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MODULATION OF CYCLIC AMP METABOLISM IN THE PROSTATE GLAND

A Thesis

Presented to

The School of Graduate Studies

of

The University of Ottawa

by

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LIST OF ABBREVIATIONS

Acetyl CoA: acetyl coenzyme A

ACTH: adrenocorticotropic hormone

5'-ADP or ADP: 5'-adenosine diphosphate

2'-AMP: 2'-adenosine monophosphate

3'-AMP: 3'-adenosine monophosphate

5'-AMP or AMP: 5'-adenosine monophosphate

5'-ATP or ATP: 5'-adenosine triphosphate

3α-androstanediol: 5α-Androstane-3α,17β-diol

3β-androstanediol: 5α-Androstane-3β,17β-diol

Androstanedione: Androstane-3,17-dione

Androstenedione: 4-Androstene-3,17-dione

Androsterone: 3α-Hydroxy-5α-androstan-17-one

C: catalytic subunit of cyclic AMP-dependent protein kinase

Coag. gland: coagulating gland

Cyclic AMP or cAMP: cyclic 3',5'-adenosine monophosphate

2',3'-cyclic AMP or 2',3'AMP: cyclic 2',3'-adenosine monophosphate

Cyclic CMP or cCMP: cyclic 3',5'-cytidine monophosphate

Cyclic GMP or cGMP: cyclic 3',5'-guanosine monophosphate

Cyclic TMP or cTMP: cyclic 3',5'-thymidine monophosphate

Cyclic UMP or cUMP: cyclic 3',5'-uridine monophosphate

Cyproterone: 1,2α-Methyl[redacted]-6-chloro,$\Delta^{4,6}$-17α -hydroxyprogesterone

Cyproterone acetate or CA: 1,2α-Methylene-6-chloro,$\Delta^{4,6}$-17α-hydroxyprogesterone acetate

DEAE: diethyl amino ethyl

Dibutyryl cyclic AMP or db-cAMP: N^6,$O^{2'}$-dibutyryl cyclic 3',5'-adenosine monophosphate

Dehydroepiandrosterone: 3β-Hydroxy-5-androsten-17-one

Dihydrotestosterone or 5α-dihydrotestosterone: 17β-Hydroxy-5α-androstan-3-one

DNA: deoxyribonucleic acid

EDTA: (ethylenedinitrilo)tetraacetic acid

Estradiol-17α: 1,3,5,(10)-Estratriene-3,17α-diol

Estradiol-17β or estradiol: 1,3,5(10)-Estratriene-3,17β-diol.

FSH: follicle stimulating hormone

G: guanine

GMP: guanosine monophosphate

HCG: human chorionic gonadotropin

ICSH: interstitial cell stimulating hormone

Isoprot: DL-isoproterenol

Km: Michaelis constant

LH: luteinizing hormone

Methyl prednisolone sodium succinate: 11β,17α,-21-Trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione 21-succinate sodium salt

mRNA: messenger ribonucleic acid

M.W.: molecular weight

NAD: nicotinamide adenine dinucleotide

NADH: dihydronicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate

NADPH: dihydronicotinamide adenine dinucleotide phosphate

Prop.: propranolol

Progesterone: Δ^6-Pregnene-3,20-dione

R: regulatory subunit of cyclic AMP-dependent protein kinase

RC: cyclic AMP-dependent protein kinase

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

$S_{20,w}$: sedimentation coefficient

Sem. Vesicle: seminal vesicle

TCA: trichloroacetic acid

Testosterone or Testos.: 17β-Hydroxy-4-androsten-3-one

Tris-HCl or Tris: Tris(hydroxymethyl)aminomethane

tRNA: transfer ribonucleic acid

TSH: thyroid stimulating hormone

U: uridine

Ven. Prostate: ventral prostate

Vmax.: maximum velocity

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I. INTRODUCTION

I. INTRODUCTION

Although the dependence of prostatic size and function upon continuous secretion of testicular hormones has been well documented, the precise mechanics of growth, differentiation and metabolism of this accessory organ is still obscure. Generally speaking, substances or conditions which affect the hypothalamus and/or the pituitary gland, or the synthesis of testosterone in the testis influence prostatic function and morphology. While the direct end organ effects of various pituitary hormones on sex accessory activity have not been as clearly established as their indirect effects mediated via the testis, the interactions of these hormones, especially prolactin, with androgens and their role in the pathogenesis of the prostate gland remain to be elucidated (1,2). Various forms of treatment of prostatic dysfunctions including the use of estrogens, progestogens, orchidectomy and hypophysectomy have been introduced in attempts to remove or reduce the influence of androgens on this hormone-dependent tissue (3). While the effectiveness of treatment with antiandrogens such as cyproterone and cyproterone acetate appears to support the notion that the androgens play an etiological role in prostatic growth, the exact molecular basis by which the androgens and/or other hormones promote the development of prostatic cancer remains unknown.

In view of the anabolic actions of androgens on target tissues that are constantly dependent on male sex steroids for normal differentiation and function, the prostate glands offer an excellent model for the study of the mechanisms underlying the control of transcription and cellular growth. Numerous investigations carried out during the past decade on the molecular actions of sex hormones have led to the concept that a co-ordinated increase in genetic transcription in their target tissues may be central to the mechanism of action of these hormones. Although the precise mechanism for the control of gene expression in androgen-dependent cells is still not fully established,

the regulation of transcription in response to hormonal stimulation is mediated by enzymatic modification of nuclear proteins associated with the chromatin (4,5). While the action of biogenic amines, polypeptide hormones and several cell regulating substances is believed to require cyclic AMP as an "intracellular messenger," evidence also exists that this ubiquitous nucleotide might be involved in some of the actions of sex steroids as well (6-10). Whereas administration of exogenous cyclic AMP or its dibutyryl analog to castrate or immature rats mimicked several of the anabolic actions of androgens (increases in seminal vesicular and prostatic weights, glycogen synthesis and induction of certain enzymes involved in glucose metabolism), these responses were effectively inhibited by actinomycin D and cycloheximide (11-14). It is noteworthy that recent investigations on the effects of various cyclic nucleotides have centered around the ability of these compounds to stimulate cyclic nucleotide-dependent protein kinases. It has been suggested that protein kinase(s) plays an important role in the hormone-mediated induction of RNA and protein biosynthesis and that phosphorylation of nuclear proteins may be a means by which the cell "turns on" its genetic machinery (15). Studies on nuclear proteins of neoplastic cells also have implicated that aberrations in gene control mechanisms may be responsible either for the initiation or continuation of tumor growth and that an increased rate of phosphorylation of various nuclear proteins (both histones and non-histone proteins) is associated with the nuclei isolated from tumorous tissue (16,17).

The present dissertation is primarily concerned with the androgenic regulation of cyclic AMP-adenylate cyclase system in the ventral prostate of mature rats. Earlier studies have indicated that whereas endogenous cyclic AMP in the rat prostate decreased following orchidectomy, administration of testosterone was capable of partially reversing this effect (12,14). In the present study, the important question whether androgenic deprivation and

testosterone repletion can alter the activity of prostatic adenylate cyclase has been investigated. Moreover, in order to establish the earliest possible time when an increase in the activity of this prostatic enzyme can be detected under in vivo conditions, the influence of intravenously administered testosterone on prostatic adenylate cyclase in orchidectomized rats was monitored at different time intervals. The effect of certain other steroidal compounds such as estradiol-17β, progesterone and methylprednisolone was also studied to establish whether this response was specific for androgens. In addition, changes in adenylate cyclase activity of rat thymus, an organ known to be unresponsive to male sex hormone, also were investigated to establish the tissue specificity of this androgenic response. Since the intracellular concentration of cyclic AMP is also dependent on the activity of cyclic AMP phosphodiesterases and evidence indicates that testosterone exerted a marked inhibitory effect on this enzyme in certain steroid-independent tissues (18), it was of interest to investigate whether this androgen also influenced the activity of these enzymes in the ventral prostate.

It has been demonstrated that prostatic cell nuclei possess the enzyme, 5α-reductase, which reduces testosterone to dihydrotestosterone and that the anabolic effects of androgens on the prostate may be preceded and probably initiated by the nuclear-binding of this metabolite, rather than the parent compound itself (19-21). In light of the possibility that dihydrotestosterone might be the active form of testosterone in prostatic tissue (19,20,22), the question whether dihydrotestosterone could produce more rapid and pronounced changes in adenylate cyclase and phosphodiesterase activities of the prostate was examined in orchidectomized rats injected intravenously with this androgen. In addition, in order to elucidate whether the androgen-induced changes in prostatic adenylate cyclase represent an activation of the pre-existing enzyme and/or de novo synthesis of new enzyme

protein, the influence of two inhibitors of RNA and protein biosynthesis, actinomycin D and cycloheximide was examined on testosterone-stimulated changes in enzyme activity.

Although evidence implicates cyclic AMP in the action of sex steroids on their target tissues, the question remains as to whether cyclic AMP acts directly as a "second messenger" or indirectly subsequent to the release and action of catecholamines (6,23-38). The effect of propranolol on androgen-induced alterations on prostatic adenylate cyclase was studied in order to determine whether the androgenic action was mediated through an activation of the β-adrenergic system. To further study the possible modulation of adenylate cyclase system by β-adrenergic stimulation, the in vivo effects of a potent β-adrenergic agonist, isoproterenol were examined on endogenous cyclic AMP levels as well as the activities of adenylate cyclase, cyclic AMP-dependent and-independent protein kinases in the ventral prostate. Furthermore, since certain steroids have been shown to elicit a "permissive effect" in the action of other hormones (29), it was of interest to study the influence of androgens on the sensitivity of prostatic cyclic AMP system to stimulation by isoproterenol in rats castrated and/or treated with testosterone.

Evidence indicates that cyclic AMP exerts its influence on various cellular processes by activating a class of enzymes called protein kinases (15,30). In order to gain deeper insight into the possible involvement of the adenylate cyclase system in the mechanism of action of male sex steroids on the prostate gland, attempts were made to partially purify and characterize the cyclic AMP-dependent protein kinase from canine prostate. In particular, the effects of several cyclic nucleotides such as cyclic AMP, cyclic GMP, cyclic CMP, cyclic IMP, cyclic UMP, cyclic TMP and dibutyryl cyclic AMP on protein kinase activity were examined to assess the cyclic nucleotide specificity. In addition, the ability of various proteins, including histones,

to serve as substrate for the prostatic enzyme was investigated. To understand the molecular interaction of cyclic AMP with its dependent protein kinase, association of the nucleotide with the prostatic enzyme was studied at equilibrium. Moreover, the effects of cyclic AMP on the Vmax and apparent Km for prostatic protein kinase were determined for both histones and ATP. In order to further examine the influence of androgens on the prostatic cyclic AMP system, the effects of orchidectomy and testosterone replacement on the activities of soluble cyclic AMP-dependent and-independent protein kinases as well as the ability of enzyme to bind (^{3}H)-cyclic AMP in vitro were examined. Furthermore, since phosphorylation of nuclear proteins may play an important role in gene activation in eukaryote cells, the in vivo effects of androgenic deprivation and repletion on the phosphorylation of crude nuclear substrate as well as on the cyclic AMP-binding capacity of this fraction were investigated.

Since the antiandrogen, cyproterone acetate is presumed to compete with testosterone for binding sites on specific "receptor" or alternatively with dihydrotestosterone binding (31,32), attention also was directed to investigate the effects of this steroidal antiandrogen on prostatic adenylate cyclase system. In particular, endogenous cyclic AMP levels, activities of adenylate cyclase, cyclic AMP-dependent and-independent protein kinases, cyclic AMP-binding capacity as well as the phosphorylation of endogenous nuclear substrates were monitored at various intervals following daily treatment with cyproterone acetate. Moreover, since cyproterone acetate also displays certain hormonal properties other than its anti-androgenic effects (33), it was of interest to examine if simultaneous administration of testosterone could antagonize or reverse these various biochemical responses in the ventral prostate. In order to elucidate whether the effects of anti-

androgen treatment persisted upon cessation of drug therapy, the influence of 10-day "withdrawal" was examined upon biochemical parameters in rats which had been pretreated with cyproterone acetate for 10 days.

Results of the present investigation demonstrate that androgens play an important role in the regulation of cyclic AMP metabolism of the prostate gland. The activity of prostatic adenylate cyclase was markedly altered following orchidectomy and testosterone replacement. The response appeared to be specific both for androgenic steroids as well as the prostate gland, and may involve stimulation of the β-adrenergic system. The regulatory influence of androgens on prostatic adenylate cyclase system also was associated with marked changes in the activity of cytosolic and nuclear prostatic protein kinases as well as the number of unsaturated cyclic AMP receptor sites of the enzymes. Since phosphorylation of endogenous nuclear substrates also appeared to be affected by androgens, the possibility remains that a functional adenylate cyclase and protein kinase system has a central place in the manifestation of at least some of the androgenic effects on the prostate gland.

II. LITERATURE REVIEW

II. LITERATURE REVIEW

1. HORMONAL DEPENDENCE OF MALE ACCESSORY TISSUES

A. Androgens

Although the use of human subjects in studies of hormone dependence of male accessory sex organs has attained some emphasis during the past two decades, the majority of investigations on the biochemistry of androgen action have been carried out on rodent male accessory tissues. In mammals, the male accessory reproductive glands consist of the prostate, seminal vesicles, bulbourethral and ampullary glands, the secretions of which provide the carrying medium for the spermatozoa upon emission. It is now well established that the secretory activity of the epithelial cells in these glands is primarily under the control of testicular androgens. Although the volume and the chemical composition of seminal plasma are influenced by many factors, marked changes have been noted in the biochemical constituents of various accessory sex glands and their secretions following androgenic deprivation and repletion, some of which have been shown to be extremely sensitive indicators of androgenicity (34-39). Moreover, most of the morphological and metabolic changes evoked by androgens are reversible in castrate rats in that if hormone administration is stopped, the glands begin to retrogress with disappearance of the induced biochemical characteristics and secretory activities. Current evidence also indicates that testicular hormones initiate and maintain growth and functional differentiation of the epithelial cells of male accessory sexual tissues by controlling the utilization and DNA-dependent synthesis of both messenger and ribosomal RNA (40-45). Studies on metabolism and ultrastructure of the prostate in orchidectomized animals have indicated that progressive involution of the initially distended ergastoplasmic sacs is accompanied by a similar decrease in the rate of incorporation of amino acids

into protein of the accessory sex gland (46). Nevertheless, involution of these organs following androgen withdrawal proceeds up to a certain point, such that even at long intervals after orchidectomy, the dwindled accessory glands do not shrivel up entirely. While the molecular events underlying the involution of such androgen-dependent structures are still poorly understood, numerous reviews on the dependence of male sex accessory organs on continued androgenic stimulation for the preservation of their normal structure as well as for the maintenance of their functional integrity have accumulated over the past years (46-60).

(i) Androgenic regulation of growth and differentiation

The dramatic changes in the size and function of responsive tissues which result from androgen treatment of juvenile, orchidectomized or hypophysectomized animals in postnatal life have focussed much attention during the last three decades on cellular growth and functional differentiation of these organs. Moore et al. (61,62) demonstrated a gradual loss in cytoplasmic volume of prostatic and seminal vesicular cells in castrated animals. Orchidectomy also resulted in decreases in secretory activity, cytoplasmic basophilia, endoplasmic reticulum volume, ribosomes and mitochondria (56,63,64). The change in the size of the nuclei however, was not significant even after 90 days of orchidectomy, although nuclei isolated 4 days following the removal of the testis appeared somewhat pycnotic with nucleolar structure being less distinct. The dramatic disintegration of cytoplasmic structure was accompanied by the loss of cytoplasmic enzymes, but more importantly, by the loss of cytoplasmic RNA's, particularly ribosomal RNA. Nevertheless, all these degradatory effects of castration were quickly and effectively reversed by the administration of androgens. While it is known that male sex hormones exhibit extraordinary organ selectivity as they elicit their steroid-specific

effects, some of these responses are achieved through the existing metabolic machinery of the cell. Recent studies have shown that at least some, if not all, of the manifestations of hormone action on target cells are the result of culmination of striking inductive effects of the steroid on the genetic expression and depend on the synthesis of new RNA and proteins (40-45). In the organized growth sequences of various target tissues, the inductive effect of these compounds lead to distinctive patterns of differentiation among the cell progeny.

In a comprehensive review, Tata (65) pointed out many examples where hormones are known to elicit marked stimulation of nuclear RNA synthesis. Whereas Williams-Ashman and his associates published in 1962 the classic observation that the administration of androgens to castrated rats increased the incorporation of cytidine triphosphate into RNA by a nuclear preparation of the ventral prostate (66), several investigators have since shown that androgens also stimulated RNA synthesis in the seminal vesicles (67,68) and that the primary stimulation was of the synthesis of ribosomal RNA (69-71). Within 1 hr after the administration of testosterone to castrate rats, Liao et al. (72) detected marked enhancement in RNA polymerase activity of isolated prostatic cell nuclei. Whereas biochemical and radioautographic studies have indicated that this early effect occurs most distinctly at the nucleolar chromatin region that synthesizes rRNA precursors, mRNA synthesis may also be affected in this early stage of androgen action (70,71,73). As recently pointed out by Coffey (47), activation of RNA synthesis may involve several processes: (a) activation of the chromatic template as implicated by Liao and Lin (71) and Mangan and coworkers (21), (b) increased availability of nucleotide precursors for RNA, (c) possible involvement of cytoplasmic or initiating factors which are known to be necessary for other mammalian systems as already discussed by Harris (74) and (d) the activation of the RNA

polymerase enzymes.

Utilizing procedures for a complete and reproducible solubilization of nuclear RNA polymerase, Liao et al. (75) and Mainwaring et al., (76) were able to separate two active components, one associated with the nucleolus and the other extranucleolar from the prostatic nuclear RNA polymerase preparations. It was also noted by Mainwaring and coworkers (76) that 2 hr after androgen administration in vivo into castrate rats, large increases in the activity of ventral prostate Mg^{2+}-stimulated nucleolar RNA polymerase (which probably reflects largely the transcription of ribosomal RNA precursors) occurred prior to the smaller elevation in the activity of Mn^{2+}-activated nucleoplasmic RNA polymerase (concerned with the transcription of mRNA and tRNA genes). This increase in the activity of the nucleolar enzyme was followed by an activation of nucleolar template. Since the testosterone-induced synthesis of nuclear 45S ribosomal RNA precursor preceded increases in the capacity of free and membrane-bound cytoplasmic polyribosomes to incorporate labeled amino acids into proteins (77,78), evidence supports the observation of Liao and Fang (69) that stimulation of ribosomal RNA synthesis may be one of the early effects of androgens on accessory sex tissues. The possibility that changes in RNA production and/or ribosomal protein synthesis may be of prime importance in androgen-induced growth of a variety of organs is also supported by the studies of Kochakian (79,80), Kassenaar (81), Williams and coworkers (21,40), Florini and Brener (82) and Pellegrino and Pollera (83). Although various processes or factors such as enhanced synthesis of the polymerase enzyme, stabilization of the enzyme, removal of an inhibitor or the direct activation of the enzyme by the sex steroid-receptor complex may possibly be attributed to this androgen-induced increase in nucleolar RNA polymerase activity, exactly how it is achieved still remains to be determined.

It is generally accepted that the mitochondria of eukaryote cells contain unique circular DNA molecules which are not only replicated independently of the nuclear DNA genome, but may also play a directive role in the synthesis of certain proteins. Pegg and Williams-Ashman (84) found that whereas the incorporation of labeled leucine and phenylalanine into mitochondrial proteins of rat ventral prostate was enhanced by testosterone administration, no effects of androgen treatment could be detected upto 12 hr after hormone injection when nuclear RNA polymerase activities were already markedly increased. It thus appears possible that alterations in the cytoplasmic environment following androgen administration may be a major factor that determines these rather slow androgen-induced changes in prostatic mitochondrial amino acid incorporations.

Studies on protein metabolism in the prostate glands of orchidectomized animals have demonstrated that progressive involution of the accessory sex organ during androgenic deprivation is accompanied by a similar decrease in the rate of incorporation of labeled amino acids into proteins (46). Post-castrate protein synthesis in the prostate is reduced to 40% of control values by the second day and by 3½ days, ribosome yield is only 29% of uncastrate values. The RNA/DNA ratio is reduced from 2.4 to 0.77, 7 days following orchidectomy (40). Liao and Williams-Ashman (85) studied the effect of androgens on the protein-synthesizing activity of prostatic ribosomes and found that the amino acid polymerizing activity (from aminoacyl tRNA) per unit amount of ribosomes was reduced by castration and restored after testosterone administration. Since the synthetic template RNA, poly UG (which was rich in codons for valine) was able to enhance significantly the incorporation of valine-^{14}C into protein when the ribosomes were obtained from orchidectomized and not from testosterone-treated animals, these investigators concluded that ribosomal preparations from the androgen-deprived rats contained

a large number of active ribonucleoprotein particles deficient in mRNA. Testosterone thus appeared to increase the amount of mRNA associated with prostatic ribosomes (85). Silverman et al. (86) obtained similar results when poly U was used to direct the prostatic ribosome-dependent polypeptide formation. These findings were subsequently confirmed by Mangan et al. (40) in the normal ventral prostate, and by Mainwaring and Williams (87) in prostate tumors. It is also of interest that decreases in oxygen uptake by ventral prostatic slices from gonadectomized rats parallel the decrease in the activities of a number of mitochondrial enzymes and can be explained by the observed reduction in prostatic mitochondrial population to one half the pre-castrate values 4 days following castration (88). The absence of androgenic stimulation of prostatic tissue following orchidectomy seems to activate or release amino peptidase, a proteolytic enzyme which plays an important role in the involutionary change seen following castration (89). This enzyme has been found to be absent in human prostatic cancer tumors but whether this has any important bearing on the mechanism of malignant growth of this tissue remains to be determined (90). The prostatic regression seen following gonadectomy can thus be explained biochemically in terms of a progressive deficiency in messenger RNA and microsomal ribosomes due to a decrease in RNA polymerase activity (91).

Probably the earliest recorded biochemical response seen following androgen treatment is the increase in prostatic NAD^+ synthesis that occurs within 30 min following testosterone injection (92). Ritter (92) proposed that the processes regulating the concentrations of NAD^+ and ATP in response to androgen stimulation redirect the energy metabolism towards a more efficient production of ATP. The transient decrease in ATP levels following testosterone was later confirmed by Coffey et al. (93) and was shown to be followed by an increase in the activity of nicotinamide mononucleotide

adenyl-transferase. This nuclear enzyme catalyzes the incorporation of radioactive ATP into acid-insoluble products believed to result from polymerization of ADP-ribose moiety of NAD and has been thought to play a role in RNA transcription. An increase in the soluble RNA content of prostatic tissue after androgen treatment has been reported by Kochakian and Harrison (80). In addition, an increase in the incorporation of formate-C^{14} into proteins of the rat prostate was seen before any organ weight increase became evident (94).

One of the earliest effects of androgen treatment on male genital tissue is believed to be the initiation of the synthesis of nuclear RNA (transcription) and subsequent protein synthesis (translation) (44,72,95). Although the precise mechanism for the control of gene expression in eukaryotic cells has not been fully established, the regulation of transcription is probably influenced by the presence of two classes of nuclear proteins, the histones (or basic proteins) and the non-histones (or acidic proteins) (96). Modification of amino acid side chains in histones by acetylation, methylation, phosphorylation or oxidation-reduction in response to hormonal stimulation may be important in regulating histone function and subsequent induction of protein synthesis (96,97). Ahmed (98) demonstrated that castration decreased the rate of incorporation of ^{32}P into nuclear phosphoproteins of the rat ventral prostate. A single injection of testosterone to gonadectomized rats restored this decrease within 30 min. Ahmed and Ishida (99) further showed that both the non-histone and histone proteins were phosphorylated in nuclei of prostatic tissue although, the rate of incorporation of radioactivity into non-histones and phosphoproteins was found to be of a much greater magnitude than that observed in the histone fraction. The question of the rate, quantity and type of reactions involved in the biotransformation of nuclear proteins needs further investigation before any precise conclusions can be drawn about

the "organizational" or "morphogenetic" actions of androgens on dependent tissues.

Bruchovsky and Wilson (19,20) greatly advanced our understanding of the molecular basis of androgen action when they demonstrated that prostate cell nuclei contain an active NADPH-dependent enzyme, 3-ketosteroid 5α-reductase, which reduces testosterone to 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone) and that subsequent events characteristic of androgen action are preceded and probably initiated by the binding of this latter compound, rather than of testosterone itself. These findings have raised the possibility that dihydrotestosterone might be the active form of testosterone in prostatic tissue with its principle action being associated with the nucleus. The intranuclear binding substance appeared, from the work of Bruchovsky and Wilson (20), to be an acidic nuclear protein although Mangan et al. (21) reported binding of radioactivity derived from testosterone-(^{3}H) directly to DNA preparations isolated from prostatic nuclei. Recently, Lesser and Bruchovsky (100) found that 24-26 hr after the beginning of treatment with dihydrotestosterone to castrate male rats, the rates of DNA synthesis and cell production increased sharply above those observed in non-treated animals. These elevated rates were maintained for 3-4 days and by the 5th day, the number of cells per prostate reached the normal levels of the intact controls. After this time, despite continued administration of the hormone, the rate of cell production fell markedly and the rate of DNA synthesis continued to decline until day 14, when it reached a negligible value. The growth response induced by testosterone was considerably slower than that seen with dihydrotestosterone indicating that the former was indeed a less potent androgen. Nevertheless, the molecular events that occur following the nuclear binding of dihydrotestosterone and the subsequent stimulation of nucleic acid and protein biosynthetic mechanisms in target cells have not yet been fully elucidated.

(ii) Androgenic stimulation of carbohydrate metabolism

Attempts during the last two decades to uncover meaningful androgenic influences on intermediary metabolism have centered around the effects of these steroids on various enzyme systems (6,10,53,55-57,59,101-103). The activities of several enzymes in androgen-responsive tissues are known to be markedly altered as a result of androgenic deprivation and repletion. However, most of these modulations of the enzymatic makeup of accessory tissues are first manifested only after a considerable lag period following the androgenic stimuli and in most instances, reflects alterations in the rate of synthesis and/or degradation of catalytic proteins.

Carbohydrate metabolism plays an important role in the maintenance of accessory reproductive tissues. Glucose, upon entry into male accessory tissues, is phosphorylated into glucose 6-phosphate which is then further chanelled either into the glycolytic pathway or to glycogen formation. A portion of the glucose 6-phosphate pool is also utilized *via* the hexose monophosphate shunt involving NADP-specific glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, two of the initial enzymes in this metabolic pathway (Figure 1). Hexokinase, phosphofructokinase and pyruvate kinase are believed to be the key, rate-limiting enzymes in the glycolytic pathway and therefore play an important role in regulating the conversion of glucose into pyruvate or lactate.

In most mammals, fructose is formed from glucose mainly in the seminal vesicles (37,104-106). The seminal vesicles in the rat however, secrete little fructose, most of which originates from the dorsal prostate and coagulating glands (107). The principal nutrient present in seminal plasma has been assumed to be glucose (108-112) until Mann and his co-workers (113-115) were able to demonstrate conclusively that the sugar was not glucose but D(-)fructose. Fructose in the seminal plasma serves as a source of energy for spermatozoa

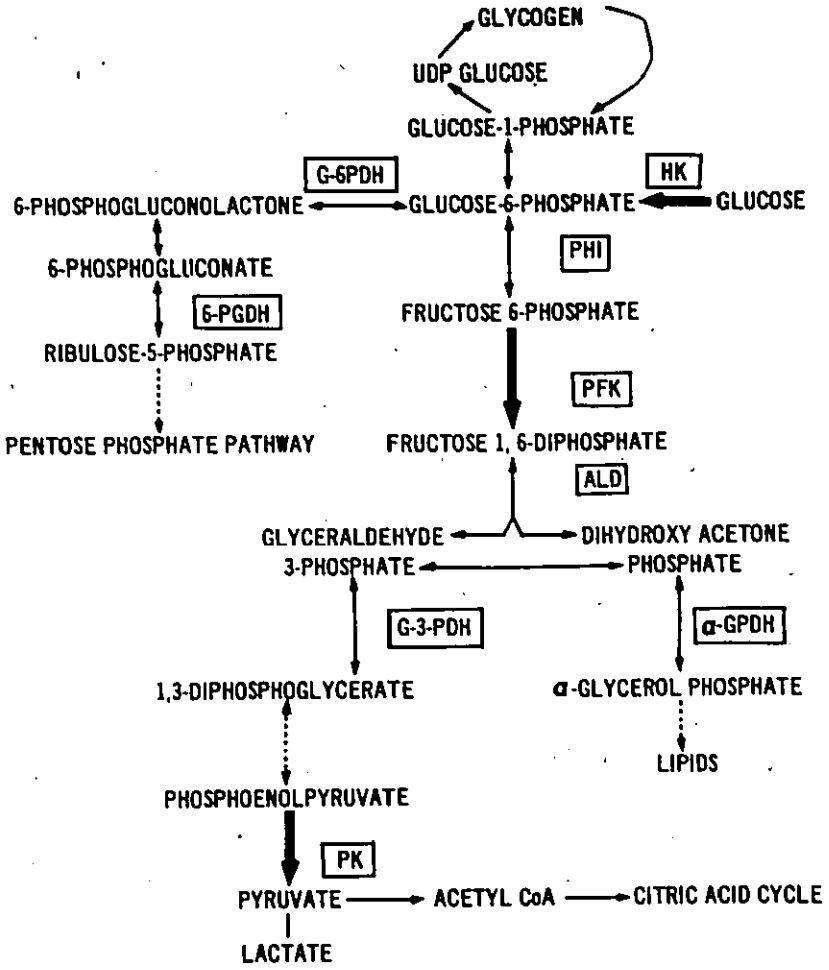

FIG. 1

Summary of major pathways involved in glucose utilization in most mammalian tissues. HK, hexokinase; PHI, phosphohexose isomerase; PFK, phosphofructokinase; ALD, aldolase; α-GPDH, α-glycerophosphate dehydrogenase; G3-PDH, glucose 3-phosphate dehydrogenase; PK, pyruvate kinase; G-6PDH, glucose 6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase. Thick arrows indicate irreversible, key rate-limiting steps; broken arrows indicate that a sequence of reactions is involved.

under both aerobic and anaerobic conditions (116) and its content in the semen and the accessory gland has been shown to be under constant modulation of the testicular hormones (39). While seminal fructose levels in sexually mature rabbits fell shortly following orchidectomy, this decrease was prevented by implantation of a testosterone pellet. It is also of interest that the increase in fructose content of the coagulating gland of castrate rats in response to testosterone was greater than the corresponding change in organ weight and may thus be a better indicator for androgenic activity (39). Mann and Lutwak-Mann (117,118) postulated that the conversion of glucose to fructose may involve an initial phosphorylation by hexokinase to form glucose 6-phosphate which is then isomerized to fructose 6-phosphate, and later dephosphorylated to fructose possibly through the action of an alkaline phosphatase. Other investigators however, found that certain fructose-secreting accessory glands of rodents contain the enzyme, aldose reductase (56) which catalyzes the reduction of glucose to sorbitol, and ketose reductase, which catalyzes the reversible oxidation of sorbitol to fructose, and thus suggested an alternative pathway for the conversion of glucose to fructose (119). Nevertheless, the relative importance of these phosphorylative and non-phosphorylative pathways for the biosynthesis of seminal fructose still remains to be determined (120). Studies by Gunaga et al. (121,122) demonstrated the presence of higher maltase activity in the dorsolateral prostate of several animal species when compared to that of other tissues. While the enzyme is believed to be rate-limiting for fructolysis (123), Gunaga et al. (122,124,125) showed that its activity also was androgen-dependent. Analysis of fructose content and maltase activity in male accessory sex glands and their secretions has been widely used as an index for androgenic influences on these steroid-dependent tissues (115,125-129).

Price and Williams-Ashman (56) conducted a detailed survey on the biochemistry of citric acid in semen and in the secretions of a variety of mammalian accessory sex glands. In some animals, like the rat and man, citric acid is produced mainly by the prostate gland, while in others (e.g. guinea pig, boar and bull), most of it originates from the seminal vesicles. The citric acid content of the seminal plasma and of the secretions of accessory reproductive tissues depends on androgenic hormones. Whereas orchidectomy resulted in loss of citric acid from these fluids, treatment with androgenic hormones was able to reverse this response (106,115). It is also worth noting that whereas the androgen-induced changes in citric acid levels are reminiscent of similar alterations in the fructose content of the accessory sex tissues, its decrease and reappearance upon androgen treatment in the post-castrate animal are usually more sluggish than that of fructose (107). Farnsworth (130) reported that incubation of ventral prostate minces with testosterone stimulated the synthesis of citrate, free fatty acids and lactate. In contrast, testosterone produced a decrease in the production of radioactive carbon dioxide from acetate. These data were interpreted to suggest that increased biosynthetic activity after testosterone was the result of facilitated removal of citric acid. Depletion of citric acid would promote a compensatory increase in the mobilization of acetyl CoA to restore the normal levels of citrate. The energy derived from increased glycolysis could then be utilized for the biosynthesis of lipids, proteins and nucleic acids required for increased cellular growth seen following androgen stimulation.

Testosterone also has been shown to exert a regulatory influence on a number of carbohydrate metabolizing enzymes in androgen-dependent tissues. The marked decline in the activities of glycolytic enzymes, phosphofructokinase, hexokinase, pyruvate kinase and glyceraldehyde 3-phosphate dehydrogenase and the activities of 2 hexose monophosphate shunt dehydrogenases (glucose 6-phosphate and 6-phosphogluconate) as well as of α-glycerophosphate

dehydrogenase in the prostate and seminal vesicles seen after castration was found to be effectively reversed by testosterone (11,131-133). Phosphofructokinase activities declined to 51% and 47% in the prostate and 9% and 6% of the normal values in seminal vesicles 4 and 8 weeks after castration, respectively. Moreover, administration of testosterone (100 µg/100 g) for 3 days reversed substantially the effects of orchidectomy and phosphofructokinase activity increased to 173% in the prostate and 536% in seminal vesicles when compared with the values of castrate controls. Time-course studies demonstrated that after a single injection of testosterone (5 mg/100 g), phosphofructokinase activity was maximally elevated to 236% in the prostate and 342% in seminal vesicles at 24 hr. While dose-response studies revealed that 2.5 mg of testosterone propionate/100 g body weight was the minimal amount necessary to induce significant increases in enzyme activity in both accessory sex organs, maximal increases were obtained with a dose of 5 mg/100 g (131,133,134). Cycloheximide, 5-fluorouracil and ethionine, inhibitors of nucleic acid and protein biosynthesis, blocked the androgen-stimulated rise in enzyme activity observed 24 hr after steroid injection. Actinomycin D, which is known to inhibit the synthesis of messenger RNA as well as the synthesis of other cellular RNA fractions, when given simultaneously with the hormone, also inhibited the testosterone-induced increases in prostatic and seminal vesicular enzyme (131). It is also of interest that 24 hr following testosterone treatment, the activities of the two hexose monophosphate shunt dehydrogenases as well as of α-glycerophosphate dehydrogenase were enhanced to 161%, 188% and 168%, respectively, when compared with the values of control rats. The observed stimulation of prostatic enzymes by testosterone was inhibited by actinomycin D and cycloheximide, suggesting that the testosterone-induced increases in various enzyme activities may represent de novo enzyme synthesis and that stimulation of the synthesis of certain RNA species may be a primary

response to androgenic stimulation (132,133). Moreover, a single intramuscular injection of testosterone propionate (5.0 mg/100 g) enhanced progressively the activities of prostatic and vesicular hexokinase and glucose 6-phosphate dehydrogenase to more than 12-fold of the control values over a period of 5 days. The observed increase in enzyme activities then gradually started to decline but even after 45 days of androgen injection, the values were significantly higher (2-fold) when compared with castrate controls (132). Furthermore, whereas administration of a single intramuscular dose of testosterone (5.0 mg/100 g) to castrate rats significantly increased prostate and vesicular glycogen at 4, 8, 12 and 24 hr, pretreatment of testosterone-injected animals with actinomycin D effectively prevented the observed androgenic response in both accessory glands; glycogen content increased initially by approximately 50% of the control values following testosterone stimulation, but then failed to rise any further during the entire 24 hr period. A similar inhibition of testosterone-stimulated prostatic and vesicular glycogen synthesis was seen when 5-fluouracil or ethionine was administered along with the hormone (135). These reports on the biochemical concomitants of certain activational effects of androgens suggest that the hormone-induced quantitative changes in specific proteins or enzymes may represent an important means for regulating metabolic processes in male secondary sexual tissues (131-134).

(iii) Receptor mechanisms of androgen action

The primary molecular processes by which steroid hormones elicit their effects on target cells are still not clear. One of the most widely used approaches to study this problem has been to examine sequential effects of sex hormones on the wide-range of biosynthetic reactions stimulated in the cells of responsive organs. Although such studies yielded plenty of information, the expectation of defining an orderly sequence of events was hampered as it became apparent that the initial triggering step might not be clearly

measurable before secondary responses intervene and thus blur the issue.

An alternative approach that has recently emerged in studying the molecular mechanism of action of sex steroids lies in attempts to study the fate of these gonadal hormones after they have been administered to animals. This approach involves the study of the transformation and inter- and intracellular transport of hormones, as well as their interaction with various cellular compounds. Although the sex steroid-dependent cells have been shown to contain specific binding proteins in both the nucleus and the cytoplasm with a high degree of affinity for androgens and estrogens, it is unknown as to how the hormone-receptor complex amplifies gene expression within the target cell. It has been pointed out that characterization of these receptors and of the physico-chemical basis of their interaction with the hormones must lie at the heart of any postulate concerning the molecular basis of sex hormone actions.

One of the most significant advances in studies of steroid receptors was made by Jensen and his coworkers (136), who demonstrated that both the uterus and vagina possess striking ability to retain estradiol-17β *in vivo*. While these biochemical responses appeared to be estrogen-specific and were inhibited by several anti-estrogenic compounds, they could not be demonstrated in non-target tissues. Estrogen receptors, later found to be involved in selective tissue retention and estrogenic function, were subsequently discovered by Jensen and DeSombre (137) and Gorski *et al.* (138).

Although specific cellular receptors for androgens are still not as well defined and characterized as for estrogens, our knowledge has been advanced since the observation by various investigators that target tissues of male sex steroids exhibited high affinity for androgen (139,140). Retention of radioactive androgen by rat ventral prostate was most evident the first 3 hr following injection of (^{3}H)-testosterone and approximately 50% of the amount

retained disappeared in about 6 hr (140,141). Moreover, analysis of the radioisotopes for testosterone, androstenedione and 5α-dihydrotestosterone revealed that whereas dihydrotestosterone was only found in the seminal vesicles and ventral prostate after 3 hr, no retention of this androgen could be detected in the spleen, liver, thymus, lung and diaphragm, suggesting that only the male accessory glands may have the ability to bind or retain dihydrotestosterone (140). Furthermore, biochemical and autoradiographic studies have indicated that while prolonged retention of dihydrotestosterone by rat ventral prostate appeared to be a result of selective retention of the androgen by prostatic cell nuclei, maximum nuclear labelling occurred at 1 hr and remained for about 3 hr (19,142-144). The formation of dihydrotestosterone from testosterone in the prostate gland is catalyzed by an NADPH-dependent Δ^4-3-ketosteroid, 5α-oxidoreductase. Although this enzyme is located in the microsomal fraction and tightly bound to endoplasmic reticular membranes (46,145), evidence indicates that purified prostatic nuclei also contain a large quantity of this enzyme (19,142). It has been suggested (19,20,22) that dihydrotestosterone may be the active metabolite of testosterone in the prostatic tissue with its principal action being associated with the nucleus. While the proliferative response of the prostate varies with the intracellular or intranuclear concentration of dihydrotestosterone, it appears possible that the enzyme which promotes its formation (5α-reductase) or metabolism may determine the degree of the growth response of this organ to androgenic stimulation (22).

With the new concept that dihydrotestosterone may indeed be the active androgen, the search for specific proteins that bind dihydrotestosterone as well as testosterone was initiated in many laboratories (20,140,146-151). Binding of radioactive androgenic steroids can occur in other subcellular fractions of the prostate, prior to binding within the nucleus (152). Several investigators (149-151) have identified an androgen receptor in the cytosol

of rat prostate with a sedimentation coefficient of approximately 8S whereas Fang et al. (140) found it to be around 3.5. Both receptors have an almost exclusive affinity for dihydrotestosterone. The disparity between the sedimentation coefficients however, did not necessarily indicate the presence of two distinct and unrelated forms of soluble androgen reception since the labile 8S form could easily be converted to the 4S form in vitro either by KCl treatment or raising the incubation temperature from 0 to 37°, and in vivo following a delay in the isolation of the receptor protein after androgen administration (152).

Studies by Bruchovsky and Wilson (20) and Liao and Fang (69) and Mainwaring (148) have demonstrated that the (^{3}H)-dihydrotestosterone retained by prostatic nuclei or chromatin in vivo binds to a protein that could be extracted with 0.3 M to 0.6 M KCl solution. The sedimentation coefficient of the dihydrotestosterone-bound protein was about 3S and more than 80% of the nuclear dihydrotestosterone retained in vivo could be recovered as the 3S protein-bound form (140,147). Fang et al. (140) further demonstrated a critical involvement of extranuclear androgen receptors in the specific nuclear retention of dihydrotestosterone in the prostate. Whereas prostatic nuclei isolated from castrated animals contained very few proteins that bind dihydrotestosterone in vitro, enhanced formation of (^{3}H)-dihydrotestosterone-protein complex was observed when these nuclei were incubated with (^{3}H)-dihydrotestosterone in the presence of a whole cytosol preparation. Evidence thus supports the concept that the prostatic nuclear dihydrotestosterone-receptor protein originates in the cytoplasm and is translocated to a specific nuclear acceptor at a nuclear chromatin site (153,154). Since the retention of dihydrotestosterone by prostatic nuclei was dependent on a cytoplasmic receptor protein, the receptor protein might simply have functioned as a carrier of the hormone to regulate its concentration at various cellular sites. On the

other hand, one may also visualize the binding of the steroid to a specific protein as being a necessary step in the translocation of the protein to another cellular locus where the receptor protein (or the protein in complex with the steroid), rather than the steroid alone, functions. It is also of interest that although the cytoplasm of the prostate contains a number of androgens, only those which form androgen-receptor complexes (i.e. testosterone and dihydrotestosterone) are incorporated into the nucleus. Other intracellular androgens such as 3α- and 3β-androstanediol, androsterone, androstanedione, androstenedione and dehydroepiandrosterone in general do not enter the nucleus but are first converted to dihydrotestosterone in the cytoplasm and transported across the nuclear membrane. Since the binding of testosterone and dihydrotestosterone to cytoplasmic receptor(s) appears to be an obligatory step in the transport process, the level of these androgens in the nucleus may depend on the number of receptors in the cytoplasm. The loss of cytoplasmic receptor with increasing time after orchidectomy has previously been documented (155-157), although its influence if at all present, on the disappearance of receptor protein from prostatic nuclei remains unclear (158).

(iv) The use of antiandrogens in studies of androgen-dependent tissues

The search for drugs that can effectively control abnormal growth of the prostate such as benign prostatic hyperplasia and prostatic adenocarcinoma has been the subject of continuing investigation. Palliative therapy with estrogen has been an important and successful method for the management of carcinoma of the prostate ever since it was first introduced by Huggins and Hodges in 1941 (159). Although it is generally accepted that estrogens elicit their effects on the prostate via the hypothalamo-pituitary-testicular axis, the problem of adverse effects during estrogen therapy, particularly feminization and loss of libido, remains unresolved. The last few years have witnessed

the development of certain powerful anti-androgenic substances (steroidal and non-steroidal) that have been used to control the growth of hyperplastic tissue in both the canine and human prostate. Amongst the popular antiandrogens being studied today, cyproterone, cyproterone acetate and flutamide have been examined in greater depth. The term "anti-androgen" has been frequently used to describe a substance which will completely prevent, or at least significantly reduce the physiological effects normally produced by androgens. Within the scope of this definition, many substances which interfere with ultimate expression of androgen action, whether by inhibiting androgen biosynthesis, by preventing gonadotropin release or reducing accessory sex structure through protein synthesis suppression or by blocking androgen receptor sites, have been termed anti-androgens.

Among the steroidal anti-androgens, cyproterone and cyproterone acetate are currently being used as experimental tools to help clarify the molecular sequence of events that occurs following the androgen stimulation of the prostate gland. Cyproterone acetate, which displays a certain amount of progestational, antiestrogenic and antigonadotropic activities, has 3 times the anti-androgenic potency of cyproterone (33). There have been numerous reports of decreased prostatic weight after cyproterone acetate administration (160, 161). Moreover, Walsh and Gittes (162) reported cyproterone acetate not only blocked the testosterone-induced growth of the prostate, but also that observed following the administration of androstenedione, dihydroepiandrosterone, adrenocorticotropic hormone and prolactin, suggesting a possible inhibition of entry of the steroid into the cell. Conversely, Giorgi (163) reported from studies with human hyperplastic and adenocarcinomatous prostatic tissue that cyproterone acetate did not inhibit the uptake of androgens.

Many non-steroidal compounds have been tested for anti-androgenic properties (164-168). Spironolactone, a diuretic, has been reported to have

some anti-androgenic activity (169) and at least one non-steroidal anti-androgenic compound has been demonstrated not to alter other endocrine functions (170). Of the non-steroidal antiandrogens, flutamide appears to be most promising. Flutamide has been reported to be equipotent to cyproterone acetate as an anti-androgen in both sex differentiation effects (171) and biochemical activity (172). Because of structural dissimilarities, it might be expected that the antiandrogenic activity of flutamide and cyproterone acetate is exerted via different mechanisms. However, Peets et al. (173), in a comparison of the two anti-androgens, observed both flutamide and cyproterone acetate fully antagonized the effects of testosterone to maintain prostate and seminal vesicle weights in orchidectomized rats. Both flutamide and cyproterone acetate inhibited the in vivo uptake of (^{3}H)-testosterone by prostate whole tissue and nuclei, and in addition, inhibited the formation of the 3S protein-^{3}H androgen complex. In contrast, Liao et al. (174) found cyproterone acetate to have 10 times the potency of flutamide in inhibiting the receptor binding of dihydrotestosterone. Evidence suggests that like testosterone, flutamide may also undergo alteration to form a more active metabolite which inhibits androgen binding in rats (173,175-176) and humans (177).

Fang and Liao (147) provided evidence to show that cyproterone acetate suppressed the uptake of radioactive androgens in vivo by the rat ventral prostate which was accompanied by a decrease in the retention of dihydrotestosterone by prostate cell nuclei suggesting a possible competition between the active androgen and the antiandrogen for nuclear binding sites. Walsh and Korenman (161) also suggested that cyproterone acetate does not act by inhibiting the transportation of androgen across the cell membrane, but rather inhibits the action of androgens in the ventral prostate by preventing the formation of protein-chromatin complex in the nucleus. Stern and Eisenfield (32) reported inhibition of testosterone binding to macromolecules present in

the supernatant of seminal vesicle homogenates by cyproterone, lending support to the suggestion that the antiandrogen blocked dihydrotestosterone binding to nuclear proteins. The significance of such biochemical studies with cyproterone acetate and its prospective role in prostate carcinoma were demonstrated in a paper by Geller et al. (178). Using 11 patients with severely advanced carcinomas (stage III or IV as classified by Whitmore), these authors observed objective remissions with cyproterone acetate in 9 out of 11 patients. The most notable biochemical change was a decrease in serum acid phosphatase levels to near normal limits. This was accompanied by a softening and/or decreased size of the prostatic tumor and a depression in plasma testosterone levels. Interestingly, it has been suggested that the decreased plasma testosterone levels following cyproterone acetate administration was the result of a direct inhibition of hormone synthesis by the testis, because plasma pituitary gonadotropin levels remained unaltered (178). Decreased acid phosphatase activity during cyproterone acetate therapy has also been described in the dog (179). Recently, Rajalakshmi and Prasad (180) reported that cyproterone acetate administration significantly decreased fructose content of the dorsolateral prostate and coagulating glands, the citric acid levels of the ventral prostate and seminal vesicles as well as the concentration of sialic acid in Cowper's glands of the rat. They also found a marked decrease in accessory sex gland secretions and DNA content of the caput and cauda epididymis. In addition, Geller et al. (160) found a reduction in the RNA/DNA ratio and a decreased acid phosphatase synthesis concomitant with prostate weight loss after the administration of cyproterone acetate to male rats.

It is known that apart from their stimulatory effects on the growth and differentiation of male accessory sex glands, androgens also exert their action on male secondary sex characteristics, the testis and on the hypothalamo-

pituitary axis. These androgenic effects have also been shown to be antagonized by the administration of cyproterone acetate or cyproterone to the intact animal. Goslar et al. (181) reported that cyproterone acetate enlarged the interstitial cell complex of the testis and increased 3β-hydroxysteroid dehydrogenase activity in the rat, suggesting an increased production of androgens. Recent studies by Tsang et al. (182) have shown that whereas rat testicular adenylate cyclase activity and cyclic AMP levels were significantly decreased 10 days following daily administration of cyproterone acetate, marked stimulation (74 and 516%, respectively) of these parameters was seen during the early phase of anti-androgen treatment. Whereas serum ICSH content following cyproterone administration has been found to increase (183), both cyproterone acetate and cyproterone were shown to raise serum levels of FSH in normal and orchidectomized rats (184). However, in vitro studies have indicated that at least some of the antiandrogenic effects of cyproterone acetate on the prostate may be mediated through the direct action of this compound on testicular steroidogenesis. While Sommerville et al. (185) reported decreased testosterone formation from rat testicular incubates containing cyproterone acetate, Engel and Karsznia (186) demonstrated a decrease in the rate of testosterone production from pregnenolone in presence of the anti-androgen. Recently, cyproterone acetate (25 µg/100 ml) has been reported to inhibit the Δ^4 pathway of androgen biosynthesis and to interfere with the biotransformation of (^{3}H)-pregnenolone to (^{3}H)-testosterone and (^{3}H)-androstenedione by rabbit testicular mince in vitro (187).

