THE EFFECTS OF ANABOLIC AND ANDROGENIC STEROIDS ON ANDROGEN-RESPONSIVE TISSUES

by

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ABSTRACT

Androgens are known to produce two different responses in target cells. In androgen-dependent tissues, androgens produce both hyperplasia and hypertrophy (androgenic actions) while in androgen-sensitive tissues, androgens produce hypertrophy only (anabolic actions). To delineate the molecular mechanism underlying this differential action, the present studies compared the actions of androgens in the ventral prostate, an androgen-dependent tissue, with the kidney, an androgen-sensitive tissue, and related them to the subcellular localization of androgen receptors (AR) in these tissues of the rat.

In the prostate, long term (7 day) castration produces a reduction in DNA synthesis, DNA content, and weight. Androgen replacement (dihydrotestosterone - DHT) results in an increase in DNA synthesis followed temporally by an increase in DNA content and weight. In short term (24 h) castrates, DHT treatment (5 mg/kg) increases prostate weight (hypertrophy) but it does not affect DNA content. In the kidney, long or short term castration does not result in a change in weight, DNA content, or DNA synthesis. DHT treatment (5 mg/kg) produces an increase in renal weight, but does not affect DNA content or synthesis. In the short

term castrated rat, treatment with an anabolic steroid (stanozolol - STAN, 5 or 25 mg/kg) prevents regression of the prostate (maintenance effect), but it fails to stimulate growth. In addition, stanozolol fails to stimulate growth in the fully regressed prostate from rats 7 days post-castration.

Both the prostate and kidney contain specific, high affinity AR, however, the subcellular localization is different. In the prostate, the majority of the AR is localized to the nucleus [nuclear salt soluble (nuclear) = 2989 ± 262 fmol/mg DNA; nuclear matrix associated (matrix) = 1671 ± 110 fmol/mg DNA; cytosolic = 1244 ± 49 fmol/mg DNA]. Correction for matrix sphere loss during preparation increases the matrix AR ≈ 3000 fmol/mg DNA. In the kidney, the majority of the AR is cytosolic (298 ± 81 fmol/mg DNA; nuclear = 149 ± 7 fmol/mg DNA) and there is no detectable matrix AR. After castration, no nuclear AR is present in the kidney, but the prostate contains residual nuclear AR (119 ± 36 fmol/mg DNA) which remains at 7 days post-castration. No prostatic matrix AR is present after castration. DHT treatment (5 mg/kg) restores prostate matrix AR to normal levels but does not induce kidney matrix AR.

Treatment of short term castrated rats with DHT or STAN (5 mg/kg) stimulates localization of AR to the prostatic

nuclear matrix, but DHT produces higher titers than does STAN. In addition, DHT produces longer retention of the AR on the matrix, and the concentration of the nuclear plus matrix AR is two-fold higher 24 hours after DHT injection.

The results of these studies demonstrate that in an androgen-dependent tissue, androgens produce both hypertrophy and hyperplasia. In contrast, androgens produce hypertrophy only in an androgen-sensitive tissue. The differential actions of androgens in these tissues may be attributable to the presence of matrix AR in androgen-dependent tissues such as the prostate. In the developed prostate gland, anabolic steroid prevents castration-induced regression, but it fails to stimulate growth. It is proposed that prolonged retention of AR on the nuclear matrix is required for full androgenic effects in androgen-responsive tissues.

