemical processes would be confidently predicted to change with the stimulation of such an acutely atrophied prostate gland. In addition, Singhal *et al.* (1971) calculated the amount of cyclic AMP with reference to organ weight, but, since androgens induce hypertrophy of the prostate gland, their results indicate an increase in cyclic AMP content rather than an actual increase in cyclic AMP concentration within the prostate gland after androgenic stimulation. In this study we have demonstrated that cyclic AMP does enter certain organs, including the accessory sexual glands, when administered together with an inhibitor of phosphodiesterase to whole animals. Even with this protection from rapid degradation, the amount of non-metabolized cyclic AMP is low but clearly sufficient to exceed the critical threshold values necessary for the activation of cyclic AMP-mediated processes, including the stimulation of glucose 6-phosphate dehydrogenase.

The most forceful distinction between the androgen- and cyclic AMP-mediated processes in the prostate gland may be drawn from studies with the antiandrogen cyproterone acetate. Currently available evidence suggests that this androgen antagonist is active by reason of its competition for the intracellular 5α-dihydrotestosterone-binding sites; it does not have any suppressive effect on the rate of formation of 5α-reduced steroids by the NADPH-dependent prostate enzyme $\Delta^4$-3-oxo steroid 5α-reductase (Fang & Liao, 1969; Belham & Neal, 1971). The use of such antiandrogens enables some insight to be gained into the biochemical events that are ultimately influenced by the binding and transfer of 5α-dihydrotestosterone into chromatin. The present results pertain to the steroid-mediated stimulation of RNA polymerase and polyamine-synthesizing enzymes and, on the basis of other evidence (Geller *et al.*, 1969), acid phosphatase also falls into this category. The common antagonism of RNA polymerase activation and the binding of 5α-dihydrotestosterone is not exhibited solely by cyproterone acetate; studies by Mangan & Mainwaring (1972) have shown that another synthetic antiandrogen, 6α-bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one, behaves in an identical manner. The sensitivity of these androgen-mediated processes to inhibitors of RNA and protein synthesis permits the conclusion that the induction or activation of many processes after the nuclear binding of 5α-dihydrotestosterone is triggered by an increase in both transcriptional and translational events.

Continued RNA and protein synthesis is also necessary for the stimulation of such cyclic AMP-mediated processes, including the activation of glucose 6-phosphate dehydrogenase. The lack of effect of cyproterone acetate on this enzymic stimulation precludes any involvement of the binding of 5α-dihydrotestosterone in the overall mechanism of activation. However, it would not rule out the involvement of other metabolites of testosterone in this process. This, together with a similar degree of stimulation by cyclic AMP, suggests that the response in the carbohydrate-metabolizing enzymes is uniquely distinct from other biochemical processes studied in the prostate gland to date during the early phase of the androgenic response. The induction of tyrosine aminotransferase in liver is similarly sensitive to both a steroid hormone, in this case cortisol, and cyclic AMP. Wicks (1971) has argued that the steroid promotes the synthesis of the RNA components necessary for an increase in enzyme synthesis, whereas cyclic AMP controls the rate of synthesis thereafter, certainly at the translational level and perhaps at the level of transcription as well. In view of the low extent of nuclear binding of cyclic AMP in the prostate gland, it seems possible that cyclic AMP would largely be implicated in the synthesis of glucose 6-phosphate dehydrogenase at the level of translation only.

One final aspect of cyclic AMP in the prostate gland warrants brief mention. Prolactin is known to have significant effects on the prostate gland (Lawrence & Landau, 1965; Grayhack & Lebowitz, 1967) and, like other polypeptide hormones, prolactin could stimulate adenylate cyclase in the prostate gland independently from the effects of androgens on this enzyme. Such a possibility was not investigated in the present study.

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