B. Pituitary Hormones

Although several studies (2) have demonstrated that pituitary hormones act directly and/or indirectly upon male accessory sex glands, the precise role of these hormones in the overall control of the growth and function of androgen-dependent organs is still unclear. The effects of these hormones

(particularly prolactin) on various androgen-dependent tissues seem to involve primarily an augmentation of androgen stimulation, although under certain conditions, prolactin was found to directly stimulate male accessory sex organ growth. Prolactin also facilitated testicular steroidogenesis by enhancing the action of LH and thus indirectly stimulated the accessory sex organs (188, 189). Whereas the direct end organ effects of these hormones on sex accessory activity have not been as clearly established as their indirect effects mediated *via* the testis, the interactions of these hormones with androgens as well as their possible role in the pathogenesis of the prostate gland remain to be elucidated.

Early studies demonstrated that hypophysectomy resulted in a reduction of testicular weight in cat (190), rat (191), guinea pig and rabbit (192). Huggins and Russell (35) also reported a significant decrease in the weights of canine testis and prostate glands after hypophysectomy. Studies by Segaloff *et al.* (193) demonstrated that prolactin enhanced LH-mediated stimulation of ventral prostate weights in hypophysectomized immature rats. Woods and Simpson (194) confirmed the findings of Segaloff *et al.* (193) about augmentation of the prostatic responses to ICSH in hypophysectomized rats, other hormones such as ACTH, TSH or L-thyroxine were found to be ineffective in this regard. Recent studies by Thomas and Keenan (2) indicated that whereas FSH and LH failed to enhance the effects of testosterone in restoring the accessory sex organ weights in 6-day castrated mice, growth hormone treatment augmented androgen activity in both the anterior prostate gland and seminal vesicles. Lostroh (195) observed similar growth hormone stimulation of androgen effects in the rat ventral prostate gland obtained from hypophysectomized-castrated rats. Grayhack *et al.* (196) observed that simultaneous administration of testosterone and prolactin to castrated-hypophysectomized rats produced greater increases in prostate gland weights than did testosterone alone. Grayhack (197) had

earlier reported that the dorsolateral prostate of hypophysectomized-castrated rats was more responsive to testosterone-prolactin interaction than other lobes. Recently, Keenan and Thomas (198) also found that whereas testosterone, but not prolactin, alone stimulated anterior prostatic and seminal vesicular weight gain in castrated mice, the two hormones given together further increased the weights of both accessory sex organs. Since administration of prolactin failed to alter prostatic or blood levels of testosterone, these investigators concluded that enhancement of male accessory sex organ weight produced by the interaction of prolactin and testosterone was not due to enhanced prostatic localization of testosterone.

The dependence of nucleic acid metabolism of male accessory sex organs on androgens has been well established (53). Recent studies by Thomas and his coworkers (199,200) have shown that augmentation by prolactin of mouse and rat accessory sex organ weights following androgen administration was associated with enhanced cellular proliferation, as reflected by elevated levels of DNA. While prolactin alone failed to elicit any effect on nucleic acid levels of these tissues, androgen-induced elevation in RNA levels was likewise enhanced by prolactin treatment. Moreover, maximal stimulation of accessory sex organ DNA synthesis, as observed after 3 days of prolactin treatment, preceded significant increases in accessory sex organ weights and DNA content (observed on the 5th day), suggesting that prolactin enhanced accessory sex organ DNA synthesis prior to stimulation of cell growth of the mouse anterior prostate and seminal vesicles (2).

It is well established that the secretory activity of the epithelial cells in male accessory sex glands is primarily under the control of testicular androgens. While marked changes in the chemical constituents of various accessory sex glands and their secretions have been noted following androgenic deprivation and repletion, several studies have employed these biochemical

parameters as indices in the evaluation of the influence of prolactin and other pituitary hormones upon androgenic responses of these tissues. Grayhack (197) reported that prolactin administered with testosterone to hypophysectomized-castrated rats enhanced the total content, but not the concentrations of fructose and citric acid in the dorsolateral lobe of the prostate gland. Although later studies by Grayhack and Lebowitz (201) did show that the prostate of hypophysectomized-orchidectomized rats exhibited marked increases in citric acid content after simultaneous treatment with testosterone and prolactin, consistent differences in either the content or concentration of fructose in the dorsal and the lateral lobes of the prostate glands could not be observed. Whereas Reddi (202) failed to demonstrate a significant influence of prolactin upon testosterone stimulation of fructose levels in the prostate gland of castrated Indian palm squirrel, increases in prostatic citric acid and seminal vesicular fructose contents following testosterone stimulation were enhanced by simultaneous administration of prolactin. Peyre et al. (203) observed that prolactin released by reserpine treatment significantly augmented the testosterone-induced stimulation of vesicular citric acid and anterior prostatic fructose content in castrated rats, neither growth hormone nor prolactin was found to enhance the stimulatory action of testosterone on prostatic fructose concentration (198). It also is of interest that the activities of prostatic alkaline and acid phosphatase decreased in hypophysectomized-castrate dogs to a much greater extent than those seen in hypophysectomized animals (35). Gunn et al. (204) reported that in mature hypophysectomized rats, the uptake of zinc by the dorsolateral prostate was a more sensitive indicator of androgenic activity than the weight of the gland. These authors also demonstrated that while ACTH, FSH or TSH failed to alter the stimulatory effect of interstitial cell stimulating hormone on the weight or zinc uptake of the dorsolateral prostate, prolactin and growth

hormone evoked significant increases in these responses to the trophic hormone (204). Rosoff and Martin (205) studied the effects of HCG, FSH, ICSH, prolactin and testosterone on ^{65}Zn uptake of dorsal, lateral and ventral prostates, seminal vesicles, coagulating glands, preputial glands and testes of intact rats. Whereas HCG increased the total ^{65}Zn uptake for all organs examined and the ^{65}Zn uptake when expressed on a per milligram tissue basis in the lateral prostate, preputial gland and testis, ICSH and FSH had opposite effects for the most part (decreasing zinc uptake for seminal vesicles, dorsal and lateral prostates and coagulating glands). Prolactin alone showed no effect except to decrease total ^{65}Zn uptake of the testis.

Recent studies by Golder et al. (206) have demonstrated that whereas high concentration of prolactin failed to elicit any effect on adenylate cyclase activity of prostate homogenates in vitro, lower concentrations of the hormone significantly enhanced the activity of this enzyme although no augmentation of the effect was observed with testosterone. This observation is in accord with the findings of Lasnitzki (207) who demonstrated that prolactin, in a low dose, sustained the growth of rat prostate in vitro, whereas high doses of the hormone failed to maintain epithelial and secretory activity. Furthermore, human luteinizing hormone, chorionic somatomammotrophin and growth hormone also were found to stimulate the activity of prostatic adenylate cyclase in vitro (206). It is of interest that while Thomas and Manandhar (200) reported a decrease in prostatic cyclic AMP in the presence of testosterone or high concentrations of prolactin, administration of testosterone with prolactin produced a small increase in the nucleotide levels.

2. CYCLIC AMP INVOLVEMENT IN HORMONE ACTION

The discovery of cyclic AMP 20 years ago and the gradual understanding of its biological significance has led to the belief that this cyclic nucleotide acts as an intracellular mediator in the action of many hormones and cellular regulatory substances. Cyclic AMP has been implicated in the mechanism

of action of hypothalamic releasing hormones, anterior and posterior pituitary hormones, insulin, glucagon, catecholamines, parathyroid hormone, prostaglandins, melatonin, serotonin and histamine (208). It appears that cyclic AMP may also be involved in certain neuronal functions as well as other physiological processes in the body (208-210).

The biological importance of cyclic AMP has been extended from its relatively simple function in liver glycogenolysis to the point where it now appears to play a key regulatory role in numerous important cellular responses. As a result of many extensive studies, the concept has evolved that many hormones act by a "two-messenger" system (24,211,212). According to this concept, the first messenger, the hormone itself, released from its specific endocrine gland, travels to the cells of the target tissues where it stimulates the formation of cyclic AMP from ATP. The cyclic nucleotide, in turn, activates subcellular effector devices including protein phosphorylation by specific enzymes to produce a sequential series of effects which may differ depending upon the target tissues involved, but in each case, are the same as those characteristically produced by the hormone.

A. Cyclic AMP

Cyclic AMP is known to be distributed widely in nature. It is found in most tissues of multicellular organisms and in a variety of mammalian fluids. In general, in the absence of stimulation by exogenous hormones, intracellular concentrations of cyclic AMP have been found to be in the order of 0.1 to 1 μmoles per gram of tissue. Assuming that the cyclic nucleotide is evenly distributed within the intracellular fluid, cyclic AMP concentration would thus be between $1x10^{-7}$M and $1x10^{-6}$M and about 1/1000 to 1/10,000 of the concentrations of other adenosine nucleotides (213). The concentrations of the cyclic nucleotide in plasma, cerebrospinal fluid and gastric juice are ordinarily quite low (approximately 10^{-8}M) but can be increased dramatically in response

to pharmacological doses of hormones (214,215). Cyclic AMP is also found in milk (216) and urine (217,218) and exists at a concentration in the order of about 10^{-6}M. Whereas the nucleotide appears to be absent in avian erythrocytes (219) and human platelets (220,221), distribution studies indicate that its presence is not merely confined to mammalian tissues (222). Cyclic AMP has been detected in several bacteria where it appears to play a role in the regulation of synthesis of inducible enzymes (223) and has been identified as the acrasin of slime molds (224). It has been reported to potentiate the anti-viral effects of interferon (225) and it is now believed that the levels of cyclic AMP increase in some plant tissues in response to gibberellic acid (226). The presence of cyclic AMP in unicellular organisms and in plants together with the evidence that it plays an important role in their cellular processes suggests that the importance of this nucleotide may indeed surpass its role in endocrine biology (24).

B. Adenylate Cyclase

The synthesis of cyclic AMP in biological systems is catalyzed by the enzyme adenylate cyclase, which is present in all animal and bacterial cells examined except dog erythrocytes (227) and a line of rat hepatoma cells (228). Nevertheless, differences do exist between the properties of mammalian and bacterial adenylate cyclase, particularly with respect to the control of enzyme activity. While the synthesis of cyclic AMP in mammalian cells is regulated by hormones produced in a cell type distinct and distant from the target cells, regulation of the synthesis of the nucleotide in bacterial system appears to be related to the nutritional state of the bacteria and no evidence as yet exists to suggest a mechanism involving cell to cell communication (229).

Early studies by Sutherland and his coworkers (227) demonstrated that adenylate cyclase activity was associated with particulate fractions of tissue homogenates. While Davoren and Sutherland (219) provided evidence from studies

with pigeon erythrocytes to suggest that adenylate cyclase was probably localized in the plasma membrane, this notion was later confirmed by a number of laboratories for most, if not all mammalian tissues. Recently, Rodbell and his coworkers (230-235) extensively characterized the adenylate cyclase of rat liver plasma membrane. Electron microscopic examination revealed numerous paired membrane sheets with typical intracellular junctions characteristic of hepatic parenchymal plasma membrane. In line with these findings were the observations provided by other investigators that ACTH, glucagon and norepinephrine, bound to large insoluble support materials (too large to enter the target cells) were also effective in stimulating adenylate cyclase and in eliciting normal physiological responses (236,237). Moreover, recent studies have demonstrated the binding of polypeptide hormone and biogenic amines to partially purified plasma membrane preparations (233,238-240). While ample evidence suggests the existence of an adenylate cyclase system in the plasma membrane of various target cells, the possibility that this enzyme may likewise be present in certain internal membranous structures such as mitochondrial and microsomal components (241-243), sarcolemma (244) and nuclear membrane (245-247) should not be overlooked. Of particular interest is the finding of Mangan et al. (246) which indicates that whereas adenylate cyclase in purified prostatic nuclei decreased markedly following androgenic deprivation, administration of androgen was able to reverse this effect in 1 hr. The significance of these findings will be discussed in greater detail in later sections.

One feature which is common to adenylate cyclase in most preparations from multicellular organisms is its sensitivity to fluoride ion (248). Fluoride, however, does not stimulate bacterial adenylate cyclase (249). The magnitude of the effect of fluoride varies markedly with enzymes from different tissues (250). Although the maximally effective concentration of

fluoride ranges between 3 to 4 and 12 to 15 mM, the sensitivity of the enzyme to fluoride stimulation is also influenced by the concentration of other ions in the assay mixture (251). Moreover, it has been demonstrated that adenylate cyclases from most tissues are not responsive to hormones in the presence of maximally effective concentrations of NaF. Since the activity of the enzyme is generally activated to a greater extent by fluoride than by hormones, it has been assumed by some workers that fluoride causes full expression of enzyme activity. However, an exception to this generalization may be obtained from the studies of Birnbaumer et al. (231), who showed that rat liver adenylate cyclase was stimulated to a greater degree by glucagon than by NaF. Subsequently, Hynie and Sharp (252) demonstrated the enzyme from frog bladder epithelium to be more responsive to vasopressin than NaF. Although the effect of fluoride has been studied to some extent in preparations of fat cell ghosts (251,253,254) and evidence suggested that it markedly increased the maximum velocity of the cyclase reaction with little or no change in apparent affinity of the enzyme for ATP, the mechanism of action of fluoride on the cyclase system is still poorly understood (250).

One of the most striking features of adenylate cyclase is its sensitivity to hormonal stimulation. Adenylate cyclase from different mammalian cells and tissues has been shown to be regulated by a variety of hormones (208,255). Moreover, it has been demonstrated that only some of these effectors can modulate adenylate cyclase activity in a given tissue. Adenylate cyclase activity in liver preparations can be stimulated by epinephrine and other catecholamines (256,257) as well as by glucagon (258,259). Glucagon is much more potent than any of the catecholamines, not only in the sense that smaller concentrations of glucagon are required to stimulate the enzyme, but also in that the maximal response in this case is much greater than the maximal response to catecholamines.

The adenylate cyclase of the rat epididymal fat pad is another system that has been extensively explored. Birnbaumer, Pohl and Rodbell (251), Vaughan and Murad (254) and Bar and Hechter (253) found that catecholamines, ACTH, glucagon and other polypeptide lipolytic hormones all stimulated adenylate cyclase activity of fat cells or fat cell ghosts prepared from rat epididymal fat pad. Moreover, when fat pads were incubated with low concentrations of epinephrine, cyclic AMP levels and the rate of lipolysis were proportionally increased (260). Other hormones with lipolytic activity also increased cyclic AMP levels, while those without lipolytic activity did not (261). Although β-adrenergic blockers antagonized the effects of catecholamines on both cyclic AMP accumulation and on lipolysis, other substances such as insulin and prostaglandins were also found to be effective antagonists. Rodbell and his coworkers (262) proposed a model wherein the hormone-sensitive membrane bound adenylate cyclase system is composed of three components: a hormone discriminator (receptor), a transducer, and an amplifier (catalytic unit). The discriminator, located on the external surface of the cell membrane, serves as receptor(s) for hormone(s) and provides hormonal specificity for the target cells. The second component is a transducer which converts signals coming from the discriminator in such a way that the amplifier (third component) recognizes them as instructions to accelerate its catalytic function. The amplifier or catalytic unit lies on the inner surface of the cell membrane which has access to ATP and can generate cyclic AMP.

C. Phosphodiesterases

The levels of cyclic AMP in biological systems are dependent upon the rate of both the synthesis and degradation of the nucleotide. The enzymes responsible for the hydrolysis of cyclic AMP to 5'-AMP are specific cyclic nucleotide phosphodiesterases (263,264) which are present in all animal tissues, except non-nucleated erythrocytes, as well as certain lower organisms and

plants (265-270). Cyclic nucleotide phosphodiesterases are the only enzymes known to directly terminate the activity of cyclic nucleotides. Various metabolites including citrate, pyrophosphate, adenosine, ATP and other nucleoside triphosphates have been shown to inhibit cyclic AMP phosphodiesterase activity in vitro and it appears that these agents act by chelating Mg^{++}. Similarly, enzymatic activity can be inhibited by various pharmacological agents such as methyl xanthines (217,271), puromycin (272-274), 4-benzyl-2-imidazolidinones (275-277), papaverine (274,278-281) and various α-adrenergic antagonists (282-284). Moreover, it is generally true that the effects of hormones which act by stimulating adenylate cyclase are potentiated in the presence of one of the phosphodiesterase inhibitors.

Although considerable effort has been devoted during the past years to study the hormonal regulation of cyclic nucleotide phosphodiesterases, the results, in general, have been disappointing. This is in part due to the fact that there are multiple cyclic nucleotide phosphodiesterases in each cell type, and it has been rather difficult to separate and purify them (270). Nevertheless, recent work utilizing better kinetic analysis, fractionation of cellular components and enzyme purification procedures has yielded valuable information regarding the hormone-phosphodiesterase relationship. A hypothesis has been proposed that hormones, for which the physiological response is paralleled by depressed cyclic AMP levels, might act through activation (285-290) or even induction of the synthesis (291) of phosphodiesterase to achieve this effect. Although the physiological role of cyclic nucleotide phosphodiesterase in various hormone-mediated responses remains to be determined, there are indications to suggest that certain hormones such as thyroid hormone, cholecystokinin and insulin, decrease cellular cyclic AMP levels through regulation of the cyclic AMP phosphodiesterases in their target cells (270, 292-295).

D. Protein Kinases

In order that one may fully understand the role of the adenylate cyclase-cyclic AMP system in the mechanism of action of hormones in their target tissues, studies of the various biochemical responses following the binding of these hormones to specific receptors and stimulation of cyclic AMP formation are necessary. Whereas cyclic AMP has been found to be present throughout the animal kingdom, protein kinases which are activated by nucleotides have been isolated from a variety of mammalian tissues as well as species of different invertebrate phyla (296-306). The activation of protein kinases appears at present to be the major if not the only mechanism by which cyclic AMP carries out its function as a "second messenger" in the transmission of hormonal signals. Although the functional role of this class of proteins in the adenylate cyclase-cyclic AMP system is still under extensive investigation, recent evidence has indicated that in the presence of cyclic AMP they catalyze the phosphorylation of other proteins having key roles in various hormone-mediated responses (307-313).

During the past 5 years, numerous studies have indicated that cyclic AMP-dependent protein kinases are composed of regulatory (R) and catalytic (C) subunits. The binding of the regulatory subunit to the catalytic component results in an inactive homoenzyme (RC). As shown below, cyclic AMP promotes dissociation of RC to yield a regulatory subunit-cyclic AMP complex and a free catalytic subunit which is then enzymatically active (Equation 1).

$$\underset{\text{(inactive)}}{\text{Cyclic AMP} + \text{RC}} \rightleftharpoons \underset{\text{(active)}}{\text{R}\cdot\text{Cyclic AMP}} + \text{C}$$

By itself, the activity of the catalytic component is unaffected by cyclic AMP, but cyclic AMP dependence may be restored by the addition of regulatory subunit to the system. Although this model was first proposed on the basis of studies with protein kinase from bovine cardiac muscle, similar mechanisms have since been reported for many other tissues (296,

303, 314-318). Since the cyclic AMP-dependent phosphorylation step precedes hormonal manifestation, a rapid control of the reaction is vital for the maintenance of normal physiological states. The effectiveness of the cyclic AMP-dependent protein kinase system in responding to hormonal stimulation is well illustrated in the regulation of glycogen metabolism in skeletal muscle. By means of the cascade of sequential activation reactions, the original hormone signal via cyclic AMP is amplified many-fold by the time its effect impinges on the glycogenolytic pathway per se. The activation of phosphorylase and inhibition of glycogen synthetase by one enzyme, protein kinase, thus provides a unique and highly efficient metabolic control mechanism.

It is also of interest that within 1 min of the addition of epinephrine or ACTH to isolated fat cells, the cyclic AMP content of the cells was elevated. The nucleotide levels continued to rise, reaching a peak at 4-7 min, and fell thereafter. A maximal rate of glycerol production was, however, established within 1-2 min, well before the peak in cyclic AMP concentration, and was maintained unaltered while the nucleotide levels continued to rise and then fell. Furthermore, incubation of epididymal fat pads with epinephrine was also found to increase protein kinase activity in tissue extracts. This activation was associated with the conversion of the inactive holoenzyme to the active catalytic subunit and the time-course of formation of the latter paralleled the epinephrine-induced increase in cyclic AMP concentration. Concentrations of epinephrine (0.1-0.3 μM) which produced small increases in both the cyclic AMP concentration and the catalytic form of protein kinase gave a maximum rate of lipolysis (308). These results demonstrate the rapidity of the enzyme system with which it responds to hormonal stimulation.

With such an effective and responsive system, the need for regulatory mechanisms that may provide rapid and instantaneous control of hormone responses is undoubtedly crucial. This aspect of endocrine function may

be regulated at various levels, and control of cyclic AMP and its dependent protein kinase seems quite important. The adjustment of steady state levels of free endogenous cyclic AMP by the actions of adenylate cyclase and phosphodiesterase has been well documented. Furthermore, it has been shown that cyclic AMP bound to the receptor subunit of protein kinase was less susceptible to degradation by phosphodiesterase, and that the rate of dissociation of cyclic AMP from the receptor protein appeared to be sufficiently rapid at physiological temperature to account for the relatively rapid equilibrium between the free and bound cyclic nucleotide. Therefore, it is possible that the proportion of the total kinase which is cyclic AMP-activated is adjusted through the enzymatic degradation of the unbound nucleotide alone (319).

(i) Multiple forms of protein kinases

There is accumulating evidence indicating the existence of multiple forms of protein kinases in various tissues. Three forms of enzyme, RC, R and C, having different physical characteristics have been isolated from adrenal cortical tissue (317,320). While RC, having a molecular weight of 144,000 and sedimentation constant of 7 S, possessed both cyclic AMP binding and kinase activities, R and C had molecular weights of 92,000 and 60,500, respectively. R also had a sedimentation constant of 4 S. It has been shown that recombination of R and C repressed the latter's activity but this inhibition could be relieved by addition of cyclic AMP (317).

Multiple forms of cyclic AMP-dependent protein kinase from skeletal muscle have been reported. Two protein kinase peaks, I and II were obtained and were dependent on cyclic AMP (302). At least three and probably four forms of cyclic AMP-dependent protein kinase have been suggested to be present in this tissue. The largest of these had a molecular weight of 123,000 consisting of a regulatory (M.W. 82,000) and a catalytic subunit (M.W. 49,000). While protein kinase of peak II has been estimated to have

a molecular weight of 76,000, peak I has been suggested to contain a total of 3 species of protein kinase, sedimenting at 6.8 S, 5.4 S and 5.0 S (322). It has also been shown that incubation of protein kinase of peak I with cyclic (^{3}H)-AMP followed by chromatography on a casein-Sepharose 2B column provided a cyclic AMP-independent kinase and a cyclic (^{3}H)-AMP receptor complex (315). It was further documented that the presence of added cyclic AMP-receptor complex increased the nucleotide requirement for the activation of RC. These findings imply the existence of a dynamic equilibrium among RC, R and C, as shown in Equation 1. Hormonal elevation of intracellular concentration of cyclic AMP shifts the equilibrium of this equation towards formation of activated kinase (C), while depression of intracellular cyclic AMP concentration leads to the formation of the repressed form of the enzyme (RC).

A cyclic AMP-dependent protein kinase has been purified from bovine heart muscle (318). Its molecular weight was 280,000, and it is composed of a cyclic AMP receptor and a cyclic AMP-independent kinase subunit having molecular weights of 55,000 and 42,000, respectively. In brain, two cyclic AMP-dependent protein kinase peaks (I and II) were obtained after column chromatography on hydroxylapatite (322,323). When protein kinase I was centrifuged in a sucrose density gradient, two peaks of cyclic AMP-dependent catalytic activity with molecular weights of 140,000 and 80,000 again were observed. Under the same condition, protein kinase II appeared as a single peak (also with cyclic AMP-dependent catalytic activity) with molecular weight of around 140,000. It has also been demonstrated that preincubation of either protein kinase I or II with cyclic AMP prior to sucrose gradient sedimentation converted the protein kinase into another protein of lower molecular weight (40,000).

In rat liver, three fractions have been resolved from cytoplasmic hepatic protein kinase by DEAE-Sephadex chromatography (324). Two of these fractions exhibited sedimentation constants of 6.8 S and isoelectric point of pH 5.2, while the third component had a 4.0 S with an isoelectric point of pH 8.6. The activity of the 6.8 S fractions was stimulated by cyclic AMP, while that of the 4.0 S kinase was independent of the nucleotide. In the presence of cyclic AMP, the 6.8 S protein kinase dissociated to yield two cyclic AMP-independent protein kinases of isoelectric points 7.6 and 8.9 each of 4.0 S, and neither of which was identical with the 4.0 S protein kinase isolated by DEAE-Sephadex chromatography. Addition of a cyclic AMP receptor to 4.0 S cyclic AMP-independent protein kinases inhibited the activity of the latter, whereas no effect was observed if the nucleotide was present. On the basis of reconstitution experiments, each 4.0 S component has been interpreted to represent a species of catalytic subunit of cyclic AMP-dependent protein kinase, while the 6.8 S protein represents a holoenzyme(s) composed of catalytic and cyclic AMP-binding regulatory subunit.

The physical characteristics of various protein kinases and their subunits from various tissues have been well documented (296,315,321,317,301,322, 302,318,325). The differences in molecular size of some of the protein kinases and subunits may be explained as a result of either aggregation or dissociation of these protein molecules, limited digestion due to contamination of small amounts of proteolytic enzymes, tissue specificity of the proteins, or a combination of any of these factors. Nevertheless, effort should be made to determine, once and for all, what these various species represent in order that one may have a clearer picture of the role of cyclic AMP-dependent protein kinase in the mechanism of hormone action.

(ii) Stimulation of protein kinases by cyclic AMP and other cyclic nucleotides in vitro

The activity of protein kinases from various sources has been studied as

a function of concentration of cyclic AMP (298). It has been documented in various bovine tissues that cyclic AMP stimulated the enzyme in a dose-related manner with a maximum response at approximately 5 μM of the nucleotide, but decreased at higher concentrations. The apparent K_m was found to range from 30-160 mM (298). These K_m values also agreed well with those described for enzymes from rabbit skeletal muscle (302), rat adipose tissue (296), rat mammary gland (301), frog bladder (297), bovine thyroid gland (304) and Escherichia coli (326). The relative ability of various analogs of cyclic AMP to stimulate protein kinase activities from different sources has also been examined (306). Of the various cyclic nucleotides examined, cyclic IMP, cyclic GMP, cyclic UMP and cyclic CMP, at high concentrations, stimulated protein kinase activity almost as effectively as cyclic AMP at optimal concentrations. In contrast, the cyclic deoxyribonucleotide, cyclic dTMP, even at high concentrations, only caused moderate increases in the activity of the enzyme from rat muscle and from lobster muscle, but exerted little or no effect on the activity of the enzymes from other tissues. The effectiveness of these cyclic nucleotides in stimulating protein kinase activity (with the exception of the enzyme from lobster tail muscle which was stimulated quite effectively by cyclic UMP and cyclic CMP on a comparative basis) at 5 μM concentration of the nucleotides followed the general pattern: cyclic AMP> cyclic GMP>cyclic UMP>cyclic CMP>cyclic dTMP (306). These findings were further substantiated by similar observation of the effects of these analogs on protein kinase activities from rat mammary gland (301), frog bladder (297), and bovine adrenal cortex (320). However, it is of interest that a cyclic nucleotide-dependent protein kinase, activated to the same extent by cyclic AMP and cyclic IMP has recently been partially purified from human polymorphonuclear leucocytes (305). Moreover, apart from a cyclic AMP-activated protein kinase, a cyclic AMP-inhibitable enzyme has also been reported to be

present in the acellular slime mold, Physarum polycaphalum. The rate of casein phosphorylation catalyzed by the latter enzyme was completely inhibited at a concentration of cyclic AMP above 1×10^{-9}M (327). Furthermore, it has been shown that many arthropod tissues were rich in cyclic GMP-dependent protein kinases and that the action of cyclic GMP in these tissues may be mediated through regulation of cyclic GMP-dependent protein kinases (299,328).

The effect of various nucleotide derivatives on the activity of protein kinases also has been studied. Adenosine 5'-monophosphate, a degradation product of cyclic AMP, was shown to inhibit protein kinases from rat and lobster muscle both in the presence and absence of cyclic AMP (306). Species differences in the sensitivity of protein kinases to various nucleotides have been reported. Whereas protein kinase activity from rat skeletal muscle was inhibited by 90% in the presence of 5 μM AMP, activity of bovine muscle protein kinase was only slightly decreased (18%). While GDP was also found to be inhibitory to the enzyme from bovine brain, rat adipose cells and lobster muscle, its effect was most pronounced on the brain and muscle enzyme. A number of compounds such as adenine, adenosine, 2'-deoxyadenosine, ADP, guanosine, GMP, riboflavin, FMN and FAD have been shown to elicit varying degrees of inhibition of protein kinases from various sources (306,298,328).

(iii) Effects of various substrates on protein kinase activity

In addition to the regulation of protein kinases by cyclic nucleotides, evidence for the activation of these enzymes by various substrates has also been reported (329,330). Reimann et al. (302) found that preincubation of skeletal muscle protein kinase (peak I) with casein at pH 6.0 or lower, increased the activity of the enzyme measured in the absence of cyclic AMP. Preincubation with histone resulted in a complete loss of the requirement for the cyclic nucleotide at all pH values. Miyamoto et al. (329) reported that preincubation of brain protein kinase with histone, protamine or poly(L-lysine)

partially activated the enzyme (2-fold as compared to 8-fold by cyclic AMP). Sucrose gradient centrifugation studies revealed that whereas brain cyclic AMP-dependent protein kinases (I and II) normally sedimented in positions corresponding to molecular weights of 140,000 and 80,000, respectively, preincubation of these enzymes with histones resulted in the formation of a cyclic AMP-independent peak with molecular weight around 40,000. Interestingly, cyclic AMP also caused the protein kinase activity to sediment in a position corresponding to molecular weight 40,000. These results suggest that histone can activate protein kinase by causing dissociation of the regulatory subunits from the holoenzymes in the absence of cyclic AMP. The regulatory subunits of protein kinases are somewhat acidic (324,30), favoring interaction with the highly basic histones. The interaction of brain protein kinase with histones however, was complicated by the fact that histones also caused a shift of the 140,000 molecular form of the enzyme to 80,000 without a change in cyclic AMP dependency, thereby implicating a structure for brain protein kinase consisting of a tetramer which, in the presence of histone or cyclic AMP, splits into dimers (molecular weight 80,000), each of which contained one catalytic and one regulatory monomeric subunit of 40,000 molecular weight (329). Subsequently, Tao (330) showed that preincubation of reticulocyte protein kinase with protamine resulted in a complete conversion of this enzyme to a slower sedimenting, cyclic AMP-independent form corresponding to that produced by incubation with cyclic AMP.

In general, the susceptibility of protein kinases to this type of activation seemed to vary considerably from one tissue to another. This may be attributed to variations in charge and in the affinity of the regulatory subunits for the catalytic components. Activation by this mechanism may contribute to variations in the degree of cyclic AMP stimulation with different substrates as has been observed in many laboratories. It remains, however,

to be shown as to how important is the substrate activation of protein kinase in vivo. Complexing of histones to DNA might prevent their interaction with the regulatory subunit of protein kinases. The phosphorylation of liver histone in vivo is highly dependent on cyclic AMP or glucagon administration (331,332). However, it is of interest that cyclic AMP also stimulated phosphorylation of rat liver nuclear acidic protein in vivo and in vitro (333), and that the rate of phosphorylation of these specific nonhistone proteins is modified by the addition of exogenous histone fractions (334,335). The interaction between histones and acidic chromosomal proteins may be evident in two ways. Phosphorylation studies indicated that different histones may also modify the structure and metabolic activity of individual nuclear phosphoproteins. Conversely, it is known that an acidic protein fraction which binds selectively to histones F_{2a1} and F_3 functions as a histone deacetylase (336). This mutual interdependence of enzyme systems regulating the modification of nuclear proteins may represent an important aspect of interlocking controls in the regulation of genetic activity.

Haddox et al. (337) observed that exposure of skeletal muscle protein kinase (peak I) to micromolar ATP in the presence of Mg^{2+} before initiating the cyclic AMP binding reaction or cyclic AMP activation of histone phosphorylation, increased the requirement of the cyclic nucleotide about 10-fold. Whereas the dissociation constant of cyclic AMP in the binding reaction was changed from 2×10^{-8} to 2×10^{-7} M and the K_m of cyclic AMP for kinase activation also increased from 3×10^{-9} to 5×10^{-8} M, 50% inhibition of cyclic AMP (10^{-8} M) binding was observed at approximately 2×10^{-7} M ATP. Furthermore, it was also noted that the binding of (^3H)-ATP was associated with the holoenzyme and could be exchanged with unlabeled ATP (but not cyclic AMP). These findings not only implied the existence of separate binding sites for the cyclic nucleotide and the triphosphate, but also suggested that the

binding site involved in the Mg^{2+}-induced inhibition of cyclic AMP reactivity may be different from that for binding substrate (ATP) since the K_m of the enzyme for ATP was in the range of 10^{-5} M (302). These observations were consistent with the report by Brostrom et al. (315) that the addition of Mg^{2+} ATP promoted the dissociation of cyclic (^{3}H)-AMP from the regulatory subunit of the kinase in the presence of catalytic subunit. While the importance of these effects on the sensitivity of protein kinase to regulation by cyclic AMP in vivo remains unclear, evidence is available indicating that a facilitation or suppression of protein kinase activity in vitro may be brought about by a mechanism not necessarily involving an alteration in intracellular cyclic AMP concentration of the tissue.

(iv) Nuclear proteins in relation to substrate specificity of protein kinases

The presence of phosphoproteins and protein phosphokinases in various tissues and their localization at various sites in the cell has stimulated extensive investigations into their biochemical functions (4,5,306,312,338-345). The discovery of active metabolism of phosphoproteins in the nucleus by Langan and Lipmann (as cited in Ref. 346) has prompted the hypothesis that these molecules might play a role in the structural organization of the chromosome and in the control of RNA transcription in vivo (346-348). The two aspects of transcriptional control (suppression of template activity of most of the DNA and the activation of RNA synthesis at particular genetic loci) require the participation of proteins with different properties, DNA binding affinities and contrasting effects on transcription. The proteins concerned include the histones, the basic suppressive components of chromatin, as well as more acidic proteins which also demonstrated strong indication for involvement in the control of RNA synthesis at specific sites on the genome. It is thus apparent that enzymatic modification of amino acid residues of these nuclear proteins, by phosphorylation, acetylation and methylation, may modify the charge and conformation of the respective poly-

peptide chains. These changes, which ultimately alter the nature and strength of the interactions between the nuclear proteins and DNA, may provide clues to the molecular events underlying the selective activation and repression of particular cistrons in different cell types at different times. The role of these nuclear phosphoproteins and their modification during gene activation has recently been reviewed (4,5,339,340,349).

The likelihood that a large number of hormonal responses are mediated by cyclic AMP-dependent protein kinases suggests that the search for substrates of these enzymes should be highly fruitful in elucidating pathways of hormone action. However, this approach has met with difficulties, due in part to the apparent low specificity of the kinases for protein substrates. It has been shown that cyclic AMP-dependent protein kinases in various mammalian tissues catalyze the incorporation of the terminal phosphate of ATP into the same specific proteins, such as glycogen phosphorylase b kinase, glycogen synthetase, lipase, histones and ribosomal proteins (350). Since only a limited number of specific substrates have been demonstrated to be phosphorylated in vivo by these enzymes, arbitrary protein substrates have been used in most in vitro studies of protein kinases in many tissues. Histones are good substrates for cyclic AMP-dependent protein kinases and have been used extensively for this purpose. With skeletal muscle protein kinase, the rate of phosphorylation of calf thymus total histones was approximately one-third of that with phosphorylase kinase (313), but when histone subfractions were used, the rate of phosphorylation was comparable to that obtained with phosphorylase kinase and glycogen synthetase (30). Phosphorylation of all histone fractions has been demonstrated in vitro. In general, while histones F_{2b} and F_1 have been shown to be phosphorylated more rapidly than F_{2a} and F_3 (296,308,351,352), isolated catalytic subunit from hepatic cyclic AMP-dependent protein kinase phosphorylated histones F_1, F_{2a} and F_3 at about

half the rate observed with F_{2b} histone (32). Kuo and Greengard (353) however, observed that protein kinases from a wide variety of organisms were more active with histone IV (subfraction of histone F_{2a}) when compared to histone IIB (F_{2b}) and Ib (F_1). Nevertheless, as pointed out by Krebs (30), since the relative rates of phosphorylation of various protein substrates are highly dependent on assay conditions, caution must be exercised in evaluating the significance of moderate differences in the rate of phosphorylation of various substrates in vitro.

The phosphorylation of rat liver lysine-rich histone by cyclic AMP-dependent protein kinase has been demonstrated in vivo by Langan (312). Characterization of the site of phosphorylation and distribution of phosphate in tryptic phosphopeptides of the phosphorylated lysine-rich histone revealed one major radioactive peptide having one of the seryl residues phosphorylated. It was also observed that the same site on the histone molecule was phosphorylated in the presence and absence of cyclic AMP. Fifteen min after the administration of glycogen or dibutyryl cyclic AMP, histone phosphorylation in rat liver was increased by 8-20 fold. The incorporation of ^{32}P into lysine-rich histone was not blocked by actinomycin D or cycloheximide, indicating that phosphorylation took place in intact histone molecules and that the observed response was not dependent on increased synthesis of histone kinase or other enzymes (331,332). The increased phosphorylation following insulin administration observed in intact rats (331) could not be demonstrated in isolated perfused liver (354), suggesting that the effect of insulin in the whole animal might be an indirect one, and probably due to glucagon or catecholamines released in response to insulin-induced hypoglycemia. By contrast, hydrocortisone and ACTH, two hormones which do not alter the levels of hepatic cyclic AMP, had no effect on histone phosphorylation. Whereas glucagon, in doses corresponding to those which stimulated histone phosphorylation has been shown to induce actinomycin-sensitive de novo synthesis of liver tyrosine

amino transferase (355), and serine dehydratase (356), these effects were also mimicked by cyclic AMP (357,358). It has also been demonstrated that modification of histones by phosphorylation influenced DNA-histone interactions and caused derepression of template activity (359,360). In their studies on the effect of phosphorylation on the ability of lysine-rich histones to block RNA synthesis on reconstituted chromatin templates, Watson and Langan (361) observed that chromatin reconstituted with lysine-rich histone phosphorylated by cyclic AMP-dependent protein kinase was 25 to 80% more active as template for RNA synthesis than chromatin reconstituted with control histone. It was proposed that the induction of RNA synthesis, following hormonal stimulation, might be the result of a change in DNA-histone interaction brought about by cyclic AMP-dependent histone phosphorylation, involving a conformational change in histone molecules with subsequent derepression of DNA template (312).

In recent years, interest has been renewed in studies of the nonhistone proteins of cell nucleus, particularly in relation to mechanisms of genetic control. Elegant studies by Kleinsmith et al. (346-349) have established that both histone and nonhistone proteins contained phosphoseryl residues, and that most of the phosphate incorporated into nuclear proteins by nondividing cell nuclei was recovered as phosphoserine in the acidic protein fraction. It was also shown that phosphorylation and dephosphorylation of these acidic nuclear proteins could take place on their intact polypeptide chains in the cell nucleus (346,348), and that the ability of these proteins to stimulate transcription in vitro was enhanced with increasing phosphorylation and was decreased upon enzymatic removal of their phosphate groups (349,362). Both the synthesis and phosphorylation of individual nuclear acidic proteins have been shown to be subject to hormonal control. Whereas cortisol as well as glucagon have been demonstrated to selectively stimulate

the synthesis of various nonhistone nuclear proteins in liver, the synthetic response to these hormonal stimuli was slow (363). Much more rapid responses were evident in the phosphorylation of these acid proteins. While enhanced phosphorylation of hepatic nuclear phosphoproteins was within 5 min of cortisol administration, kinetic analysis of ^{32}P incorporation into these proteins showed a highly complex time course of phosphate uptake and release (339,349). Moreover, administration of dibutyryl cyclic AMP to adrenalectomized rats has been shown to alter the pattern of phosphorylation of these proteins in liver tissue (333). Within 15 min after the administration of the dibutyryl cyclic nucleotide, significant increases in the rate of phosphorylation (>200%) were observed in specific protein fractions (molecular weight ranges 15,000-40,000 and 60,000-85,000). Selectivity in the effects of cyclic AMP was evident in that many protein bands, particularly a peak at molecular weight 57,000, showed the same activity in control and treated animals.

Effects of cyclic AMP on the phosphorylation of nuclear acidic proteins also were demonstrated in isolated rat liver nuclei. While the magnitude of stimulation observed in isolated nuclei was considerably lower than that observed when dibutyryl cyclic AMP was administered *in vivo*, the *in vitro* stimulation of phosphorylation by cyclic AMP was also selective for specific acidic proteins. Moreover, when isolated acidic proteins were incubated with (γ-^{32}P) ATP, a significant amount of endogenous protein kinase activity was detected, which could be stimulated (2-fold) in the presence of exogenous cyclic AMP. This is of interest in the light of recent findings by Rikans and Ruddon (364) that four peaks of protein kinase activity and two peaks of cyclic AMP binding activity could be separated by phosphocellulose chromatography from acidic nuclear proteins of rat liver nuclei. The activity of the major kinase peak (KIV) was inhibited in the presence of these cyclic AMP binding fractions, but could also be partially reversed by the addition

of 5 μM cyclic AMP to the incubation mixture (364). It was demonstrated that the soluble cyclic AMP-dependent protein kinases were fully capable of phosphorylating nuclear acidic proteins and that cyclic AMP stimulated this phosphorylation about 2-fold (333). These findings are consistent with the hypothesis that nuclear kinases might be catalytic subunits of the cyclic AMP-dependent holoenzymes and are separated from their inhibitory or regulatory subunits either as a result of dissociation and concomitant activation by nucleohistones (329,334,335), or merely during the process of purification and preparation of the enzymes.

The phosphorylation of E. coli RNA polymerase (365) and polynucleotide phosphorylase (366) by mammalian cyclic AMP-dependent protein kinase has also been reported. In both cases, an increase in enzyme activity was associated with phosphorylation. E. coli RNA polymerase phosphorylation occurred on the sigma factor. Recently, a cyclic AMP-stimulated protein kinase from rat liver nuclei was found to stimulate hepatic RNA synthesis (367). The stimulatory effect was seen primarily with nucleolar RNA polymerase and only when rat liver DNA was used as the template. Jungmann et al. (311) also reported that a cyclic AMP-dependent protein kinase from calf ovary cytosol stimulated the incorporation of ^{32}P from (γ-^{32}P)-ATP into fractions containing nuclear RNA polymerases Ia, Ib and II. Chromatography on DEAE-cellulose indicated a coincidence of phosphorylated proteins with three polymerase activities, although no evidence for phosphorylation of the RNA polymerases themselves was provided. The fraction containing RNA polymerase II activity was the preferred substrate as judged by the significant higher incorporation of ^{32}P. The cyclic AMP-dependent phosphorylation of RNA polymerase preparations was associated with an increase of the RNA-synthesizing capacity of all three enzyme preparations examined. Whereas the activity of the polymerase II was increased 9-fold after phosphorylation, only 2 to [redacted]-fold stimulation of RNA

synthesis was seen by the phosphorylated polymerases Ia and Ib. These results suggest that cyclic AMP and protein kinases may play an important role in RNA synthesis through regulation and perhaps phosphorylation of nuclear RNA polymerases.

E. Protein Modulators of Cyclic Nucleotide-Dependent Protein Kinases

Although it seems clear that activation by cyclic AMP constitutes a major mechanism for regulation of cyclic AMP-dependent protein kinase, information is now available concerning various protein modulators that may also have a potential role in the regulation of the activity of this enzyme. Heat-stable protein from various sources that alter the activity of cyclic nucleotide-dependent protein kinases from several tissues has been reported (368-373,323,326,328). Although the physiological role of these proteins in the regulation of protein kinases is yet to be elucidated, their potential importance in the adenylate cyclase-cyclic AMP system justifies further studies of these proteins.

Crude skeletal muscle extracts have been found to contain a trypsin-labile inhibitor of the phosphorylase kinase activation reactions (274,275). The inhibitor was demonstrated to affect only the component of the activation that required cyclic AMP, later found to be phosphorylase kinase. The inhibitor has also been shown to inhibit the conversion of glycogen synthetase from the I to the D form, a process involving phosphorylation of glycogen synthetase I in the presence of the cyclic nucleotide (276). Subsequent studies on the effects of this protein on the inhibition of glycogen synthetase and the activation of phosphorylase kinase confirmed that phosphorylase kinase and glycogen synthetase kinase were in fact a single enzyme (313,314,296,298). Cyclic AMP-dependent protein kinases from a wide variety of tissues have been shown to be sensitive to inactivation by the rabbit skeletal muscle protein inhibitor (369). Similar inhibitory factors have also been found during the course of purification of the cyclic AMP-dependent enzyme from

brain, E. coli and various other tissues and species of arthropoda (323,326, 328). Moreover, in a survey of the amount of protein inhibitor present in several tissues of the rabbit, highest levels were detected in skeletal muscle, brain, diaphragm and heart (377).

A heat-stable inhibitor of cyclic AMP-dependent protein kinase from rabbit skeletal muscle has recently been characterized by Walsh et al. (369). Although the inhibitor had a molecular weight of 26,000 and a partial specific volume of 0.74, sucrose density studies demonstrated a sedimentation coefficient of 1.5 (369). Kinetic analysis of casein phosphorylation by RC in the presence of the inhibitor showed that the latter interacted noncompetitively with the two enzyme substrates, ATP and casein and with the enzyme activator, cyclic AMP (369). The same phenomenon was observed with respect to these substrates when C was used (377). The degree of inhibition of protein phosphorylation catalyzed by either C or RC in presence of cyclic AMP was identical when casein, histone f_1, histone f_{2a} or histone f_3 was used as the protein substrate, but was hower for protamine (377,378). The inhibitor had no effect on the activity of the cyclic AMP-dependent protein kinase in the absence of the nucleotide.

Elucidation of the mechanism by which the protein inhibitor exerted its effects has shown that it did not inhibit protein phosphorylation by acting as adenosine triphosphatase (377), nor did it function as a phosphoprotein phosphatase by reversal of phosphorylation reaction (369). Whereas the inhibitor has been demonstrated to possess no affinity for cyclic AMP, kinetic studies have shown that it enhanced the binding affinity of RC to cyclic AMP (369,379). It appears to be specific for cyclic AMP-dependent protein kinases, having no effect on phosphorylase kinase, phosphofructokinase, hexokinase, pyruvate kinase and a number of other enzymes examined (376).

It has been demonstrated that addition of C to cyclic (^{3}H)-AMP-receptor complex enhanced the release of cyclic (^{3}H)-AMP, resulting in a decrease in the protein-bound nucleotide (315). Studies on the effect of the inhibitor on cyclic (^{3}H)-AMP release from the protein complex have shown that prior addition of the inhibitor to C prevented this catalytic subunit-stimulated release of cyclic (^{3}H)-AMP from R. Moreover, reconstitution of R and C generated RC with simultaneous inhibition of the activity of the enzyme, a process reversible by cyclic AMP (302). Inhibition of C by the protein inhibitor, however, was not overcome by the cyclic nucleotide (369). Studies on the interaction of the inhibitor with C and RC by Ashby and Walsh (378) suggested that dissociation of the latter enzyme was a necessary prerequisite for inhibitor activity. In the presence of cyclic AMP, interaction of inhibitor with RC mimicked that obtained with C, and a greater amount of inhibitor was required to titrate C and RC together than was needed to titrate the enzymes individually. A mechanism of action of the protein inhibitor involving direct interaction of this protein with C was later proposed by Ashby and Walsh (378). This mechanism agrees well with the effect of the inhibitor on protein phosphorylation catalyzed by C or RC in the presence of cyclic AMP. It also accounts for the increase in the association constant of cyclic AMP to RC without any change in the number of binding sites in the presence of the inhibitor. It has been demonstrated that for a given ratio of inhibitor to catalytic subunit, the inhibitor was less effective at low concentration of the enzyme, implying reversibility of the catalytic subunit-inhibitor interaction (378). Furthermore, the mechanism also conformed with the observation that the inhibitor prevented the catalytic subunit-mediated release of cyclic AMP from the cyclic AMP-receptor complex. Although both the inhibitor and the R demonstrated similar inhibitory activity on C and RC in the presence of cyclic nucleotide, comparison of their properties suggested two nonidentical species (302,321,369,379,380).

Recently, a heat-stable, trypsin-labile protein having stimulatory and inhibitory effects on protein phosphorylation by cyclic AMP-dependent and cyclic GMP-dependent protein kinases has been isolated from lobster tail muscle (370). This modulator had a molecular weight of 34,000 and was found to be a highly charged, acidic protein. It is of interest that this modulator altered the substrate specificity of cyclic AMP- and cyclic GMP-dependent protein kinases. Whereas the protein inhibited phosphorylation of lysine-rich histone and histone mixture catalyzed by both the cyclic AMP-dependent and cyclic GMP-dependent protein kinases, it had opposite effects on the two enzymes when arginine-rich histone was used as the protein substrate. Moreover, the activities of both enzymes in protamine phosphorylation were stimulated in the presence of the modulator (370). While kinetic analysis of the phosphorylation of arginine-rich histone in presence of the modulator revealed an increase in the apparent K_m of both cyclic AMP-dependent and cyclic GMP-dependent protein kinases for the protein substrate, the effect of the modulator on the activity of these enzymes from bovine heart muscle and lobster tail muscle was not associated with any change in the apparent K_m of these enzymes for ATP. These effects of the modulator were also observed when isolated catalytic subunits were studied from the respective holoenzymes (371).