INTRODUCTION

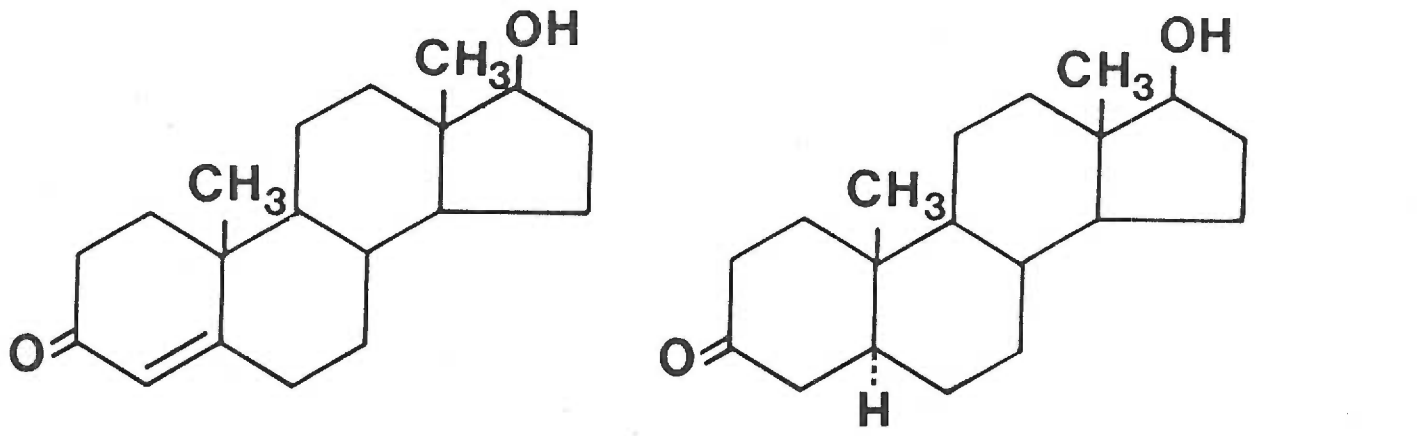
Androgen Physiology

Androgens are sex steroids with masculinizing action. Although typically considered a male hormone, females also produce small amounts of androgens. The major endogenous androgen in the male, testosterone, is produced primarily by the Leydig cells of the testes (95%) and, to a lesser extent, by the adrenals (5%). In females, testosterone is produced in approximately equal parts by the ovaries, the adrenals, and by peripheral conversion of other hormones (1).

Adult males produce approximately 8 mg of testosterone per day resulting in a plasma concentration of about 600 ng/dl (normal range 300 - 900 ng/dl). Female testosterone levels in plasma are approximately 30 ng/dl. In humans, the majority of the circulating testosterone is bound to serum proteins. Approximately 65% is bound to sex hormone binding globulin (SHBG), and most of the remaining is bound to albumin. Only 2% remains free and is capable of entering cells and producing androgen actions (1).

In many target tissues, testosterone is converted to dihydrotestosterone (DHT) by the microsomal or nuclear enzyme 5α-reductase (Figure 1). This enzyme has been shown to be present in most androgen-responsive tissues, but some

Figure 1
Chemical structures of testosterone and 5α-dihydrotestosterone.



variation occurs between species. For example, while the kidney in the rat contains appreciable amounts of 5α-reductase (2), the kidney in mice is devoid of this enzyme (3). However, DHT is usually the major androgen in target tissues, and it is 2 - 2.5 times more potent than testosterone (4).

The major pathway for degradation of testosterone is by hepatic reduction of the ketone and the double bond in the A ring. This reduction produces inactive metabolites (androsterone, epiandrosterone, and etiocholanolone) which are subsequently conjugated and excreted in the urine (1).

Androgen Actions

Androgens produce a variety of androgenic and anabolic actions (Table 1). While androgens are not involved in gonadal differentiation, they are responsible for the *in utero* development of the male reproductive tract. Androgens stimulate the differentiation of the mesonephric (Wolffian) ducts into seminal vesicles, ejaculatory ducts, epididymis, and ductus deferens. In addition, androgens are responsible for the development of the scrotum from the labioscrotal swellings, the penis from the phallus and urogenital folds, and the prostate from the urogenital sinus (5-7). Finally, in most mammals, imprinting of the non-cyclic pattern of hypothalamic-pituitary function in males is believed to be

Table 1
Androgen actions.

Development of male reproductive tract *in utero*
Growth of penis, scrotum, and male accessory sex organs at puberty
Maintenance of male secondary sex characteristics
Stimulation of long bone growth
Induction of epiphyseal plate closure at puberty
Stimulation of libido (males and females)
Muscle development
Stimulation of erythropoiesis

mediated by the action of androgens *in utero* (8). It appears that the undifferentiated hypothalamic-pituitary axis is inherently female, i.e., gonadotropin-releasing hormone (GnRH) and, consequently, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are released in a cyclical fashion required for normal ovulatory function. Gonadotropin release is converted to a tonic pattern under the influence of androgens in the male. The current data indicate, however, that it is estradiol-17β, produced by aromatization of testosterone in the hypothalamic cells, which actually leads to non-cyclical release of GnRH. Females retain the cyclic pattern of gonadotropin release because ovarian production of estrogens is low *in utero*. The small amount which is produced is bound to α-fetoprotein and is not available to influence hypothalamic function (8). In contrast to other mammals, permanent masculinization of the hypothalamus in humans and subhuman primates does not

occur. The potential for cyclic gonadotropin release remains intact (9).