Recently, a heat-stable, trypsin-labile inhibitory factor of cyclic AMP-dependent protein kinase was isolated and partially purified from bovine uterine tissues (372). Whereas subcellular distribution studies of the myometrial inhibitor indicated that this protein was located exclusively in the soluble fraction, a small amount of inhibitory activity was also found to be associated with the 650 x g pellet. Sucrose gradient ultracentrifugation of the uterine inhibitor demonstrated an $S_{20,w}$ of approximately 1.5 while the Stokes radius of this protein, as determined by filtration, was 23Å. By Scatchard plot analysis and in the presence of a concentration of inhibitor

sufficient to completely inhibit the activity of RC, the association constant of the enzyme to cyclic AMP at pH 4.0, 0°C was increased by 2-fold with no significant change in the number of binding sites. This characteristic of the uterine inhibitor protein, however, was not demonstrated at pH 5.0, a condition at which RC exhibited maximal cyclic AMP binding (381). In agreement with the data on the protein inhibitor from rabbit skeletal muscle (369), the uterine protein inhibited both endometrial as well as myometrial cyclic AMP-dependent protein kinases in the presence of cyclic AMP in a dose-dependent manner. Moreover, the uterine protein inhibitor was equally effective in blocking the activity of the catalytic subunit of the enzyme assayed in the absence of cyclic AMP. However, contrary to the finding of the skeletal muscle protein inhibitor by Ashby and Walsh (377), the uterine protein activated histone phosphorylation catalyzed by RC in the absence of cyclic AMP.

More recently, two heat-stable proteins (modulators I and II) which altered the substrate specificity for cyclic AMP-dependent protein kinase from rat mammary glands were partially purified by Majumder (373). Modulator I stimulated specifically the rate of phosphorylation of F_1 and F_{2a} histones catalyzed by cyclic AMP-dependent protein kinase and had no significant effect on that of F_{2b} and F_3 histones. Whereas maximal stimulatory effect was elicited with modulator I when F_1 histone was used as the protein substrate, little stimulation of the enzyme was seen with phosphorylation of total histones. In contrast, modulator II exhibited an inhibitory effect on mammary protein kinase activity when total histones or histone subfractions were used as substrates. It has been postulated that the process of cell differentiation may confer upon the cells tissue-specific distribution pattern of protein kinase modulator, and that these proteins may interact with protein kinases to achieve a high degree of specificity in the phosphorylation of

various sites in the chromosomal proteins during cellular differentiation (373).

Protein kinase inhibitor and modulator activities have now been demonstrated in various animal tissues (369,370,372,373). Although direct evidence for the physiological role of these proteins is still lacking, Walsh and Ashby (382) demonstrated that the tissue concentration of these proteins was sensitive to alterations in the physiological state of the animal as well as in the hormonal environment of the specific tissue. Whereas prolonged starvation decreased the level of the inhibitor in rat heart by 30%, refeeding for 72 hr restored inhibitor activity to a value approximately 40% above normal. It was also reported that while treatment with alloxan also resulted in a decrease in the level of this protein, the effect was antagonized within 1 hr following insulin administration (382).

3. CYCLIC AMP INVOLVEMENT IN SEX STEROID ACTION

A. Criteria for Establishing Cyclic AMP Involvement in Hormone Action

As a result of many elegant studies, Sutherland and Robison (212) provided a set of criteria which must be fulfilled before the role of cyclic AMP as an intracellular mediator in the action of a given hormone or drug can be firmly established. Although some of these have been satisfied for a score of hormones, only a few seem to have achieved all of the criteria suggested. These criteria, most of which are self-evident, can be briefly summarized as follows: first, the effects of the hormone should be reproduced by exogenous cyclic AMP. This is generally considered to be the most difficult criterion to meet since cyclic AMP penetrates cells very poorly, and in some cases, probably not at all. Nevertheless, cyclic AMP has been shown to mimic hormonal actions in a variety of body tissues including the adrenal cortex, liver, testis, prostate and uterus (12,383-386). The acylated derivative such as the $N^6,O^{2'}$-dibutyryl analog of cyclic AMP has been found to be more

potent than the parent compound and therefore, when tissues treated with dibutyryl cyclic AMP show the same response as seen with the hormone, this is taken as strong evidence that the hormone in question works through the cyclic AMP system. Secondly, the inhibitors of phosphodiesterase such as the methyl xanthines should be able to potentiate the effects produced by a sub-maximal dose of the hormone. Indeed, the lipolysis in fat cells caused by ACTH, catecholamines and glucagon (261,387-389), the effects of vasopressin on the toad bladder (390), the cardiac inotropic responses to norepinephrine (391), ovarian steroidogenesis by luteinizing hormone (392), melanocyte dispersion on frog skin by melanocyte stimulating hormone (393) and the effects of isoproterenol on DNA synthesis in parotid glands (394) have all been shown to be potentiated by methyl xanthines. Thirdly, the first messenger (i.e. the hormone) should induce a measurable change in the concentration of tissue cyclic AMP. The endogenous levels of target tissue cyclic AMP should decrease upon hormonal deprivation such as should be the case following castration in male and female rats. Administration of the hormone should restore the drop in tissue cyclic AMP levels observed after castration. Ideally, the changes in cyclic AMP concentration should preceed the metabolic responses that are presumed to be mediated by cyclic AMP. This change in the concentration of cyclic AMP should be tissue- and hormone-specific and correlate temporally and quantitatively with the physiological effects of the hormone. Finally, administration of the hormone should either enhance the activity of tissue adenylate cyclase or inhibit phosphodiesterase or do both. The hormone should also stimulate the conversion of ATP (or adenosine) to cyclic AMP when added to broken cell preparations or slices of the target organ. It is not essential however, that the full metabolic response that occurs in intact cells be evoked in broken cells as this may be dependent upon total cellular integrity. Physiological concentrations of the hormone should be effective

in such preparations and where a series of compounds have similar physiological effects on the intact tissue, their order of potency should correspond to the order of potency on in vitro cyclic AMP synthesis.

B. Estrogenic Action on the Uterus

(i) Estrogenicomimetic effects of cyclic AMP

Estrogens are known to stimulate uterine glycolytic and hexose monophosphate shunt enzymes in immature and ovariectomized animals. The question whether cyclic AMP could also elicit similar enzymatic responses in the uterus was examined (383). Administration of exogenous cyclic AMP to immature or castrate rats resulted in significant increases in uterine weights, glycogen content and in the activities of hexokinase, phosphofructokinase, pyruvate kinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. These responses were further enhanced when cyclic AMP and an inhibitor of phosphodiesterase, theophylline, were injected together. Administration of dibutyryl cyclic AMP and theophylline produced greater increases in uterine weights, glycogen content and various enzyme activities examined (383,395-397). The time-course of the cyclic AMP-induced uterotropic, glycogenic and enzymatic responses was similar to that observed previously for estradiol-17β. After treatment with cyclic AMP and theophylline, uterine weights increased progressively in a time-dependent manner reaching a maximal response (200% of control values) by the 16th hr. Glycogen content of the uterus increased significantly to 159% by the 2nd hr and to a maximum of 558% also by the 16th hr. Similarly, whereas activities of various key glycolytic and hexose monophosphate shunt enzymes were increased significantly at the 2nd hr, maximal increases were observed 16 hr after the administration of the cyclic nucleotide. Increases in various uterine parameters were related to the dose of cyclic AMP and 1 mg of the cyclic nucleotide was sufficient to elevate the activities of most enzymes investigated (383). Uterine glycogen, on the other hand, was increased significantly by as little as 0.2 mg of cyclic AMP.

The possibility of adrenal involvement in any of these uterine responses induced by cyclic AMP was ruled out since similar results were observed in adrenalectomized-ovariectomized animals (383). Moreover, treatment of ovariectomized as well as adrenalectomized-ovariectomized rats with either actinomycin D or cycloheximide blocked the cyclic AMP and theophylline-stimulated induction of uterine hexokinase, phosphofructokinase, pyruvate kinase and the two hexose monophosphate shunt dehydrogenases. These results indicate that like estrogens, the cyclic AMP-induced enzymatic increases in the uterus might be the result of new RNA and protein synthesis (383,397). Two uterine enzymes, isocitrate dehydrogenase and α-glycerophosphate dehydrogenase, which did not respond to estradiol, also failed to display any appreciable response to this cyclic nucleotide, indicating that the uterine response to cyclic AMP was specific and not just due to an overall stimulation of protein synthesis. Also, the observed uterotropic, glycogenic and enzymatic responses were specific to cyclic AMP since neither adenosine nor 2',3'-cyclic AMP, 2'-AMP, 3'-AMP, 5'-AMP, 5'-ADP and 5'-ATP produced any measurable effects. The observed cyclic AMP-stimulated uterine responses also were tissue-specific since lung and thymus, two tissues known to be unresponsive to estrogens, failed to respond to cyclic AMP and theophylline (383).

An early indication that cyclic AMP might be involved in sex steroid-mediated responses came in 1968 when Griffin and Szego (398) reported that cyclic AMP, like estrogens, enhanced the incorporation of labeled amino acids into protein of uterine segments of ovariectomized rats. Uterine accumulation of L-leucine-^{14}C *in vitro* was enhanced as early as 3 min in presence of 5.0 mM cyclic AMP. Lower concentrations of the dibutyryl derivative of cyclic AMP were shown to augment the uptake of trace amounts of both L-leucine-^{14}C and L-lysine-^{14}C during 5 min incubations. Since 5'-AMP affected only modestly the uterine uptake of isotopic L-leucine without exerting any

effect on the accumulation of L-lysine-^{14}C, these authors concluded that cyclic compound is the "more significant metabolic modulator." Evidence that cyclic AMP can stimulate the synthesis of RNA in vivo was presented by Mohla and Prasad (399). These investigators had earlier shown that estrogen activates the synthesis of RNA, DNA and protein in the blastocyst and uterus during delayed implantation and postulated that activation of the delayed blastocyst might be due to the direct action of estrogens (400). It was noted that within 5 min of treatment with an estrogen, there was a marked increase in the incorporation of H^3-uridine into RNA in the blastocyst and the uterus. It was later shown autoradiographically that cyclic AMP could mimic this early action of estrogens in stimulating RNA synthesis in the uterus and in the delayed blastocyst. In the uterus, cyclic AMP caused a 3-fold increase in the percentage of labeled epithelial cells and a 4-fold increase in the number of grains per cell; the grains being located in the nuclei of uterine epithelial cells (399).

Sharma and Talwar (401) studied the action of cyclic AMP in vitro on the uptake and incorporation of uridine into RNA of castrate rat uterus. Cyclic AMP (and dibutyryl cyclic AMP in very low concentrations) was found to be effective in stimulating the uptake by the tissue of uridine-5-H^3 and L-leucine-4,5-H^3 as well as their incorporation into RNA and proteins in uteri of ovariectomized rats. This effect of cyclic AMP was rapid and had some degree of specificity since other nucleotides such as AMP and GMP did not produce any stimulation at least in the first 15 min of incubation. On prolonged exposure however, AMP and GMP also caused effects similar to those of cyclic AMP although the magnitude was considerably smaller. It was also shown that actinomycin D in very low concentrations could inhibit almost completely the synthesis of RNA in the uterus stimulated by cyclic AMP. In contrast, cycloheximide in concentrations higher than those sufficient

to inhibit leucine incorporation by 94% did not block the stimulation of RNA synthesis brought about by cyclic AMP. Hechter et al. (402) also demonstrated that exogenous cyclic AMP could induce estrogen-like stimulation in the biosynthesis of uterine RNA, proteins, lipids and glycogen. These anabolic effects of cyclic AMP were inhibited by actinomycin D, suggesting that the actions of the nucleotide depend, at least in part, upon new RNA synthesis at the gene locus.

(ii) Theophylline-induced potentiation of estrogen action on the uterus

The methyl xanthine, theophylline, is a potent inhibitor of phosphodiesterase. As a result, the cyclic AMP formed by adenylate cyclase in response to hormonal stimulation is not metabolized to 5'-AMP quite as rapidly and therefore tends to accumulate in the treated cells. If cyclic AMP is indeed acting as a mediator between the hormone and the response being measured, treatment with theophylline would be expected to enhance the influence of a small dose of the hormone. Results obtained by Lafreniere and Singhal (403) have demonstrated that theophylline and estradiol both produced significant increases in uterine weights as well as in the activities of the three key glycolytic enzymes. Administration of theophylline increased uterine weights to 150%, hexokinase to 182%, phosphofructokinase to 176% and pyruvate kinase to 155% of the control values. Likewise, estradiol (0.1 µg/rat) produced small yet statistically significant increases in the activities of these uterine enzymes. However, when the same dose of estradiol was injected concurrently with theophylline, the increases observed in the activities of uterine glycolytic enzymes were of a much greater magnitude and were comparable to those observed with the high dose of estradiol (5.0 µg). The results indicate that theophylline is capable of potentiating the stimulatory effects of a submaximal dose of estradiol-17β on the activity of uterine hexokinase, phosphofructokinase and pyruvate kinase.

Since estrogen treatment is known to enhance the activity of uterine glucose 6-phosphate and 6-phosphogluconate dehydrogenase, the question whether theophylline could also potentiate the action of the hormone on the two hexose monophosphate shunt dehydrogenases also was investigated. Whereas theophylline stimulated glucose 6-phosphate and 6-phosphogluconate dehydrogenase to 194% and 191%, 0.10 µg dose of estradiol elevated the two hexose monophosphate shunt enzymes to 169% and 218% respectively, when compared to the values of control rats. Concurrent administration of theophylline potentiated the observed action of estradiol and increased glucose 6-phosphate dehydrogenase to 291% and 6-phosphogluconate dehydrogenase to 409% of controls. The data indicate that when a submaximal dose of estradiol (0.10 µg) is administered along with theophylline, the increases produced in all uterine enzymes studied are of the magnitude similar to that observed with the 5.0 µg dose of the hormone (403). It is of interest that administration of theophylline also was capable of potentiating the effects exerted by a small dose of estradiol-17β on glycogen formation in uteri of immature rats (6).

(iii) Effect of estrogens on uterine cyclic AMP levels and on "in vitro" biotransformation of (^{3}H)-adenosine into (^{3}H)-cyclic AMP

Direct evidence indicating the involvement of adenylate cyclase-cyclic AMP system in the action of estrogenic hormone was first reported by Szego and her colleagues. In 1967, Szego and Davis (404) demonstrated that uterine levels of cyclic AMP were strikingly depleted after ovariectomy. By the third week following ovariectomy, total uterine cyclic AMP was approximately one seventh of the intact control value. It was also demonstrated that 15 sec after an intravenous injection of estradiol-17β to ovariectomized rats, uterine cyclic AMP was almost doubled. This acute elevation of uterine cyclic AMP was maintained for at least 5 min before gradually returning to preinjection levels by the 60th min. A typical S-shaped dose-response curve was obtained

with minimal response being observed with the 0.06 μg and maximal response with 0.5 μg/100 g dose of the hormone. Diethylstilbestrol also had a potent stimulatory effect on the intracellular level of cyclic AMP, whereas the 17α-epimer of estradiol was ineffective. The β-adrenergic blocking agent, propranolol, prevented the estrogen effect on uterine cyclic AMP content in castrate rats (28). In addition, this increase in uterine cyclic AMP was tissue specific since estradiol-17β did not produce any significant change in cyclic AMP levels in the diaphragm and the mesometrial fat pad, two tissues which do not respond to estrogens (404). Szego's group (404) thus linked cyclic AMP to the chain of metabolic events resulting from estrogen administration *in vivo* and demonstrated the ultra-acute onset, organ selectivity, sensitivity to doses of a physiological order and to estrogenic compounds in accordance with their established degree of estrogenicity. These findings were recently confirmed by Dupont-Mairesse *et al.* (23) who demonstrated that whereas uterine cyclic AMP levels in spayed rats were increased by 2-fold 5 min after the injection of 17β-estradiol, pretreatment with propranolol effectively blocked this biochemical response to estrogen. Chew and Rinard (405) also observed small but significant increases in uterine cyclic nucleotide levels 6 and 48 hr after the administration of estradiol-17β, although a more acute response (30 sec and 15 min) was not evident. Contrary to these findings, Sanborn *et al.* (303,381) and Zor *et al.* (406) have recently found that estradiol treatment failed to produce an alteration in cyclic AMP levels of the uterus. Using a suspended uterine preparation *in vitro*, estradiol was found to be ineffective under conditions in which isoproterenol produced a marked increase in the concentration of cyclic AMP (303,381).

An additional approach in studying the regulation of cyclic AMP metabolism in accessory reproductive tissue by gonadal sex steroids was used by Thomas and his colleagues (407,408). The sex accessory glands of mouse,

guinea pig and dog were all capable of biotransforming (^{3}H)-adenosine into cyclic (^{3}H)-AMP. The ability of rat uteri to biotransform (^{3}H)-adenosine to (^{3}H)-cyclic AMP *in vitro* was recently examined by Thomas *et al.* (26) and the effect of pretreatment with propranolol on estradiol-stimulated (^{3}H)-cyclic AMP formation was investigated in uteri of immature rats injected with estradiol, propranolol or both. It is of interest that the capacity of the uterus to synthesize cyclic AMP-H^3 was increased to 49% of the control values in estradiol-treated rats. In contrast, propranolol alone decreased uterine cyclic AMP formation by 30%. When estradiol-injected rats were pretreated with propranolol, the β-blocking agent failed to inhibit the stimulation of cyclic AMP formation seen with the estrogen alone (26).

(iv) Stimulation of uterine adenylate cyclase by estrogenic compounds

The mechanism underlying the elevation of uterine cyclic AMP levels following estrogen treatment appears primarily to be the hormonal stimulation of the adenylate cyclase activity *per se*. Szego (409) using an isotopic precursor method for determining the activity of adenylate cyclase found a significant impairment in its activity following ovariectomy. It was also found that adenylate cyclase activity could be restored by an estrogen. The intravenous injection of estrogen upto 5 min preceding organ removal led to a non-significant increase in adenylate cyclase activity. Statistical significance was precluded due to the 15% to 200% variability of the response to estrogen. However, studies carried out in Singhal's laboratory (6) showed that oophorectomy produced a marked decrease in fluoride-stimulated adenylate cyclase activity of rat uterine homogenates. Enzyme activity fell from 1.99 in normal intact rats to 1.49, 0.90 and 0.69 (picomoles of cyclic AMP formed per milligram protein per hr) after 3, 14 and 21 days of castration, respectively. It was also shown that when ovariectomized rats were injected intraperitoneally with the 10 µg dose of estradiol-17β , uterine adenylate cyclase significantly

increased in 5 min by more than 2-fold of the control values. Moreover, whereas a single intraperitoneal injection of estradiol-17β to immature rats also produced a significant stimulation of uterine adenylate cyclase in 5 min, estradiol-17α , a relatively inactive epimer, when given in the same dose (10 μg/rat) failed to produce appreciable change in the uterine enzyme. The stimulatory action of estradiol-17β on uterine enzyme activity seemed specific to this tissue since hepatic adenylate cyclase remained completely unaffected in animals treated with this potent estrogen (6). Rosenfeld and O'Malley (27) showed that diethylstilbestrol (DES) acutely activated adenylate cyclase in uteri of ovariectomized animals. Intravenous administration of this estrogen (5 μg/100 g) consistently elevated adenylate cyclase activity at 5 min, but by 2 hr after estrogen administration, the activity of the enzyme had returned to control levels. Recently, Sim and Chantharaksri (410) reported that the activity of uterine adenylate cyclase was low at proestrus but increased to peak values at metestrus and then gradually declined until the following proestrus. Firm evidence therefore exists that estrogens are not only capable of elevating endogenous levels of uterine cyclic AMP but can actually stimulate the in vivo activity of uterine adenylate cyclase both in ovariectomized and immature rats (6).

Although numerous studies implicate cyclic AMP in estradiol action on uterine tissue, the question has been raised as to whether cyclic AMP acts directly as a "second messenger" or indirectly subsequent to the release and action of catecholamines. Estrogens have been shown to alter the concentration of epinephrine in the uterus (411) and epinephrine is known to enhance the activity of uterine adenylate cyclase (260). Szego and Davis (28) reported that the rapid elevation of uterine cyclic AMP after estradiol was blocked by a β-adrenergic blocking agent, propranolol. Rosenfeld and O'Malley (27) found that the acute activation of uterine adenylate cyclase by diethylstilbestrol also was propranolol inhibitable. The effect of propranolol on

estradiol-stimulated uterine cyclic AMP-H^3 formation from tritiated adenosine was investigated by Thomas et al. (26). When ovariectomized rats injected with estradiol for 4 hr were pretreated with propranolol, the β-blocking agent failed to inhibit the stimulation of uterine cyclic AMP-H^3 synthesis seen with the estrogen alone. Robison (24,25) also found that an intraperitoneal injection of estradiol-17β in immature rats produced a slow and sustained rise in uterine cyclic AMP levels which could not be prevented by propranolol. A similar lack of effect of propranolol and of dichloroisoproterenol was observed on estrogen-induced uterotropic, glycogenic and various enzymatic responses seen at 4 and 16 hr (6). Moreover, Dupont-Mairesse et al. (23) have recently reported that whereas the early increase in uterine cyclic AMP following estradiol was abolished by propranolol, pretreatment or repeated treatment with propranolol did not modify several sustained responses of the uterus to estradiol (e.g. hypertrophy of luminal epithelium, increases in RNA and protein content) in immature or in spayed rats. Since the β-adrenergic blocking drugs did not interfere with estradiol-induced metabolic responses as well as with the synthesis of uterine cyclic AMP-H^3, it has been suggested that the more sustained biochemical changes in uterine metabolism induced by estrogens may be independent of catecholamine release (6). The data on the ability of exogenous cyclic AMP to imitate effects of estrogens and of a methyl xanthine to potentiate the action of a submaximal dose of the hormone, together with the finding that β-adrenergic antagonists do not prevent the later effects of estradiol on cyclic AMP-H^3 synthesis, lend support to the concept that this nucleotide is involved in estrogenic stimulation of at least some of the uterine anabolic responses (6).

(v) Estrogenic regulation of uterine cyclic AMP-dependent protein kinases

Convincing evidence has accumulated to support the hypothesis (298,306, 328,412) that in many systems, cyclic AMP-dependent protein kinase mediates

the intracellular effects of the cyclic nucleotide. Whereas the regulation of protein kinases is becoming a focal point in the elucidation of the mechanism of action of various hormones, conclusive data concerning the role of this enzyme in estrogenic responses in the uterus are still incomplete. In this context, Reddi et al. (413) failed to observe any change in the activities of uterine cytosol protein kinases in the presence or absence of cyclic AMP over the first 24 hr after the injection of 1 µg of estradiol to immature rats. Ichii et al. (414) however, observed that whereas uteri obtained from adult rats exhibited higher protein kinase activity assayed in the absence of cyclic AMP than that of the immature rats, administration of 5 µg of estradiol to immature animals resulted in a significant decrease in the activity of the cyclic AMP-dependent enzyme without any change in the independent enzyme. While kinase from immature uteri also exhibited higher cyclic AMP binding capacity compared with that of the adult animals, estrogen administration to immature rats caused a significant decrease in the observed biochemical parameter. Arnaud et al. (415) have also reported that a specific estradiol-receptor in the endometrium was phosphorylated in cyclic AMP-dependent manner by a cytosol myometrial kinase, resulting in increased nuclear RNA polymerase activity when the steroid-phosphorylated receptor complex reached the nucleus. Rao and Talwar (416) studied the incorporation of ^{32}P in vitro in total phosphoproteins of the uterine tissue and observed that the labeling of the nucleoproteins increased in ovariectomized animals treated with estradiol in vivo. In addition, it has also been reported by King and Gordon (417) that rat uterine nuclear phosphokinase activity, being distinctly different from that of the cytosol enzyme (i.e. active toward dephosphorylated phosvitin but not exogenous histone as the substrate) was significantly diminished following oophorectomy but markedly increased in spayed animals injected intraperitoneally with an estrogen.

C. Androgenic Action on the Prostate and Seminal Vesicles

Although the relationship between estrogens and cyclic AMP metabolism is more convincing, evidence has emerged during the past 5 years in support of the concept that androgens also may be capable of affecting the adenylate cyclase-cyclic AMP system in the cells of their target organs (11-14,408,418-422). Although no clear-cut relationship between the mechanism of action of androgens and cyclic AMP has yet been established, both adenylate cyclase and cyclic AMP are present not only in male accessory sex glands, but also in testis, sperm and seminal plasma. The seminal secretions of dogs, monkeys and humans have been shown to contain significant amounts (10^{-5}-10^{-4}M) of cyclic AMP (407,423-425). It has been reported that cyclic AMP in ejaculates of humans or dogs is largely of the vesicular origin and is not derived from the prostate (423). Dog prostates however, can effectively biotransform tritiated adenosine into cyclic AMP-H^3 (407). Both adenylate cyclase and cyclic AMP are present in monkey spermatozoa and can be increased by testosterone or dihydrotestosterone in presence of triiodothyronine (424). Cyclic AMP has been shown to be a potent activator of certain enzymes involved in spermatozoal metabolism (426). In analogy to estrogens, a number of recent observations also suggest that cyclic AMP might also play an important role in testosterone-stimulated responses of androgen-dependent tissues. Administration of exogenous cyclic AMP or its dibutyryl analog to castrate or immature rats was found to mimic several anabolic actions of androgens such as increase in seminal vesicular and prostatic weights, glycogen synthesis and glucose metabolism (7,11,103). Whereas Smith (421) described significant increases in mouse prostatic cyclic AMP-H^3 formation *in vitro* by testosterone, studies by Mawhinney *et al.* (407) indicated that prostatic tissue obtained from dogs afflicted with benign prostatic hyperplasia (BPH) biosynthesized considerably less tritiated cyclic nucleotide than did the prostate glands of normal dogs. The work of Golder *et al.* (206) suggested that pituitary hormones may also

influence the interaction of testosterone and cyclic AMP in the prostate gland.

(i) In vivo effects of cyclic AMP on male accessory sex organs

To investigate whether cyclic AMP plays any role in the triggering of biochemical responses induced by androgens, Singhal and coworkers (12) focussed their attention on examining the ability of exogenously administered cyclic AMP to produce testosterone-like stimulation of certain prostatic enzymes involved in glucose metabolism. Animals were castrated bilaterally via the scrotal route and 2 weeks later, injected either with testosterone propionate (5 mg/100 g, i.m.) or cyclic AMP and theophylline (10 mg/rat of each i.p., in 2 equally divided doses) and killed 24 hr later. Administration of cyclic AMP and theophylline produced 83% increase in prostate weight compared to the 47% increase seen with testosterone. Similarly, cyclic AMP treatment resulted in marked enhancement of prostatic hexokinase, phosphofructokinase, pyruvate kinase, glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase which in each case was comparable to the increases seen with the androgen. Cyclic AMP administration was also able to mimic the stimulatory action of testosterone on glycogen synthesis of both the prostate glands and seminal vesicles. When the $N^6,O^{2'}$-dibutyryl analog of cyclic AMP was injected with theophylline, it produced increases in prostate weights as well as various enzyme activities greater than those observed with the parent nucleotide. There was specificity for the action of cyclic AMP on prostatic metabolism since adenosine as well as several related adenine nucleotides (5'-AMP, 2',3'-AMP, 2'-AMP, 3'-AMP, 5'-ADP and 5'-ATP) failed to produce any appreciable change in any of the parameters examined. A certain degree of tissue specificity was also present since two tissues (lung and thymus), which do not respond to androgens, also failed to demonstrate any significant response to cyclic AMP.

Treatment of immature rats with cyclic AMP over a dose range of 2.5-20 mg/rat also resulted in a dose-dependent increase in various prostatic and seminal vesicular enzymes tested. Time-course studies showed detectable increases in organ weights and enzyme activities after 4 hr, which rose to peak levels for hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase after 16 hr, whereas pyruvate kinase and α-glycerophosphate dehydrogenase reached maximal activity after 24 hr. The rise in prostatic and vesicular weights and enzyme activities induced by cyclic AMP or testosterone was prevented by treatment with actinomycin D or cycloheximide (11-14). Adrenalectomized-orchidectomized rats yielded results similar to those obtained for castrate rats indicating that the observed anabolic responses to cyclic AMP are independent of the presence of adrenal glands (12). Interestingly enough, actinomycin D was also shown to interfere with the induction of rat liver serine dehydratase observed after the administration of the dibutyryl analog of cyclic AMP (358). Indeed, it is now known that cyclic AMP can induce the synthesis of tyrosine amino transferase (427,428) and phosphoenolpyruvate carboxykinase (428,429) in hepatic tissue which can also be blocked by actinomycin D. Although the mechanism by which cyclic AMP induces hepatic enzyme synthesis has not yet been fully clarified, recent work suggests that cyclic AMP may do so, at least in part, by an indirect effect upon transcription by way of phosphorylation of nuclear histones and perhaps by controlling a post-transcriptional process (309,351).

Although exogenous cyclic AMP was capable of increasing accessory reproductive organ weights similar to that seen with androgen treatment (12), Mangan et al. (246) observed no change in the height and structure of the secretory epithelium of the prostate taken from castrate rats treated with cyclic AMP. However, they were able to confirm the similarity of the effect of cyclic AMP and testosterone on the activity of glucose 6-phosphate dehydrogenase in castrate rats. They also showed that the activity of

prostatic glyceraldehyde 3-phosphate dehydrogenase was enhanced equally by the injection of either cyclic AMP or testosterone. Their finding that these enzymatic changes after cyclic AMP can be blocked by cycloheximide and actinomycin D were also in complete accord with the earlier data of Singhal and coworkers (12). Recent studies by Brandes (48) nevertheless have shown that cyclic AMP can also mimic the action of testosterone in the restitution or stimulation of the structural components of the seminal vesicular cells. Administration of dibutyryl cyclic AMP or testosterone produced similar stimulatory effects on the epithelium of the seminal vesicles of immature animals. While the rough endoplasmic reticulum was stimulated and the cisternae became more numerous and dilated following the nucleotide administration, the lumen of the vesicular gland also appeared to be dilated and filled with secretory material (48). Mangan et al. (246) also showed that castration produced marked decreases in the activity of RNA polymerase, ornithine decarboxylase and S-adenosylmethionine decarboxylase in the rat prostate which were reversed by testosterone but not by cyclic AMP. These observations led Mangan et al. (246) to conclude that cyclic AMP probably does not mimic all the known effects of testosterone on prostatic metabolism. Whether or not the prostatic responses to cyclic AMP are limited only to carbohydrate-metabolizing enzymes is, of course, a subject of additional experimentation. However, it is interesting to note that in other steroid-sensitive organs, such as the uterus, cyclic AMP has been shown to be as effective as estradiol not only in stimulating carbohydrate-metabolizing enzymes, but also in inducing RNA, protein and phospholipid synthesis (401,402,416).

(ii) Potentiation of androgen action by methyl xanthines

The ability of a methyl xanthine to potentiate the action of a submaximal dose of testosterone on prostatic and vesicular carbohydrate metabolism was investigated by Singhal and coworkers (12). It was demonstrated that whereas

theophylline (10 mg/100 g) and testosterone (1 mg/100·g) produced small increases in both prostatic and vesicular weights as well as the activities of hexokinase, phosphofructokinase, pyruvate kinase, glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and α-glycerophosphate dehydrogenase when administered separately, increases of a much greater magnitude were observed when the hormone was given in conjunction with the methyl xanthine. Moreover, it was shown that while treatment of orchidectomized rats with theophylline or a small dose of testosterone propionate (1 mg/100 g) increased prostatic glycogen levels by 27 and 45% of control values respectively, glycogen content in the prostate was elevated over 200% of the control values when the low dose of the hormone was given together with theophylline (7,12). The ability of theophylline to mimic and to potentiate the influence of submaximal doses of androgens on accessory sex organ responses lends supportive evidence to the concept that cyclic AMP is involved in the sequence of events which result following the stimulation of the target tissue by the steroid hormone. Christiansen and Clemens (430) recently reported that the effects of the doses of testosterone that were ineffective in maintaining or inducing male sexual behaviour in laboratory animals, were potentiated by concurrent administration of theophylline and concluded that cyclic AMP may play a mediating role in the regulation of masculine sexual behaviour by androgens. These data are in accord with previous findings showing that methyl xanthines potentiate responses in other tissues which are now known to involve mediation by cyclic AMP (387,388,390,391,394).

(iii) Effects of androgens on cyclic AMP levels and on "in vitro" bioconversion of (^{3}H)-adenosine into (^{3}H)-Cyclic AMP

Among the various criteria which must be fulfilled before accepting the role of cyclic AMP in the action of a given hormone, it is important to show that the hormone can induce a detectable change in the concentration of target

tissue cyclic AMP. Also, the endogenous levels of the cyclic nucleotide should decrease upon hormonal deprivation as should be the case after castration. Studies by Singhal and coworkers (6,12) indicated that 14 days after castration, total cyclic AMP levels decreased to 24% in the prostate and 9% in seminal vesicles when compared with the values of normal rats of corresponding age and body weight. It was also found that a single intramuscular injection of testosterone proprionate (5 mg/100 g) partially reversed the effects of orchidectomy, as cyclic AMP concentration increased at 8 and 24 hr to 140% and 145% in the prostate and to 143% and 210% respectively, of controls in seminal vesicles (12,14). These results indicate that whereas castration resulted in a marked drop in the endogenous levels of target tissue cyclic AMP, testosterone treatment of castrate rats produced statistically significant increases in cyclic AMP concentration of the prostate gland and seminal vesicles (11,12,14). Moreover, preliminary findings (9) also demonstrated that following an intravenous injection of dihydrotestosterone (5 mg/100 g) to 120 hr castrate rats, the endogenous levels of prostatic cyclic AMP increased by 27% over castrate controls as early as 1 hr. However, a complete time-course study over the first 60 min after an intravenous injection of the hormone would therefore seem to be necessary in order to know whether a more rapid response similar to that noted by Szego and Davis (404) for the uterus can be obtained in prostate glands and seminal vesicles of castrate animals.

The sex accessory glands of mouse, guinea-pig and dog all were capable of biotransforming adenosine-H^3 into cyclic AMP-H^3 (407,408,420,422,431). The mouse prostate gland and seminal vesicles exhibited greater ability to synthesize cyclic AMP-H^3 from adenosine than the accessory organs from the dog or the guinea-pig. Castration was found to produce a marked decrease in the formation of cyclic AMP-H^3 by mouse anterior prostate and seminal vesicles. The tissue levels of cyclic AMP-H^3 in 24 hr castrate mice were

lowered by 30% whereas the amount detected in the incubation medium decreased by 85% when compared to normal controls. A similar decrease in the formation of cyclic AMP-H^3 was seen in the ventral prostate and seminal vesicles of castrate rats. In vitro addition of an androgen resulted in significant increases in the levels of cyclic AMP-H^3 formed from adenosine-H^3 in prostate glands and seminal vesicles as well as in the incubation medium. While cyclic AMP-H^3 levels in the anterior prostate were significantly enhanced by dihydrotestosterone (10^{-10}M - 10^{-8}M), the response of this tissue to testosterone was not as pronounced. The levels of cyclic AMP-H^3 in the incubation medium were elevated at each testosterone concentration although the tissue levels were increased only by 10^{-9}M testosterone. On the contrary, although the seminal vesicle response to dihydrotestosterone was significant between 10^{-10} to 10^{-8}M, this hormone did not produce the marked increase in cyclic AMP-H^3 levels in the incubation medium which was seen with testosterone. It is of interest that the guinea-pig seminal vesicles also responded to in vitro testosterone by an elevation in the levels of cyclic AMP-H^3 synthesized from adenosine-H^3. Studies on guinea-pig seminal vesicle preparations further indicated that epithelium was the most probable source of cyclic AMP which appears in sex accessory gland secretions. The epithelium was shown to be capable of converting more adenosine-H^3 into cyclic AMP-H^3 than the muscle layer (421).

Regardless of the sex accessory organ used, testosterone caused a greater degree of extrusion of cyclic AMP-H^3 into the incubation medium than that seen with dihydrotestosterone. On the other hand, dihydrotestosterone proved to be more effective in elevating tissue levels of cyclic AMP-H^3 in both prostate glands and seminal vesicles. The important question as to whether cyclic AMP-H^3 actively extrudes into the incubation medium or simply leaks out of the tissue thereby escaping the action of phosphodiesterase, has not yet been resolved. Both avian erythrocytes and E. coli appear to be able to pump cyclic AMP out against a concentration gradient (219,432). Since seminal

plasma contains cyclic AMP (425), it is quite possible that prostatic secretions could derive their cyclic AMP through such a pump-like mechanism. In experiments using the seminal vesicle sac preparation, cyclic AMP-H^3 formed in seminal vesicles appeared to be accumulated on the mucosal side of the sac (422). Androgens are known to stimulate citric acid synthesis and like cyclic AMP, this compound is also extruded into the incubation medium by ventral prostate (130). Both citric acid and cyclic AMP are found not only within the sex accessory organs but in seminal plasma as well. The effects of castration and/or androgen stimulation on the levels of cyclic AMP-H^3 in seminal plasma have not been examined just as the physiological role of cyclic AMP-adenylate cyclase system in accessory gland secretions has not yet been established. Nonetheless, results obtained by Smith (421) and Thomas and Singhal (419) demonstrate that both prostate glands and seminal vesicles of several species are capable of synthesizing significant amounts of cyclic AMP-H^3 from adenosine-H^3 *in vitro*. Furthermore, several factors including castration and addition of a small amount of an androgen can significantly affect the accessory sex gland's ability to synthesize cyclic AMP-H^3. It is of interest that prostatic tissue obtained from dogs afflicted with benign prostatic hyperplasia formed considerably less cyclic AMP-H^3 from adenosine than prostate glands of normal dogs. Although the precise pathological significance of the observed reduction in the ability of hyperplastic prostate to synthesize normal amounts of cyclic AMP is currently unknown, the effect seemed specific since several other biochemical parameters remained completely unaltered in this organ (407,431).

(iv) Influence of androgens on adenylate cyclase and protein kinases in male accessory sex glands

If androgens indeed exert their metabolic effects on accessory sex organs with cyclic AMP as an intracellular mediator, there is clearly a need to establish that the activity of adenylate cyclase in these steroid-

dependent cells does respond to androgenic deprivation and repletion. Even though there is a relationship between androgens and the cyclic AMP-adenylate cyclase system of male reproductive organs, there still exists some disparity with regard to the direct action of androgens on adenylate cyclase activity of the male sex-steroid dependent tissues. Rosenfeld and O'Malley (27) were unable to demonstrate any *in vivo* or *in vitro* stimulation of adenylate cyclase in the ventral prostate by testosterone. Studies by Liao *et al.* (245) on nuclear adenylate cyclase revealed a 10-20% (in 4 out of 12 experiments) non-significant increase in enzyme activity following treatment with dihydrotestosterone. Hechter and Soifer (58) once raised the possibility that distinctive adenylate cyclase is present in the nucleus, and that steroid hormones might influence the activity of this nuclear cyclase. Cyclic AMP so generated and utilized locally in the nucleus may influence gene transcription by an alternate mechanism which may not involve the cyclic AMP-dependent histone phosphorylation in the nucleus. Indeed, Liao *et al.* (245) and Hechter and Soifer (58) were able to demonstrate the existence of adenylate cyclase in isolated nuclei from both the ventral prostate and liver. More recently, Mangan *et al.* (246) were able to show that prostatic nuclear adenylate cyclase was markedly decreased (68%) 72 hr after castration and was restored within 1 hr by testosterone administration.

With regard to the hormonal control of protein kinases from male accessory sexual tissues, little information is available concerning the regulation of their activities by androgenic hormones, particularly with reference to mediation through cyclic AMP. Reddi *et al.* (413) were able to demonstrate the phosphorylation of certain histones of the rat ventral prostate in response to cyclic AMP although neither androgenic deprivation nor stimulation exerted any significant influence on the rates of these protein phosphokinase reactions. However, Ichii *et al.* (414) recently demonstrated

that whereas castration caused significant elevation in the activity of both prostatic cyclic AMP-dependent and-independent protein kinase, administration of testosterone to androgen-deprived rats was capable of reversing these effects. Ahmed and his coworkers (99) demonstrated that purified nuclei from rat ventral prostate were able to incorporate ^{32}P into their phosphoproteins in the presence of (γ-^{32}P)-ATP. The non-histone phosphoproteins were about 4 times more active than the histone phosphoproteins. The ability of the nuclei to incorporate ^{32}P from (γ-^{32}P)-ATP *in vitro* was greatly reduced as a result of androgen deprival of the animals by orchidectomy; this was reversed by *in vivo* androgen replacement. These changes were target tissue specific, as no such changes were observed in the metabolism of nuclear phosphoproteins of liver from animals of varying androgenic status. However, addition of cyclic AMP, testosterone and 5α-dihydrotestosterone *in vitro* did not increase the phosphorylation of nuclear proteins under their experimental conditions (4,99). Moreover, Wilson and Ahmed (433) were able to demonstrate the presence of distinct androgen-sensitive protein kinases from nuclei of rat ventral prostate. The activity of the nucleolar protein kinase, being different from that of the extranucleolar enzyme, was optimal at pH 6.8 but decreased rapidly with increased pH. The extranucleolar protein kinase had a broad pH curve with optimal activity between pH 7.0-7.7. Although both the nucleolar and chromatin enzymes phosphorylated histones, chromatin kinase activity assayed with lysine-rich and arginine-rich histones was 3% and 0.3%, respectively of that in presence of phosvitin. Furthermore, this chromatin-associated protein kinase was sensitive to the androgen status of the animal. Whereas orchidectomy resulted in a rapid decline in the enzyme activity (approximately 50% within 24 hr and over 80% within 48 hr), testosterone replacement *in vivo* prevented this androgen-induced enzymatic alteration (434).

III. MATERIALS AND METHODS

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1. ANIMAL PREPARATION

Adult male rats (325-350 g) of the Sprague-Dawley strain, housed in pairs and maintained on Master Laboratory Chow and water ad libitum were used in the present study. The animals were obtained from Canadian Breeding Farm and Laboratories Limited, Montreal, Quebec and maintained under constant environmental conditions (24°C temperature, 60% relative humidity and regular alternate cycles of 12 hr light and darkness). Bilateral orchidectomy was performed via the trans-scrotal route under pentobarbital anaesthesia as described previously (133). Sham-operated animals were used as controls. Rats were sacrificed by cervical dislocation, the ventral prostate glands rapidly excised, freed of extraneous tissue, weighed and immediately frozen in liquid nitrogen. The prostatic tissue was stored at -40° and enzymatic assays were performed within 1 week of sacrificing the animals. For the partial purification and biochemical characterization of canine prostatic cyclic AMP-dependent protein kinase, dogs (15-20 kg) obtained from the Ottawa Humane Society were used and sacrificed with an overdose of sodium pentobarbital.

2. PREPARATION OF SAMPLES FOR ASSAYING ADENYLATE CYCLASE AND CYCLIC AMP PHOSPHODIESTERASE

Prostatic tissue was finely minced with scissors and a 10% (w/v) homogenate prepared in a homogenizing buffer containing 20 mM NaCl, 1 mM $MgSO_4$ and 2 mM glycyl glycine (pH 7.6). Homogenization (15 strokes in 60 sec) was carried out at 0° with a Potter-Elvehjem homogenizer (Fisher Scientific Co., Fairlawn, N.J.) fitted with a plastic Teflon pestle spinning at about 700 rpm. An appropriate aliquot of the homogenate was then used for the assay of adenylate cyclase and cyclic AMP phosphodiesterase activity.

A. Measurement of Adenylate Cyclase Activity

Adenylate cyclase activity was measured by using a slight modification of the method described by Sutherland et al. (220). The assay was based on

the biotransformation of adenosine triphosphate into cyclic AMP which was determined by an established protein-binding method. In order to prevent the metabolism of cyclic AMP thus formed, a potent inhibitor of phosphodiesterase, theophylline, was also added to the incubation mixture. 0.2 ml of 10% homogenate was added to 0.8 ml of assay medium in order that the final concentration of each reactant was as follows: Tris, 50 mM (pH 7.5); $MgCl_2$, 5 mM; ATP, 4 mM; theophylline, 20 mM; phosphoenolpyruvate, 10 mM; pyruvate kinase, 2.0 µg protein. Incubation was carried out at 37° in a Dubnoff metabolic shaker for 15 min. The reaction was terminated by heating the sample for 5 min in a boiling water bath. The boiled mixture was then diluted with double distilled water to a volume of 5 ml and centrifuged at 3,000 x g for 20 min. A 20 µl aliquot of the supernatant was used for assaying cyclic 3',5'-AMP content by the protein binding method of Gilman (379), as described later in part 4 of this section. Adenylate cyclase activity was calculated as picomoles of cyclic AMP formed per min per mg of tissue or per prostate.

B. Assay of Cyclic AMP Phosphodiesterase Activity

The activity of cyclic AMP phosphodiesterase was assayed according to the method of Weiss (435) based on the conversion of cyclic AMP to 5'-AMP which was then metabolized to inorganic phosphate by the action of alkaline phosphatase. Fifty microliters of 10% homogenate was added to 0.95 ml of assay medium in order that the final concentration of each reactant was as follows: 50 mM Tris buffer (pH 8.0); 1 mM cyclic AMP; 3 mM $MgSO_4$; 0.1 mg of protein of alkaline phosphatase. Incubation was carried out at 37° for 10 min and the reaction was stopped by the addition of 0.2 ml of 55% trichloroacetic acid. The precipitate was removed by centrifugation (3,000 x g for 20 min) and inorganic phosphate was measured in the supernatant according to the method of Fiske and Subbarow (436) using a Klett-Summerson Photoelectric Colorimeter (Klett Manufacturing Co., N.Y.). The activity of cyclic AMP

phosphodiesterase was expressed as micromoles of cyclic AMP metabolized per hr per milligram of tissue or per prostate.

3. PREPARATION OF SAMPLES FOR ASSAYING CYCLIC AMP-DEPENDENT AND-INDEPENDENT PROTEIN KINASES AND CYCLIC AMP BINDING CAPACITY

5% homogenates of rat ventral prostate were prepared in a homogenizing buffer containing 0.05 M Tris-HCl, pH 7.4, 0.25 M sucrose, 0.05 M KCl and 5 mM $MgCl_2$. Homogenization was carried out at 0^o with a Potter-Elvehjem homogenizer (Fisher Scientific Co., Fairlawn, N.J.) fitted with a plastic Teflon pestle spinning at about 700 rpm (15 strokes in 60 sec). The homogenate was then filtered through 3 layers of cheese cloth and centrifuged at 650 x g at 0^o for 20 min in a Sorvall RC2B refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The supernatant was centrifuged at 100,000 x g at 0^o for 1 hr in an International Preparative model B-60 Ultracentrifuge (International Equipment Co., Needham Heights, Mass.). Fifty microliters of cytosol (100,000 x g supernatant) was used for assaying protein kinase activity and cyclic AMP-binding capacity. The 650 x g pellet was washed 3 times with the homogenizing buffer and later resuspended in the same buffer containing 0.1% Triton X-100. Fifty microliter aliquots of the suspension were used to assay particulate protein kinase activity and cyclic AMP binding capacity. Protein was determined by the method of Lowry et al. (437) using bovine serum albumin as the standard.

A. Measurement of Cyclic AMP-Dependent and-Independent Protein Kinase Activities

Soluble protein kinase activity (100,000 x g supernatant) was determined by measuring the incorporation of ^{32}P into histone mixture (Type IIA, Sigma) by a slight modification of the method of Sanborn et al. (303) as described by Tsang and Singhal (438). The assay medium (final volume of 0.13 ml, pH 6.0) contained 46 mM sodium acetate, 1.5 mM NaF, 3.1 mM theophylline, 0.24 mg histone, 17 mM $MgCl_2$, 85 µM ATP, 5 µM cyclic AMP (when measuring the activity of cyclic AMP-dependent enzyme) and 50 µl of cytosolic extract (approximately

150 µg protein). The reaction was initiated by addition of (γ-^{32}P)-ATP-$MgCl_2$ (2×10^5 cpm) followed by incubation at 30° for 7 min and terminated by the addition of 5 ml of 20% trichloroacetic acid. The protein precipitate was then filtered through 25 mM cellulose ester Millipore filters (HAWP, 0.45 µM, Millipore Ltd., Mississauga, Ont.), washed thrice with 5% TCA and counted for radioactivity. The filter was counted using a scintillation cocktail containing 7 parts Omnifluor solution (4 g Omnifluor per liter of scintanalyzed toluene) to 3 parts ethylene glycol monoethyl ether. Appropriate blanks without histone and enzyme preparation were run and subtracted from the experimental values. Protein kinase activity was expressed in nanomoles ^{32}P incorporated into histone mixture per 7 min per mg protein or per prostate. Kinase activity of the crude nuclear preparation (650 x g pellet, washed 3 times and resuspended in 0.1% Triton X-100) was assayed by measuring the incorporation of ^{32}P from (γ^{32}P)-ATP into endogenous nuclear substrates as described for the soluble enzyme and also expressed as nmoles ^{32}P incorporated per 7 min per mg protein or per prostate. The kinase activity ratio was defined as the ratio of protein kinase activity assayed in absence of cyclic AMP to that in the presence of the nucleotide.

B. Estimation of Cyclic AMP Binding Capacity

The *in vitro* (^{3}H)-cyclic AMP binding studies were performed with slight modification of the method of Sanborn *et al.* (303) as reported previously by Tsang and Singhal (439). Fifty microliters of the cytosolic or crude nuclear preparation was added to a mixture (final volume 0.3 ml, pH 5.0) containing 0.05 M sodium acetate, 1 mM EDTA, 10 mM theophylline and 1.5×10^{-7} M (^{3}H)-cyclic AMP (saturating concentration). The reaction mixture was then incubated at 0° for 3 hr and passed through 25 mM cellulose ester Millipore filters (HAWP, 0.45 µM, Millipore Ltd., Mississauga, Ont.) to separate the bound from the free (^{3}H)-cyclic AMP. The filters were dried at 90° and dissolved in

scintillation cocktail containing 7 parts Omnifluor solution (4 g of Omnifluor per liter of scintillation grade toluene) to 3 parts of ethylene glycol monoethyl ether for radioactivity counting. Cyclic AMP binding capacity was expressed in pmoles (^{3}H)-cyclic AMP bound per mg protein or per prostate.

4. PREPARATION OF TISSUE SAMPLES, PROTEIN KINASE INHIBITOR AND PROTEIN KINASE FOR ASSAYING ENDOGENOUS LEVELS OF CYCLIC AMP

Approximately 100 mg of prostatic tissue was homogenized in 3 ml of ice cold 5% (w/v) trichloroacetic acid with a Potter-Elvehjem homogenizer (Fisher Scientific Co., Fairlawn, N.J.), fitted with a plastic Teflon pestle spinning at 700 rpm (15 strokes in 60 sec). The homogenate was then centrifuged for 15 min at 1,000 x g in a clinical centrifuge (International Equipment Co., Needham Heights, Mass.). 0.2 ml of 1 N HCl was then added to 2 ml of the supernatant and the resulting solution was extracted 5 times with 3 volumes of anhydrous ethyl ether in order to remove trichloroacetic acid. The aqueous phase was then lyophilized with a freeze-drying lyophilizer (Virtis Co., Gardiner, N.Y.), and resuspended in 2 ml of distilled water. Fifty microliters of this solution was used for assaying cyclic AMP concentration by the protein-binding method of Gilman (379). To monitor recovery of cyclic AMP, a known quantity of (^{3}H)-cyclic AMP was homogenized in TCA with a prostatic sample. The supernatant obtained was processed as mentioned above. A 50 µl aliquot of the final solution was dried on Millipore filters and counted, using the scintillation cocktail used for the assay of cyclic AMP.

A. Preparation of Protein Kinase Inhibitor

Protein kinase inhibitor was prepared according to the method of Appleman et al. (376). Fresh bovine skeletal muscle obtained from a near-by slaughter house was minced and homogenized in 3 volumes of 10 mM Tris buffer (pH 7.5). Homogenization was carried out at maximum speed in a Sorvall Omni-mixer Homogenizer (Ivan Sorvall Inc., Norwalk, Conn.) for 3 one min periods at 0°.

The homogenate was then boiled for 10 min, filtered on 3 layers of cheese cloth and centrifuged at 10,000 x g for 15 min in a Sorvall RC2B refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). After removal of the particulate material, the activity in the supernatant was precipitated with 1/9 volume of 50% trichloroacetic acid. The precipitate was collected at 15,000 x g (International preparative model B-60 ultracentrifuge, International Equipment Co., Needham Heights, Mass.), dissolved in distilled water (1/10 volume of homogenizing buffer) and the pH adjusted to 7.0 with 1 N NaOH. The resulting solution was dialyzed against distilled water at room temperature for 16 hr. The suspension was then spun for 30 min at 15,000 x g in an International preparative model B-60 ultracentrifuge and the remaining supernatant diluted with distilled water to attain a final concentration of 3 mg protein per ml.

B. Preparation of Cyclic AMP Binding Protein

To prepare the binding protein for cyclic AMP (cyclic AMP-dependent protein kinase), the method of Miyamoto et al. (323) was adopted. Fresh bovine muscle was minced and homogenized in 3 volumes of 4 mM EDTA (pH 7.0). Homogenization was carried out at 0° in a Sorvall Omni-mixer Homogenizer (Ivan Sorvall Inc., Norwalk, Conn.) for 3 one-min periods. The homogenate was centrifuged for 30 min at 27,000 x g at 0° in a refrigerated International preparative model B-60 ultracentrifuge (International Equipment Co., Needham Heights, Mass.). The supernatant solution was adjusted to pH 4.8 by adding 1 M acetic acid with constant stirring. After 15 min, the precipitate was removed by centrifugation at 27,000 x g for 30 min in an International Preparative model B-60 ultracentrifuge. The pH of the clear supernatant was then readjusted to 6.5 with 1 M potassium phosphate buffer (pH 7.2). Protein kinase activity was then precipitated with solid ammonium sulfate (32.5 g/100 ml). After stirring for 30 min, the precipitate was collected by centrifuging at 27,000 x g for 20 min and dissolved in 5 mM potassium phosphate buffer containing 2 mM

EDTA (pH 7.0). All potassium phosphate buffers subsequently used contained 2 mM EDTA. The resulting solution was dialyzed overnight at 0° and its 27,000 x g supernatant was applied to a column of DEAE-cellulose (Whatman DE11) 32 x 2.6 cm) previously equilibrated with a buffer containing 5 mM potassium phosphate and 2 mM EDTA, pH 7.0. Following the elution with 100 mM potassium phosphate (pH 7.0), the cyclic AMP binding protein was eluted off the column with 300 mM potassium phosphate (pH 7.0). The active fractions were pooled and dialyzed overnight at 0° against 3 x 20 volumes of 5 mM potassium phosphate buffer (pH 7.0) and used for cyclic AMP determination.

C. Cyclic AMP Determination

Cyclic AMP levels were measured according to the protein-binding method of Gilman (379). Cyclic AMP was allowed to compete with a saturating amount of tritiated cyclic AMP for binding sites on a cyclic AMP-dependent protein kinase in the presence of a protein kinase inhibitor. The following components at the designated final concentrations, were added to the reaction mixture (final volume, 110 µl) in the given sequence: 50 mM Na acetate (pH 4.0); 30 µg of protein kinase inhibitor preparation; 2 picomoles of tritiated cyclic AMP; cyclic AMP standards (1-10 pmoles) or 50 µl of extracts from unknown sample. The reaction was initiated by the addition of binding protein (sufficient to bind 30% of the added (^{3}H)-cyclic AMP and allowed to proceed for 90 min at 0°. At equilibrium, the mixture was diluted to approximately 1.0 ml with cold 20 mM potassium phosphate, pH 6.0, and passed through a 25 mM cellulose ester Millipore filter (HAWP 0.45 µm; Millipore Ltd., Mississauga, Ont.) to separate the free from the bound cyclic AMP. The filter was immediately washed with 10 ml of the same buffer, dried at 90° and dissolved in a scintillation cocktail containing 7 parts Omnifluor solution (4 g of Omnifluor per liter of scintillation grade toluene) to 3 parts of ethylene glycol monoethyl ether for radioactivity counting. Counts bound to the filter

were independent of both the filtration speed and the volume of rinse (5 to 20 ml). In the absence of binding protein, blank values of approximately 30 cpm were detected on the filter. The concentration of cyclic AMP in the unknown sample was calculated from the binding of the set of standards and expressed as picomoles of cyclic AMP per mg wet weight of tissue or per prostate.

5. ISOLATION AND PARTIAL PURIFICATION OF A CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM CANINE PROSTATE

Cyclic AMP-dependent protein kinase was partially purified by modification of reported procedures (302,303,440). A dog (15-20 kg body weight) was killed by an intravenous injection of sodium pentobarbital and the prostate gland was quickly removed and trimmed of all extraneous tissue. The gland was then homogenized in 3 volumes of buffer (0.05 M Tris-HCl, pH 7.4 - 0.25 M sucrose - 0.05 M KCl - 5 mM $MgCl_2$) and the homogenate was centrifuged at 650 x g for 30 min. The supernatant was decanted and centrifuged at 130,000 x g for 90 min. The supernatant containing the soluble cyclic AMP-dependent protein kinase was then subjected to precipitation at pH 5.5, and the resulting supernatant was readjusted to pH 7.4 and subjected to $(NH_4)_2SO_4$ precipitation (0.32 g/ml). The protein precipitate was then dissolved in a volume of buffer (0.01 M Tris-HCl, 1 mM 2-mercaptoethanol, pH 7.4), equivalent to approximately 1/5 of that used for homogenization of the tissue, and dialyzed overnight against 3 changes (6 litres each) of the same buffer. The solution was then centrifuged at 10,000 x g for 10 min to remove any precipitate and was applied to a DEAE-cellulose column previously equilibrated with the same buffer just before loading. The column, after it has been washed with the same buffer again (10 x bed volume of column) was subsequently eluted with a linear gradient of 0.05 - 0.35 M NaCl containing 0.01 M Tris-HCl, 1 mM 2-mercaptoethanol, pH 7.4. Fractions comprising both peaks containing cyclic AMP

binding and cyclic AMP-dependent kinase activities were pooled, dialyzed against the column buffer without NaCl and stored in small portions in liquid nitrogen.

6. CHEMICALS

All reagents were of the purest grade available and were dissolved in glass distilled water unless stated otherwise. Histone (Type IIA), α-casein, protamine, pyruvate kinase, phosphoenolpyruvate, theophylline, actinomycin D, DEAE-cellulose, DL-isoproterenol, testosterone, 5α-dihydrotestosterone, estradiol-17β and all unlabelled nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.). While cycloheximide and methyl prednisolone sodium succinate were products of Upjohn Co. of Canada (Don Mills, Ontario), propranolol was obtained from Ayerst Laboratories (Montreal, Quebec). Cyproterone acetate and phentolamine (Regitime Mesylate) were gifts from Schering AG, Berlin and CIBA Pharmaceuticals respectively, whereas progesterone was purchased from Nutritional Biochemicals Co. (Cleveland, Ohio). (^{3}H)-Cyclic AMP (28 Ci/mmole) was obtained from Schwarz-Mann (Orangeburg, New York) and (γ-^{32}P)-ATP (25.5 Ci/mmole) was purchased from New England Nuclear (Dorval, Quebec). All histone subfractions were products of Worthington Biochemical Corp. (Freehold, New Jersey).

7. STATISTICAL ANALYSIS

Results were expressed as means ± standard error of the mean (S.E.M.) in experiments where more than three values were measured for a single point. The significance of difference between the mean values of various experimental groups was calculated by using the Student's t distribution (441). No statistical significance was indicated when the p value was >0.05.

IV. RESULTS

IV. RESULTS

1. ANDROGENIC REGULATION OF PROSTATIC ADENYLATE CYCLASE AND PHOSPHODIESTERASE ACTIVITIES OF THE RAT PROSTATE GLAND

A. Effects of Castration on Prostatic Adenylate Cyclase Activity

The question whether androgen deprivation following orchidectomy produces any effect on the activity of prostatic adenylate cyclase was investigated. The data presented in Table 1 show that adenylate cyclase activity in the rat ventral prostate decreased progressively after orchidectomy. Although the enzyme activity, when expressed per mg tissue, was only 37% of the controls 144 hr after castration, a more pronounced change was observed when calculated on total organ weight basis.

B. Effects of Castration and Testosterone Replacement on Prostatic Adenylate Cyclase

Table 2 depicts the influence of testosterone administration on prostatic adenylate cyclase activity in 120 hr castrate rats. While activity of the enzyme was markedly decreased from 4.41±0.40 to 2.03±0.20 pmoles cyclic AMP formed per min per mg of tissue, a single intramuscular dose of testosterone (5 mg/100 g) effectively stimulated prostatic adenylate cyclase activity in 4 hr. Values seen in the testosterone-treated group were statistically non-significantly different from those of the normal, intact controls. Similar findings were also attained when the activity of the enzyme was expressed on total organ weight basis (Table 2).

C. Influence of Intravenously Administered Testosterone on Prostatic Adenylate Cyclase

In order to establish the earliest possible time when an increase in adenylate cyclase activity could be measured under *in vivo* conditions, the effects of intravenously administered testosterone were examined. Figure 2 shows the time-course response following a single injection of free testosterone (5 mg/100 g body weight) given via the external jugular vein to 120 hr castrate rats.

TABLE 1

EFFECTS OF CASTRATION ON PROSTATIC ADENYLATE CYCLASE ACTIVITY

Values represent the means ± S.E.M. of 6 animals in each group. Animals were castrated under light pentobarbital anesthesia and sacrificed after 96, 120 and 144 hr. Adenylate cyclase activity was calculated as pmoles of cyclic AMP formed per min and expressed as per mg of tissue and per prostate. Data are also given in percentages (in parentheses) taking the values of the control group as 100%.

Treatment	Adenylate Cyclase Activity (pmoles cyclic AMP formed/min)	
	per mg tissue	per prostate
Control	4.41±0.40 (100)	992±141 (100)
Castrate		
96 hr	3.69±0.21 (83)*	572±43 (58)*
120 hr	2.03±0.20 (45)*	352±34 (35)*
144 hr	1.62±0.24 (37)*	198±10 (20)*

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

TABLE 2

EFFECTS OF CASTRATION AND TESTOSTERONE REPLACEMENT ON PROSTATIC ADENYLATE CYCLASE

Values represent the means ± S.E.M. of 6 animals in each group. Animals were castrated and sacrificed after 120 hr. Free testosterone (5 mg/100 g) was administered intramuscularly to castrate rats with the control animals receiving an equal volume of the vehicle. Adenylate cyclase activity was calculated as pmoles of cyclic AMP formed per min and expressed as per mg of tissue and per prostate. Data are also given in percentages (in parentheses) taking the values of the normal, intact control group as 100%.

Treatment	Adenylate Cyclase Activity (pmoles cyclic AMP formed/min)	
	per mg tissue	per prostate
Normal	4.41±0.40 (100)	992±141 (100)
Castrate	2.03±0.20 (45)*	352±34 (35)*
Castrate + Testosterone	3.85±0.51 (87)†	797±34 (80)†

*Statistically significant difference when compared with the values of normal intact rats ($p < 0.05$).

†Statistically significant difference when compared with the values of rats castrated for 120 hr ($p < 0.05$).

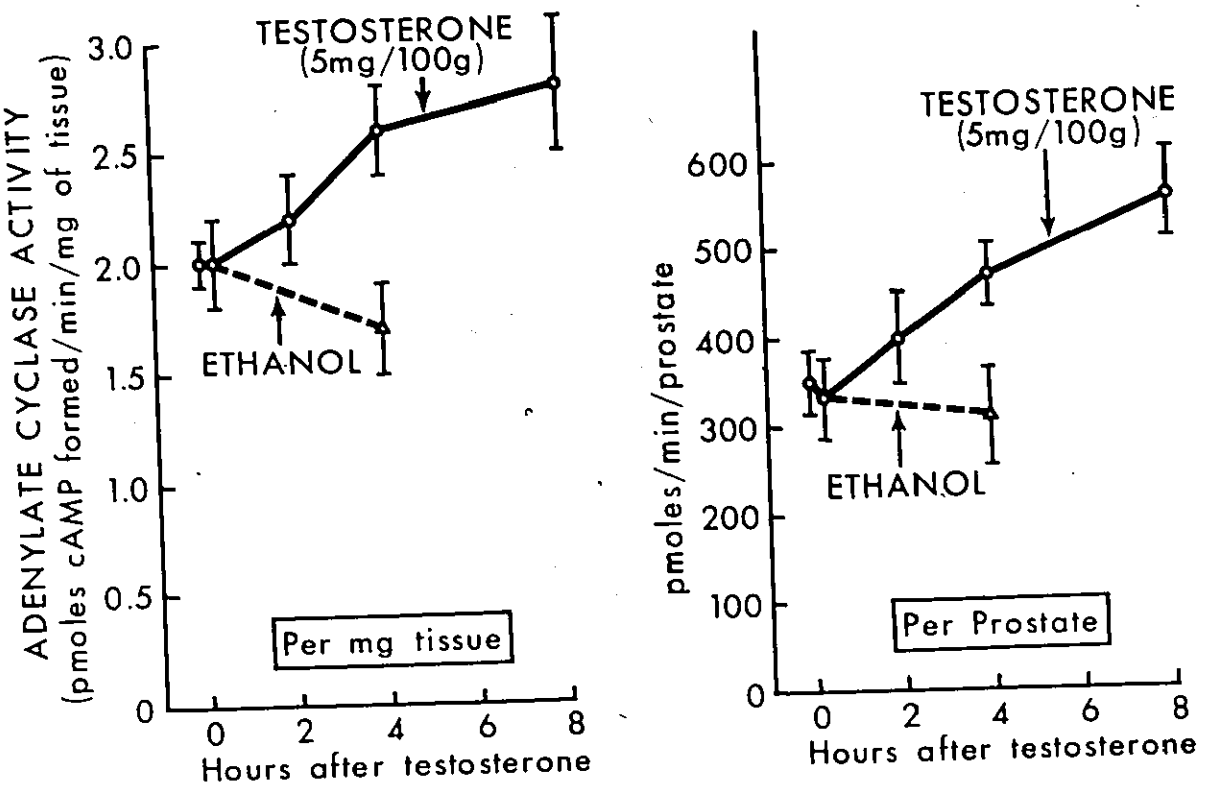


FIG. 2

Effect of intravenous testosterone on prostatic adenylate cyclase activity of 120 hr castrate rats. Each point represents the mean ± S.E.M. of 6-10 separate determinations of adenylate cyclase activity in prostate glands. Free testosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol, injected into the external jugular vein of castrate rats anesthetized with pentobarbital and sacrificed after 0.25, 2, 4 and 8 hr. Another group of rats was injected with an equal volume of 95% ethanol and sacrificed 4 hr later. Adenylate cyclase activity was calculated as pmoles of cyclic AMP formed per min and expressed as per mg of tissue and per prostate. The difference in enzyme activity between ethanol- and testosterone-treated rats at 4 hr was statistically highly significant ($p < 0.01$).

A slight increase in enzyme activity was noted 2 hr after hormone injection and by the 4th and 8th hr, statistically significant ($p < 0.01$) increases could be recorded regardless of whether the activity was expressed on a per mg tissue or per prostate basis. In contrast, administration of ethanol, the vehicle used for dissolving the androgen, failed to produce any statistically significant change in enzyme activity of the rat prostate at 4 hr. The observed increase in adenylate cyclase activity 4 hr after androgen administration was statistically highly significant ($p < 0.01$) when compared with the values seen in castrate controls injected with ethanol.

D. Effect of Two Different Intravenous Doses of Free Testosterone on Prostatic Adenylate Cyclase Activity of 120 hr Castrate Rats

The effect of two different doses of free testosterone administered by the intravenous route on prostatic adenylate cyclase activity is shown in Figure 3. A 2.5 mg/100 g dose of the hormone produced 15% and 37% increase in enzyme activity at 4 hr when expressed per mg of tissue or per prostate, respectively. However, greater increases in adenylate cyclase activity were obtained (35% and 55%) with the 5 mg/100 g dose of free testosterone regardless of the basis of expression used (Fig. 3).

E. Effect of Intravenously Administered Dihydrotestosterone on Prostatic Adenylate Cyclase

Since dihydrotestosterone is believed to be the active metabolite of testosterone (19), the question whether dihydrotestosterone could produce a more rapid change in enzyme activity than testosterone was next examined. Data presented in Figure 4 show that within 1 hr of an intravenous injection of dihydrotestosterone (5 mg/100 g), prostatic adenylate cyclase activity expressed as per mg of tissue increased by 50%. The stimulatory effect of dihydrotestosterone was maintained up to 4 hr following hormone treatment. When data were expressed as total activity per organ rather than per mg of tissue, dihydrotestosterone produced similar responses in adenylate cyclase of the

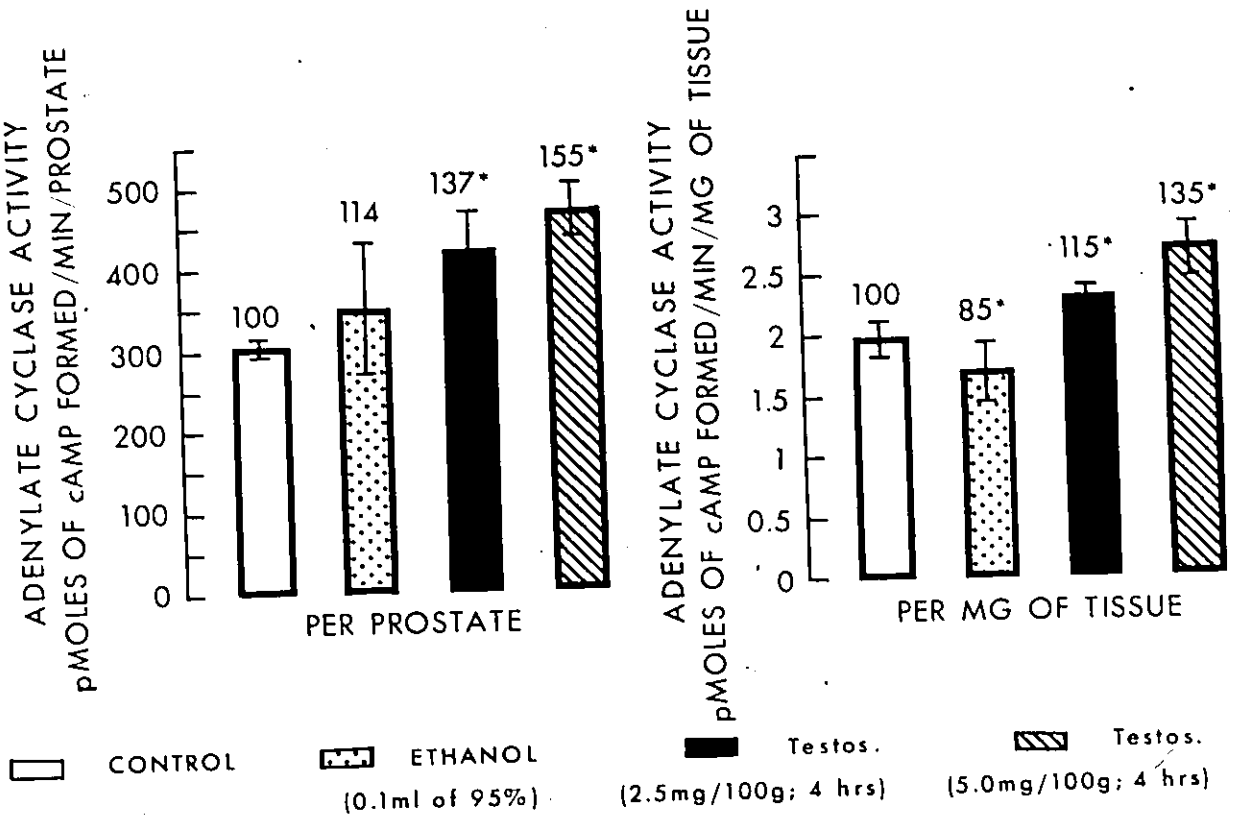


FIG. 3

Effect of two different intravenous doses of free testosterone on prostatic adenylate cyclase activity of 120 hr castrate rats. Each bar represents the mean ± S.E.M. of 6 separate determinations of enzyme activity in prostate glands pooled from 2-3 rats. Free testosterone (2.5 or 5.0 mg/100 g) was dissolved in 0.1 ml of 95% ethanol, injected into the external jubular vein of rats anesthetized with pentobarbital and killed 4 hr later. Another group of castrate rats was injected with an equal volume of 95% ethanol and also killed 4 hr later. Adenylate cyclase activity was calculated as pmoles of cyclic AMP formed per min and expressed as per mg of tissue and per prostate.

*Statistically significant difference when compared with the values of castrate controls ($p < 0.05$).

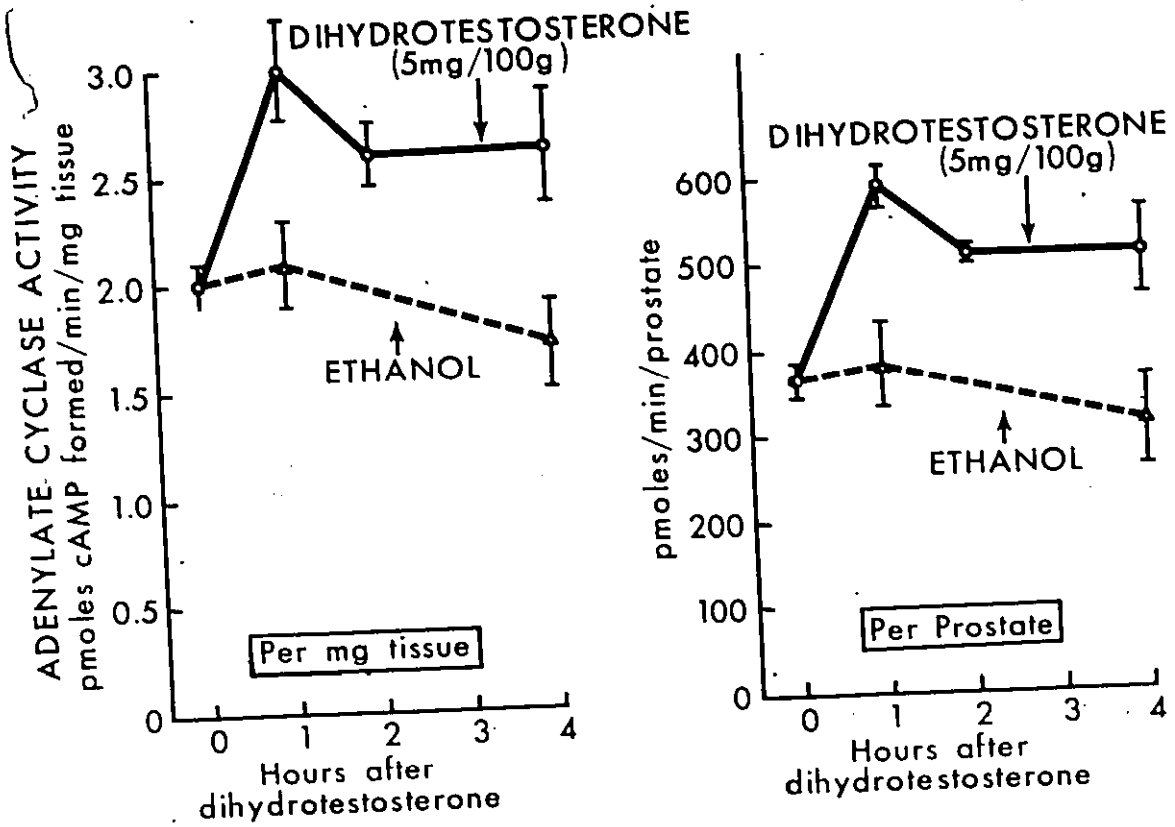


FIG. 4

Effect of intravenous dihydrotestosterone on prostatic adenylate cyclase activity of 120 hr castrate rats. Each point represents the mean ± S.E.M. of 6-10 separate determinations of adenylate cyclase activity in prostate glands. Dihydrotestosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol, injected into the external jugular vein of 120 hr castrate rats anesthetized with pentobarbital and sacrificed at 1, 2 and 4 hr. Groups of rats were also injected with an equal volume of 95% ethanol and sacrificed 1 and 4 hr later. Adenylate cyclase activity was calculated as pmoles cyclic AMP formed per min and expressed as per mg of tissue and per prostate. The difference in enzyme activity between ethanol- and dihydrotestosterone-treated rats at 1 and 4 hr was statistically highly significant ($p < 0.01$).

the prostate gland. Treatment with ethanol alone failed to exert any significant effect on the prostatic enzyme (Fig. 4).

F. Specificity of Androgenic Response of Prostatic Adenylate Cyclase

Data presented in Table 3 compare the influence of testosterone on prostatic adenylate cyclase with that obtained after the injection of certain related steroidal compounds. In contrast to significant stimulation produced by the androgen, intravenous injection of estradiol-17β, progesterone or methyl prednisolone failed to exert any measurable effect on the enzyme activity.

G. Tissue-Specific Stimulation of Prostatic Adenylate Cyclase by Dihydrotestosterone

The question whether the observed effects of androgen on adenylate cyclase activity were specific to the prostate was examined by studying the influence of dihydrotestosterone on the activity of the enzyme in the thymus, a tissue known to be unresponsive to androgenic hormones (Table 4). While a single administration of dihydrotestosterone (5 mg/100 g, i.v.) markedly increased (75%) prostatic adenylate cyclase activity from 2.03±0.21 to 3.51±0.12 pmoles cyclic AMP formed per min per mg of tissue, the activity of the enzyme in the thymus was not significantly altered by androgen treatment.

H. Effect of Cyproterone Acetate on Testosterone-Induced Elevation in Prostatic Adenylate Cyclase Activity

Since the anti-androgen, cyproterone acetate, is presumed to compete with testosterone for binding sites on specific "receptors" or alternatively with 5α-dihydrotestosterone binding (31,32), attention was directed to investigate the influence of this steroidal anti-androgen on the testosterone-stimulated increase in prostatic adenylate cyclase. Table 5 demonstrates that when cyproterone acetate was administered alone, a marked decrease (60-70%) in the prostatic enzyme was produced. Furthermore, pretreatment of testosterone-injected rats with this anti-androgen completely inhibited the androgen-induced increase in adenylate cyclase activity and the enzyme values were even lower

TABLE 3

EFFECT OF INTRAVENOUS STEROID HORMONES ON PROSTATIC ADENYLATE CYCLASE ACTIVITY

Each value represents the mean ± S.E.M. of 6 animals per group. All steroids were dissolved in 0.1 ml of 95% ethanol and injected via the external jugular vein of 120 hr castrate rats anesthetized with pentobarbital and killed 4 hr later. Control animals received an equal volume of the vehicle. Adenylate cyclase activity was calculated as pmoles of cyclic AMP formed per min and expressed as per mg of tissue or per prostate.

Treatment	Adenylate Cyclase Activity (pmoles cyclic AMP formed/min)	
	per mg tissue	per prostate
Control (castrate)	2.03±0.21	352±34
Testosterone (5 mg/100 g)	2.90±0.23*	572±35*
Estradiol-17β (10 µg/100 g)	2.20±0.11	419±25
Progesterone (5 mg/100 g)	2.34±0.32	384±44
Methyl prednisolone (5 mg/100 g)	1.74±0.20	308±30

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

TABLE 4

TISSUE SPECIFIC STIMULATION OF PROSTATIC ADENYLATE CYCLASE BY DIHYDROTESTOSTERONE

Each value represents the mean ± S.E.M. of 6 animals per group. Dihydrotestosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol and injected via the external jugular vein of 120 hr castrate rats anesthetized with pentobarbital and killed 1 hr later. Control animals received an equal volume of the vehicle. Adenylate cyclase activity was calculated as pmoles of cyclic AMP formed per min per mg of tissue. Data are also given in percentages (in parentheses) taking the values of control group as 100%.

Tissue	Adenylate Cyclase Activity (pmoles cyclic AMP formed/min/mg tissue)	
	Control (castrate)	Dihydrotestosterone
Prostate	2.03±0.21 (100)	3.51±0.12 (175)*
Thymus	1.06±0.12 (100)	1.23±0.11 (120)

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

TABLE 5

EFFECT OF CYPROTERONE ACETATE ON THE TESTOSTERONE-STIMULATED INCREASE IN PROSTATIC ADENYLATE CYCLASE ACTIVITY OF CASTRATE RATS

Each value is the mean ± S.E.M. of 6 rats. Cyproterone acetate (5 mg/100 g) was given intraperitoneally 30 min prior to the intravenous injection of free testosterone (5 mg/100 g) and rats were sacrificed 4 hr later. Data are also given in percentages (in parentheses) taking the control (120 hr castrate) values as 100%.

Treatment	Adenylate Cyclase Activity (pmoles of cyclic AMP formed/min)	
	per mg tissue	per prostate
Control (castrate)	2.32±0.21 (100)	425±30 (100)
Testosterone	3.50±0.15 (152)*	698±49 (164)*
Cyproterone Acetate	0.85±0.10 (37)*	133±21 (31)*
Testosterone + Cyproterone Acetate	0.96±0.12 (41)*†	173±36 (40)*†

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

†Statistically significant difference when compared with the values of rats given testosterone alone ($p < 0.05$).

than those seen in castrate controls.

I. Influence of Actinomycin D and Cycloheximide on Testosterone-Stimulated Increases in Prostatic Adenylate Cyclase Activity

Experiments were carried out to study the sensitivity of the testosterone-stimulated changes in adenylate cyclase activity to actinomycin D and cycloheximide, compounds known to inhibit RNA and protein biosynthesis. The results presented in Table 6 show that when free testosterone (5 mg/100 g) was administered to castrate rats, significant increases in prostatic adenylate cyclase were observed at 4 hr. Whereas actinomycin D alone elicited little or no effect on the activity of the prostate enzyme, concurrent administration of this antibiotic with the androgen produced an almost complete inhibition of the steroid-sensitive enzyme response. Likewise, cycloheximide also blocked the testosterone-stimulated rise in prostatic adenylate cyclase activity when administered simultaneously with the androgen. These results indicate that the rise in adenylate cyclase seen after testosterone treatment might be contingent upon continuous synthesis of RNA and protein in androgen-dependent tissues.

J. Effect of Propranolol on Testosterone-Stimulated Increase in Prostatic Adenylate Cyclase Activity

The influence of a β-adrenergic blocking agent, propranolol, on the androgen-stimulated increase in prostatic adenylate cyclase was also investigated. Data presented in Figure 5 show that whereas intravenous injection of free testosterone (5 mg/100 g) to 120 hr castrate rats resulted in approximately 35% increase in adenylate cyclase within 4 hr, propranolol, by itself, produced a slight inhibition of enzyme activity. However, when propranolol was given 30 min prior to the hormone, the stimulation in prostatic adenylate cyclase obtained in response to testosterone was effectively blocked.

K. Influence of Propranolol on Dihydrotestosterone-Stimulated Increase in Prostatic Adenylate Cyclase Activity

The influence of a β-adrenergic blocking agent, propranolol, on dihydro-

TABLE 6

INFLUENCE OF ACTINOMYCIN D AND CYCLOHEXIMIDE ON THE TESTOSTERONE-INDUCED INCREASE IN PROSTATIC ADENYLATE CYCLASE ACTIVITY AT 4 HR

Each value represents the mean ± S.E.M. of 5-6 rats. Free testosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol and injected via the external jugular vein of 120 hr castrate rats anesthetized with pentobarbital. Actinomycin D (25 µg/100 g) or cycloheximide (70 µg/100 g) was given intraperitoneally at the same time as the androgen and all rats were sacrificed 4 hr later. Data are also given in percentages (in parentheses) taking the control values as 100%.

Treatment	Adenylate Cyclase Activity (pmoles of cyclic AMP formed/min)	
	per mg tissue	per prostate
Control (castrate)	2.03±0.21 (100)	352±34 (100)
Testosterone	2.72±0.20 (135)*	472±35 (134)*
Actinomycin D	2.09±0.14 (105)	403±41 (114)
Testosterone + Actinomycin D	1.94±0.19 (95)†	345±37 (98)†
Cycloheximide	2.01±0.17 (100)	301±30 (86)
Testosterone + Cycloheximide	2.08±0.18 (105)†	292±20 (83)†

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with testosterone alone ($p < 0.05$).

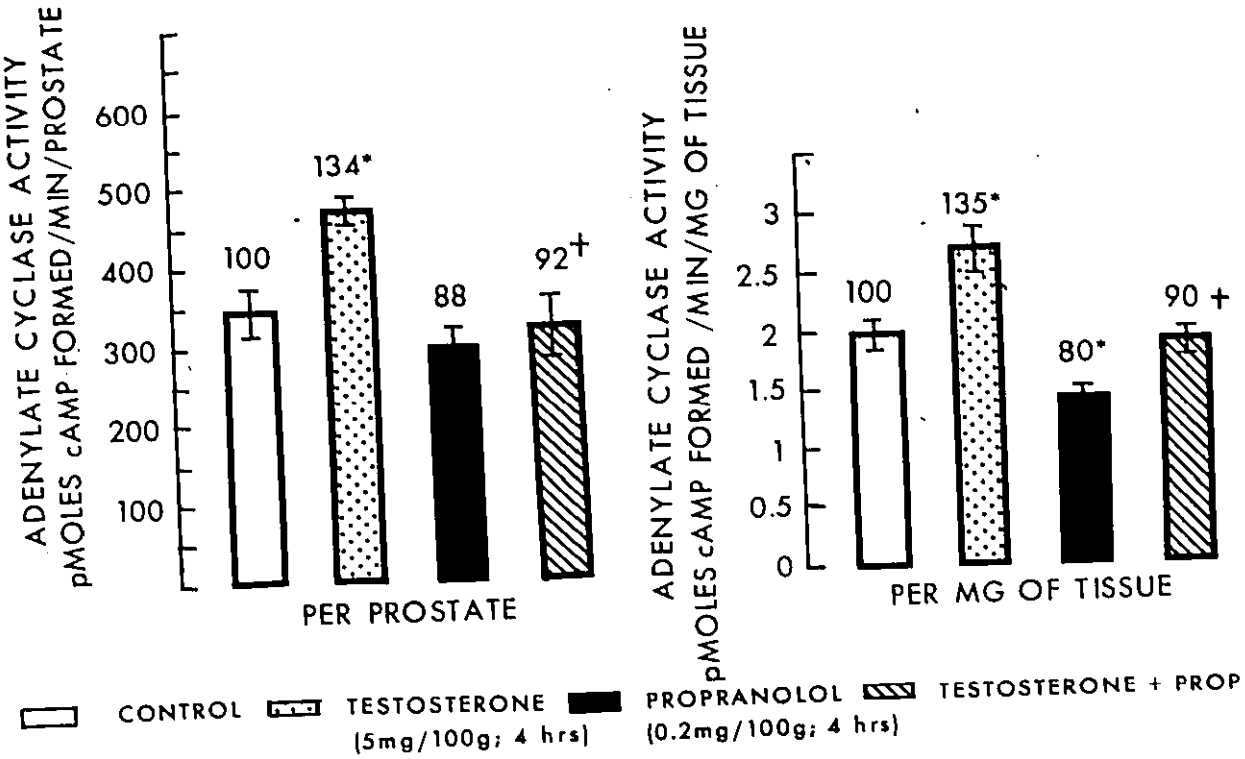


FIG. 5

Effect of propranolol on testosterone-stimulated increase in prostatic adenylate cyclase activity at 4 hr. Each bar represents the mean ± S.E.M. of 8 separate determinations of adenylate cyclase activity in prostate glands pooled from 2-3 rats. Testosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol, injected into the external jugular vein of 120 hr castrate rats anesthetized with pentobarbital and sacrificed after 4 hr. Propranolol (0.2 mg/100 g) was given either alone or concurrently with testosterone by the same route. Enzyme activity was calculated as pmoles of cyclic AMP formed per min and expressed as per mg of tissue and per prostate. Data are also given in percentages taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with testosterone ($p < 0.05$).

testosterone-stimulation of prostate adenylate cyclase was also investigated and the data are shown in Figure 6. Whereas androgen treatment (5 mg/100 g, i.v.) produced a significant stimulation of the prostatic enzyme at 1 hr regardless of the basis of expression used, propranolol alone (0.2 mg/100 g, i.v.) produced little or no effect on the enzyme activity. However, when propranolol was given concurrently with dihydrotestosterone, the stimulation of prostatic adenylate cyclase in response to the hormone was blocked.

L. Effect of Intravenously Administered Androgens on Prostatic Cyclic AMP Phosphodiesterase Activity

In order to examine whether androgens may affect prostatic cyclic AMP levels through catabolism of the nucleotide, the influence of testosterone or dihydrotestosterone on the activity of cyclic AMP phosphodiesterase in the rat ventral prostate was examined. Results presented in Table 7 show that intravenous administration of a single dose of free testosterone (5 mg/100 g) to castrate rats failed to noticeably affect the activity of prostatic cyclic AMP phosphodiesterase examined 4 and 8 hr after the androgen treatment. Although enzyme activity was slightly depressed when compared with the castrate controls, the differences observed were statistically non-significant ($p > 0.05$). In contrast, intravenous administration of the active metabolite of testosterone, dihydrotestosterone (5 mg/100 g) produced a slight but significant decrease in the activity of the enzyme at 1 hr (Table 8). Whereas cyclic AMP phosphodiesterase activity remained depressed by 2 and 4 hr when expressed per mg tissue, normal values were obtained when activity of the enzyme was calculated on total organ weight basis.

M. The "In Vitro" Effects of Androgens on Prostatic Adenylate Cyclase From Intact Adult Rats

The finding that addition of an androgen could significantly enhance the synthesis of cyclic AMP-H^3 from tritiated adenosine by prostatic slices (421) prompted studies on the influence of testosterone and dihydrotestosterone

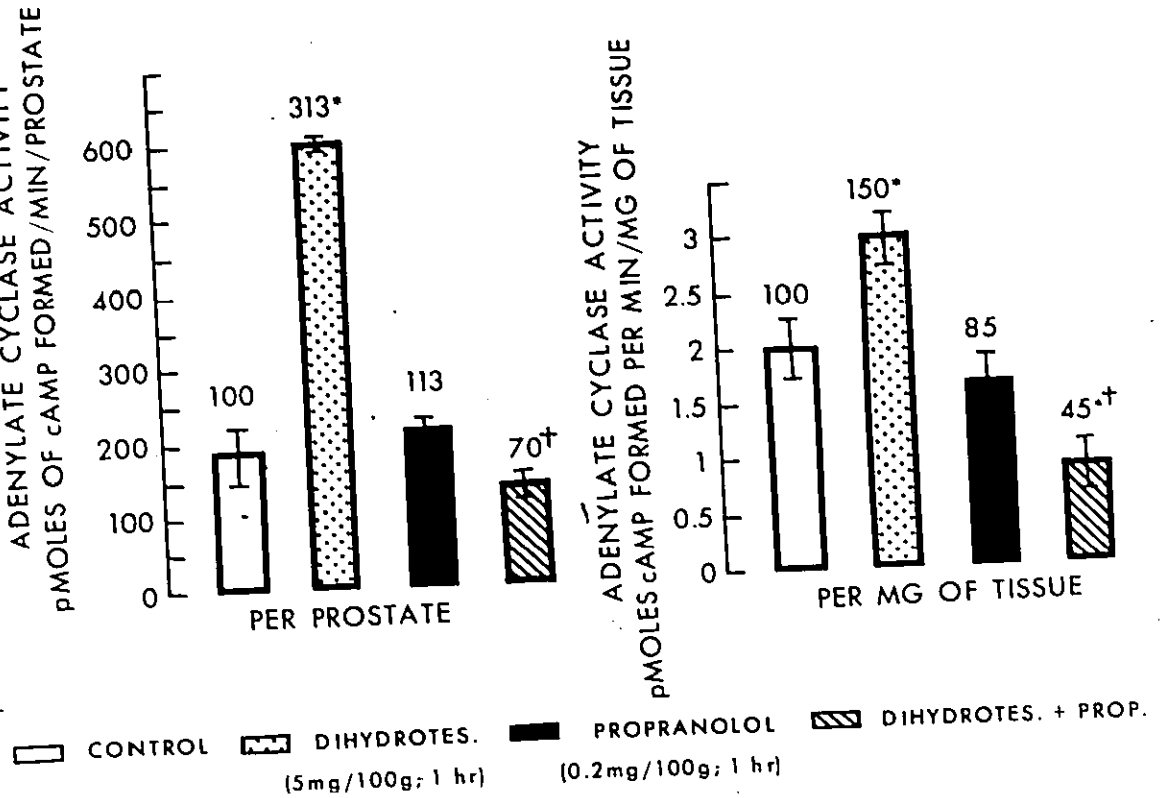


FIG. 6

Effect of propranolol on dihydrotestosterone-stimulated increase in prostatic adenylate cyclase activity at 1 hr. Each bar represents the mean ± S.E.M. of 6-7 separate determinations of adenylate cyclase activity in prostate glands pooled from 2-3 rats. Dihydrotestosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol, injected into the external jugular vein of castrate rats anesthetized with pentobarbital and sacrificed after 1 hr. Propranolol (0.2 mg/100 g) was given either alone or concurrently with dihydrotestosterone by the same route. Enzyme activity was calculated as pmoles of cyclic AMP formed per min and expressed as per mg of tissue and per prostate. Data are also given in percentages taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with dihydrotestosterone ($p < 0.05$).

TABLE 7

EFFECTS OF A SINGLE INTRAVENOUS INJECTION OF TESTOSTERONE ON PROSTATIC CYCLIC AMP PHOSPHODIESTERASE ACTIVITY

Values represent means ± S.E.M. of 6-8 separate determinations of enzyme activity in prostate glands. Free testosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol, injected into the external jugular vein of 120 hr castrate rats anesthetized with pentobarbital and sacrificed at 4 and 8 hr. Cyclic AMP phosphodiesterase activity was expressed as μmoles of substrate metabolized per hr per mg of tissue and per prostate. Data are also given in percentages (in parentheses) taking the control values as 100%.

Groups	Enzyme Activity (μmoles of cAMP metabolized/hr)	
	per mg tissue	per prostate
Control (castrate)	0.48±0.01 (100)	90±5 (100)
Testosterone		
4 hr	0.45±0.01 (94)	86±5 (96)
8 hr	0.46±0.01 (96)	89±1 (99)

All values were not statistically different from control group ($p < 0.05$).

TABLE 8

CHANGES IN PROSTATIC PHOSPHODIESTERASE ACTIVITY FOLLOWING A SINGLE INTRAVENOUS INJECTION OF DIHYDROTESTOSTERONE

Values represent means ± S.E.M. of 6-8 separate determinations of enzyme activity in prostate glands. Dihydrotestosterone (5 mg/100 g) was dissolved in 0.1 ml of 95% ethanol, injected into the external jugular vein of 120 hr castrate rats anesthetized with pentobarbital and sacrificed at 1, 2 and 4 hr. Phosphodiesterase activity was expressed as µmoles of substrate metabolized per hr per mg of tissue or per prostate. Data are also given in percentages (in parentheses) taking the control values as 100%.

Time	Enzyme Activity (µmoles of cAMP metabolized/hr)	
	per mg tissue	per prostate
Control (castrate)	0.48±0.01 (100)	90±5 (100)
Dihydrotestosterone		
1 hr	0.39±0.01 (81)*	73±2 (81)*
2 hr	0.40±0.04 (83)*	89±2 (99)
4 hr	0.42±0.01 (88)*	89±4 (99)

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

on adenylate cyclase activity of prostatic homogenates *in vitro* (Table 9). When the integrity of this accessory sex organ was disrupted by 10 strokes on a Teflon homogenizer and enzyme activity measured in the homogenate, the responsiveness of the enzyme to either androgen was completely lost. The direct addition of dihydrotestosterone, testosterone propionate or free testosterone (up to 10^{-4}M) to homogenates produced no significant increase in adenylate cyclase activity over the values seen in ethanol blanks. It is of interest that ethanol, the vehicle used for dissolving the hormone, exerted a slight stimulatory effect of its own (Table 9). Even the prior incubation of prostate homogenates for periods up to 30 min at 37°C with androgens did not result in any significant stimulation of enzyme activity. Additionally, when ventral prostates were used from 48 hr castrate rats, addition of the hormone *in vitro* failed to produce any significant response.

N. The "In Vitro" Effects of L-Epinephrine, FSH, ICSH and NaF on Prostatic Adenylate Cyclase Activity From Intact Adult Rats

In order to determine whether adenylate cyclase was at all responsive to stimulation under *in vitro* conditions, the influence of epinephrine, follicle stimulating hormone, interstitial cell stimulating hormone and sodium fluoride was investigated and the results are shown in Table 10. Whereas addition of sodium fluoride or L-epinephrine to the incubation medium produced significant stimulation (2-4 fold) of prostatic adenylate cyclase activity, follicle stimulating hormone and interstitial cell stimulating hormone elicited a slight but significant inhibition of the activity of the prostatic enzyme.

2. PARTIAL PURIFICATION AND CHARACTERIZATION OF CANINE PROSTATIC CYCLIC AMP-DEPENDENT PROTEIN KINASE

A. Subcellular Localization of Canine Prostatic Protein Kinase and Cyclic AMP Binding Activities

Of the four subcellular fractions examined, the cytosol showed the highest amount of total activity as well as the highest specific enzyme activity (Table 11). Maximum enzyme stimulation by cyclic AMP was also

TABLE 9

THE IN VITRO EFFECTS OF ANDROGENS ON PROSTATIC ADENYLATE CYCLASE

Each value represents the mean ± S.E.M. of 4-5 rats. Both dihydrotestosterone and testosterone were dissolved in 95% ethanol and added to the incubation medium just prior to assay. Adenylate cyclase activity is expressed as pmoles of cyclic AMP formed per min per mg of tissue.

Treatment	Final Conc.	Adenylate Cyclase Activity (pmoles of cAMP formed/min/mg tissue)
Normal Control		4.41±0.70
Ethanol Blank	5%	5.32±0.71
Free Testosterone	1×10^{-4} M	4.61±0.32
Testosterone Propionate	1×10^{-5} M	6.09±0.40
Dihydrotestosterone	1×10^{-5} M	5.43±0.41
	1×10^{-7} M	5.41±0.45
	1×10^{-9} M	5.40±0.32

All values obtained in the presence of the hormone were not statistically different from ethanol blanks ($p > 0.05$).

TABLE 10

EFFECTS OF L-EPINEPHRINE, FOLLICLE STIMULATING HORMONE, INTERSTITIAL CELL STIMULATING HORMONE AND SODIUM FLUORIDE ON PROSTATIC ADENYLATE CYCLASE ACTIVITY IN VITRO

Values represent means ± S.E.M. of at least 4 values. Various compounds (in final concentrations as shown) were added to the incubation medium just prior to the assay of adenylate cyclase activity. Enzyme activity is expressed as pmoles of cyclic AMP formed per min per mg of tissue.

Treatment	Final Conc.	Enzyme Activity (pmoles of cAMP formed/ min/mg tissue)
Normal Control		3.81±0.40
L-Epinephrine	10^{-4} M	9.00±1.31*
FSH	4 µg/ml	3.33±0.32
	400 µg/ml	3.09±0.21*
ICSH	4 µg/ml	3.34±0.33
	400 µg/ml	2.92±0.22*
NaF	15 mM	15.08±1.02*

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

TABLE 11

SUBCELLULAR LOCALIZATION OF CANINE PROSTATE PROTEIN KINASE ACTIVITY

Prostatic tissue was homogenized in 3 volumes of buffer (0.05 M Tris-HCl, pH 7.4, 0.25 M sucrose, 0.05 M KCl and 5 mM $MgCl_2$) with twelve strokes of a motor-driven Potter-Elvehjem homogenizer. Following filtration through four layers of cheesecloth, the homogenate was subjected to differential centrifugation. All fractions were washed twice with the homogenizing buffer and once with the same buffer without sucrose. Protein kinase assay was performed as described in the text, employing 100 µg of protein in the presence and absence of 4.6 µM cyclic AMP. In the fractions treated with Triton-X 100, the detergent was added in order to attain a final concentration of 0.1%. Specific activity of protein kinase was expressed in nmoles ^{32}P incorporated into histone mixture (Type IIA) per mg protein whereas the total activity was given in terms of mmoles ^{32}P per 5 g wet weight of the canine prostate. Values represent means of 3 separate determinations of protein kinase activity.