At puberty, androgens stimulate growth of the penis, scrotum, and male accessory sex organs. In addition, they develop and maintain male secondary sex characteristics, normal reproductive function, and sexual performance ability. Other androgenic actions include stimulation of long bone growth, epiphyseal plate closure, and a poorly understood effect on the central nervous system to increase libido and influence behavior (1). The anabolic effects of androgens include muscle development and stimulation of erythropoiesis by increasing renal erythropoietin production and by direct effects on bone marrow (10).

In females, androgens are responsible for development of pubic hair at puberty and probably for the stimulation of libido. Androgen excess can result in virilization characterized by hirsutism, clitoromegaly, acne, and menstrual irregularities (1).

Androgen-Dependent Versus Androgen-Sensitive Tissues

Androgens are capable of producing two distinct responses in target cells (Table 2). In androgen-dependent tissues such as the prostate gland and seminal vesicles, castration produces tissue atrophy characterized by a loss in cell number and decrease in cell size (11-13).

Table 2
Androgen-dependent versus androgen-sensitive tissues.

TYPE	TISSUE	ACTION
Dependent	Prostate Seminal Vesicle	Hyperplasia (RNA & DNA synthesis)
Sensitive	Kidney Skeletal Muscle	Hypertrophy (RNA synthesis)

Histological examination of the prostate gland in castrated rats reveals that this atrophy is characterized by a loss of epithelial cells while the stromal cell population remains relatively constant (14,15). The biochemical steps involved in this cell loss include loss of nuclear androgen receptor retention, fragmentation of nuclear DNA into low molecular weight oligomers by calcium-magnesium-dependent endonuclease, and eventual complete digestion of these DNA oligomers (16). Androgen replacement restores the tissue to its normal size by producing hyperplasia and hypertrophy (androgenic actions). Just as the cell loss is most evident in the epithelium, the majority of the hyperplastic response also occurs in this cell type. It is important to note that once maximal prostate size has been attained, further androgen treatment does not result in continued increase in DNA content (17). Only in abnormal conditions such as benign prostatic hyperplasia and prostate cancer is there a failure of this control and a resultant overgrowth of the gland.

In androgen-sensitive tissues such as the kidney, androgen depletion produces a decrease in cell size without concomitant cell loss. Androgen stimulation causes the cells to hypertrophy but does not cause cell proliferation (anabolic actions) (13,18,19). The response of the DNA to either castration or androgen stimulation is both slow (no change prior to two weeks) and minimal (<10% variation in

DNA content with hormonal manipulation) (13). To date, the mechanisms by which androgens produce hyperplastic responses in some tissues but only hypertrophic responses in others have not been elucidated.

Many synthetic androgen analogs have been manufactured in an attempt to dissociate anabolic actions from androgenic actions. To date, complete elimination of the androgenic activity in the anabolic steroids has been unsuccessful (Table 3). Most anabolic steroids are produced by modifications of testosterone designed to increase the anabolic:androgenic ratio or to retard catabolism and enhance oral activity. Increases in the anabolic:androgenic

Table 3
Anabolic steroids and their anabolic:androgenic ratios.

Preparation	Anabolic:Androgenic* Ratio
Dromostanolone	4:1
Methandriol	4:1
Methandrostenolone	3:1
Nandrolone	5:1
Oxandrolone	13:1
Oxymetholone	3:1
Stanozolol	6:1

*Approximate values based on testosterone which exhibits an anabolic:androgenic ratio of 1:1 (values obtained from reference 8)

ratio are accomplished by removal of the C-19 methyl group (to produce 19-nortestosterone derivatives) or by modification of the A-ring of the steroid nucleus. Oral activity is increased by alkylation at the 17α position. Unfortunately, this latter modification, which slows catabolism by the liver, also makes the steroids hepatotoxic and hepatocarcinogenic (8). The most favorable anabolic:androgenic ratios are produced by modifications in the A-ring of the steroid nucleus (e.g., stanozolol and oxandrolone). While oxandrolone offers a higher anabolic:androgenic ratio, it is not available commercially.