	Protein Kinase Activity							
	Non-Treated				Triton-X 100 Treated			
	Specific Activity		Total Activity		Specific Activity		Total Activity	
	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP
650 X g Pellet	.09	.25	4.7	12.5	.25	.61	12.6	30.3
20,200 X g Pellet	.05	.07	2.7	3.7	.06	.10	2.9	5.5
100,000 X g Pellet	.16	.22	3.7	5.3	.39	.60	9.4	14.3
100,000 X g Supernatant	.67	1.97	136.8	424.0	.62	1.85	111.8	398.2

detectable with this high-speed supernatant as well as the nuclear pellet. These results agree with the finding that cyclic AMP binding activity was found predominantly in the cytosol with significant activity also associated with the 650 x g pellet (Table 12). In view of the possibility that the enzyme activities in these particulate fractions might be present in an occluded form, the effect of Triton X-100 on both kinase and cyclic AMP binding activities of the subcellular fractions was investigated. A comparison of the enzyme activities in the untreated fractions with those in fractions treated with Triton X-100 revealed that there were latent activities in all three untreated particulate fractions that could be unmasked by addition of the detergent. It is noteworthy that Triton X-100 (0.1%) not only failed to enhance enzyme activities in the high-speed supernatant, but rather caused a slight decrease in specific, as well as total, catalytic and cyclic AMP binding activities (Tables 11 and 12).

B. Partial Purification of Soluble Prostatic Cyclic AMP-Dependent Protein Kinase

Cyclic AMP-dependent protein kinases were isolated and partially purified from the high speed supernatant of dog prostate glands (Table 13). Two peaks (fractions I and II), both containing the kinase and the cyclic AMP binding activities, were obtained during purification with DEAE-cellulose ion-exchange chromatography (Fig. 7). The major activity peak (fraction II), eluted at 0.17 M NaCl, was stimulated 4-fold by 5×10^{-6} M concentration of cyclic AMP. In contrast, the minor activity peak (fraction I), which was eluted at a lower salt concentration, was stimulated 2-3 fold by the cyclic nucleotide. Occasionally, a cyclic AMP-independent protein kinase was also observed. Although both kinase and cyclic AMP binding activities were stable during storage for 6 months in liquid nitrogen, over 50% of its cyclic AMP binding capacity was lost after 4 months when the samples were stored at -20°C.

TABLE 12

SUBCELLULAR LOCALIZATION OF CYCLIC AMP BINDING ACTIVITY FROM CANINE PROSTATE

Experimental conditions were the same as described in the legend to Table 11. The cyclic AMP binding capacity was assayed in presence of 1.5×10^{-7}M ^{3}H-cyclic AMP, 5 mM theophylline and 100 to 150 µg of protein from each fraction. In fractions treated with Triton-X100, the detergent was added in order to attain a final concentration of 0.1%. Specific activity for cyclic AMP binding was expressed as pmoles cyclic AMP bound per mg protein whereas the total activity was given in terms of pmoles cyclic AMP bound per 5 g wet weight of the canine prostate. Values represent means of 3 separate determinations of cyclic AMP binding activity.

Fractions	Cyclic AMP Binding Activity			
	Non-Treated		Triton-X100 Treated	
	Sp. Activity	Total Activity	Sp. Activity	Total Activity
650 X g Pellet	.41	20.1	.66	32.7
20,200 X g Pellet	.15	8.0	.28	14.8
100,000 X g Pellet	.31	7.4	.62	14.9
100,000 X g Supernatant	1.93	416.4	1.86	407.1

TABLE 13

PURIFICATION OF SOLUBLE PROSTATIC CYCLIC AMP-DEPENDENT PROTEIN KINASE

	Kinase Activity				Cyclic AMP Binding Activity	
	-Cyclic AMP		+Cyclic AMP			
	Specific Activity (nmoles ^{32}P incorp./mg protein)	Yield (%)	Specific Activity (nmoles ^{32}P incorp./ mg protein)	Yield (%)	Specific Activity (pmoles ^{3}H bound/mg protein)	Yield (%)
100,000 X g Supernatant	.43	100	2.02	100	1.03	100
Supernatant pH 5.5 precipitation	.42	89	2.17	98	1.34	119
$(NH_4)_2SO_4$ precipitate	1.21	69	8.91	107	3.13	74
DEAE Cellulose Fraction I	2.43	13	6.72	8	1.31	3
DEAE Cellulose Fraction II	2.52	30	11.87	20	6.92	34

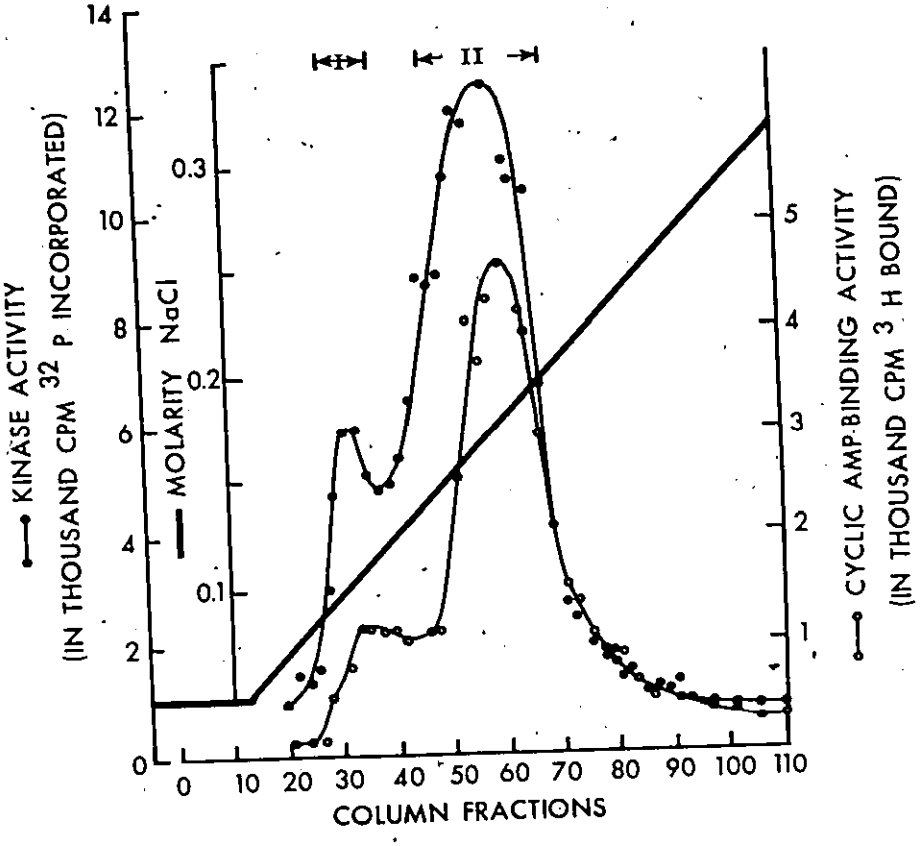


FIG. 7

Chromatographic behaviour of canine prostatic protein kinase and cyclic AMP binding activities. The enzyme (0.3 g of protein) was applied to a DEAE-cellulose column (16x30 cm) and eluted with a linear gradient of 0.05 - 0.35 M NaCl solution (200 ml of each) after extensive washing with 0.05 M NaCl. The buffer contained 0.01 M Tris-HCl and 1 mM β-mercaptoethanol, pH 7.4. Units were expressed as counts/min/aliquot. (●——●) ^{32}P incorporated into histone mixture in the presence of cyclic AMP (4.6×10^{-6}M) without correction for sample blank; (o——o) (^{3}H)-cyclic AMP bound; (——) molarity of NaCl in elution buffer.

C. Effects of Various Incubation Conditions on Prostatic Protein Kinase Activity

The time-course of the phosphorylation of histone (Type IIA) by soluble prostatic protein kinase in the presence and absence of cyclic AMP is illustrated in Figure 8. In the presence of 5×10^{-6}M concentration of cyclic AMP, maximum stimulation was seen at 7 min during which the enzyme activity was found to be linear with time. In contrast, when assayed without the addition of cyclic AMP, protein kinase activity was linear with time up to 20 min (Fig. 8). In studying the effect of various amounts of enzyme on prostatic protein kinase activity, the incorporation of ^{32}P into the histone mixture was linear up to approximately 60 µg of protein of the enzyme preparation both in the presence and absence of cyclic AMP (Fig. 9).

D. Effect of Varying Amounts of Cyclic AMP on Prostatic Protein Kinase Activity

The effect of various cyclic AMP concentrations on histone phosphorylation catalyzed by prostate protein kinase was also studied and the data are presented in Figure 10. Although the enzyme was half maximally activated by 5×10^{-8}M cyclic AMP, maximum stimulation was noted with 5×10^{-6}M concentration of the cyclic nucleotide. Higher concentrations of cyclic AMP appeared to exert a somewhat smaller effect on the enzyme.

E. Nucleotide Specificity of Prostatic Protein Kinase

The effect of several 3',5'-cyclic mononucleotides on the activity of prostatic protein kinase is shown in Figure 11. At low concentrations, cyclic AMP was far more effective than any of the other nucleotides in activating the enzyme. Although kinase activity was at its maximum with 1 µM cyclic AMP, cyclic IMP, the most potent activator among the analogues tested, produced only 30% of the kinase activity elicited by cyclic AMP at equimolar concentration. Nevertheless, various cyclic mononucleotides including those of guanosine, inosine, cytosine, uridine as well as the $N^6,O^{2'}$-dibutyryl derivative of

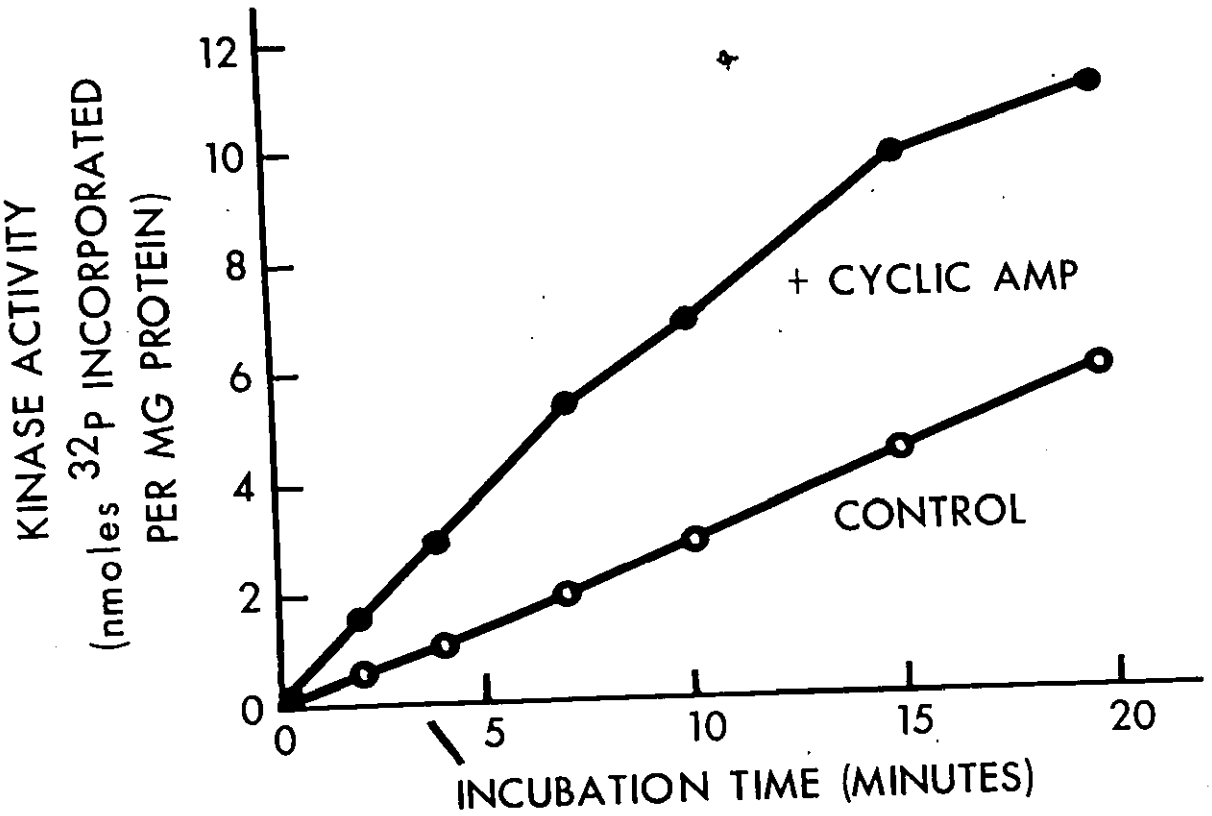


FIG. 8

Effect of incubation time on histone phosphorylation catalyzed by soluble prostatic cyclic AMP-dependent protein kinase. Assays were carried out under standard conditions as described in the text with the exception of the varying incubation times. Kinase activity was expressed as nmoles ^{32}P incorporated into histone mixture (Type IIA) per mg protein in the absence (o——o) and presence (●——●) of 4.6×10^{-6} M cyclic AMP.

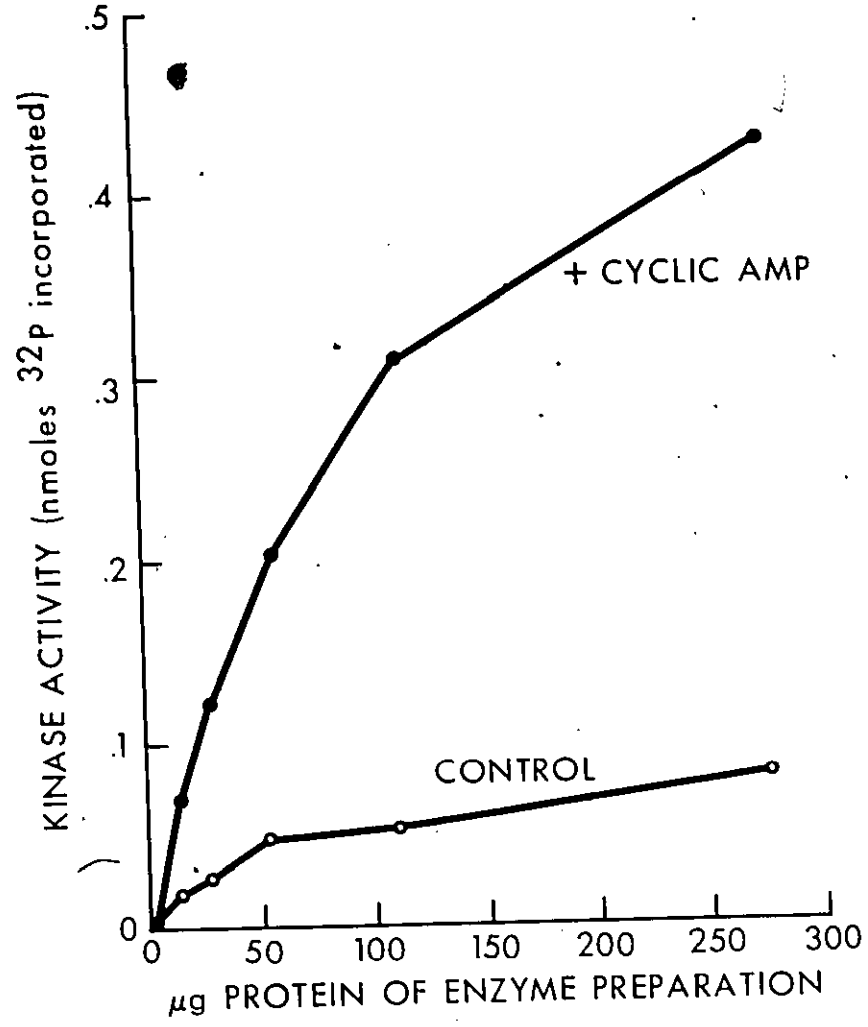


FIG. 9

Effect of varying enzyme concentrations on cyclic AMP-dependent protein kinase activity. Assay conditions were identical to those described in the text with the exception of the concentration of enzyme preparation after DEAE-cellulose chromatography. Kinase activity in the absence (o—o) and presence of 4.6×10^{-6} M cyclic AMP (●—●) was expressed as nmoles ^{32}P incorporated into histone mixture.

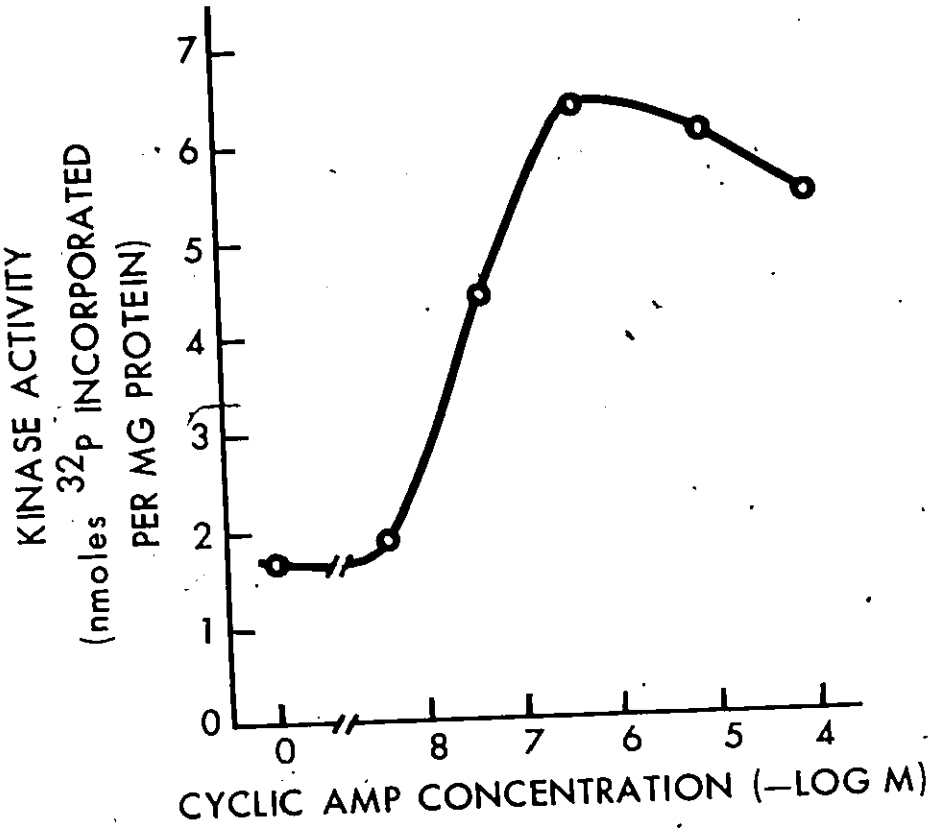


FIG. 10

Effect of varying cyclic AMP concentrations on histone (Type IIA) phosphorylation catalyzed by soluble cyclic AMP-dependent protein kinase from dog prostate.

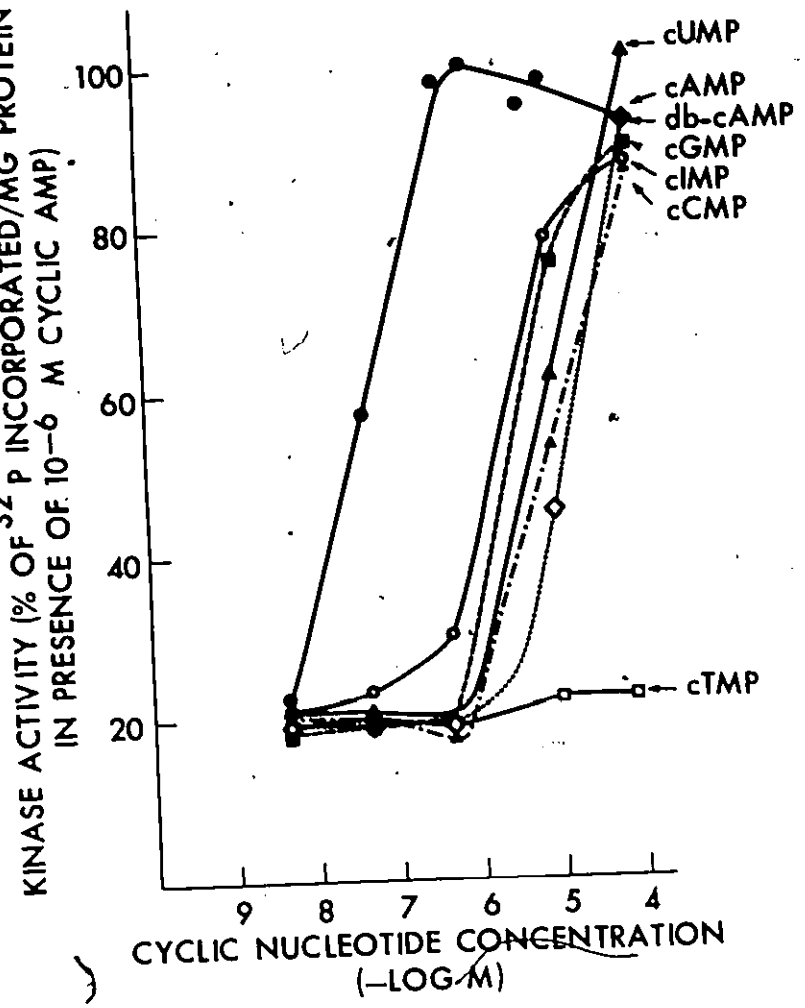


FIG. 11

The effect of various cyclic 3',5'-mononucleotides on prostatic protein kinase activities. Data were expressed as percentages of kinase activity observed in presence of micromolar concentration of cyclic AMP. Assays were carried out under standard conditions as described in the text, except for the variation in the concentration of the indicated cyclic nucleotide. The various cyclic 3',5'-mononucleotides tested were cyclic AMP (●——●, cAMP), cyclic TMP (□——□, cTMP), cyclic CMP (▲–·–▲, cCMP), cyclic IMP (○——○, cIMP), cyclic GMP (■– –■, cGMP), dibutyryl cyclic AMP (◇·····◇, db-cAMP) and cyclic UMP (△——△, cUMP).

cyclic AMP, at the highest concentration examined, were also capable of stimulating enzyme activity to near maximal values. In contrast, the effect of cyclic TMP on kinase activity was only slightly stimulatory.

F. Ability of Various Proteins to Serve as Substrate for Prostatic Cyclic AMP-Dependent Protein Kinase

Several proteins, including histone mixture, arginine- and lysine-rich histones, protamine and casein were tested for their ability to serve as the substrate for protein kinase. Data presented in Figure 12 demonstrate that histones were much superior substrates for both the basal and the cyclic AMP-stimulated forms of enzyme activity. Cyclic AMP (5 μM) markedly enhanced ^{32}P transfer to calf thymus histones, especially fractions F_1 and F_3, as classified by the terminology of Johns (442). Although both basal and cyclic AMP-stimulated activities of the enzyme was highest with F_{2b} histones, the cyclic AMP stimulation of the enzyme in this fraction was considerably lower. Although basal enzyme activity was measurable with both protamine and α-casein, little or no stimulation could be demonstrated with cyclic AMP (Fig. 12).

G. Equilibrium Studies on Cyclic AMP Binding

To understand further the molecular interaction of cyclic AMP with its dependent protein kinase, association of the nucleotide with the enzyme was studied at equilibrium. Scatchard-plot analysis of the binding data (443) demonstrated non-cooperativity and one order of cyclic AMP-binding sites (Fig. 13). The association constant ($K_{assoc.}$) at $0^{o}C$ and pH 5.0 was about $7.8 \times 10^{7}\ M^{-1}$, and the concentration of binding sites was 18 pmol/mg of protein of the enzyme preparation.

H. Apparent Km of Prostatic Protein Kinase for Histones and ATP

The effects of various histone and ATP concentrations on protein kinase activity were examined by Lineweaver-Burk plots (Figs. 14 and 15). Although cyclic AMP did not significantly change the apparent K_m for histone (0.5 mg/ml),

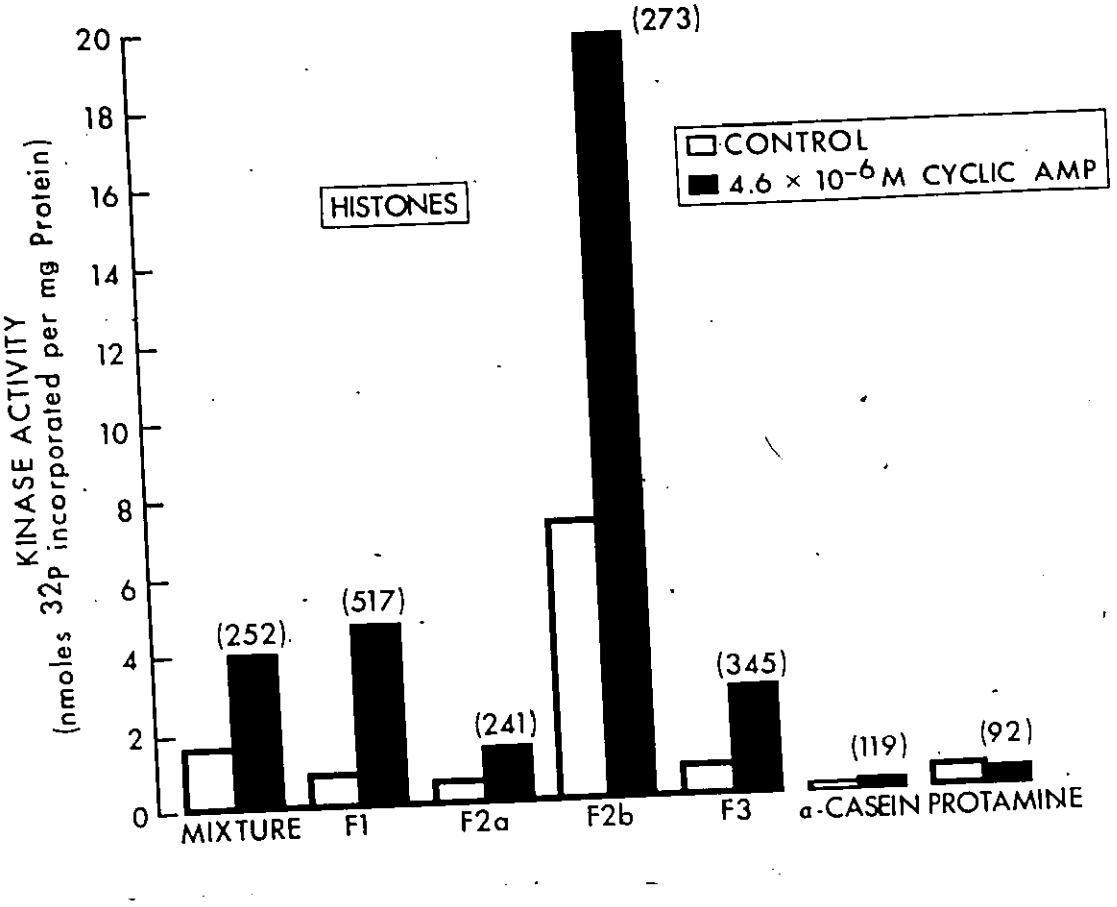


FIG. 12

Substrate specificity of soluble prostatic cyclic AMP-dependent protein kinase. Standard assay conditions were employed except for the addition of other protein substrates (240 µg). α-Casein was dissolved by the method of Reimann et al. (302). Values in parentheses represent percent stimulation in kinase activity in the presence of 4.6×10^{-6}M cyclic AMP, taking the control as 100 percent.

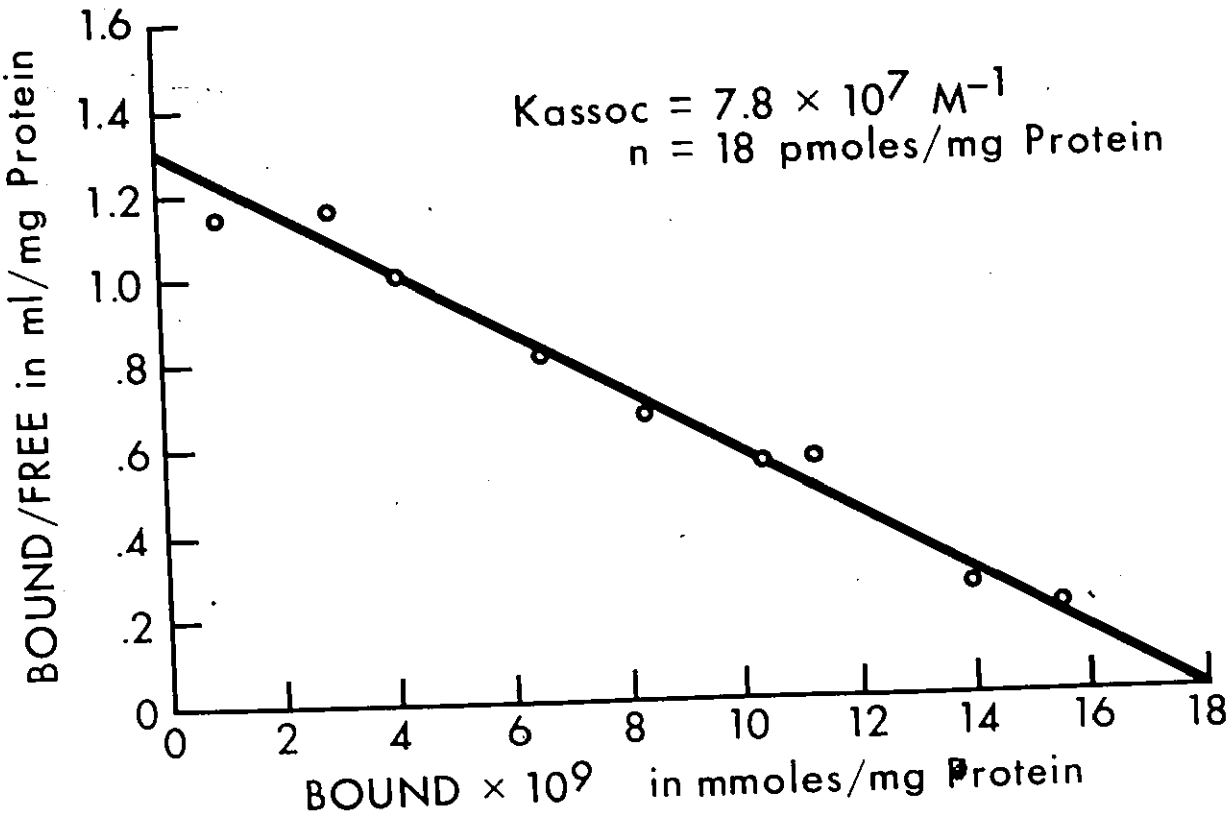


FIG. 13

Scatchard Plot describing the interaction at equilibrium between prostatic cyclic AMP-dependent protein kinase and cyclic AMP. Cyclic AMP-binding activity was assayed in an incubation medium (final volume 0.25 ml, pH 5.0) containing 2.4 nM (^{3}H)-cyclic AMP, 0 to 72 nM unlabeled cyclic AMP, 0.05 M sodium acetate, 1 mM EDTA and 100 µg protein of fraction II enzyme preparation after DEAE-cellulose chromatography. Incubation was carried out at 0°C for 10 hr and protein bound cyclic AMP was separated by Millipore filtration as described in the text.

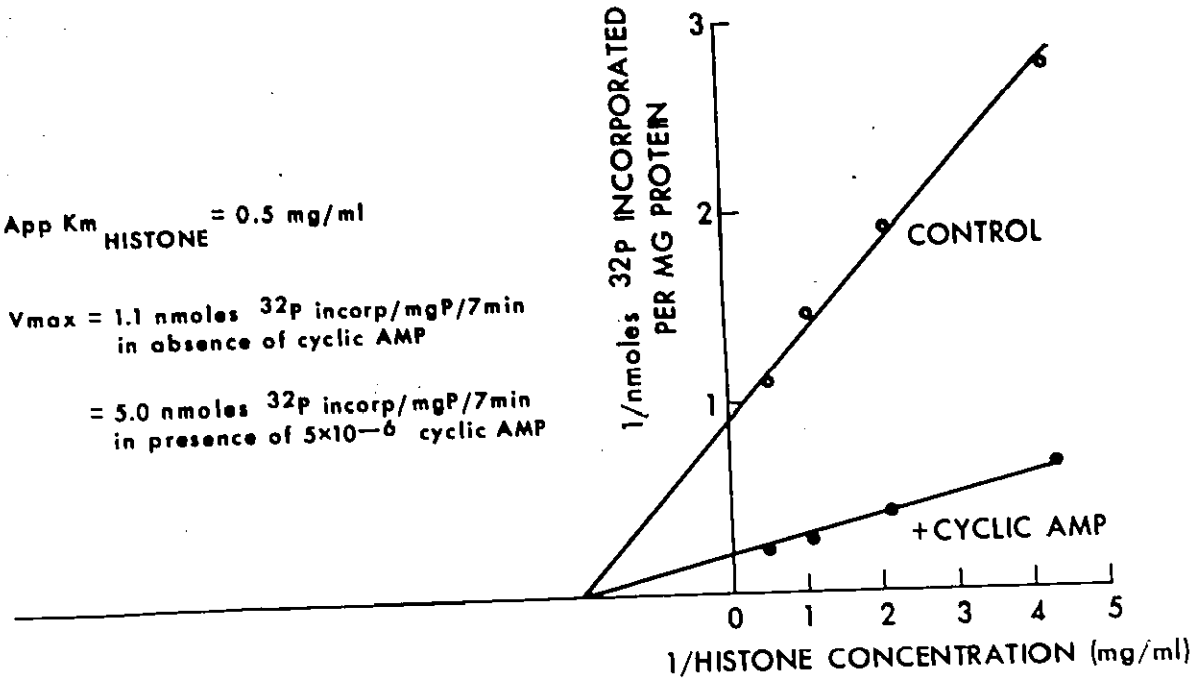


FIG. 14

Lineweaver-Burk plots illustrating the effect of histone concentration on prostatic protein kinase activity. The enzyme activity was assayed in the absence (o—o) and presence (•—•) of 4.6 x 10^{-6}M cyclic AMP and expressed over a period of 7 min. Assay conditions were the same as described in the text except for the variation in histone concentration.

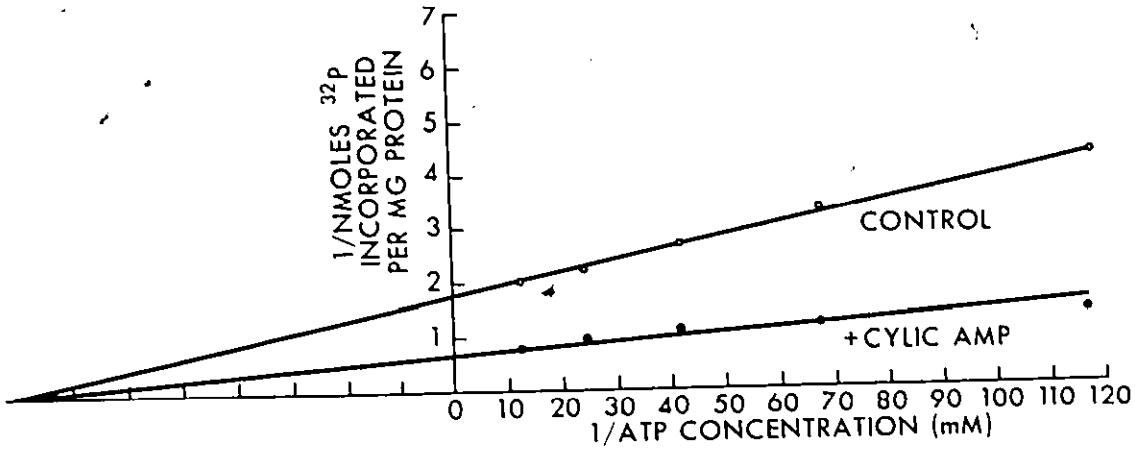


FIG. 15

Lineweaver-Burk plots illustrating the effect of ATP concentration on prostatic protein kinase activity. The enzyme activity was assayed in the absence (o——o) and presence (●——●) of 4.6 x 10^{-6}M cyclic AMP and expressed over a period of 7 min. $MgCl_2$ concentration was held constant at 17 mM. Assay conditions were the same as described in the text except for the variation in ATP concentration.

the maximum velocity was increased approximately 5-fold by the nucleotide. Values of 1.1 and 5.0 nmol of ^{32}P incorporated/7 min per mg of protein were obtained in the absence and presence of cyclic AMP, respectively (Fig. 14). As seen in the case of histones, the concentration of ATP needed for half-maximal activity was not affected significantly by the addition of cyclic AMP, whereas maximal activity was greatly increased. The apparent K_m for ATP was 1.2×10^{-5}M in the presence and absence of the nucleotide. Addition of cyclic AMP, however, again increased maximal velocity from 0.57 to 1.5 nmol ^{32}P incorporated/7 min per mg of protein (Fig. 15).

I. Influence of Certain Divalent Metal Ions on Prostatic Protein Kinase Activity

Studies on the effect of certain bivalent metal ions on protein kinase activity demonstrated an absolute requirement of the enzyme for a bivalent metal (Table 14). Although minimal basal activity was observed in the absence of any bivalent cations, little or no stimulation by cyclic AMP could be detected. However, in the presence of 1.7 mM Mg^{2+}, kinase activity was markedly increased and could be further enhanced 3.5-fold in the presence of the cyclic nucleotide. Although increasing the Mg^{2+} concentration to 17 mM further augmented basal and cyclic AMP-stimulated kinase activity, the percentage stimulation remained relatively unaltered. When equimolar concentrations of Mn^{2+} were tested instead of Mg^{2+}, enzyme activities were somewhat decreased. Though cyclic AMP caused a slight increase in the activity of the enzyme in the presence of 1.7 mM Ca^{2+}, higher concentration of the metal (17 mM) inhibited both the basal and the cyclic AMP-stimulated prostatic protein kinase activities (Table 14).

3. ANDROGENIC EFFECTS ON PROSTATIC PROTEIN KINASES

A. Influence of Castration and Testosterone Treatment on Soluble Prostatic Protein Kinases

Data presented in Table 15 illustrate the effects of castration and

TABLE 14

EFFECT OF CERTAIN DIVALENT METAL IONS ON CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY OF THE CANINE PROSTATE

Protein kinase activity was assayed under standard conditions as described in the text both in the presence and absence of 4.6×10^{-6}M cyclic AMP. Enzyme activity was expressed as nmoles ^{32}P incorporated into histone (Type IIA) per mg protein of the enzyme preparation following DEAE-cellulose purification.

Cation		Protein Kinase Activity	
		-Cyclic AMP	+Cyclic AMP
None		0.19	0.20
Mg^{++}	1.69 mM	0.61	2.18
	16.9 mM	1.46	5.43
Mn^{++}	1.69 mM	0.32	0.97
	16.9 mM	0.18	0.56
Ca^{++}	1.69 mM	0.17	0.33
	16.9 mM	0.06	0.04

TABLE 15

EFFECT OF CASTRATION AND TESTOSTERONE TREATMENT ON SOLUBLE PROSTATIC PROTEIN KINASES

Values represent the mean ± S.E.M. of 7-8 rats per group. Rats were sham-operated or castrated for 7 days and daily testosterone administration (5 mg/100 g/day, i.m.) for 3 and 5 days was initiated on the 7th day after castration. Protein kinase activity was expressed as nmoles ^{32}P incorporated into histone mixture (Type IIA) per mg protein and per prostate in the absence or presence of exogenous cyclic AMP (5×10^{-6}M). Kinase activity ratio was defined as the ratio of protein kinase activity assayed in the absence of cyclic AMP to that in the presence of the nucleotide. Results are also expressed in percentages (in parentheses) taking the control values as 100%.

Groups	Protein Kinase Activity (nmoles ^{32}P incorporated)				Kinase Activity Ratio
	Per mg protein		Per prostate		
	-cAMP	+cAMP	-cAMP	+cAMP	
Control	0.53±0.03 (100)	1.09±0.09 (100)	16.00±1.57 (100)	35.00±2.58 (100)	0.49±0.04 (100)
Castrate	0.78±0.04 (145)*	1.88±0.12 (173)*	1.44±0.20 (9)*	4.64±0.67 (13)*	0.39±0.01 (80)
Castrate + Testosterone					
3 days	0.77±0.03 (145)*	1.98±0.11 (183)*	5.12±0.34 (32)*	13.81±0.89 (39)*†	0.37±0.01 (76)
5 days	0.63±0.04 (119)†	1.86±0.06 (171)*	7.27±0.43 (45)*†	21.63±1.13 (62)*†	0.36±0.02 (73)

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

†Statistically significant difference when compared with the values of castrate rats ($p < 0.05$).

androgen replacement on soluble prostatic protein kinase. When expressed on a per mg protein basis, protein kinase activity was significantly increased both in the absence (45%) and the presence (73%) of cyclic AMP 7 days following castration. Although administration of testosterone appeared to reverse this effect on the cyclic AMP-independent enzyme (-cAMP), the total enzyme activity (+cAMP) remained relatively unaffected by androgenic repletion. In contrast, protein kinase activity, when expressed on per total prostate basis, was markedly depressed in the absence (91%) and the presence (87%) of the cyclic nucleotide. Moreover, testosterone administration for 3 days partially reversed the effect of castration. Although administration of testosterone for 5 days further enhanced protein kinase activity in both the absence and the presence of exogenous cyclic AMP, complete restoration to normal enzyme levels was not achieved. Castration also resulted in a slight decrease (20%) in the kinase activity ratio of soluble protein kinase which remained unaffected in rats treated with testosterone.

B. Effect of Castration and Testosterone Treatment on Cytosolic Cyclic AMP Binding Capacity of Rat Ventral Prostate

Androgenic deprivation and repletion elicited marked effects on the cyclic AMP binding capacity of soluble prostatic protein kinase, a parameter indicative of the number of unsaturated receptor sites being present (Table 16). When expressed on a per mg protein basis, the cyclic AMP binding capacity of the soluble enzyme in castrate rats was 3 times as high as that of the sham-operated controls, and was affected only slightly by the administration of testosterone. However, when cyclic AMP binding capacity of the enzyme was expressed on per prostate basis, castration was found to produce a marked depressing effect (77%). In addition, while administration of testosterone for 3 days reversed this effect only partially, the ability of the enzyme to bind (^{3}H)-cyclic AMP *in vitro* was completely restored after 5 days of androgenic stimulation.

TABLE 16

EFFECT OF CASTRATION AND TESTOSTERONE TREATMENT ON CYTOSOLIC CYCLIC AMP BINDING CAPACITY OF RAT VENTRAL PROSTATE

Values represent the means ± S.E.M. of 7-8 rats per group. Rats were sham-operated or castrated for 7 days and daily testosterone administration (5 mg/100 g/day, i.m.) for 3 and 5 days was initiated on the 7th day after castration. Cyclic AMP binding capacity was expressed as pmoles (^{3}H)-cyclic AMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the control values as 100%.

Groups	cAMP Binding Capacity (pmoles (^{3}H)-cAMP bound)	
	per mg protein	per prostate
Control	6.43±0.61 (100)	204.61±15.04 (100)
Castrate	19.06±1.55 (296)*	46.85±6.65 (23)*
Castrate + Testosterone		
3 days	14.23±0.86 (221)*†	95.04±7.76 (47)*†
5 days	15.43±0.81 (240)*	177.75±7.31 (87)†

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

†Statistically significant difference when compared with the values of castrate rats ($p < 0.05$).

C. Influence of Castration and Testosterone Treatment on Particulate Prostatic Protein Kinase Activity

There is increasing evidence to suggest that nuclear protein phosphorylation plays an important role in gene activation in eukaryotic cells (4,349). Since the cellular growth and differentiation of the prostate is constantly under the control of testicular androgens, the influence of castration and testosterone treatment also was examined on the phosphorylation of endogenous substrates in crude nuclear preparations of this tissue. As shown in Table 17, androgenic deprivation resulted in marked decreases in protein kinase activity of the particulate fraction in the rat ventral prostate. While enzyme activity was significantly lowered from 1.10±0.03 to 0.49±0.03 nmoles ^{32}P incorporated per mg protein, the amount of labelled phosphate incorporated per prostate was markedly decreased by 95% of the control values 7 days after castration. Moreover, although full recovery of specific enzyme activity appeared to have been achieved as early as 3 days following the initiation of testosterone treatment, total activity per prostate was not normalized until after 5 days of androgen administration. Studies on the kinase activity ratio revealed a lack of dependence of the enzyme on cyclic AMP (activity ratio being close to unity) as well as the insensitivity of this parameter to androgenic deprivation and repletion (Table 17).

D. Influence of Castration and Testosterone Treatment on Cyclic AMP Binding Capacity in Crude Prostatic Nuclear Preparation

Results in Table 18 demonstrate the ability of crude nuclear preparation from rat ventral prostate to bind (^{3}H)-cyclic AMP *in vitro*. Whereas castration led to significant decreases in both the specific (30%) and the total (90%) cyclic AMP binding activity of the particulate fraction, replacement therapy with testosterone completely abolished the effects of castration on the cyclic AMP binding capacity of the ventral prostate (Table 18).

TABLE 17

INFLUENCE OF TESTOSTERONE TREATMENT ON PARTICULATE PROTEIN KINASE ACTIVITY OF RAT VENTRAL PROSTATE

Values represent the means ± S.E.M. of 7-8 rats per group. Rats were sham-operated or castrated for 7 days and daily testosterone administration (5 mg/100 g/day, i.m.) for 3 and 5 days was initiated on the 7th day after castration. Protein kinase activity of the crude nuclear preparation (650 x g pellet, washed 3 times and resuspended in 0.1% Triton X-100) was assayed by measuring the incorporation of ^{32}P from (^{32}P) ATP into endogenous nuclear substrates and was expressed as nmoles ^{32}P incorporated per mg protein and per prostate. Kinase activity ratio was defined as the ratio of protein kinase activity assayed in the absence of cyclic AMP to that in the presence of 5.6×10^{-6}M of the nucleotide. Results are also expressed in percentages (in parentheses) taking the control values as 100%.

Groups	Protein Kinase Activity (nmoles ^{32}P incorporated)		Kinase Activity Ratio
	Per mg protein	Per prostate	
Control	1.19±0.03 (100)	7.70±0.90 (100)	1.01±0.02 (100)
Castrate	0.49±0.03 (41)*	0.37±0.02 (5)*	0.93±0.06 (94)
Castrate + Testosterone			
3 days	1.46±0.11 (122)†	3.87±0.44 (50)*†	1.02±0.02 (101)
5 days	1.32±0.09 (111)†	5.77±0.47 (75)†	0.98±0.02 (96)

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

†Statistically significant difference when compared with the values of castrate rats ($p < 0.05$).

TABLE 18

INFLUENCE OF TESTOSTERONE TREATMENT ON CYCLIC AMP BINDING CAPACITY IN CRUDE NUCLEAR FRACTIONS OF RAT VENTRAL PROSTATE

Values represent the means ± S.E.M. of 7-8 rats per group. Rats were sham-operated or castrated for 7 days and daily testosterone administration (5 mg/100 g/day, i.m.) for 3 and 5 days was initiated on the 7th day after castration. Cyclic AMP binding capacity of the crude nuclear preparation (650 x g pellet, washed 3 times and resuspended in 0.1% Triton X-100) was expressed as pmoles (^{3}H)-cyclic AMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the control values as 100%.

Groups	cAMP Binding Capacity (pmoles (^{3}H)-cAMP bound)	
	per mg protein	per prostate
Control	6.58±0.58 (100)	44.41±8.61 (100)
Castrate	4.63±0.36 (70)*	4.58±0.58 (10)*
Castrate + Testosterone		
3 days	9.49±0.89 (144)*†	24.51±1.69 (55)*†
5 days	6.64±0.45 (101)†	31.11±1.47 (70)†

*Statistically significant difference when compared with the values of control rats ($p < 0.05$).

†Statistically significant difference when compared with the values of castrate rats ($p < 0.05$).

4. BETA-ADRENERGIC STIMULATION OF PROSTATIC ADENYLATE CYCLASE-CYCLIC AMP SYSTEM

A. Stimulation of Prostatic Adenylate Cyclase Activity and Cyclic AMP Levels in vivo by Isoproterenol

The effect of isoproterenol (1 mg/kg, i.p.) on prostatic cyclic AMP metabolism was examined at various time intervals following the administration of a β-adrenergic agonist. Administration of isoproterenol resulted in a rapid increase in the activity of adenylate cyclase in rat ventral prostate (Fig. 16). Whereas enzyme activity was significantly increased from 5.60±0.60 to 7.89±0.72 pmoles cyclic AMP formed/min/mg tissue by 3 min, maximal activation of the enzyme (116%) was observed 5 min following the injection of isoproterenol. By 10 min, the activity of the prostatic enzyme was almost back to normal levels. As shown in Figure 16, the sequential changes in the endogenous cyclic AMP levels following isoproterenol were similar to those seen in the synthesizing enzyme. Although the cyclic nucleotide levels at 3 min following isoproterenol treatment were increased by 58%, this augmentation was statistically non-significant. Cyclic AMP levels at 5 min were maximally elevated to approximately 7-fold of those found in the saline-injected controls and normal values were attained in 10 min following the administration of the agonist. It is of interest that by 15 min, both adenylate cyclase activity as well as the cyclic nucleotide levels were non-significantly decreased to 73% and 87%, respectively below those of the zero-time controls.

B. Effect of Various Doses of Isoproterenol on Prostatic Adenylate Cyclase and Cyclic AMP

Figure 17 shows the influence of various doses of isoproterenol on adenylate cyclase activity and cyclic AMP levels in the rat ventral prostate 5 min following the administration of the β-adrenergic agonist. Whereas injection of a 0.5 mg/kg dose increased endogenous cyclic AMP from 2.29±0.32 to 10.65±0.41 pmoles/mg tissue, maximal elevation (up to 7-fold) of the

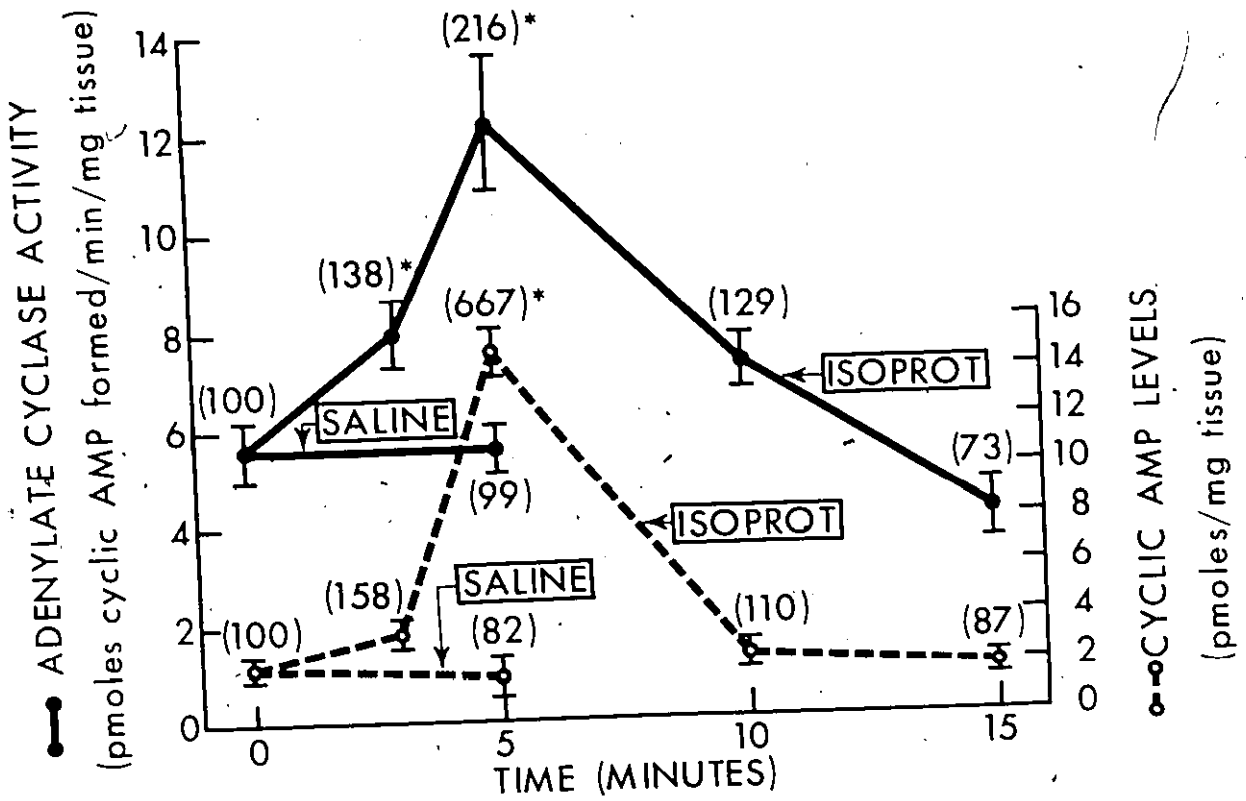


FIG. 16

Beta-adrenergic stimulation of prostatic adenylate cyclase activity and cyclic AMP levels. Each point represents the mean ± S.E.M. of 7 animals in each group. DL-isoproterenol (1 mg/kg) was administered intraperitoneally and animals were sacrificed at the indicated time interval. Whereas adenylate cyclase activity (●——●) was expressed in pmoles cyclic AMP formed per min per mg of tissue, endogenous cyclic AMP levels (o----o) were calculated as pmoles cyclic AMP per mg tissue. Data are also expressed in percentages (in parentheses) taking the control values as 100%.

*Statistically significant difference when compared with the control values ($p < 0.05$).

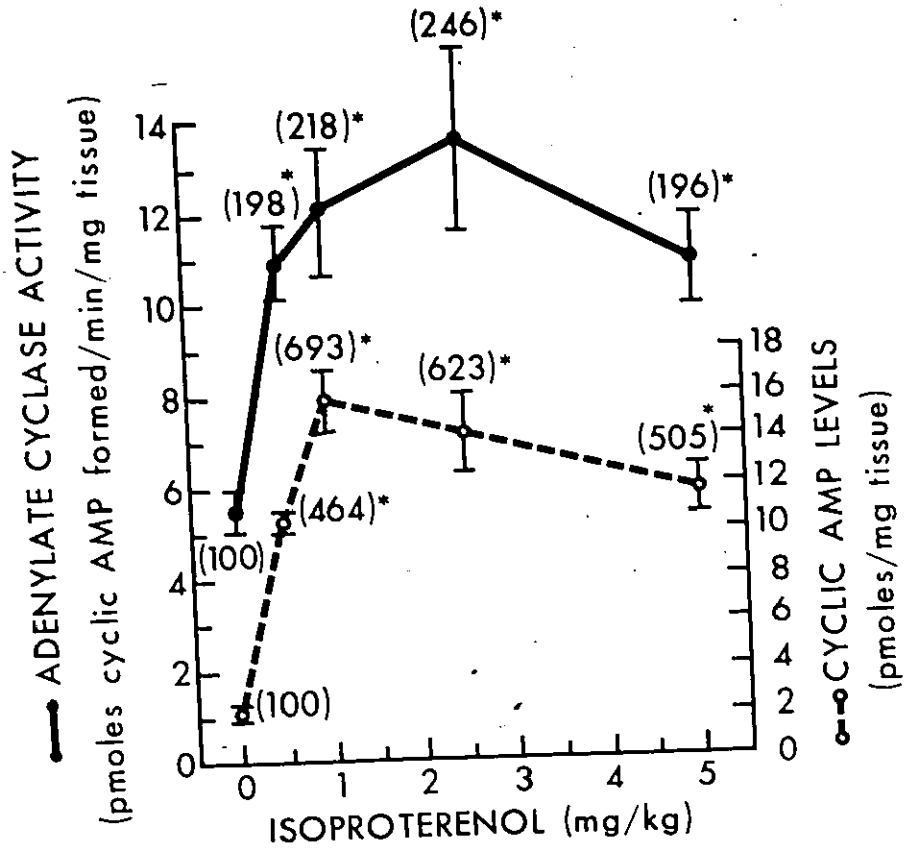


FIG. 17

Effect of various doses of isoproterenol on prostatic adenylate cyclase and cyclic AMP. Each point represents the mean ± S.E.M. of 7 animals in each group. Rats were injected intraperitoneally with different doses of DL-isoproterenol and sacrificed 5 min after the administration of the agonist. Whereas adenylate cyclase activity (●——●) was calculated as pmoles cyclic AMP formed per mg of tissue, endogenous cyclic AMP levels (o- - -o) were expressed as pmoles cyclic AMP per mg tissue. Data are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

cyclic nucleotide was observed with 1 mg/kg dose of isoproterenol. Administration of higher dosages of the agonist was found to be less stimulatory. The responsiveness of the prostatic adenylate cyclase to stimulation by isoproterenol was, in some respect, similar to the pattern of the elevation of the cyclic nucleotide levels. While maximal stimulation of adenylate cyclase activity (98%) was statistically obtained with 0.5 mg/kg isoproterenol, increasing the dose of the agonist to 1.0, 2.5 and 5.0 mg/kg produced little or no further increase in the activity of the enzyme. Although the prostatic adenylate cyclase activity was significantly elevated at all dose levels examined, the percent stimulation was lower than that observed for the cyclic nucleotide.

C. Effects of Propranolol and Phentolamine on Isoproterenol-Stimulated Prostatic Adenylate Cyclase and Cyclic AMP

The influence of propranolol on isoproterenol-stimulated adenylate cyclase activity and endogenous cyclic AMP levels in the rat ventral prostate is shown in Figure 18. Five min following the administration of isoproterenol (1 mg/kg), prostatic adenylate cyclase activity was significantly increased to 219% of the saline-injected controls. Whereas propranolol (3 mg/kg) alone had little or no effect on the activity of the prostatic enzyme, treatment with this antagonist 30 min prior to isoproterenol significantly reduced enzyme activity when compared to that seen with the β-adrenergic effector alone. Similarly, although propranolol caused only a slight reduction in the endogenous cyclic AMP levels of the prostate gland, stimulation by isoproterenol was significantly inhibited when the animals were pretreated with this β-adrenergic antagonist (Fig. 18). In contrast, administration of phentolamine, even at a dose of 5 mg/kg, failed to block the effect of isoproterenol on the activity of the prostatic enzyme (Table 19).

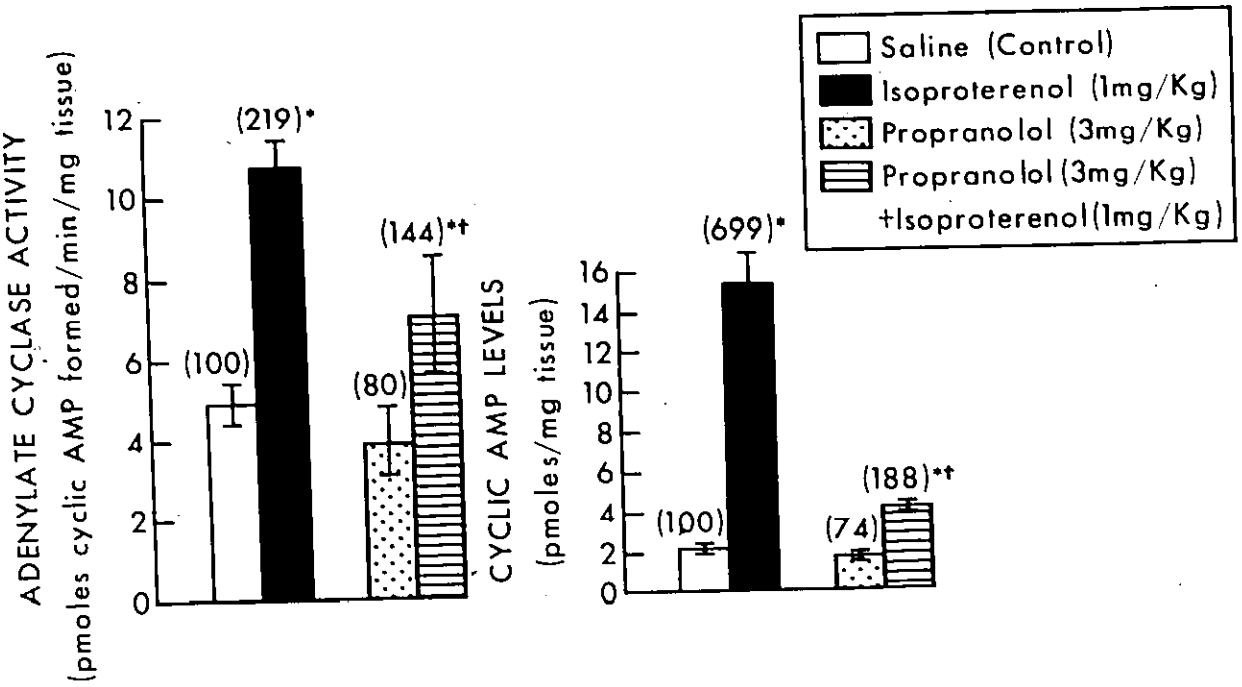


FIG. 18

Effects of isoproterenol and propranolol on prostatic cyclic AMP metabolism. Each bar represents the mean ± S.E.M. of 7 animals in each group. DL-isoproterenol was administered intraperitoneally at a dose of 1 mg/kg and the animals killed 5 min later. Propranolol (3 mg/kg, i.p.) was administered 30 min prior to sacrifice. Whereas adenylate cyclase activity was expressed as pmoles cyclic AMP formed per min per mg tissue, endogenous cyclic AMP levels were calculated as pmoles cyclic AMP per mg tissue. Data are also given in percentages (in parentheses) taking the values of control animals as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of isoproterenol-treated rats ($p < 0.05$).

TABLE 19

EFFECTS OF ISOPROTERENOL AND PHENTOLAMINE ON PROSTATIC ADENYLATE CYCLASE ACTIVITY

Values represent means ± S.E.M. of 7 animals per group. DL-Isoproterenol (1 mg/kg) was injected intraperitoneally and animals were killed 5 min later. Phentolamine (5 mg/kg, i.p.) was administered 30 min prior to sacrifice. Data are also given in percentages taking the control values as 100%.

	Adenylate Cyclase Activity (pmoles cyclic AMP formed/ min/mg tissue)	% of Control
Control (saline)	4.91±0.46	100
Isoproterenol (1 mg/kg)	10.77±0.61	219*
Phentolamine (5 mg/kg)	4.24±0.29	86
Isoproterenol (1 mg/kg) + Phentolamine (5 mg/kg)	12.57±1.36	256*

*Statistically significant difference when compared with the control values ($p < 0.05$).

D. Effects of Isoproterenol and Propranolol on Soluble Protein Kinase Activity in Rat Ventral Prostate

In view of the evidence suggesting that protein kinases play an important role in mediating the action of cyclic AMP, the influence of isoproterenol (1 mg/kg) and propranolol (3 mg/kg) was investigated on the activities of soluble prostatic cyclic AMP-dependent and -independent protein kinases 5 min following the administration of the agonist. As shown in Table 20, stimulation of the prostatic protein kinase by isoproterenol was associated with a decrease (33%) in the activity of the cyclic AMP-dependent protein kinase and a concomitant increase (25%) in that of the cyclic nucleotide-independent enzyme. The kinase activity ratio, obtained by dividing protein kinase activity in the absence of cyclic AMP by that in the presence of the nucleotide, was significantly increased from 0.51±0.05 to 0.95±0.08. Whereas propranolol alone (given 30 min prior to sacrifice) had little or no effect on cyclic AMP-dependent and -independent protein kinase activity and on the kinase activity ratio, it not only partially inhibited the isoproterenol-induced alterations in cyclic AMP-dependent protein kinase, but completely blocked the increases in the kinase activity ratio as well as in the activity of cyclic AMP-independent enzyme seen with isoproterenol (Table 20).

E. Influence of Isoproterenol and Propranolol on Cyclic AMP Binding Capacity of Prostatic Soluble Protein Kinase

Results presented in Figure 19 illustrate the effects of isoproterenol and propranolol on the cyclic AMP binding capacity of soluble prostatic protein kinase, a parameter that could be interpreted to indicate the number of non-saturated cyclic AMP binding sites present. Five min following the injection of isoproterenol (1 mg/kg), the ability of the enzyme to bind (^{3}H)-cyclic AMP *in vitro* was significantly decreased (54%) from 4.88±0.35 to 2.23±0.13 pmoles cyclic AMP bound/mg protein. Whereas propranolol alone exerted little or no effect on the number of cyclic AMP binding sites, the effects of isoproterenol

TABLE 20

EFFECTS OF ISOPROTERENOL AND PROPRANOLOL ON SOLUBLE PROTEIN KINASE ACTIVITY IN RAT VENTRAL PROSTATE

Values represent means ± S.E.M. of 7 animals per group. Protein kinase activity was expressed in pmoles ^{32}P incorporated into histone (Type IIA, Sigma) per mg protein in the presence or absence of cyclic AMP ($5x10^{-6}M$). Kinase activity ratio was calculated by dividing protein kinase activity in the absence of cyclic AMP by that in the presence of the nucleotide. Data are also given in percentages (in parentheses) taking the control values as 100%. See legend to Fig. 18 for experimental details.

	Protein Kinase Activity		Kinase Activity Ratio
	-Cyclic AMP	+Cyclic AMP	$\frac{\text{-Cyclic AMP}}{\text{+Cyclic AMP}}$
Control (saline)	54.96±5.80 (100)	108.42±7.62 (100)	0.51±0.05 (100)
Isoproterenol (1 mg/kg)	68.68±3.74 (125)*	72.36±2.22 (67)*	0.95±0.08 (186)*
Propranolol (3 mg/kg)	48.72±4.70 (89)	99.82±1.88 (92)	0.49±0.05 (96)
Isoproterenol (1 mg/kg) + Propranolol (3 mg/kg)	58.81±5.80 (107)	88.09±3.39 (81)*	0.67±0.08 (131)

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of isoproterenol-treated rats ($p < 0.05$).

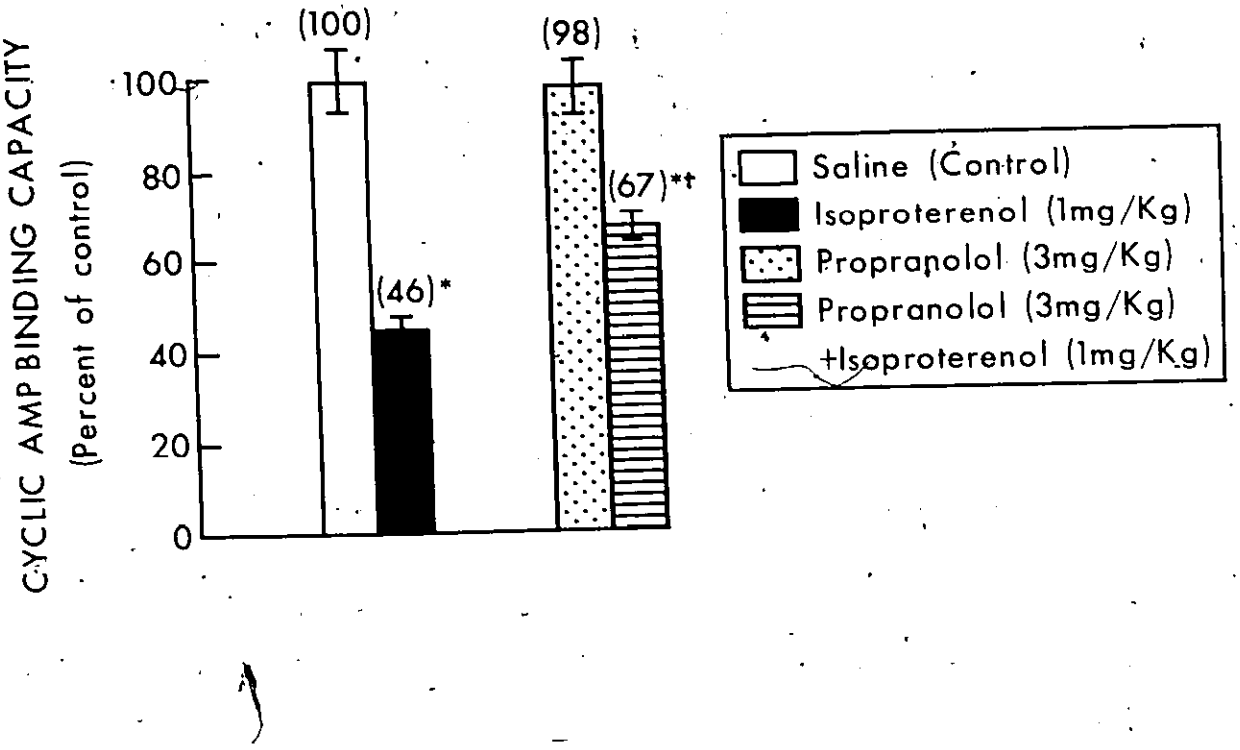


FIG. 19

Influence of isoproterenol and propranolol on cyclic AMP binding capacity of prostatic soluble protein kinase. Each bar represents the mean ± S.E.M. of 7 animals in each group. Data are given in percentages taking the values of control rats as 100%. See legend to Fig. 18 for experimental details.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of isoproterenol-treated rats ($p < 0.05$).

were significantly blocked when the animals were pretreated with this antagonist.

F. Beta-Adrenergic Stimulation of Prostatic Adenylate Cyclase Following Castration and Testosterone Replacement

Data presented in Table 21 illustrate the in vivo effects of isoproterenol on prostatic adenylate cyclase activity during androgenic deprivation and repletion. Intraperitoneal administration of isoproterenol (1 mg/kg) markedly stimulated prostatic adenylate cyclase in intact controls, castrates as well as in castrates given testosterone, regardless of whether enzyme activity was expressed on a per unit tissue weight or a per organ weight basis. While the percent stimulation of prostatic enzyme in the castrate group appeared to be only slightly lower than that of controls, the enzyme activity in the saline- as well as the isoproterenol-treated, androgen-deprived animals was considerably lower than intact controls. Testosterone replacement not only restored the responsiveness of prostatic adenylate cyclase to β-adrenergic stimulation, but significantly enhanced the activity of this enzyme both in the presence and absence of isoproterenol treatment (Table 21).

G. Effect of Isoproterenol on Prostatic Cyclic AMP Levels in Androgen-Deprived Rats

To further examine the influence of β-adrenergic stimulation on prostatic cyclic AMP metabolism, the effects of isoproterenol (1 mg/kg, i.p.) on the endogenous cyclic AMP levels of intact controls, castrates and castrates treated with testosterone (5 mg/100 g/day, i.m.) were investigated. As shown in Table 22 , administration of isoproterenol markedly elevated prostatic cyclic AMP levels in all three groups studied, regardless of the basis of expression used. Although the degree of stimulation elicited by the β-adrenergic agonist in the castrate group was only slightly different from intact controls, the cyclic nucleotide levels after isoproterenol administration were however, considerably lower. While administration of testosterone not only restored

TABLE 21

EFFECT OF ISOPROTERENOL ON PROSTATIC ADENYLATE CYCLASE ACTIVITY FOLLOWING CASTRATION AND TESTOSTERONE REPLACEMENT

Values represent the mean ± S.E.M. of 7-8 rats per group. Rats were sham-operated or castrated for 96 hr and daily testosterone administration (5 mg/100 g/day, i.m.) for 4 days was initiated on the day of castration. Animals not receiving testosterone were given an equal volume of the vehicle (corn oil). Five min prior to sacrifice, rats were injected intraperitoneally with either DL-isoproterenol (1 mg/kg) or normal saline. Adenylate cyclase activity was expressed as pmoles of cyclic AMP formed per min per mg of tissue and nmoles of the nucleotide formed per min per prostate. Data are also given in percentages (in parentheses) taking the values of the saline-treated groups as 100%.

Treatment	Adenylate Cyclase Activity			
	pmoles cAMP formed/min/mg tissue		nmoles cAMP formed/min/prostate	
	Saline	Isoproterenol	Saline	Isoproterenol
Control	4.89±0.46 (100)	10.79±0.61 (221)*	2.21±0.21 (100)	4.74±0.24 (214)*
Castrate	2.83±0.37 (100)	4.53±0.59 (160)*	0.75±0.06 (100)	1.17±0.14 (156)*
Castrate + Testosterone	5.26±0.41 (100)	13.05±1.34 (248)*	4.97±0.30 (100)	14.42±1.48 (290)*

*Statistically significant difference when compared with the values of the saline-treated rats in their respective groups ($p < 0.05$).

TABLE 22

EFFECT OF ISOPROTERENOL ON PROSTATIC CYCLIC AMP LEVELS FOLLOWING CASTRATION AND TESTOSTERONE REPLACEMENT

Values represent the means ± S.E.M. of 7-8 rats per group. Rats were sham-operated or castrated for 96 hr and daily testosterone administration (5 mg/100 g/day, i.m.) for 4 days was initiated on the day of castration. Animals not receiving testosterone were given an equal volume of the vehicle (corn oil). Five min prior to sacrifice, rats were injected intraperitoneally with either DL-isoproterenol (1 mg/kg) or normal saline. Prostatic cyclic AMP levels were expressed as pmoles cyclic AMP per mg of tissue or nmoles of the nucleotide per prostate. Data are also given in percentages (in parentheses) taking the values of the saline-treated groups as 100%.

Treatment	Cyclic AMP Levels			
	pmoles/mg tissue		nmoles/prostate	
	Saline	Isoproterenol	Saline	Isoproterenol
Control	2.29±0.32 (100)	15.89±1.30 (693)*	1.03±0.14 (100)	7.15±0.59 (728)*
Castrate	1.58±0.42 (100)	9.77±0.99 (618)*	0.42±0.11 (100)	3.08±0.25 (733)*
Castrate + Testosterone	1.20±0.10 (100)	15.79±1.85 (1315)*	1.13±0.08 (100)	17.45±2.00 (1544)*

*Statistically significant difference when compared with the values of the saline-treated rats in their respective groups ($p < 0.05$).

prostatic cyclic AMP levels in the isoproterenol-treated animals, the responsiveness of the adenylate cyclase-cyclic AMP system to stimulation by the adrenergic agonist in androgen-treated animals was greater when compared to those seen in the androgen-deprived group (Table 22).

5. INFLUENCE OF CYPROTERONE ACETATE ON PROSTATIC CYCLIC AMP METABOLISM

A. Effects of Cyproterone Acetate on Body and Male Sex Organ Weights

The influence of daily administration of cyproterone acetate on body and sex organ weights of adult male rats at various time intervals (1, 3, 5 and 10 days) is presented in Table 23 . Cyproterone acetate (1.25 mg/100 g/day, s.c.) significantly retarded both growth in a time-dependent manner and by the 10th day, the body weight was reduced to 87% of controls. In contrast, testicular weights were not significantly altered during the course of the study (Table 23).

The effects of cyproterone acetate on male accessory sex gland weights are shown in Figure 20. Daily administration of cyproterone acetate significantly decreased the weights of the ventral prostate, seminal vesicles and coagulating glands. While the coagulating glands appeared to be most sensitive to cyproterone acetate treatment (organ weight significantly lowered to 77% and 48% of controls in 24 hr and 10 days respectively), the seminal vesicular and prostatic weights were also lowered (58% and 53% respectively) after the 10 day treatment (Fig. 20).

B. Effect of Cyproterone Acetate Withdrawal and Simultaneously Administered Testosterone on Male Sex Organ Weights

Table 24 shows the effects of cyproterone acetate withdrawal and simultaneously administered testosterone on body and male accessory gland weights. Whereas cyproterone acetate administration for 10 days resulted in marked decreases in seminal vesicular, prostatic and coagulating gland weights, subsequent withdrawal of the antiandrogen treatment for 10 days significantly increased the weights to 79%, 65% and 64%, respectively. It

TABLE 23

EFFECTS OF CYPROTERONE ACETATE ON TESTICULAR AND BODY WEIGHTS

Values represent means ± S.E.M. of 10 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

Groups		Body Weight (g)	Testis Weight (g)
Control		411±14 (100)	3.10±0.14 (100)
Cyproterone Acetate,	1 day	403±11 (98)	3.09±0.11 (100)
	3 days	374±7 (91)*	2.96±0.10 (95)
	5 days	367±6 (89)*	3.00±0.10 (97)
	10 days	359±8 (87)*	3.03±0.09 (98)

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

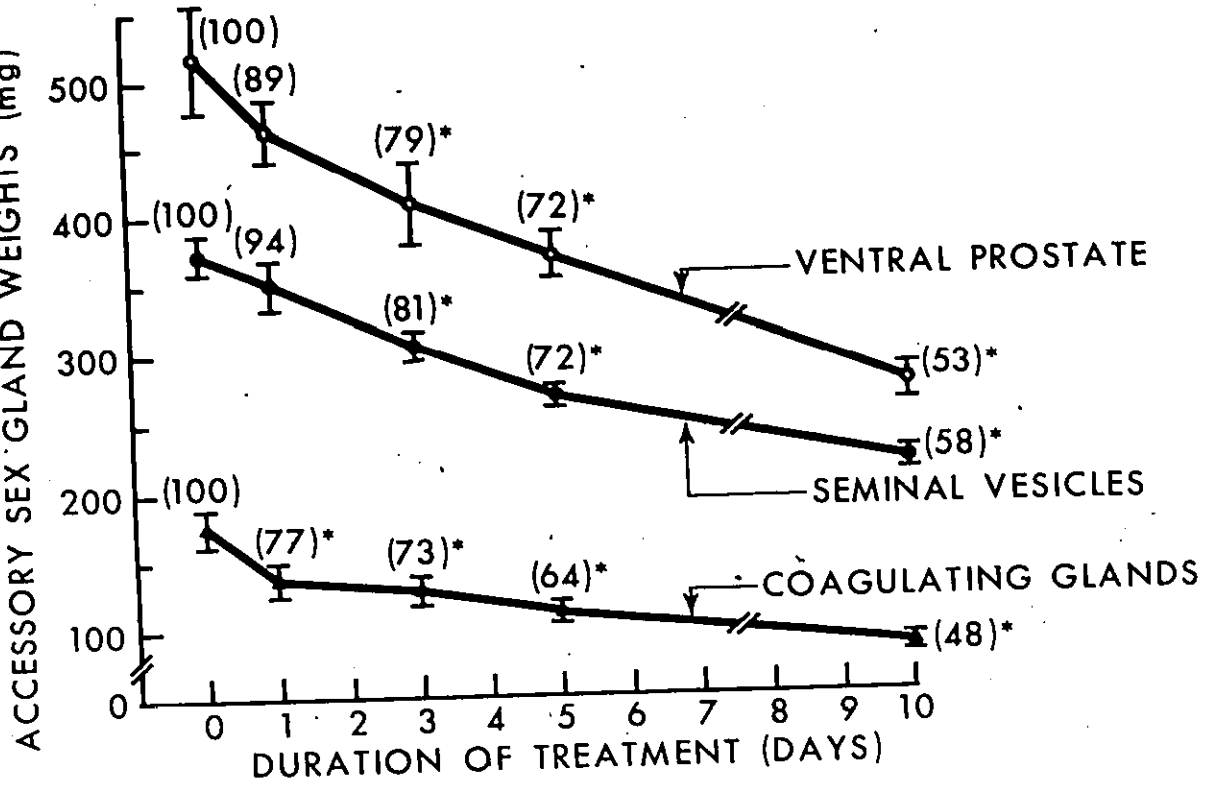


FIG. 20

Effects of cyproterone acetate on accessory sex gland weights. Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

TABLE 24

EFFECTS OF CYPROTERONE ACETATE WITHDRAWAL AND SIMULTANEOUS ADMINISTRATION OF TESTOSTERONE ON BODY AND SEX ACCESSORY GLAND WEIGHTS

Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. Another group of rats were maintained for an additional 10 days without any further treatment with the anti-androgen. Control animals received an equal volume of the vehicle. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

Groups	Body (g)	Sem. Ves. (mg)	V. Prost. (mg)	Coag. Gland (mg)
Controls	412±14 (100)	372±15 (100)	516±31 (100)	176±13 (100)
Cyproterone acetate	359±8 (87)*	217±7 (58)*	273±13 (53)*	85±4 (48)*
Cyproterone acetate + withdrawn	333±5 (81)*	294±16 (79)*†	337±27 (65)*†	112±11 (64)*†
Cyproterone acetate + Testosterone	358±9 (87)*	671±25 (180)*†	778±50 (151)*†	348±21 (197)*†

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

should however, be noted that while significant recovery in organ weights was seen during anti-androgen withdrawal in all three cases, normal levels were not achieved even after 10 days. In contrast, simultaneous administration of testosterone (5 mg/100 g/day) not only effectively antagonized these effects of cyproterone acetate, but significantly stimulated weight gain in these sex glands by 50-100%. On the contrary, while cyproterone acetate also significantly decreased body weight, neither withdrawal of the anti-androgen nor concurrent administration of testosterone appeared to have any effects on the weight gain of these animals (Table 24).

C. Effects of Cyproterone Acetate on Prostatic Adenylate Cyclase Activity and Cyclic AMP Levels

The influence of cyproterone acetate on adenylate cyclase activity and cyclic AMP levels in the ventral prostate of adult rats was examined at 1, 3, 5 and 10 days following the daily administration of this anti-androgen (5 mg/rat/day, s.c.). As demonstrated in Table 25, cyproterone acetate progressively decreased prostatic adenylate cyclase activity, regardless of whether enzyme activity was expressed on a per mg tissue or a total organ weight basis. While adenylate cyclase activity dropped markedly (55%) during the first 24 hr, only 15-25% of the initial activity remained after the 10 day administration of the anti-androgen (Table 25).

Analogous to adenylate cyclase, cyproterone acetate treatment also decreased the cyclic AMP content of the prostate gland (Fig. 21). Twenty-four hr following the first injection of the anti-androgen, prostatic cyclic AMP was significantly decreased from 849±39 to 605±65 pmoles per prostate and by the 5th day, it was only about 35% of the control values. Continuation of anti-androgen treatment for another 5 days failed to elicit any further decrease in the cyclic nucleotide content of the prostate gland. Although cyclic AMP levels as expressed on a per mg tissue basis, were slightly lowered during

TABLE 25

EFFECTS OF CYPROTERONE ACETATE ON PROSTATIC ADENYLATE CYCLASE ACTIVITY

Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Adenylate cyclase activity was calculated as pmoles cyclic AMP formed per min and expressed as per mg of tissue and per prostate. Data are also given in percentages (in parentheses) taking the values of the control group as 100%.

Groups	Adenylate Cyclase Activity (pmoles cyclic AMP formed/min)	
	per mg tissue	per prostate
Controls	4.82±0.23 (100)	2482±131 (100)
Cyproterone Acetate, 1 day	2.22±0.19 (46)*	1191±103 (48)*
3 days	1.54±0.17 (32)*	769±58 (31)*
5 days	1.59±0.10 (33)*	521±52 (21)*
10 days	1.25±0.11 (26)*	422±40 (17)*

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

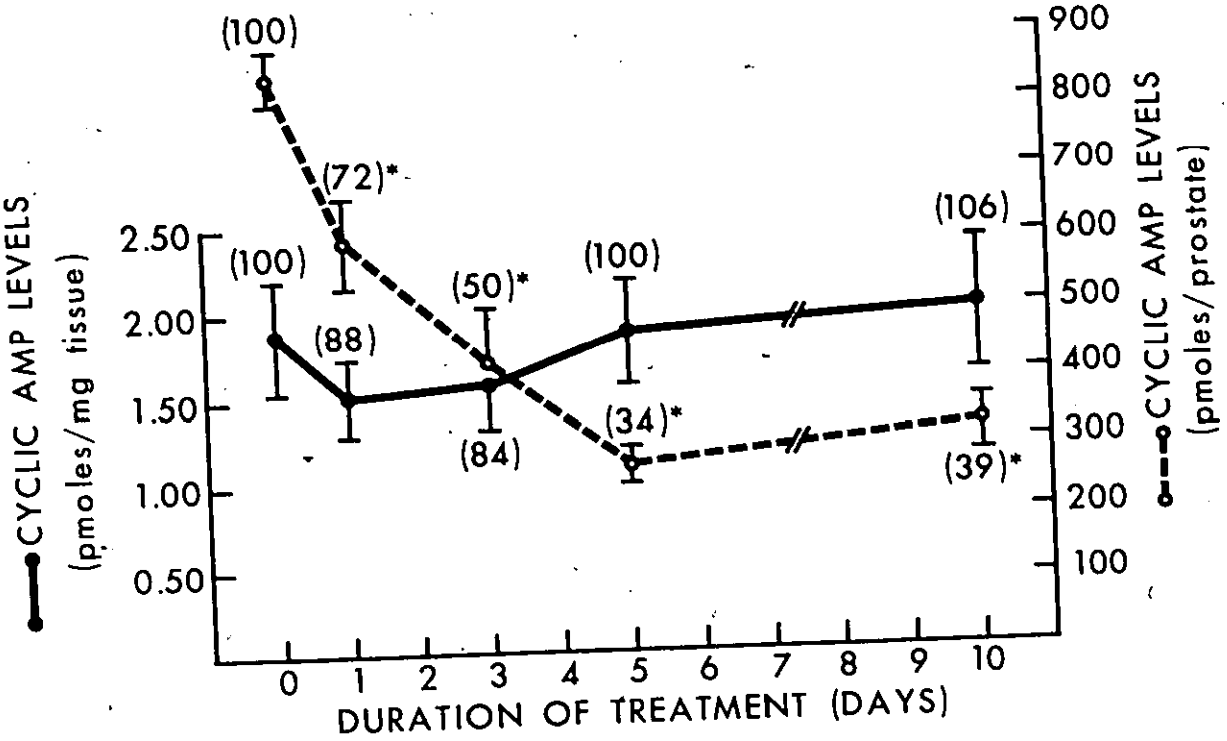


FIG. 21

Effects of cyproterone acetate on prostatic cyclic AMP levels. Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Endogenous cyclic AMP levels were expressed as pmoles cyclic AMP per mg of tissue and per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

the first 3 days of cyproterone acetate treatment, the differences between the "treated groups" and the "controls" were statistically non-significant.

D. Effects of Cyproterone Acetate Withdrawal and Simultaneous Testosterone Administration on Adenylate Cyclase and Cyclic AMP

In order to determine if the observed alterations persist after the cessation of anti-androgen therapy, prostatic adenylate cyclase and cyclic AMP were examined in rats that had been given cyproterone acetate for 10 days and subsequently maintained without further treatment for another 10 days. Data in Figure 22 show that daily administration of cyproterone acetate (1.25 mg/100 g) for 10 days resulted in a marked decrease in the activity of prostatic adenylate cyclase whether expressed on the basis of per mg of tissue (74%) or per prostate (83%). Although subsequent withdrawal of cyproterone acetate significantly increased the enzyme activity by approximately 2-fold of that observed in the cyproterone acetate-treated group, it was still significantly lower than that of controls. In contrast, concurrent administration of testosterone (5 mg/100 g/day, s.c. for 10 days) during anti-androgen treatment not only antagonized the effect of cyproterone acetate on prostatic adenylate cyclase (activity expressed per mg of tissue), but significantly enhanced the enzyme activity by 40% of the control group (when expressed on a per prostate basis) (Fig. 22).

Results in Figure 23 show that whereas cyclic AMP levels (per prostate) were markedly lowered (62%) following the 10 day cyproterone acetate administration, normal values were attained after subsequent withdrawal of the anti-androgen treatment for 10 days. However, when the nucleotide levels were expressed on a per mg tissue basis, neither cyproterone acetate treatment nor its discontinuation appeared to have any significant effect (Fig. 23). Moreover, whereas concurrent administration of testosterone (5 mg/100 g/day, 10 days) during anti-androgen treatment resulted in a further decrease in prostatic cyclic AMP levels from 1.88±0.37 to 0.99±0.08 pmoles per mg of tissue,

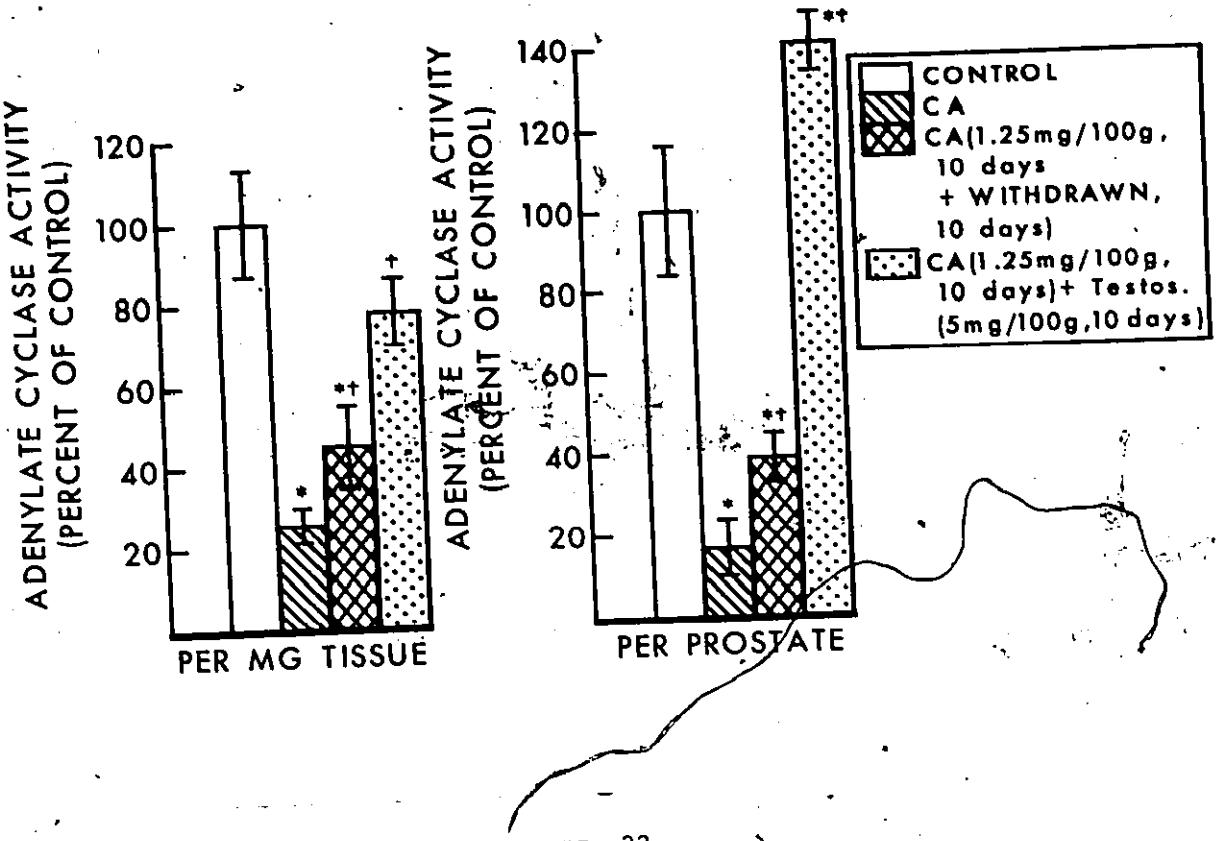


FIG. 22

Effects of cyproterone acetate withdrawal and simultaneous administration of testosterone on prostatic adenylate cyclase. Each bar represents the mean ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. In another group of rats, free testosterone (5 mg/100 g/day) dissolved in 0.3 ml of the same vehicle was injected simultaneously with cyproterone acetate via the subcutaneous route. Another group of rats treated with cyproterone acetate for 10 days was maintained for an additional 10 days without any further treatment with the antiandrogen. Control animals received an equal volume of the vehicle. Adenylate cyclase activity was calculated as pmoles cyclic AMP formed per min per mg of tissue and per prostate and expressed in percentages taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

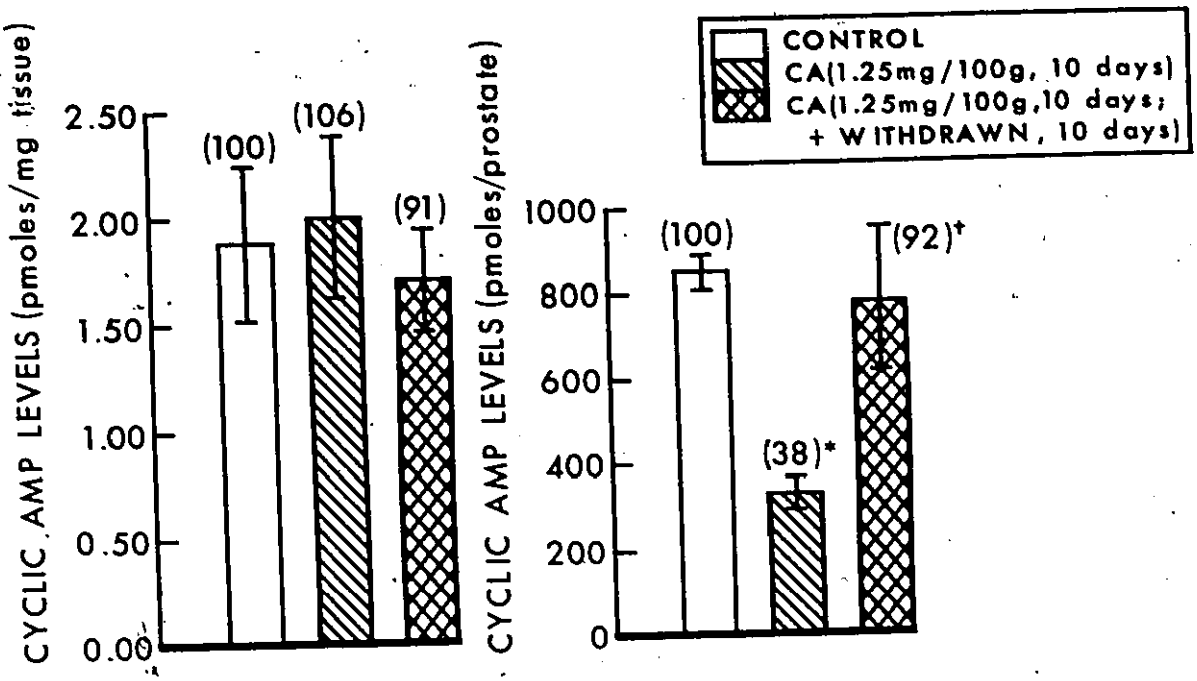


FIG. 23

Effects of cyproterone acetate administration and subsequent withdrawal on prostatic cyclic AMP levels. Each bar represents means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. Another group of rats were maintained for an additional 10 days without any further treatment with the antiandrogen. Control animals received an equal volume of the vehicle. Endogenous cyclic AMP levels were expressed as pmoles cyclic AMP per mg of tissue or per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

the effect of cyproterone acetate was effectively blocked when the cyclic nucleotide levels were expressed on a total prostate basis (Table 26).

E. Effects of Cyproterone Acetate on Soluble Prostatic Protein Kinase and Cyclic AMP Binding Activities

The effects of daily administration of cyproterone acetate (1.25 mg/100 g/day, 10 days) on cytosolic protein kinase activities and cyclic AMP binding capacity of the ventral prostate also were investigated. Figure 24 shows that the specific activity of the soluble prostatic protein kinase assayed in both the absence and presence of exogenous cyclic AMP increased progressively with the duration of cyproterone acetate treatment. However, the total cyclic AMP-dependent and independent protein kinase activity (expressed on the total organ weight basis) was markedly decreased (58% and 74%, respectively) by the 10th day. Although protein kinase activity ratio was also lowered significantly during the first 3 days of cyproterone acetate administration, this change was not apparent when the anti-androgen was given for 5 or 10 days (Fig. 24).

The influence of cyproterone acetate on soluble prostatic cyclic AMP binding capacity of protein kinase was similar to that seen on the cyclic AMP-dependent protein kinase activity (Fig. 25). While the ability of the enzyme to bind (^{3}H)-cyclic AMP *in vitro* was significantly enhanced with the duration of cyproterone acetate treatment when expressed on a per unit protein basis, a marked decrease (37%) was seen in the total cyclic AMP binding capacity of the prostate after 10 days. Although this response appeared to be slightly greater during the early phase of the study, the differences between 1 and 3 day cyproterone acetate treated rats and controls were statistically non-significant (Fig. 25).