It is not known if the anabolic and androgenic actions of androgens are mediated via the same mechanism or receptor. However, most receptor-binding studies favor the concept of a single androgen receptor with differences in binding affinity or efficacy producing the differential actions observed (20). The concept of a single androgen receptor is further supported by several studies which reveal that a mutation in the androgen receptor gene on the X-chromosome eliminates androgen responses in both androgen-sensitive and androgen-dependent tissues (3,21,22)

A review of the literature reveals that most investigations of anabolic steroids have been concerned with protein metabolism or athletic performance (23-26). A few investigators have studied the interaction of anabolic

steroids with the androgen receptor (8,20,27), but these experiments have been limited to studies involving the soluble, cytosolic form of the receptor. Few studies to date have investigated the interaction of anabolic steroids with nuclear androgen receptors, and no studies have examined anabolic steroid induction of matrix-associated androgen receptors.

Mechanisms of Androgen Action

Androgens, like all steroid hormones, produce their effects by associating with specific receptors in target cells. Androgen receptors are acidic proteins and exhibit a high specificity and affinity (K_D = 0.2 - 2.0 nM) for androgens. They have a limited capacity (i.e., they are saturable) and are found only in tissues which respond to androgens.

In addition, evidence indicates that these receptors are necessary for androgens to produce their effects. In the testicular feminization (Tfm) syndrome, androgen receptors (AR) are absent or abnormal, and testosterone levels are elevated. The lack of active receptors results in an inability to respond to androgens, and an individual who is genotypically male (46XY) appears phenotypically female (6,7,28). The insensitivity to androgens results in degeneration of the Wolffian ducts internally and,

consequently, lack of development of the vas deferens, epididymis, and seminal vesicles. Further, the absence of androgen action results in development of female external genitalia and the lower portion of the vagina.

While the Tfm syndrome was initially described in man (29), the syndrome has subsequently been noted in rats (30), mice (3,31), and cattle (32). The majority of the studies on the Tfm syndrome have utilized the mouse model. One of the problems in these studies has been that affected animals do not develop androgen-dependent tissues such as the prostate. Therefore, most studies have examined androgen-sensitive tissues such as the kidney or submaxillary gland (3). These studies have shown that the Tfm syndrome results from an abnormal gene which is transmitted by X-linked inheritance (31). In addition, this genetic abnormality leads to production of a defective androgen receptor in the Tfm mice (3,21,22). It is now known that the Tfm syndrome in humans is also the result of a genetic defect in the androgen receptor gene located on the X chromosome (33,34).

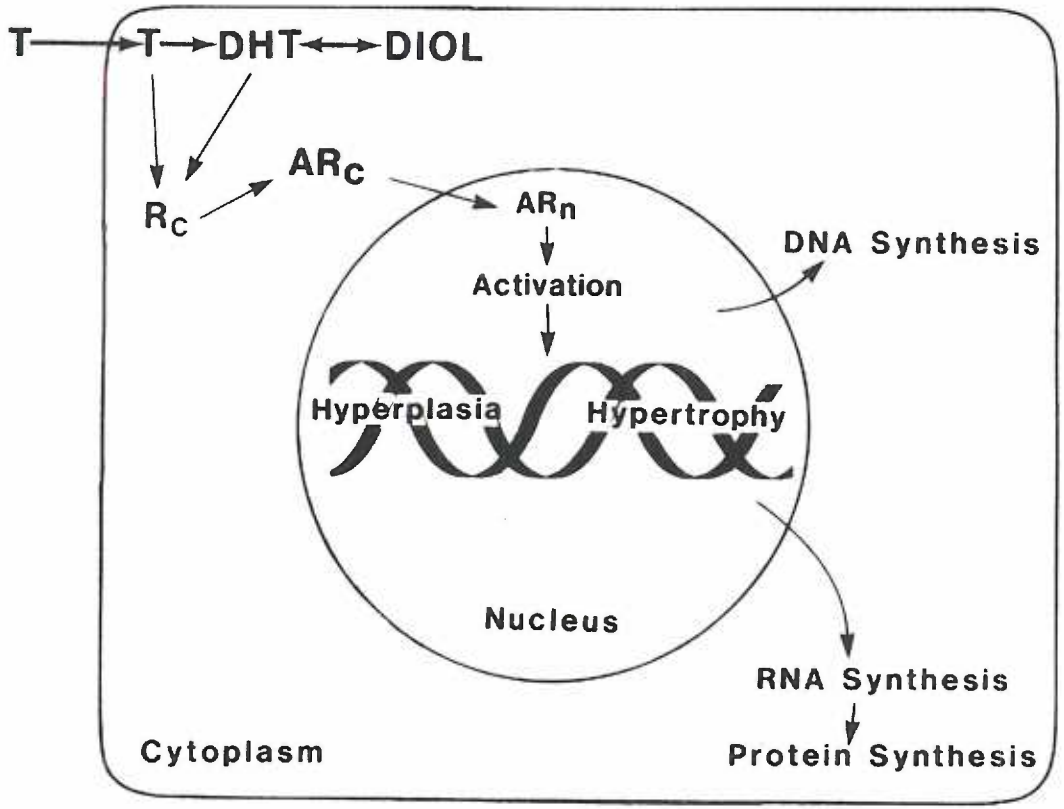
Over the years, the results of numerous studies have led to the development of a general model for steroid hormone action. While certain aspects of this model have recently changed as a result of data obtained from more sophisticated laboratory methods (e.g., immunocytochemistry and gene cloning), the classic scheme is presented below for