F. Influence of Cyproterone Acetate Administration and Subsequent Withdrawal on Cytosolic Prostatic Protein Kinase Activity and Cyclic AMP Binding Capacity

Data presented in Figure 26 illustrate the effects of 10 day cyproterone acetate administration and subsequent discontinuation of the anti-androgen

TABLE 26

EFFECTS OF TESTOSTERONE ON CYPROTERONE ACETATE-INDUCED ALTERATIONS IN PROSTATIC CYCLIC AMP LEVELS

Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. In another group of rats, free testosterone (5 mg/100 g/day) dissolved in 0.3 ml of the same vehicle was injected simultaneously with cyproterone acetate via the subcutaneous route. Control animals received an equal volume of the vehicle. Endogenous cyclic AMP levels were expressed as pmoles cyclic AMP per mg of tissue or per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

Groups	Cyclic AMP Levels (pmoles)	
	per mg tissue	per prostate
Control	1.88±0.37 (100)	849±39 (100)
Cyproterone acetate	2.00±0.38 (106)	326±39 (38)*
Cyproterone acetate + Testosterone	0.99±0.08 (53)*	838±88 (98)†

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

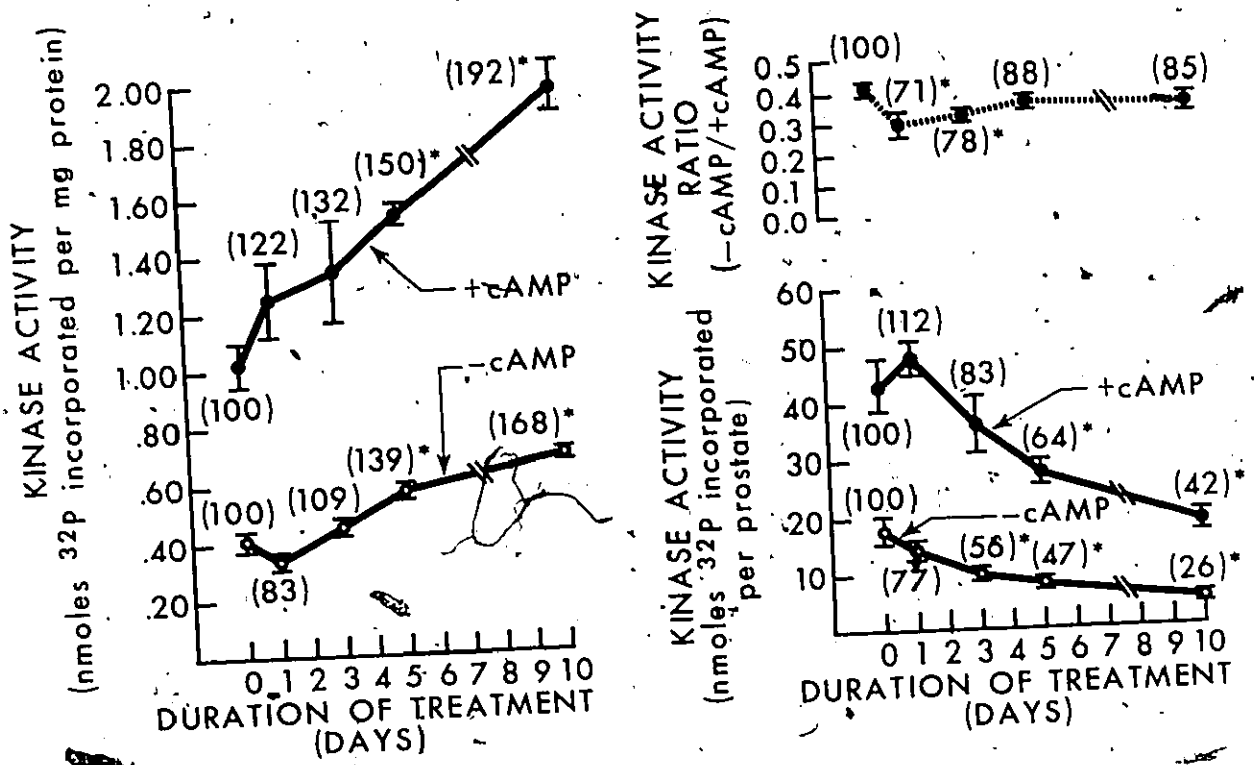


FIG. 24

Effects of cyproterone acetate on soluble prostatic protein kinase activity. Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Protein kinase activity was expressed as nmoles ^{32}P incorporated into histone mixture (Type IIA) per mg protein and per prostate in the absence (-) or presence (+) of cyclic AMP (5×10^{-6}M). Kinase activity ratio was defined as the ratio of protein kinase activity assayed in the absence of cyclic AMP to that in the presence of the nucleotide. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

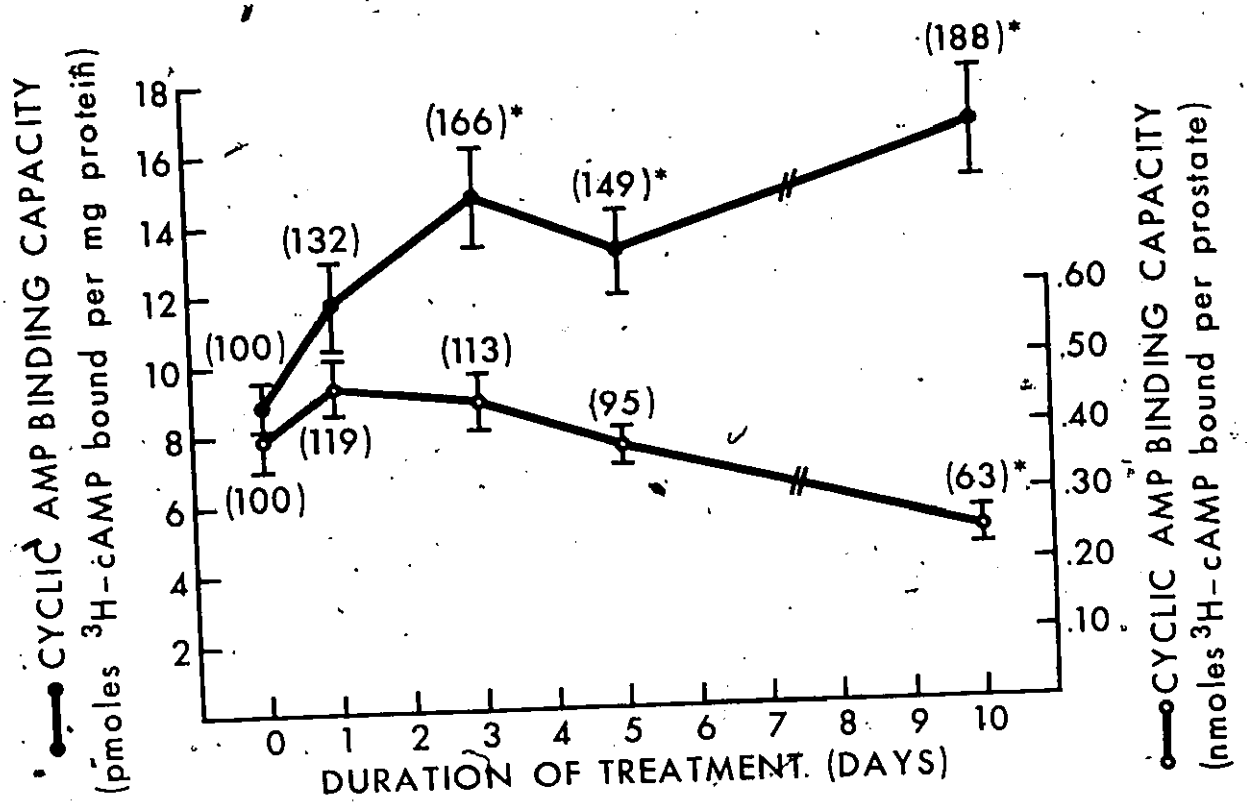


FIG. 25

Effects of cyproterone acetate on soluble prostatic cyclic AMP binding capacity. Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Cyclic AMP binding capacity was expressed as pmoles (^{3}H)-cyclicAMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

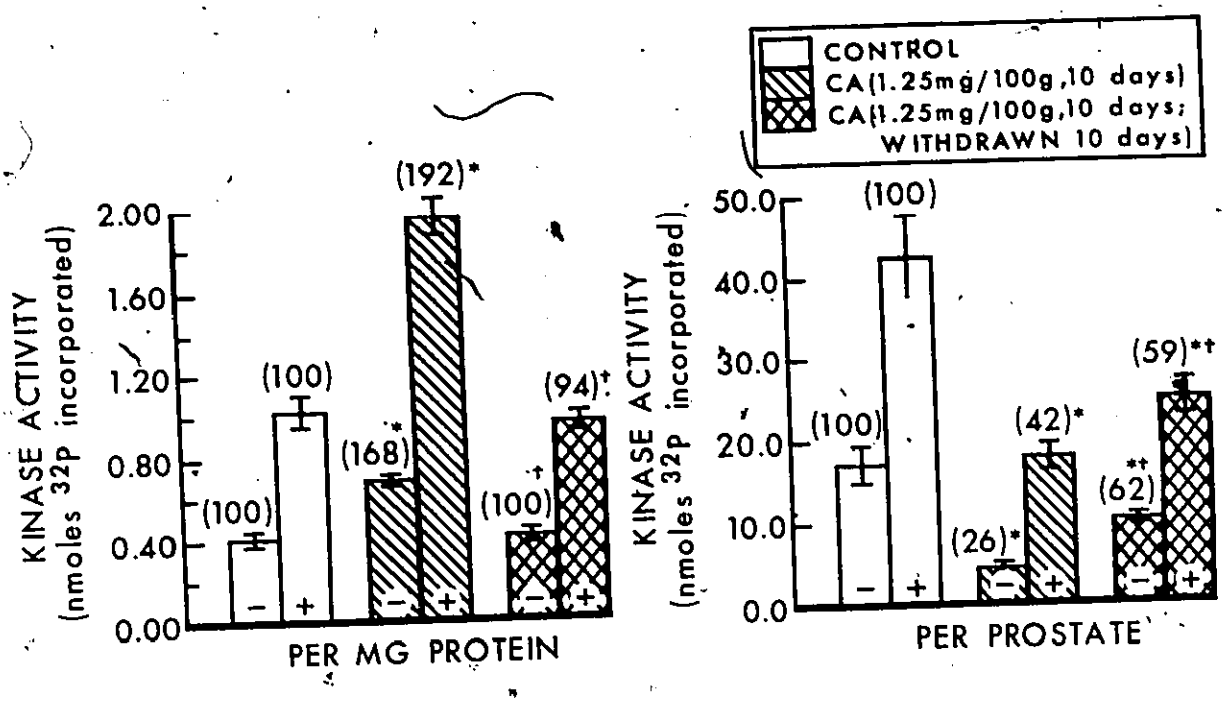


FIG. 26

Influence of cyproterone acetate administration and subsequent withdrawal on soluble prostatic protein kinases. Each bar represents the mean ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. Another group of rats were maintained for an additional 10 days without any further treatment with the antiandrogen. Control animals received an equal volume of the vehicle. Protein kinase activity was expressed as nmoles ^{32}P incorporated into histone mixture (Type IIA) per mg protein and per prostate in the absence (-) or presence (+) of cyclic AMP (5×10^{-6} M). Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

treatment on cytosolic cyclic AMP-dependent and-independent protein kinases. While the specific activities of the prostatic enzymes were markedly elevated from 1.02±0.07 and 0.41±0.03 to 1.96±0.09 and 0.69±0.03 nmoles ^{32}P incorporated per mg protein, respectively after 10 days of cyproterone acetate treatment, normal values were achieved 10 days following the withdrawal of the anti-androgen. In contrast, cyproterone acetate markedly decreased total protein kinase activity (expressed on a per prostate basis) to 26 and 42% of the controls when assayed in the absence and the presence of exogenous cyclic AMP, respectively. Although withdrawal of the anti-androgen significantly stimulated enzyme activities, complete recovery was not achieved after 10 days discontinuation of cyproterone acetate treatment (Fig. 26).

The influence of daily administration of cyproterone acetate and subsequent withdrawal on soluble prostatic cyclic AMP binding capacity was also examined. Table 27 shows that although cyproterone acetate markedly increased the specific cyclic AMP binding capacity of the cytosol from 8.78±0.71 to 16.49±1.53 pmoles (^{3}H)-cyclic AMP bound per mg of protein, a significant decrease was seen following the withdrawal of anti-androgen. Moreover, while the total capacity of the prostatic cytosol to bind (^{3}H)-cyclic AMP *in vitro* was significantly decreased by 37% after cyproterone acetate treatment, full recovery was achieved when the rats were maintained for additional 10 days without any further treatment with the anti-androgen.

G. Effects of Concurrent Administration of Testosterone on Cyproterone Acetate Induced Alterations in Cytosolic Protein Kinase Activity and Cyclic AMP Binding Capacity

Results in Figure 27 demonstrate the effects of simultaneously administered testosterone (5 mg/100 g, 10 days) on cyproterone acetate-induced changes in cytosolic cyclic AMP-dependent and-independent protein kinase activities. Whereas daily administration of cyproterone acetate for 10 days produced marked alterations in the activities of soluble protein kinases

TABLE 27

EFFECTS OF CYPROTERONE ACETATE AND SUBSEQUENT WITHDRAWAL ON SOLUBLE PROSTATIC CYCLIC AMP BINDING CAPACITY

Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. Another group of rats were maintained for an additional 10 days without any further treatment with the antiandrogen. Control animals received an equal volume of the vehicle. Cyclic AMP binding capacity was expressed as pmoles (^{3}H)-cyclic AMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

Groups	Cyclic AMP Binding Capacity (pmoles (^{3}H)-cAMP bound)	
	per mg protein	per prostate
Control	8.78±0.71 (100)	393.21±39.13 (100)
Cyproterone Acetate	16.49±1.53 (188)*	246.10±20.52 (63)*
Cyproterone Acetate + Withdrawn	12.51±0.56 (142)*†	327.05±29.36 (83)†

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

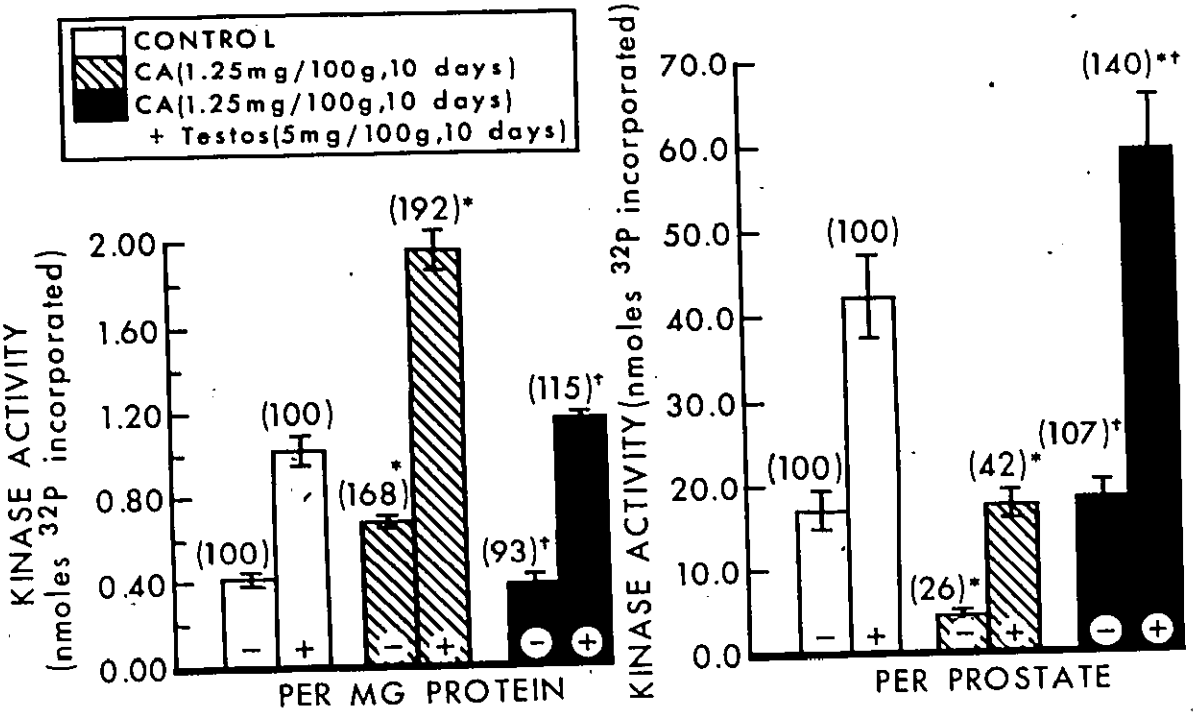


FIG. 27

Effects of testosterone on cyproterone acetate-induced alteration in soluble prostatic protein kinase activities. Each bar represents the mean ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. In another group of rats, free testosterone (5 mg/100 g/day) dissolved in 0.3 ml of the same vehicle was injected simultaneously with cyproterone acetate via the subcutaneous route. Protein kinase activity was expressed as nmoles ^{32}P incorporated into histone mixture (Type IIA) per mg protein and per prostate in the absence (-) or presence (+) of cyclic AMP (5×10^{-6}M). Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

(specific and total), concurrent administration of testosterone effectively antagonized these enzymatic responses (Fig. 27). Likewise, as shown in Figure 28 , alterations in the ability of the cytosol to bind (^{3}H) cyclic AMP in vitro in response to cyproterone acetate treatment were also blocked by the androgen.

H. Effects of Cyproterone Acetate on Protein Kinase Activity and Cyclic AMP Binding Capacity of Crude Nuclear Preparation from Rat Ventral Prostate

Since nuclear protein phosphorylation is known to play an important role in gene activation in eukaryotic cells (4), the influence of cyproterone acetate on the phosphorylation of endogenous substrates was examined in crude nuclear preparations of this tissue. As shown in Figure 29 , daily administration of this anti-androgen progressively lowered the activity of particulate prostatic protein kinase, as indicated by a decrease in the incorporation of ^{32}P from (γ-^{32}P)-ATP into endogenous nuclear substrates. This response was consistent regardless of whether the enzyme activity was expressed on a per protein or per organ weight basis. Studies on the kinase activity ratio demonstrated a lack of dependence of this particulate enzyme on cyclic AMP (kinase activity ratio being close to unity) as well as an insensitivity of this parameter to cyproterone acetate treatment (Fig. 29). However, as demonstrated in Figure 30 , crude nuclear preparations from the ventral prostate also possessed the ability to bind (^{3}H)-cyclic AMP in vitro. The specific cyclic AMP binding capacity decreased progressively with the duration of anti-androgen treatment and by the 10th day of cyproterone acetate administration, it was only 50% of control values. Although the total cyclic AMP binding capacity (per prostate) also decreased progressively with cyproterone acetate treatment, changes in the total affinity were not quite as pronounced as those seen with the specific capacity.

I. Effects of Cyproterone Acetate Administration and Subsequent Withdrawal on Particulate Protein Kinase Activity and Cyclic AMP Binding Capacity of Rat Ventral Prostate

Table 28 demonstrates that whereas daily administration of cyproterone

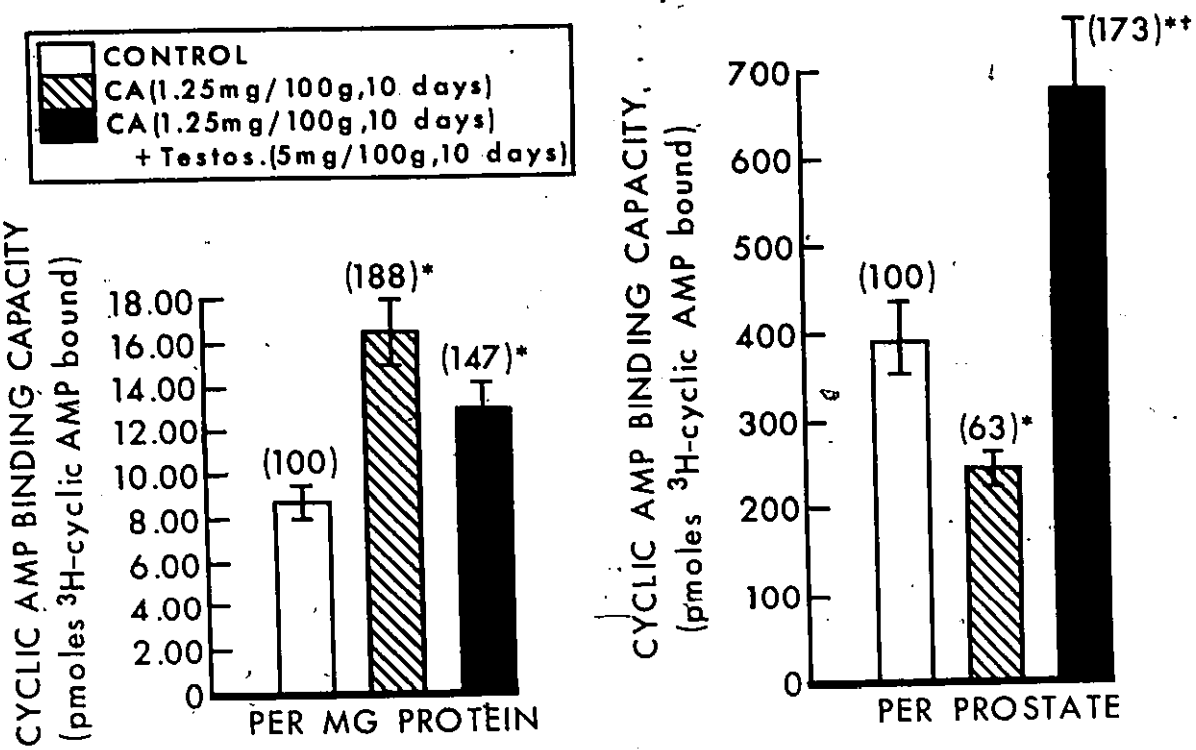


FIG. 28

Effect of testosterone on cyproterone acetate-induced alteration in soluble prostatic cyclic AMP binding capacity. Each bar represents the mean ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. In another group of rats, free testosterone (5 mg/100 g/day) dissolved in 0.3 ml of the same vehicle was injected simultaneously with cyproterone acetate via the subcutaneous route. Cyclic AMP binding capacity was expressed as pmoles (^{3}H)-cyclic AMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

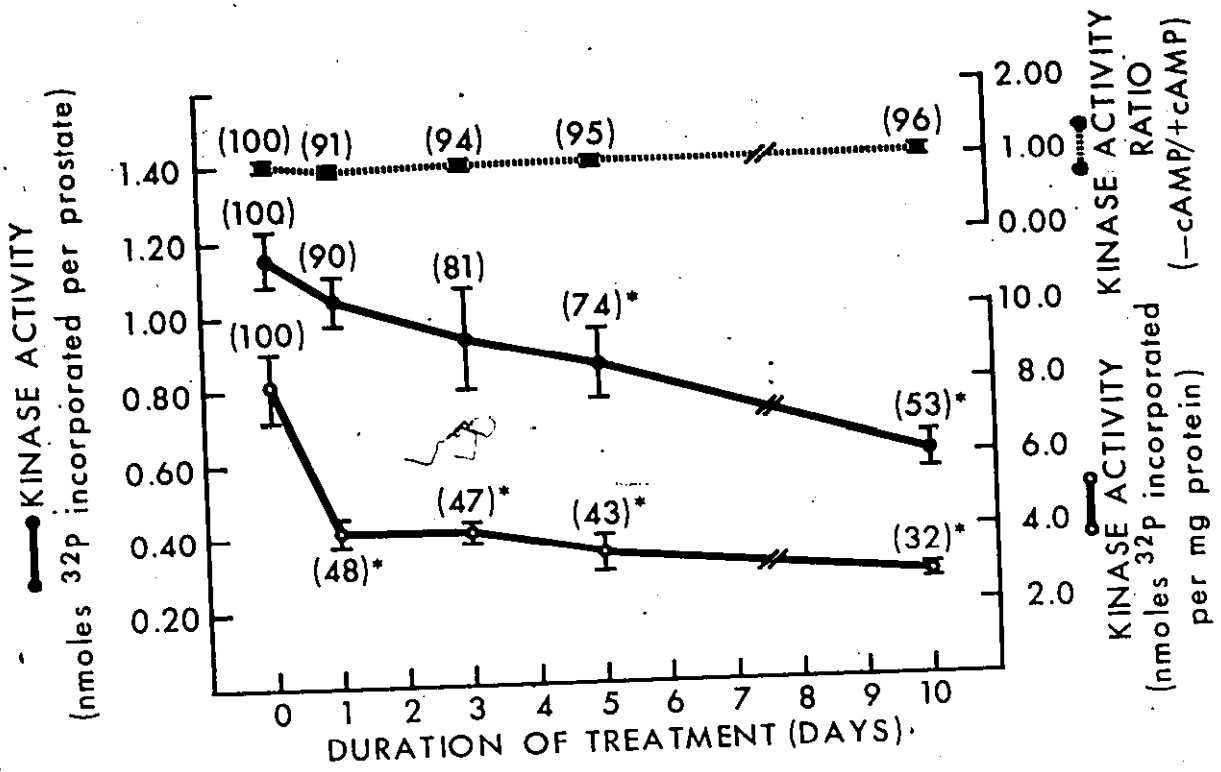


FIG. 29

Effects of cyproterone acetate on particulate protein kinase activity of rat ventral prostate. Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Protein kinase activity was expressed as nmoles ^{32}P incorporated into crude nuclear substrates per mg protein or per prostate in the absence of cyclic AMP. Kinase activity ratio was defined as the ratio of protein kinase activity assayed in the absence of cyclic AMP to that in the presence of the nucleotide (5×10^{-6}M). Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

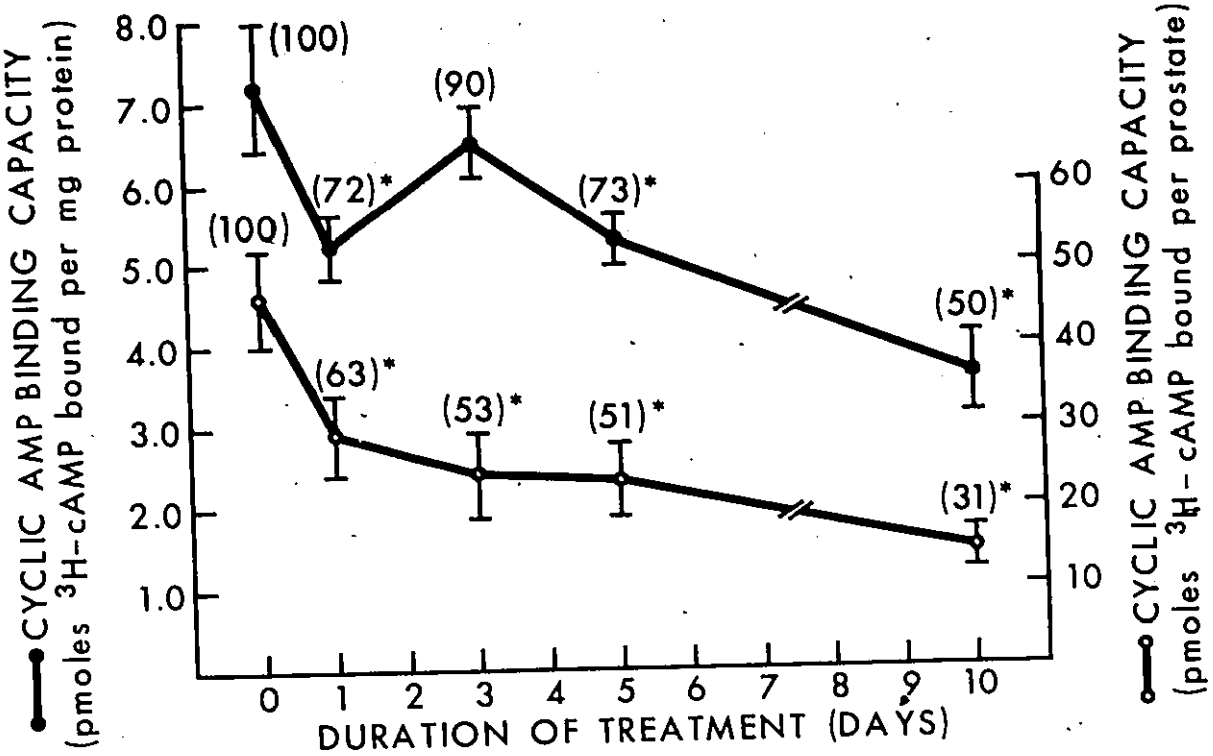


FIG. 30

Effect of cyproterone acetate on cyclic AMP binding capacity of crude nuclear fraction from rat ventral prostate. Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 1, 3, 5 and 10 days after the first injection. Control animals received an equal volume of the vehicle. Cyclic AMP binding capacity was expressed as pmoles (^{3}H)-cyclic AMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

TABLE 28

EFFECT OF CYPROTERONE ACETATE ADMINISTRATION AND SUBSEQUENT WITHDRAWAL ON PARTICULATE PROTEIN KINASE OF RAT VENTRAL PROSTATE

Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. Another group of rats were maintained for an additional 10 days without any further treatment with the antiandrogen. Control animals received an equal volume of the vehicle. Protein kinase activity was expressed as nmoles ^{32}P incorporated into crude nuclear substrates per mg protein and per prostate in the absence of cyclic AMP. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

Groups	Protein Kinase Activity (nmoles ^{32}P incorporated)	
	per mg protein	per prostate
Control	1.16±0.07 (100)	8.08±0.90 (100)
Cyproterone Acetate	0.61±0.05 (53)*	2.80±0.21 (32)*
Cyproterone Acetate + Withdrawn	0.81±0.06 (70)*	4.14±0.04 (51)*

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate ($p < 0.05$).

acetate for 10 days markedly decreased particulate protein kinase activity (1.16±0.07 to 0.61±0.05 nmoles ^{32}P incorporated per mg protein and 8.08±0.90 to 2.80±0.21 nmoles ^{32}P incorporated per prostate), phosphorylation of endogenous substrates in crude nuclear preparations was significantly stimulated upon withdrawal of the anti-androgen. Similarly, the ability of the nuclear preparation to bind (^{3}H)-cyclic AMP in vitro was depressed after 10 days of cyproterone acetate treatment, and was significantly increased in the "withdrawal" group regardless of the basis of expression used (Table 29). Nevertheless, it may be noted that complete recovery in both protein kinase activity and cyclic AMP binding capacity was not achieved even 10 days following the cessation of cyproterone acetate administration (Tables 28 and 29).

J. Influence of Simultaneously Administered Testosterone on Cyproterone Acetate Induced Alterations in Particulate Protein Kinase Activity and Cyclic AMP Binding Capacity of Rat Ventral Prostate

The effects of concurrent administration of testosterone on cyproterone acetate-induced changes in particulate protein kinase activity as well as cyclic AMP binding capacity were also investigated in the nuclear preparation of the ventral prostate. Data presented in Figure 31 demonstrate that whereas cyproterone acetate significantly decreased the incorporation of ^{32}P into endogenous nuclear substrates regardless of the basis of expression used, this response was effectively blocked by simultaneous administration of testosterone (5 mg/100 g/day). Likewise, while the ability of the nuclear preparation to bind (^{3}H)-cyclic AMP in vitro was decreased to 50% when expressed per mg protein and to 31% of controls when calculated on a total organ weight basis, concurrent treatment with testosterone effectively prevented these cyproterone acetate-induced changes (Fig. 32).

TABLE 29

EFFECT OF CYPROTERONE ACETATE ADMINISTRATION AND SUBSEQUENT WITHDRAWAL ON PARTICULATE CYCLIC AMP BINDING CAPACITY OF RAT VENTRAL PROSTATE

Values represent means ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. Another group of rats were maintained for an additional 10 days without any further treatment with the antiandrogen. Control animals received an equal volume of the vehicle. Cyclic AMP binding capacity was expressed as pmoles (^{3}H)-cyclic AMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

Groups	Cyclic AMP Binding Capacity (pmoles (^{3}H)-cyclic AMP bound)	
	Per mg protein	Per prostate
Control	7.20±0.92 (100)	45.81±5.82 (100)
Cyproterone acetate	3.60±0.47 (50)*	14.42±2.60 (31)*
Cyproterone acetate + Withdrawn	5.39±0.60 (75)†	22.85±3.84 (50)*†

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

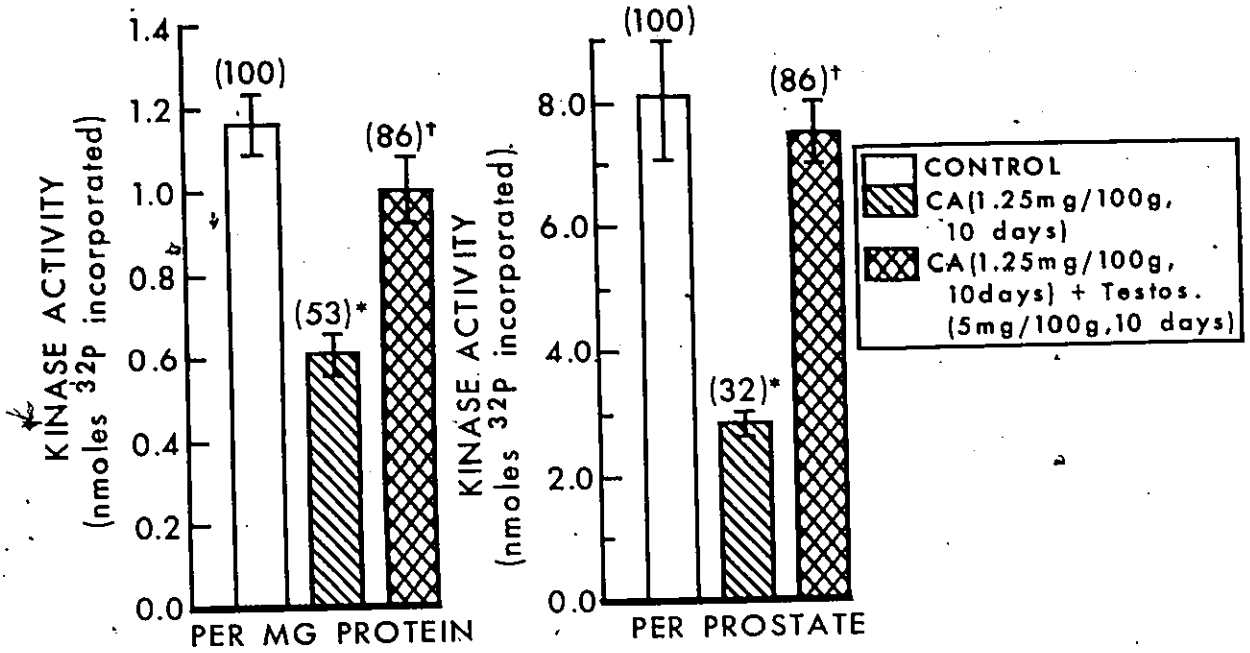


FIG. 31

Influence of simultaneously administered testosterone on cyproterone acetate-induced alteration in particulate prostatic protein kinase activity. Each bar represents the mean ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. In another group of rats, free testosterone (5 mg/100 g/day) dissolved in 0.3 ml of the same vehicle was injected simultaneously with cyproterone acetate via the subcutaneous route. Protein kinase activity was expressed as nmoles ^{32}P incorporated into crude nuclear substrates per mg protein and per prostate in the absence of cyclic AMP. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

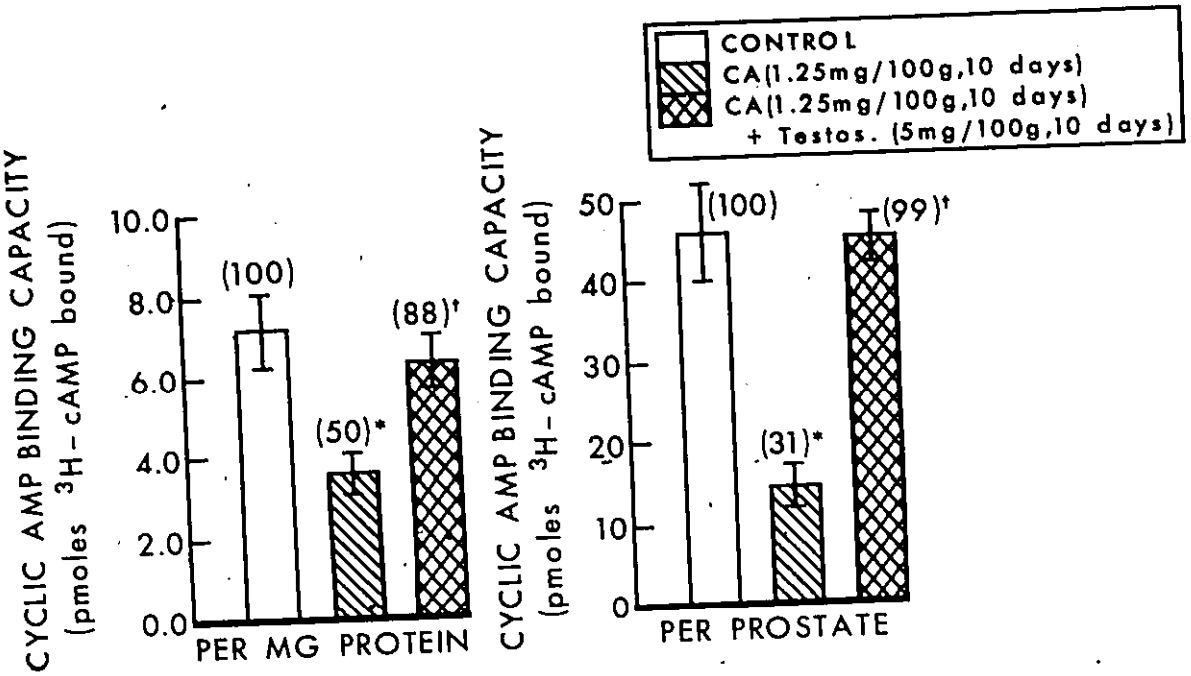


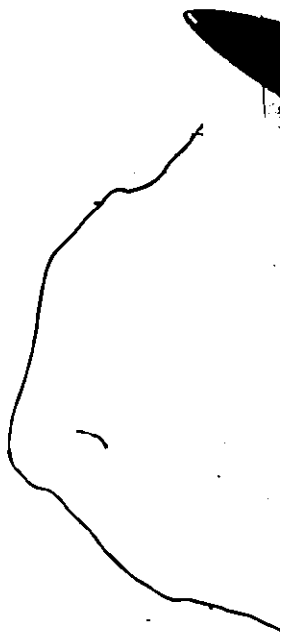
FIG. 32

Effect of testosterone on cyproterone acetate-induced alteration in cyclic AMP binding capacity of particulate fraction of rat ventral prostate. Each bar represents the mean ± S.E.M. of 8 animals per group. Cyproterone acetate (1.25 mg/100 g/day), dissolved in 0.3 ml of corn oil, was injected subcutaneously and rats were sacrificed 10 days after the first injection. In another group of rats, free testosterone (5 mg/100 g/day) dissolved in 0.3 ml of the same vehicle was injected simultaneously with cyproterone acetate via the subcutaneous route. Cyclic AMP binding capacity was expressed as pmoles (^{3}H)-cyclic AMP bound per mg protein and per prostate. Results are also expressed in percentages (in parentheses) taking the values of control rats as 100%.

*Statistically significant difference when compared with the values of control animals ($p < 0.05$).

†Statistically significant difference when compared with the values of rats treated with cyproterone acetate for 10 days ($p < 0.05$).

V. DISCUSSION



V. DISCUSSION

1. ANDROGENIC REGULATION OF PROSTATIC ADENYLATE CYCLASE AND CYCLIC AMP LEVELS

Although studies on the mode of action of gonadal steroids in eukaryote cells have occupied the attention of many investigators, our knowledge concerning the initial event(s) triggered by these anabolic hormones remains incomplete. While the action of several biogenic amines and peptide hormones has been conclusively linked with cyclic AMP, evidence that this nucleotide might also be involved in some of the actions of sex steroids on their target tissues is beginning to emerge. Previous studies by Singhal and coworkers (12) demonstrated that administration of exogenous cyclic AMP to castrate rats was capable of producing a marked "andromimetic" effect on a number of seminal vesicular and prostatic enzymes involved in carbohydrate metabolism. Whereas castration resulted in marked decreases in endogenous cyclic AMP of both the prostate gland and the seminal vesicles, cyclic nucleotide levels in these accessory sex tissues were significantly elevated following a single intramuscular injection of testosterone propionate (12). Likewise, results obtained by Smith and co-workers (408,421,422) have shown that while the capacity of both mouse and rat prostate glands to synthesize labeled cyclic AMP from tritiated adenosine *in vitro* decreased upon androgenic deprivation, subsequent treatment with testosterone or dihydrotestosterone resulted in a significant increase in the synthesis of the ^{3}H-labeled cyclic nucleotide.

Results from the present study demonstrate that whereas castration resulted in a progressive decrease in adenylate cyclase activity of the rat ventral prostate, a single intramuscular dose of testosterone (5 mg/100 g) reversed the observed effects of gonadectomy within 24 hr. Although these results are at variance with an earlier report of Golder *et al.* (206) who failed to demonstrate any significant stimulation of the prostate enzyme in

normal rats by testosterone, it is noteworthy that similar findings on the androgenic regulation of adenylate cyclase have been reported for the rat seminal vesicles by Thomas and Singhal (419). It is of interest that Rosenfeld and O'Malley (27) were unable to demonstrate any in vivo effect of testosterone on adenylate cyclase activity of the ventral prostate of hypophysectomized rats. In their study, administration of a 2 mg dose of testosterone to rats which had been hypophysectomized for 3 weeks did not produce any significant increase in prostatic adenylate cyclase activity up to 24 hr. However, it is known that the ventral prostate in a rat hypophysectomized for 3 weeks undergoes a greater degree of histological involution and is less responsive to testosterone stimulation than that seen in orchidectomized rats (46,444). The possibility thus exists that the 2 mg dose of the hormone and the 24 hr interval used by Rosenfeld and O'Malley (27) may not have been adequate to cause measurable activation of adenylate cyclase in this tissue. Perhaps an important finding reported in this study is the rapidity with which androgens can produce consistently reproducible increases in adenylate cyclase activity of the prostate gland. The significant increase in adenylate cyclase at 1 hr with dihydrotestosterone in contrast to the 4 hr with testosterone supports the contention that dihydrotestosterone is the active metabolite of testosterone in androgen-sensitive tissues (19). These results thus demonstrate that under appropriate experimental conditions, it is possible to obtain measurable stimulation of the prostatic enzyme in vivo as early as 1 hr.

The rapid stimulation of adenylate cyclase activity by testosterone or dihydrotestosterone was found to be specific to male sex steroids since intravenous injection in castrate rats of either estradiol-17β, progesterone or methyl prednisolone was without any significant effect on the prostate enzyme. Actinomycin D and cycloheximide, two inhibitors of RNA and protein synthesis, were able to abolish the response of adenylate cyclase to testosterone. Likewise,

the β-adrenergic blocker, propranolol, prevented the androgenic stimulation of prostatic adenylate cyclase in castrate rats. It is of interest that Szego and Davis (28) reported a similar inhibition of the instantaneous increase in uterine cyclic AMP levels when ovariectomized rats were treated with estradiol and certain β-adrenergic blocking agents. In contrast to the stimulatory effect of androgens on adenylate cyclase, dihydrotestosterone was found to produce a slight inhibition of phosphodiesterase activity in the rat prostate. However, the order of magnitude of this decrease was much smaller than that reported by Weinryb et al. (18) for the effect of testosterone on phosphodiesterase activity of rat brain and cat heart. In this connection, it is of interest that whereas testosterone or dihydrotestosterone (10^{-4} - 10^{-6} M) failed to exert any significant effect on prostatic phosphodiesterase activity in vitro (data not shown), addition of estradiol-17β, progesterone, testosterone or dihydrotestosterone led to marked inhibition of enzyme activity in chromatin preparations of Krebs 2 ascites tumor cells obtained from a mouse mammary carcinoma (445).

An alternative approach to studying the mechanism of action of androgens is to examine their binding to specific cellular components of androgen-sensitive tissues following the administration of a labeled androgen. With the new concept that dihydrotestosterone may indeed be the active metabolite of testosterone (19), the search for specific protein receptors with high affinity for androgens in various sex accessory tissues as well as the investigation into the biochemical interaction of these protein molecules with certain anti-androgens have further advanced our knowledge regarding the molecular basis of androgen action. Among the steroidal anti-androgens, cyproterone and cyproterone acetate are currently being used as experimental tools to help clarify the molecular sequence of events that occurs following androgen stimulation of the prostate gland. Numerous reports have indicated that cyproterone acetate exerts its anti-androgenic effects by inhibiting the uptake and retention of

testosterone and dihydrotestosterone by their target tissues (31,140,147,161). Since androgenic deprivation following orchidectomy resulted in a marked decrease in adenylate cyclase activity of the ventral prostate, blockade of the androgen receptor sites in these target cells with anti-androgens should mimic the effects of orchidectomy if these 'high affinity androgen binding proteins' are indeed the receptors or the regulatory subunits of adenylate cyclase complex (10). The present investigation on the effects of cyproterone acetate on prostatic cyclic AMP metabolism demonstrated that when the anti-androgen (5 mg/100 g, i.p.) was administered alone to mature castrate rats, a marked decrease was noted in the activity of prostatic adenylate cyclase at approximately 4 hr. Pretreatment of testosterone-injected rats (5 mg/100 g, i.v.) with this anti-androgen completely inhibited the androgen-induced increase in adenylate cyclase activity observed at 4 hr. Moreover, the present study demonstrated that whereas daily administration of cyproterone acetate resulted in progressive decrease in both adenylate cyclase activity and cyclic AMP content of the ventral prostate of normal mature rats, subsequent withdrawal of anti-androgen treatment or concurrent administration of high doses of testosterone effectively reversed these effects of cyproterone acetate. Recently, Mangan et al. (246) examined the influence of cyproterone acetate on the cycloheximide and actinomycin D-sensitive elevation of glucose 6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase activities in the prostate gland following both testosterone and cyclic AMP administration. It is of interest that simultaneous administration of the anti-androgen failed to inhibit the increase in the activities of these two enzymes promoted by either cyclic AMP or testosterone. While this lack of effect of cyproterone acetate precludes involvement of dihydrotestosterone in the overall mechanism of stimulation of these prostatic enzymes, the possible participation of other metabolites of testosterone in this process cannot be ruled out. These findings merely suggest that the response of the carbohydrate

metabolizing enzymes may be uniquely distinct from other biochemical processes examined to date in the prostate gland during the early phase of androgen action (246).

The finding that addition of an androgen could significantly enhance the synthesis of labeled cyclic AMP from tritiated adenosine by prostatic slices (408) prompted us to examine the influence of testosterone and dihydrotestosterone on adenylate cyclase activity in prostatic homogenates in vitro. When the integrity of the accessory sex glands was disrupted by homogenization, the responsiveness of the enzyme to androgenic stimulation was completely lost. The direct addition of dihydrotestosterone, testosterone propionate, or free testosterone (up to 10^{-5} M) to the homogenate produced no detectable increase in adenylate cyclase activity over the values seen in ethanol blanks. Moreover, when ventral prostates from 48 hr castrate rats were used, addition of the androgens in vitro also failed to produce any significant response. Since L-epinephrine and NaF produced significant stimulation of the prostatic enzyme under the same in vitro conditions, results indicate that adenylate cyclase activity of prostatic homogenates is certainly capable of responding to certain treatment in vitro. Our observations on the lack of an in vitro effect of androgens on prostatic adenylate cyclase are in accord with those reported earlier by Golder et al. (206) and by Rosenfeld and O'Malley (27) who also failed to observe any stimulation with androgen over ethanol blanks. The failure to demonstrate measurable alterations in adenylate cyclase activity, when androgens are added in vitro to ventral prostate homogenates, as opposed to the intact tissue, may reflect a possible deterioration of the hormone-receptor adenylate cyclase interaction which remains intact under in vivo conditions. This could be particularly true if a pre-binding of androgen to a cytosol-binding protein occurs before the activation of nuclear adenylate cyclase. Nevertheless, it has long been demonstrated that partial purification of adenylate

cyclase can result in loss of responsiveness of the enzyme to hormonal stimulation (227,446). It is also of interest that androgen addition to broken cell preparations in vitro generally fails to produce many of the biosynthetic responses which are known to be induced in accessory sex glands after in vivo administration of the hormone (103).

2. PROSTATIC PROTEIN KINASES: CHARACTERISTICS AND ANDROGENIC REGULATION

Recent studies on the mechanism by which cyclic AMP exerts its diverse effects have been centered around the ability of this cyclic nucleotide to stimulate protein kinases (296,302-304,310,314,322). Numerous studies have indicated that cyclic AMP-dependent protein kinases are composed of regulatory and catalytic subunits. The binding of the regulatory subunit to the catalytic component results in an inactive holoenzyme. Cyclic AMP is presumed to mediate the action of the hormones by promoting the dissociation of the holoenzyme to yield a regulatory subunit cyclic AMP complex and free catalytic subunits (cyclic AMP-independent protein kinase) which are then enzymatically active. With this concomitant dissociation and activation of protein kinases, various enzymes and structural proteins having key roles in hormone mediated responses are phosphorylated.

Studies presented in this dissertation demonstrate the presence of cyclic AMP-dependent protein kinases in canine and rat prostate glands. Although the prostate enzyme was found predominantly in the cytosol, 3-4% of the total catalytic as well as the cyclic AMP-binding activities were associated with the 650 g pellet and could be enhanced by the presence of 0.1% Triton X-100. In lactating rat mammary glands (301), subcellular distribution studies have demonstrated that as much as 98% of the total kinase activity was recoverable in the soluble fraction. Similar findings also have been reported for protein kinases from bovine endometrial (303) and rat liver (324). It is of interest that in the anterior pituitary (447), brain (448) and adrenal cortex (310), kinases with high specific activity measured without the addition of exogenous

substrate, were localized in particulate fractions and enzyme activity could be further increased by treatment with the detergent. Nevertheless, 50% of the activity towards exogenously added histone was shown to be present in the soluble fraction of these tissues.

The properties of cyclic AMP-stimulated protein kinases have now been studied in a variety of tissues. Although the assay conditions used were not identical with those used in the present study, it is of interest to compare some of their characteristics with our findings for the canine prostate gland enzyme. In general, the kinetic and physical properties of the prostatic cyclic AMP-dependent protein kinase seem to resemble those described for the enzymes from other mammalian tissues (296,301-304,310,314,324,332,447-449). Canine prostate possessed a protein kinase (Fraction II) with cyclic AMP-binding and protein kinase activities, as demonstrated on DEAE cellulose chromatography. Although no cyclic AMP binding protein devoid of kinase activity was found, a small peak of cyclic AMP dependent kinase (fraction I) was occasionally observed. Only fraction II was used in studies reported in this dissertation. Although the similarities and the differences in kinetic and physical properties of the two activity peaks remain to be determined, it is of interest that multiple forms of cyclic AMP-dependent protein kinase have recently been reported in skeletal muscle (321), liver (324), cardiac tissue (318) and adrenals (317).

Studies on the influence of varying cyclic AMP concentrations on histone phosphorylation catalyzed by prostatic protein kinase has demonstrated that whereas the enzyme was half-maximally activated by 5×10^{-8} M cyclic AMP, maximum stimulation was noted with about 5×10^{-6} M concentration of the cyclic nucleotide. Higher concentrations of cyclic AMP appeared to be less stimulatory. These findings agree well with those reported earlier for the enzyme from rabbit skeletal muscle (302), rat adipose tissue (450), rat mammary gland (301), frog

bladder (297), bovine thyroid gland (304) as well as bovine uterus (303). The results on the ability of various other cyclic nucleotides to stimulate the prostatic enzyme are also in line with those reported earlier for protein kinases from several other tissues (302,306,314,322,450). In general, cyclic IMP has been shown to be most effective at low concentrations. This is not surprising considering the high degree of structural similarity between cyclic IMP and cyclic AMP. Cyclic GMP has also been found to stimulate protein kinases, although much higher concentrations are needed than those in the case of cyclic AMP. An exception to this however, was demonstrated with the recently reported cyclic GMP-stimulated protein kinase from lobster muscle (299). This enzyme was found to have lower apparent Km for cyclic GMP than for cyclic AMP. Although dibutyryl cyclic AMP, the lipid-soluble analogue of cyclic AMP widely used for the purpose of facilitating passage of cyclic AMP across cell membranes, was much less active than cyclic AMP, it did activate the enzyme fully at high concentrations. The possibility can not be excluded however, that some hydrolysis of the dibutyryl derivative to free cyclic AMP occurs during the incubation period of the assay.

As anticipated from its cyclic nucleotide dependence, the prostatic enzyme exhibited high affinity for cyclic AMP. One single order of binding sites exhibiting an association constant (Kassoc.) of approximately 0.78×10^8 M^{-1} at pH 5.0, 0^oC was observed for the major activity peak obtained on DEAE ion-exchange chromatography. It is of interest that under the same experimental conditions, cyclic AMP-dependent protein kinase from bovine endometrium also demonstrated a single class of cyclic AMP binding sites with Kassoc. of 0.6 to 1.0×10^8 M^{-1}. Although Kassoc. decreased slightly to 0.4×10^8 M^{-1} at 20^oC, the number of binding sites per mg of protein remained unaltered in the case of the uterine enzyme (303). Adrenal tissue yielded separate binding and kinase components in addition to a cyclic AMP-dependent protein kinase upon

elution from DEAE (310). Nevertheless, Kassoc. for the holoenzyme and cyclic AMP at physiological pH was 0.8 x 10^8 M^{-1} (440). In addition, two cyclic AMP-dependent protein kinases exhibiting a slight difference in the affinity for cyclic AMP at pH 7.5 have also been recently reported in rat hepatic tissue (Kassoc. = 0.25 x 10^8 M^{-1}, 1 x 10^8 M^{-1}) (324).

The effect of varying ATP concentrations on protein kinase activity has been reported for the enzyme from a variety of tissues including bovine heart (314), brain (322) and skeletal muscle (302). The heart enzyme exhibited an apparent Km of 5.9 x 10^{-5} M in the presence and absence of cyclic AMP. While half-maximal velocity, independent of the cyclic AMP presence, was achieved at 1.5 x 10^{-5} M ATP with skeletal muscle protein kinase, the brain enzyme also demonstrated an apparent Km of 1.3 x 10^{-5} M in the presence of cyclic AMP. In the absence of the cyclic nucleotide, double reciprocal plots failed to yield a linear relationship and the apparent Km for the brain protein kinase could not be accurately determined. Nevertheless, the concentrations of ATP at half-maximal velocity of these various enzymes agree well with the value of 1.2 x 10^{-5} M reported in the present study for canine prostate. It may be noteworthy that enzymes from certain bovine tissues such as pancreas, kidney and lung required significantly higher concentrations of ATP for half-maximal velocity in the absence than in the presence of cyclic AMP (353). The reason for these kinetic differences remains to be established.

With regard to the protein substrate specificity, like the enzymes from heart (314), adipose tissue (296), liver (332), brain (322), trout testis (451), skeletal muscle (302) and bovine thyroid (304), the prostatic protein kinase was found to phosphorylate histones much more readily than casein and protamine. Of several proteins tested as substrates for the adipose tissue enzyme, muscle glycogen synthetase, muscle phosphorylase and histone (F_{2b}) incorporated phosphate at a much higher rate than the other proteins (296). Histone F_1,

which has been shown to be phosphorylated *in vitro* by this enzyme, was also phosphorylated *in vitro* and *in vivo* by the liver protein kinase (331,332). An exception to this generality however, has been observed in the case of cyclic AMP-stimulated protein kinases from certain lower species such as lobster, fish and roundworm. These enzymes phosphorylated casein more readily than histone (306). The effect of protein substrate concentration on the activity of cyclic AMP-dependent protein kinase from various tissues has also been studied. In our study, half-maximal activity of the prostatic enzyme was achieved at approximately 500 µg histone per ml in the presence and absence of cyclic AMP. This is in agreement with that for the brain enzyme reported earlier by Miyamoto *et al.* (322). Likewise, using the heart protein kinase, Brostrom *et al.* (314) found no effect of cyclic AMP on the apparent Km for casein. Similar findings have been reported for the enzyme isolated from skeletal muscle (302), mammary gland (301) and epididymal spermatozoa (452).

Recently, Ahmed (98) demonstrated that purified nuclei from rat ventral prostate were able to incorporate ^{32}P into phosphoproteins in the presence of (γ-^{32}P) ATP. Mg^{2+} was essential for the reaction, whereas cations such as Zn^{2+}, Mn^{2+} and Cu^{2+} proved to be inhibitory. Both the histone and non-histone phosphoproteins yielded O-phosphorylserine and some phosphothreonine on acid hydrolysis. Wilson and Ahmed (433) were able to demonstrate the presence of distinct androgen-sensitive protein kinases from nuclei of rat ventral prostate. The activity of the nucleolar protein kinase was different from that of the extranucleolar enzyme and was optimal at pH 6.8, but decreased rapidly with increasing pH. The extranucleolar protein kinase had a broad pH curve with optimal activity being between pH 7.0 and 7.7. Utilizing dephosphorylated phosvitin as substrate, the nucleolar enzymes showed 3 apparent Km values (0.36, 0.9 and 3.7 mg/ml), whereas that isolated from chromatin demonstrated only one (1.0 mg/ml). While the enzymes in the nucleoli had 3 apparent Km values towards ATP as substrate (0.03, 0.1 and 1.1 mM), the chromatin-associated

protein kinase(s) had 2 (0.04 and 0.4 mM). Although both the nucleolar and chromatin enzymes phosphorylated histones, chromatin kinase activity assayed with lysine-rich and arginine-rich histones was 3 and 0.3%, respectively compared with that in presence of phosvitin (433,434).

Although the functional importance of cyclic AMP-dependent protein kinases in certain hormone-mediated responses has been well documented, their role in the mechanism of action of androgens on male accessory sexual tissues remains to be elucidated. While Smith (421) reported significant increases in prostatic cyclic (^{3}H) AMP synthesis from (^{3}H)-adenosine in the presence of testosterone or dihydrotestosterone, results from the present study also demonstrated rapid increases in adenylate cyclase activity of the rat ventral prostate following the administration of either of these hormones. Further, the administration of exogenous cyclic AMP has been found to produce testosterone-like stimulation of prostatic weights and the induction of several rate-limiting enzymes involved in carbohydrate metabolism (12). Although cyclic AMP-dependent protein kinase was found predominantly in the cytosol of the prostate, small amounts of the enzyme were consistently found to be associated with the nuclear fraction. This may be noteworthy since a small amount of adenylate cyclase activity has also been reported to be present in the nuclear fraction purified from rat prostate; enzyme activity decreased significantly after gonadectomy and was restored to normal within 1 hr of androgen injection (246). Reddi et al. (413) studied the effects of orchidectomy and subsequent androgen administration on the activity of cytosol cyclic AMP-stimulated protein phosphokinase in the rat ventral prostate, but observed that androgen depletion or repletion only had little influence on the phosphorylation of calf thumus F_{2b} histone and protamine. Data in the present study demonstrate that both cytosolic and particulate prostatic protein kinases are responsive to androgenic deprivation and repletion. Seven days following castration, specific activities of soluble prostatic

cyclic AMP-dependent and -independent protein kinases were markedly increased above those of the sham-operated controls. Although our findings are in accord with the earlier data of Ichii et al. (414), interpretation of these results must be attempted with caution. Since the total protein content of the prostate decreases following androgenic deprivation, the possibility that the high specific activity observed for the soluble enzyme may merely reflect a relatively longer biological half-life for the enzyme cannot as yet be excluded, particularly in light of the fact that the total activity of the enzyme was in fact markedly decreased after castration. Although daily administration of testosterone appeared to reverse this effect of orchidectomy on cyclic AMP-independent enzyme, the specific protein kinase activity assayed in the presence of the nucleotide was relatively unaffected by androgenic repletion. It is of interest that while total protein kinase activity (per prostate) did not recover fully following testosterone treatment, there was a significant stimulation of the prostatic enzyme in these rats. In this connection, it is noteworthy that whereas daily administration of the anti-androgen, cyproterone acetate, resulted in a progressive increase in the specific activity of soluble prostatic cyclic AMP-dependent and independent protein kinases as well as the specific cyclic AMP binding capacity, total activity as expressed on a per prostate basis was however, decreased. These responses appeared to be androgen-specific as concurrent administration of testosterone not only effectively blocked the influence of the anti-androgen, but significantly increased the activity of the enzymes in excess of that observed for controls. Whether testosterone exerts its anabolic effects through induction of protein kinases and cyclic AMP binding protein, as suggested for the hormonal control of proteins in developing mammary glands by Majumder and Turkington (453) or indirectly via the activation of prostatic adenylate cyclase, remains to be established.

The phosphorylation of nuclear proteins by cyclic AMP-dependent protein kinase has been demonstrated both in vitro and in vivo (312,333,339,349). Moreover, the ability of the proteins (both basic and acidic) from various tissues to stimulate transcription in vitro was enhanced with increasing phosphorylation and decreased upon enzymatic removal of their phosphate groups (362,349,361). Ahmed and Ishida (99) have shown that the incorporation of ^{32}P into non-histone phosphoproteins of prostatic nuclei from castrate rats was significantly enhanced as early as 30 min following testosterone treatment, suggesting that the phosphorylation of these proteins may be involved in the events related to the control of gene action in the prostate in response to androgenic stimulation. The present study also demonstrates that whereas castration resulted in a marked decrease in the phosphorylation of endogenous nuclear substrates, testosterone replacement therapy readily reversed these effects. Moreover, daily administration of cyproterone acetate to normal mature rats significantly decreased the organ weight as well as the nuclear protein kinase activity of the ventral prostate. While the incorporation of ^{32}P from (γ-^{32}P)-ATP to endogenous nuclear substrates was markedly lowered in the anti-androgen-treated group, subsequent withdrawal of cyproterone acetate treatment or concurrent administration of testosterone effectively prevented the observed enzymatic responses. Whether the incorporation of ^{32}P into endogenous substrates was catalyzed by the catalytic subunit of a soluble cyclic AMP-dependent protein kinase which translocated to the cell nucleus or by a membrane-bound phosphoprotein transferase independent of the cyclic AMP-adenylate cyclase system, is at present unclear. Although our findings are in accord with the recent report of Wilson and Ahmed (454) demonstrating the responsiveness of various nuclear protein phosphokinases (both extra-nucleolar and nucleolar) in rat ventral prostate to depletion and repletion of testicular androgens, the nature as well as the exact location of these enzymes remains

to be determined. Earlier, Ahmed (98) demonstrated that purified nuclei from rat ventral prostate were able to incorporate ^{32}P into their phosphoproteins in the presence of (γ-^{32}P)-ATP. The non-histone phosphoproteins were about 4 times more active than the histone phosphoproteins. It was also shown that phosphorylation of these proteins isolated from the nuclei of orchidectomized rats was greatly reduced when compared with nuclei isolated from orchidectomized rats treated with testosterone (99). These changes were target tissue specific, as no such effect was observed in the metabolism of nuclear phosphoproteins of liver from animals of varying androgenic status. However, the addition of testosterone, 5α-dihydrotestosterone or cyclic AMP in vitro did not increase the phosphorylation of nuclear proteins (4,99).

More recently, protein phosphokinase activity endogenous to rat ventral prostate chromatin has also been reported (454). Whereas orchidectomy resulted in a rapid decline in the enzyme activity (approximately 50% within 24 hr and over 80% within 48 hr), testosterone replacement in vivo reversed these changes produced by androgen deprivation. This decrease in kinase activity following androgen depletion appeared to precede measurable changes in the protein and RNA complements of chromatin. Furthermore, the chromatin-associated protein phosphokinase activity towards lysine- and arginine-rich histones also was sensitive to the androgenic status of the animal.

Although our data indicate that phosphorylation of nuclear endogenous substrates was unaffected by the presence of exogenous cyclic AMP and that the kinase activity ratio for the particulate fraction was independent of the androgenic status of the animal, the cyclic AMP-binding capacity of the particular fraction was responsive to androgenic deprivation and stimulation. Whether this affinity for cyclic AMP represents the binding activity of a prostatic cyclic AMP-dependent protein kinase (RC) or that of the dissociated regulatory subunits (R) of the holoenzyme remains to be determined. However,

it is of interest that in purified calf thymus and rat liver nuclei, both the cyclic AMP-dependent protein kinase and proteins possessing the physical and biochemical characteristics of the regulatory subunit (R) have been noted to be present (363,455). It has also been demonstrated that while the catalytic subunit (C) of the cyclic AMP-dependent protein kinase from calf thymus binds extensively to calf thymus DNA, the R subunit has a high affinity for nucleohistones (455). The ability of the R protein to bind to these basic nuclear proteins may represent an important aspect of protein kinase activation in the nucleus. It is known that, apart from cyclic AMP, histones also can induce partial dissociation and activation of cyclic AMP-dependent protein kinase (329,455) and that different histones are specific in their ability to induce differential changes in phosphorylation of non-histone proteins in isolated rat liver nuclei (334). It is possible that histones bound at discrete sites along DNA can activate protein kinase activity at these sites by binding to the R subunit of the enzyme. Results of the present investigation indicated that whereas cyproterone acetate administration significantly increased the specific activity of soluble prostatic protein kinase assayed in the presence of cyclic AMP, it markedly decreased that of the particulate enzyme. Subsequent withdrawal of the anti-androgen treatment or concurrent administration of testosterone was capable of reversing these enzymatic responses. These findings raise the possibility that following the stimulation of prostatic adenylate cyclase, androgen may exert its influence on the target cells by promoting a translocation of the cytoplasmic protein kinase(s) to some yet unknown site in the nucleus where it eventually catalyzes the phosphorylation of nuclear proteins.

It may be of interest, particularly in the light of the findings by Jungmann et al. (456,457) who also suggested a cyclic AMP-dependent translocation of cytoplasmic cyclic AMP-binding protein and protein kinase to nuclear acceptor

sites following gonadotropin stimulation of ovarian cells. Support to this hypothesis can be derived from the work of Korenman et al. (458) who observed that uterine stimulation by isoproterenol in ovariectomized rats resulted in a marked decrease in the total uterine cytosol protein kinase with a concomitant increase in cyclic AMP-independent protein kinase activity of the microsomal fraction. Indeed, these results have been interpreted to imply translocation of cytosol protein kinase to microsomal substrate acceptor sites. While the role of prostatic cyclic AMP binding protein in androgenic action in the nucleus still remains to be defined, detailed studies with purified ventral prostate nuclei may provide deeper insight into the role of the cyclic AMP-adenylate cyclase-protein kinase system in the overall action of male gonadal hormones on growth and differentiation of androgen-dependent organs. Definition of the cellular locus and identification of the nuclear protein kinase substrate will undoubtedly be of importance.

3. BETA-ADRENERGIC STIMULATION OF PROSTATIC ADENYLATE CYCLASE-CYCLIC AMP SYSTEM

Results of the present study demonstrated that whereas the activity of adenylate cyclase in the rat ventral prostate decreased after gonadectomy, intravenous administration of testosterone and particularly of dihydrotestosterone produced a rapid elevation of the prostatic enzyme in androgen-deprived rats. Since the androgen-stimulated increases in cyclase activity proved to be sensitive to pretreatment with the β-adrenergic antagonist, propranolol, the question as to whether the prostatic adenylate cyclase was under the control of the adrenergic system was further investigated. Although the autonomic nervous control of this sex gland appears to be mainly cholinergic in nature (459), activation of the adenylate cyclase-cyclic AMP system in many tissues has been shown to be closely associated with the stimulation of β-adrenergic receptors (256,261,375,394,460-462).

The present study demonstrates the presence of an adenylate cyclase system in the rat ventral prostate gland which is sensitive to β-adrenergic stimulation. Administration of isoproterenol resulted in rapid elevation in the activity of adenylate cyclase and the endogenous cyclic AMP levels. These effects were found to be time- and dose-dependent, with maximum stimulation being seen 5 min after treatment with 1.0 mg/kg dose of the β-adrenergic agonist. Whereas pretreatment with propranolol antagonized the observed isoproterenol-induced alterations, administration of the α-adrenergic antagonist, phentolamine, failed to elicit any appreciable effect. These results are in accord with our findings that whereas follicle stimulating hormone and interstitial cell stimulating hormone were ineffective in stimulating prostatic adenylate cyclase of crude homogenate in vitro, addition of L-epinephrine to the incubation medium enhanced the activity of the prostatic enzyme. Similarly, Zepp and Thomas (463) have shown that whereas acetylcholine (10^{-5} M) failed to alter the concentration of cyclic AMP in rat prostate gland and seminal vesicles in vitro, incubation in the presence of isoproterenol (10^{-5} M) resulted in significant increases in the endogenous cyclic nucleotide levels of these accessory tissues.

The present study has also shown that increases in prostatic adenylate cyclase and cyclic AMP levels following isoproterenol administration resulted in the activation of a soluble cyclic AMP-dependent protein kinase. Changes in the activity of the prostatic holoenzyme were found to be associated with a decrease in the total activity of protein kinase (+cyclic AMP) with concomitant increase in cyclic AMP-independent enzyme activity. Whereas the protein kinase activity ratio (-cAMP/+cAMP) was markedly elevated 5 min following the administration of isoproterenol (time at which maximal stimulation was observed in adenylate cyclase activity and cyclic AMP levels), the ability of the enzyme to bind cyclic (^{3}H) AMP in vitro was significantly reduced. These results are

in agreement with the concept that activation of protein kinase following β-adrenergic stimulation may involve conversion of the inactive cyclic AMP-dependent holoenzyme to an active catalytic subunit. Similar findings have been reported for the effects of FSH on seminiferous tubules (464) and of epinephrine, ACTH and glucagon on isolated epididymal fat pads (308).

During the past 10 years, investigations of steroid hormone action on its target cells have directed much attention to the possible interaction with various peptide hormones and biogenic amines. The so-called "permissive" effect of glucocorticoids have been proven essential to the action of a variety of hormones, especially those known to act through the adenylate cyclase-cyclic AMP system. Shafir and Steinberg (465) reported that the injection of epinephrine failed to increase plasma free fatty acid levels in adrenalectomized rats unless the animals were subsequently treated with cortisol. Similar phenomena have been detected during experiments on the role of cyclic AMP in glycogen synthesis and degradation, lipolysis, gluconeogenesis, enzyme induction, catecholamine actions on the cardiovascular system and stimulation of urea formation by glucagon (466). The intimate relationship between glucocorticoids and cyclic AMP has been demonstrated by several workers who observed that these steroids sensitized a variety of tissues to the action of exogenously administered cyclic AMP (467-471). Evidence also indicates that these steroid hormones had no effect on the basal levels of cyclic AMP or the ability of other hormones to increase the levels of this cyclic nucleotide (467). Although interaction between sex steroids and other cellular regulators in their target cells have also attracted attention during the past years, exactly how these interactions are brought about remains to be elucidated. Data from the present investigation have demonstrated that while isoproterenol administration significantly stimulated prostatic adenylate cyclase-cyclic AMP system in orchidectomized as well as normal intact rats,

prostatic adenylate cyclase activity, cyclic AMP levels and their degree of stimulation by the β-adrenergic agonist in androgen-deprived animals were lower than those seen in intact controls. While testosterone replacement restored the responsiveness of this enzyme system to β-adrenergic stimulation, it also significantly increased both the absolute enzyme activity and cyclic nucleotide levels in the presence as well as in the absence of isoproterenol. While the physiological significance of these findings in the overall metabolic control of this accessory sex gland awaits further experimentation, it is of interest that pretreatment of ovariectomized rats with estrogen significantly increased the sensitivity of both uterine cyclic AMP and phosphorylase activity to stimulation by epinephrine. When the uterus is under estrogenic domination, epinephrine administered in vivo or in vitro stimulated greater uterine cyclic AMP formation and phosphorylase activation than it did in uteri from estrogen-deprived animals (405).

In conclusion, the present investigation demonstrates the existence of a functional adenylate cyclase-cyclic AMP system in the rat ventral prostate. While androgenic deprivation resulted in marked changes in prostatic adenylate cyclase activity, cyclic AMP levels and protein kinase complex, administration of testosterone or dihydrotestosterone was capable of significantly reversing these responses. Although the regulation of prostatic cyclic AMP system also appeared to be under the influence of β-adrenergic stimulation, the functional significance of this modulation remains to be shown. In order to stimulate future research into the molecular mechanisms of action of sex steroids, a hypothetical model to help visualize the extent of cyclic AMP involvement in androgen action is proposed (Fig. 33). This sequence is not only consistent with the concepts already developed for the action of other cyclic AMP-dependent hormones, but is supplementary to our existing knowledge of androgen interaction with receptors. Wilson (472) found that testosterone

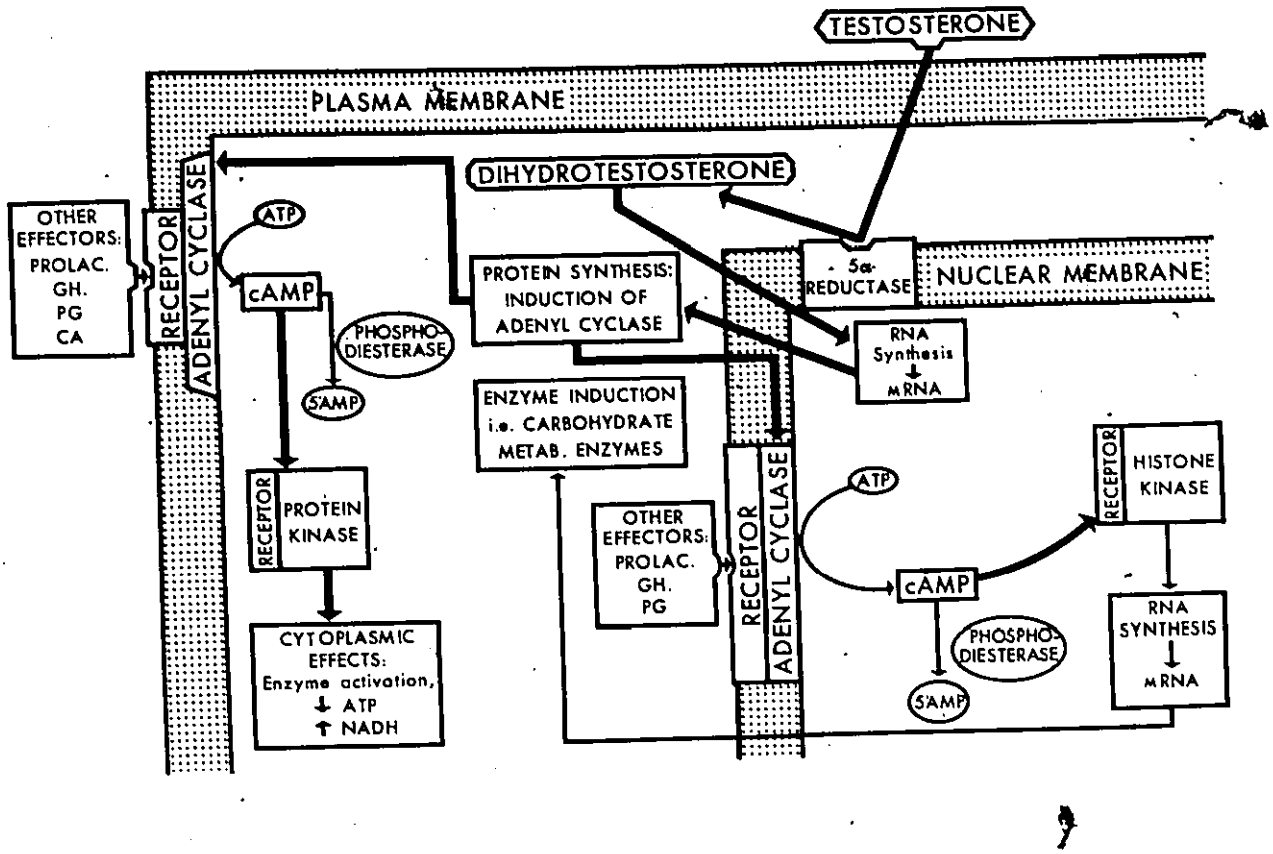


FIG. 33

Hypothetical model showing the regulation of the cyclic AMP-adenylate cyclase-protein kinase system in male gonadal steroid-dependent tissues. Testosterone enters the cell and is metabolized into dihydrotestosterone, which then enters the nucleus and probably induces adenylate cyclase. Whereas androgen may be necessary for the maintenance of basal adenylate cyclase, other effectors such as prolactin (PROLAC), growth hormone (GH), prostaglandins (PG), and catecholamines (CA) may be essential for stimulating the enzyme for specific hormonal responses. Stimulation of the nuclear adenylate cyclase may result in an increase in nuclear cyclic AMP, which activates histone kinase for the synthesis of new RNA and proteins. The more rapid cytoplasmic changes (e.g. those seen in NADH and ATP) might be related to stimulation of adenylate cyclase associated with the plasma membrane.

enters prostate tissue by diffusion and is then metabolized by 5α-reductase to dihydrotestosterone. Villee (473) also showed that testosterone could be converted into dihydrotestosterone by isolated nuclei from the prostate glands of immature or castrate rats. These authors found that the in vitro stimulation of RNA synthesis by testosterone occurred only when adequate NADPH was present to enable 5α-reductase to convert testosterone to dihydrotestosterone. Wilson (472) demonstrated that the newly formed dihydrotestosterone binds to a specific nuclear protein, which somehow initiates the transcription of messenger RNA. Recent studies indicate that one of the responses to androgens is the induction of target tissue adenylate cyclase activity. This is supported by our findings that whereas castration decreased adenylate cyclase activity in prostatic homogenates, testosterone and even more so, dihydrotestosterone caused marked increases in the enzyme activity which were sensitive to both actinomycin D and cycloheximide. Moreover, Mangan et al. (246) demonstrated that the activity of prostatic nuclear adenylate cyclase decreased following gonadectomy, and that testosterone administration was able to reverse this effect within 1 hr.

It is conceivable that androgens may be necessary for maintenance of basal adenylate cyclase activity, while other effectors such as prolactin, growth hormone, prostaglandins, and catecholamines are necessary for stimulating the enzyme for specific hormonal responses. Stimulation of nuclear adenylate cyclase would result in an increase in nuclear cyclic AMP which may activate a histone kinase for the induction of RNA and protein synthesis in a manner similar to that proposed by Langan for the action of glucagon on the biosynthesis of hepatic proteins (309,312,331). Reddi et al. (413) demonstrated the phosphorylation of certain histones of the rat ventral prostate in response to cyclic AMP, although neither androgenic deprivation nor stimulation exerted any significant influence on the rates of these protein phosphokinase reactions.

However, our present studies as well as those of Ichii et al. (414) have shown that whereas castration caused significant elevation in the activity of both prostatic cyclic AMP-dependent and cyclic AMP-independent protein kinase, administration of testosterone to androgen-deprived rats was capable of reversing these effects. Moreover, Ahmed and Ishida (99) reported that the incorporation of ^{32}P into nuclear phosphoproteins from ventral prostates of orchidectomized rats was greatly reduced when compared with incorporation into nuclei from orchidectomized rats treated with testosterone or from normal intact animals.

Our hypothesis is consistent with the data that actinomycin D and cycloheximide can block prostate growth as well as the induction of several important enzymes in response to cyclic AMP (12). It may be emphasized that even though adenylate cyclase is present in nuclei of the ventral prostate, a major portion of the total enzyme activity is associated with cytoplasmic particulates (246). It is possible that the more rapid cytoplasmic changes, e.g. those seen in NADH and ATP after testosterone (92) and which are not blocked by actinomycin D, might be related to the stimulation of adenylate cyclase bound to the plasma membrane. This of course, needs to be verified experimentally. However, it would be presumptuous to suggest that all aspects of gonadal action are related to the stimulation of cyclic AMP-adenylate cyclase system in the prostate gland. For example, Na^+ and K^+ changes are probably membrane permeability effects which are not cyclic AMP-mediated, even though androgens have been shown to affect the activity of accessory sex gland ATPase. Similarly, ^{65}Zn uptake, which is certainly an androgen-dependent process, is in all probability a membrane event. Moreover, since the prostate gland constitutes a mixture of various target cell populations, it is possible that the actions of these steroids implicate cyclic AMP mediation for only some of the target tissue responses. The observation that cyclic AMP did not produce the morphologic

expression in the prostate gland would seem to support this notion (246,274). Nevertheless, recent evidence indicates that intraluminar infusions of cyclic AMP caused morphological changes in the uterus similar to those brought about by estradiol (475,476). In addition, Brandes (48) demonstrated that cyclic AMP can also mimic the action of testosterone in the restitution or stimulation of the structural components of the seminal vesicular cells. Administration of dibutyryl cyclic AMP produced similar stimulatory effects on the epithelium of the seminal vesicles of immature animals, as did testosterone. While the rough endoplasmic reticulum was stimulated and the cisternae became more numerous and dilated following the nucleotide administration, the lumen of the vesicular gland also appeared to be dilated and filled with secretory material (48).

Evidence suggest that cyclic GMP may be involved in promoting cellular events that are antagonistic to those mediated through an elevation of cyclic AMP. The concept of 'dualism' between the two cyclic nucleotides, as first postulated by Goldberg et al. (477) is supported by observations that the cellular events induced by agents or conditions which promote cyclic AMP generation are opposed by those that are associated with an accumulation of endogenous cyclic GMP. Kuehl et al. (478) observed a significant elevation in uterine cyclic GMP in ovariectomized rats treated with estradiol benzoate (90 min) or diethylstilbestrol (3 hr). Moreover, when endogenous uterine cyclic GMP and cyclic AMP concentrations were monitored at each stage of the estrus cycle, cyclic GMP concentrations were found to be highest (while that of the adenosine nucleotide being lowest) during proestrus when plasma estrogen levels also were maximal. These results raise the possibility that an enhanced cellular accumulation of cyclic GMP may also be involved in the expression of some of the actions of estrogens on uterine tissue. However, even to date, there is no evidence in support of this hypothesis for the action of androgens

on the prostate. It would therefore be of interest to investigate if guanylate cyclase-cyclic GMP system in the prostate is indeed responsive to androgen deprivation and repletion and if prostatic cyclic GMP levels respond differently from those of cyclic AMP following various hormonal manipulations.

While the precise relationship between non-gonadotropic hormones and cyclic AMP metabolism in the overall control of growth and function of androgen-dependent tissues still remains to be established, the possibility of synergism between these steroids and compounds such as prolactin and growth hormone also needs to be fully explored. Detailed examination of the hormone sensitivity of various protein kinases in the cell cytosol and nucleus is undoubtedly necessary, particularly in view of evidence for the presence of the multiplicity of protein kinases and phosphatases in eukaryote cells (4,479). Although the importance of investigating the synthesis and modification (phosphorylation, acetylation or methylation) of nuclear proteins by enzymes located in situ in the nucleus cannot be minimized, emphasis should also be placed on recognizing the specific substrates for various enzymes involved in the modification of nuclear proteins.

VI. SUMMARY

SUMMARY

Although studies on the mode of action of gonadal steroids in androgen-dependent cells have occupied the attention of many investigators, our knowledge concerning the initial event(s) that could be considered as important triggering device(s) to set into motion the multiferous morphologic and metabolic alterations in the prostate is incomplete. While cyclic AMP has long been suggested to act as an intracellular mediator for a variety of hormones and cellular regulators, evidence that this nucleotide may also be involved in the action of sex steroids on their target cells has recently begun to emerge. In order to deliniate the involvement of cyclic AMP system in the mechanism(s) of action of androgens, the influence of androgenic deprivation and repletion was investigated on the activities of adenylate cyclase, cyclic AMP-dependent and -independent protein kinases of the rat ventral prostate.

Whereas prostatic adenylate cyclase activity decreased progressively following orchidectomy attaining 37% of the values found in normal, intact animals, a single intramuscular dose (5 mg/100 g) of free testosterone to 120 hr castrate rats effectively restored enzyme activity within 24 hr. An intravenous injection of this androgen (5 mg/100 g) produced a slight elevation in the activity of the enzyme after 2 hr, and statistically significant increases were noted by 4 and 8 hr whether expressed on a per mg of tissue weight or per prostate basis. In contrast, dihydrotestosterone (5 mg/100 g, i.v.), an active metabolite of testosterone, elicited a significant increase in enzyme activity within 1 hr and the observed stimulation was maintained up to 4 hr following the hormone treatment. The observed rapid response appeared specific to androgens since estradiol, progesterone and methyl prednisolone exerted little or no effect on the prostatic enzyme. Moreover, the testosterone-stimulated increase in adenylate cyclase activity appeared

specific for the prostate gland as the enzyme activity in the thymus, an organ known to be unresponsive to androgenic stimulation, was not significantly affected by the hormone. These increases in prostatic adenylate cyclase activity were effectively prevented by pretreatment with actinomycin D and cycloheximide as well as a β-adrenergic blocking agent, propranolol. Cyproterone acetate, a potent anti-androgen, when given concurrently with testosterone, also inhibited the observed response of adenylate cyclase to androgen stimulation in vivo. Administration of this anti-androgen, by itself, exerted a marked inhibitory effect on the prostatic enzyme activity of 120 hr castrate rats. Furthermore, whereas NaF and L-epinephrine significantly stimulated the activity of the prostatic enzyme in vitro, the direct addition of dihydrotestosterone, testosterone propionate or free testosterone (10^{-6} - 10^{-4} M) to ventral prostate homogenates failed to produce any significant response. Although the activity of prostatic phosphodiesterase was slightly depressed 1 hr after an intravenous injection of dihydrotestosterone (5 mg/100 g), administration of the same dose of testosterone to castrate rats produced no statistically significant change in the cyclic AMP metabolizing enzyme.

Recent studies on the diverse effects of cyclic AMP has centred around the ability of this nucleotide to activate a class of enzymes known as protein kinases. Results of the present investigation also demonstrate that canine prostate possesses a cyclic AMP-dependent protein kinase which is located predominantly in the cytosol. The soluble enzyme, stimulated 2 to 4-fold by cyclic AMP, was isolated and partially purified. Two peaks, each exhibiting both cyclic AMP binding and kinase activities, were separated by using diethylaminoethylcellulose chromatography. While the major activity peak (fraction II) was eluted at 0.17 M NaCl, the minor peak (fraction I) was observed at a lower salt concentration. In the presence of cyclic AMP, maximum stimulation of the enzyme (fraction II) was observed at 7 min during which enzyme activity

was found to be linear with time. The enzyme was maximally stimulated (4-fold) by micromolar concentration of cyclic AMP although half maximal activation of the enzyme was observed in presence of 5×10^{-8} M concentration of the cyclic nucleotide. Equilibrium studies at pH 5.0 indicated the presence of one major class of binding site for cyclic AMP, and the association constant of this nucleotide to the enzyme was approximately 10^8 M^{-1}. The stimulation of canine prostate protein kinase was also observed with the 3',5'-monophosphate derivatives of cytidine, inosine, guanosine and uridine as well as with dibutyryl 3',5'-adenosine monophosphate, but higher concentrations of these cyclic nucleotides were required to provide the same degree of activation as that seen with cyclic AMP. In comparing the ability of α-casein, protamine, and various histone subfractions to serve as the substrate for protein kinase, highest cyclic AMP stimulation was demonstrated with histones. Although maximum velocity of the enzyme was enhanced approximately 5-fold in presence of cyclic AMP, kinetic studies indicated that the apparent Km for histone (0.5 mg per ml) remained the same whether determined in presence or absence of the cyclic nucleotide. In addition, cyclic AMP did not significantly change the apparent Km for ATP (1.2×10^{-5} M) although it caused a 3-fold increase in the maximal velocity of the enzyme. The purified enzyme was found to have an absolute requirement for divalent metal ion. Substitution of Mn^{2+} for Mg^{2+} decreased basal protein kinase activity as well as the stimulation noted with cyclic AMP. Similarly, the basal activity was lowered when Mg^{2+} was replaced by Ca^{2+} and cyclic AMP produced only little stimulation of the prostatic enzyme.

Androgenic deprivation and repletion resulted in marked alterations in cytosolic and particulate protein kinase activities and cyclic AMP-binding capacity of the rat ventral prostate. Whereas orchidectomy for 7 days exhibited significant enhancement in the specific activity of cytosolic cyclic AMP-dependent and independent protein kinases as well as cyclic AMP-binding

protein, administration of testosterone (5.0 mg/100 g, i.m., 5 days) exerted little or no effect in reversing these responses. In contrast, when expressed as total enzyme activity per prostate, castration led to marked decreases in protein kinase activity assayed in the presence and absence of the cyclic nucleotide. Likewise, the cyclic AMP-binding capacity of the soluble enzyme was depressed following androgenic deprivation. Although testosterone treatment for 3 days significantly reversed these effects, complete restoration was not achieved even after 5 days of androgen replacement therapy. Moreover, while exogenous cyclic AMP had no effect on protein kinase activity from crude nuclear preparations, the phosphorylation of endogenous nuclear substrates was dependent on the androgenic status of the animals. Whereas the castration produced decreases in the specific and total activity of prostatic particulate protein kinase as well as the cyclic AMP-binding protein, testosterone replenishment was effective in abolishing these alterations seen in orchidectomized rats.

Daily injection of cyproterone acetate (1.25 mg/100 g/day, s.c.) to mature rats resulted in marked impairment of the weight gain of various male sex accessory glands as well as significant alterations in cyclic AMP metabolism of the ventral prostate. Progressive decreases in the organ weights of the seminal vesicles, coagulating gland and ventral prostate were noted during daily administration of the anti-androgen. Likewise, prostatic adenylate cyclase activity was decreased during the anti-androgen treatment regardless of whether enzyme activity was expressed on a per mg tissue or a total organ weight basis. Although discontinuation of cyproterone acetate treatment significantly increased weight gain of these accessory sex glands as well as the activity of prostatic adenylate cyclase, normal levels were not achieved even after 10 days. In contrast, concurrent administration of testosterone (5 mg/100 g/day, s.c.) with the anti-androgen not only effectively antagonized these effects of cyproterone acetate, but significantly stimulated prostatic

adenylate cyclase as well as weight gain in these sex glands. As in the case of adenylate cyclase, cyproterone acetate treatment also decreased the cyclic AMP content of the prostate gland. It is of interest that normal values could be attained after subsequent withdrawal of the anti-androgen treatment for 10 days or simultaneous administration of testosterone during cyproterone acetate therapy.

Treatment with cyproterone acetate also resulted in marked alterations in soluble cyclic AMP-dependent and independent protein kinase activities and cyclic AMP-binding capacity of the ventral prostate. Whereas the specific activity of the soluble enzyme assayed in both the absence or presence of cyclic AMP increased progressively with the duration of the anti-androgen treatment, the total activity of the enzyme as expressed on a per organ weight basis was markedly lowered. Similarly, while the ability of the soluble enzyme to bind (^{3}H)-cyclic AMP <u>in vitro</u> was significantly enhanced when expressed on a per unit protein basis, a marked decrease was observed in the total cyclic AMP binding capacity of the prostate during cyproterone acetate treatment. Although protein kinase activity ratio was also lowered during the first 3 days of cyproterone acetate administration, this response was not apparent when the anti-androgen was given for 5 or 10 days. Moreover, administration of cyproterone acetate significantly lowered protein kinase activity (as indicated by a marked decrease in the incorporation of ^{32}P from (γ-^{32}P) ATP into endogenous nuclear substrates as well as the cyclic AMP binding capacity in crude nuclear preparation from the rat ventral prostate regardless of the basis of expression used. However, these enzymatic responses were normalized with cessation of the anti-androgen treatment or concurrent administration of testosterone. Studies on the protein kinase activity ratio of the nuclear preparation demonstrated a lack of dependence of this particulate enzyme on cyclic AMP (ratio being close to unity) as well as an insensitivity of this

parameter to cyproterone acetate treatment.

Administration of isoproterenol (1 mg/kg, i.p.) resulted in rapid elevation of adenylate cyclase activity and cyclic AMP levels in the ventral prostate of normal mature rats. The observed isoproterenol-stimulated changes in cyclic AMP metabolism were time-dependent and maximal stimulation was seen 5 min after treatment with this β-adrenergic agonist. The increases in prostatic adenylate cyclase and cyclic AMP also were related to the dose of isoproterenol administered and maximal enhancement of these parameters was seen with 1 mg/kg dose of the agonist. Whereas pretreatment of rats with propranolol (3 mg/kg, i.p.) partially reversed these alterations, administration of an α-adrenergic antagonist, phentolamine, even at a dose of 5 mg/kg, failed to elicit any appreciable effect. Although isoproterenol treatment also stimulated prostatic adenylate cyclase-cyclic AMP system in orchidectomized rats, adenylate cyclase activity, cyclic AMP levels as well as their percent stimulation by the β-adrenergic agonist was lower than that seen for intact controls. Testosterone replacement not only restored the responsiveness of this enzyme system to β-adrenergic stimulation, but significantly increased both enzyme activity and cyclic nucleotide levels in the presence as well as in the absence of isoproterenol treatment. Furthermore, changes in the activity of the prostatic soluble protein kinase following isoproterenol stimulation were associated with a decrease in the total activity of protein kinase (+cAMP) with concomitant increase in the cyclic AMP-independent enzyme. Whereas the ability of the enzyme to bind (^{3}H) cyclic AMP in vitro was decreased following isoproterenol treatment, the protein kinase activity ratio was significantly elevated. Although propranolol alone had little or no effect on these parameters, it inhibited partially the isoproterenol-induced alterations in cyclic AMP-dependent protein kinase and the cyclic AMP binding capacity. Treatment with propranolol also blocked the increases in the kinase activity

ratio and in the activity of cyclic AMP-independent enzyme seen with isoproterenol. The present investigation lends support to the hypothesis that changes in the cyclic AMP-adenylate cyclase-protein kinase system play an important role in the overall mechanism(s) by which male sex steroids exert their diverse anabolic effects on the rat ventral prostate. Our results also suggest directions along which future work may be carried out concerning the role of cyclic nucleotides and nuclear protein phosphorylation in the androgenic control of this hormone-dependent organ.

VII. REFERENCES

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PUBLICATIONS

(a) Research Papers

Tsang, B.K. and Singhal, R.L. Cyclic 3',5'-adenosine monophosphate-dependent protein kinase from dog prostate. Can. J. Physiol. Pharmacol. 51, 941 (1973).

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(b) Abstracts

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ABSTRACT

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Whereas prostatic adenylate cyclase activity decreased progressively following orchidectomy, a single intramuscular dose (5 mg/100 g) of free testosterone to 120 hr castrate rats effectively restored enzyme activity within 24 hr. While intravenous administration of this androgen (5 mg/100 g) produced a slight elevation in the activity of the enzyme after 2 hr, statistically significant increases were noted by 4 and 8 hr. In contrast, dihydrotestosterone (5 mg/100 g, i.v.), the active metabolite of testosterone, elicited a significant increase in enzyme activity within 1 hr and the observed stimulation was maintained up to 4 hr following the hormone treatment. The observed rapid response appeared specific to androgens since estradiol, progesterone and methyl prednisolone exerted little or no effect on the prostatic enzyme. Moreover, the testosterone-stimulated increase in adenylate cyclase activity was specific for the prostate gland as the enzyme activity from the thymus, an organ known to be unresponsive to androgenic stimulation, was not significantly affected by the hormone treatment. Cyproterone acetate, a potent anti-androgen, when given concurrently with testosterone, inhibited the observed response of adenylate cyclase to androgen stimulation in vivo. Although the activity of prostatic phosphodiesterase was slightly depressed 1 hr after an intravenous injection of dihydrotestosterone (5 mg/100 g), administration of the same dose of testosterone to castrate rats produced no significant change in the cyclic AMP-metabolizing enzyme.

Canine prostate possesses a cyclic AMP-dependent protein kinase which is located predominantly in the cytosol. The enzyme was maximally stimulated (4-fold) by micromolar concentration of cyclic AMP, although half maximal activation of the enzyme was observed in presence of 5×10^{-8}M concentration of the cyclic nucleotide. Equilibrium studies at pH 5.0 indicated the presence

of one major class of binding site for cyclic AMP and the association constant of this nucleotide to the enzyme was approximately 10^8 M^{-1}. The stimulation of canine prostate protein kinase was also observed with the 3',5'-monophosphate derivatives of cytidine, inosine, guanosine and uridine as well as with dibutyryl 3',5'-adenosine monophosphate, but higher concentrations of these cyclic nucleotides were required to provide the same degree of activation as that seen with cyclic AMP. In comparing the ability of α-casein, protamine and various histone subfractions to serve as the substrate for protein kinase, highest cyclic AMP stimulation was demonstrated with histones. Although maximum velocity of the enzyme was enhanced approximately 5-fold in presence of cyclic AMP, kinetic studies indicated that the apparent Km for histone (0.5 mg per ml) remained the same whether determined in presence or absence of the cyclic nucleotide. In addition, cyclic AMP did not significantly change the apparent Km for ATP (1.2×10^{-5}M) although it caused a 3-fold increase in the maximal velocity of the enzyme. The purified enzyme was found to have an absolute requirement for divalent metal ion. Substitution of Mn^{2+} for Mg^{2+} decreased basal protein kinase activity as well as the stimulation noted with cyclic AMP. Similarly, the basal activity was lowered when Mg^{2+} was replaced by Ca^{2+} and cyclic AMP produced only little stimulation of the prostatic enzyme.

Androgenic deprivation and repletion resulted in marked alterations in cytosolic and particulate protein kinase activities and cyclic AMP-binding capacity of the rat ventral prostate. Whereas orchidectomy for 7 days exhibited significant enhancement in the specific activity of cytosolic cyclic AMP-dependent and -independent protein kinases as well as cyclic AMP-binding protein, administration of testosterone (5.0 mg/100 g, i.m., 5 days) exerted little or no effect in reversing these responses. In contrast, when expressed as total enzyme activity per prostate, castration led to marked decreases in protein kinase activity assayed in the presence and absence of the cyclic

nucleotide. Likewise, the cyclic AMP-binding capacity of the soluble enzyme was depressed following androgenic deprivation. Although testosterone treatment for 3 days significantly reversed these effects, complete restoration was not achieved even after 5 days of androgen replacement therapy. Moreover, while exogenous cyclic AMP had no effect on protein kinase activity from crude nuclear preparations, the phosphorylation of endogenous nuclear substrates was dependent on androgenic status of the animals.

Daily injection of cyproterone acetate (1.25 mg/100 g/day, s.c.) to mature rats resulted in marked impairment of the weight gain of various male sex accessory glands and produced significant alterations in cyclic AMP metabolism of the ventral prostate. Prostatic adenylate cyclase activity was markedly decreased during the anti-androgen treatment, regardless of whether enzyme activity was expressed on a per mg tissue or a total organ weight basis. Although discontinuation of cyproterone acetate treatment significantly increased weight gain of these accessory sex glands as well as the activity of prostatic adenylate cyclase, normal levels were not achieved even after 10 days. In contrast, concurrent administration of testosterone (5 mg/100 g/day, s.c.) during anti-androgen treatment not only effectively antagonized these effects of cyproterone acetate, but significantly stimulated prostatic adenylate cyclase as well as weight gain in these sex glands. Treatment with cyproterone acetate also resulted in marked alterations in soluble cyclic AMP-dependent and -independent protein kinase activities and cyclic AMP-binding capacity of the ventral prostate. Whereas the specific activity of the soluble enzyme assayed in both the absence or presence of cyclic AMP as well as its specific cyclic nucleotide binding capacity increased progressively with the duration of the anti-androgen treatment, the total activity of the enzyme as expressed on a per organ weight basis was markedly lowered. Moreover, administration of cyproterone acetate significantly lowered protein kinase activity (as

indicated by a marked decrease in the incorporation of ^{32}P from (γ-^{32}P)-ATP into endogenous nuclear substrates) as well as the cyclic AMP binding capacity in crude nuclear preparation from the rat ventral prostate regardless of the basis of expression used. As seen with adenylate cyclase, these enzymatic responses were normalized with cessation of the anti-androgen treatment or concurrent administration of testosterone.

Administration of isoproterenol (1 mg/kg, i.p.) resulted in rapid elevation of adenylate cyclase activity and cyclic AMP levels in the ventral prostate of normal mature rats. The observed isoproterenol-stimulated changes in cyclic AMP metabolism were time- and dose-dependent with maximal stimulation being seen at 5 min after the 1 mg/kg dose of the β-adrenergic agonist. Whereas pretreatment of rats with propranolol (3 mg/kg, i.p.) partially reversed these alterations, administration of an α-adrenergic antagonist, phentolamine, even at a dose of 5 mg/kg, failed to elicit any appreciable effect. Although isoproterenol also stimulated prostatic cyclic AMP system in orchidectomized rats, adenylate cyclase activity, cyclic AMP levels as well as their percent stimulation by the β-adrenergic agonist were lower than that of the intact controls. Testosterone replacement not only restored the responsiveness of this enzyme system to β-adrenergic stimulation, but significantly increased both enzyme activity and cyclic nucleotide levels in the presence as well as in the absence of isoproterenol treatment. Furthermore, changes in prostatic soluble protein kinase after isoproterenol stimulation was associated with a decrease in the total activity of protein kinase (+cAMP) with a concomitant increase in the activity of the nucleotide-independent enzyme. Whereas the ability of the enzyme to bind (^{3}H)-cyclic AMP in vitro was decreased following isoproterenol treatment, the protein kinase activity ratio was significantly elevated. Although propranolol alone had little or no effect on these parameters, it significantly inhibited the isoproterenol-induced alterations in cyclic AMP-dependent and

-independent protein kinases, kinase activity ratio and the cyclic AMP binding capacity. Data from the present investigation support the concept that changes in cyclic AMP-adenylate cyclase-protein kinase system play an important role in the overall mechanism(s) by which male sex steroids exert their diverse anabolic effects on the ventral prostate.

Addendum

1. Re: Measurement of Adenylate Cyclase Activity (page 84)

In preliminary experiments carried out to study the effect of orchidectomy and testosterone replacement on prostatic adenylate cyclase, enzyme activity was expressed on per mg tissue, per prostate as well as per mg protein basis. Since the responsiveness of the prostatic enzyme to androgenic deprivation and repletion was similar and independent of the basis of the expression of the enzyme used, activity of the enzyme reported in this thesis was thus expressed on a per mg tissue and per prostate basis.

2. Re: Beta-Adrenergic Stimulation of Prostatic Adenylate Cyclase-Cyclic AMP System (page 193)

While results from this investigation demonstrate the presence of a functional adenylate cyclase-cyclic AMP system in the rat ventral prostate which is responsive to androgenic steroids, the question has been raised as to whether this response observed following *in vivo* administration of the hormone was a direct effect of the androgens or a secondary response as a result of synthesis and release of catecholamine by the hormones. Although increase in prostatic adenylate cyclase activity following androgenic stimulation was effectively blocked by a β-adrenergic antagonist, propranolol, the possibility exists that the observed effect of propranolol may be non-specific and independent of its β-adrenergic receptor blocking activity. Dissociation of this androgenic response from the adrenergic effects on the prostatic adenylate cyclase was evident from the observation (page 148) that whereas orchidectomy resulted in a marked decrease in the activity of the prostatic enzyme (from 4.89 to 2.83 pmoles cAMP formed/min/mg tissue), administration of isoproterenol (1 mg/kg, i.p.) failed to stimulate adenylate cyclase activity to the levels observed in the intact, isoproterenol-treated controls (10.79 pmoles cAMP formed/min/mg tissue). Further experiments

are needed in order to delineate, with certainty, the extent to which biogenic amines may play in the androgen regulation of adenylate cyclase-cyclic AMP system in the prostate gland.

3. Re: Dosage of Testosterone Used to Study Effect of Cyproterone Acetate On Prostatic Cyclic AMP Levels (page 161)

While testosterone at a dose of 5 mg/100 g/day failed to increase prostatic cyclic AMP levels when concomitantly administered with cyproterone acetate, the possibility exists that a different response may be achieved if higher doses of the androgen were administered to reverse the effects of the antiandrogen.

4. Re: In Vitro Effects of High Concentration of FSH and ICSH on Prostatic Adenylate Cyclase (page 114)

Although FSH and ICSH, at a concentration of 400 µg/ml, significantly decreased the activity of prostatic adenylate cyclase in vitro, these differences were only marginal and may have questionable physiological significance.