its historical value and for completeness (Figure 2). In this model (shown for the interaction of androgens with androgen receptors), testosterone enters the target cell from the blood and is converted to DHT. The DHT binds to a cytoplasmic androgen receptor which then undergoes changes in conformation to produce an activated complex. This complex, which is capable of binding to nuclear acceptor sites, is translocated to the nucleus where it binds and stimulates a variety of biochemical events. Finally, the androgen-receptor complex breaks down and is displaced from the nucleus (35). Each of these processes will be subsequently considered in detail with new evidence and changes in the general scheme presented when appropriate.

Transport of testosterone into the cell

One of the least understood aspects of steroid binding is the mode by which steroids enter target cells. Although steroid hormones are lipid soluble and will pass into cells by passive diffusion, it appears that entry into target cells is preferentially facilitated (35). Giorgi, et al, have demonstrated the existence of specific transport mechanisms for androgens in the canine and human prostate gland (36-38). Active transport increases the uptake of testosterone along its concentration gradient and prevents the passive diffusion of DHT out of the cell. The existence

Figure 2
Schematic representation of the mechanism of androgen action. DHT, 5α-dihydrotesterone; Diol, 5α-androstane-3α,17ß-diol; R_c, cytoplasmic receptor; R_n, nuclear receptor; T, testosterone; A, androgen.



of a facilitated transport mechanism has not been demonstrated in all androgen target tissues, but such a system is believed to exist in at least the male accessory sex organs (35).

Conversion of testosterone to DHT

Once testosterone has entered a target cell, it is converted to DHT by the enzyme 5α-reductase. In tissues such as the seminal vesicles and prostate, this conversion occurs rapidly. On the other hand, in tissues such as the kidney and brain, this process may be slower or absent, and testosterone may be the major androgen present (39). Further, as discussed previously, the quantity of 5α-reductase may vary in the same target tissue from different species.

Binding of DHT to the cytoplasmic androgen receptor, activation, and translocation

The classic model of steroid hormone action states that after DHT is formed, it binds to high affinity androgen receptors in the cytoplasm. These receptors, first characterized in 1969 by investigators working with rat prostate (40-42), are highly specific for DHT but will, to a lesser extent, bind testosterone (43,44). After binding hormone, the steroid-receptor complexes undergo activation,

and are subsequently translocated to the nucleus. Recent studies indicate, however, that cytoplasmic receptors probably do not exist *in vivo* (45-49).

The data implying translocation of androgen receptors were derived from indirect evidence obtained in experiments comparing intact (i.e., non-castrated) and castrated animals. In these studies, it was noted that 24 hours post-castration there was a large increase in the concentration of cytosolic androgen receptors and, concomitantly, an equal decrease in the concentration of nuclear receptors (50-52). From these observations, it was concluded that the appearance of cytosolic androgen receptors was the result of the release or recycling of nuclear receptors into the cytoplasm (52,53). Further, within minutes after an injection of testosterone or DHT into a castrated animal, the nuclear receptor was replenished and the cytosolic receptor concentration decreased accordingly (54). Therefore, it was postulated that in the absence of androgen the receptors were localized in the cytoplasm, but after binding to androgens they were translocated to the nucleus. While the exact mechanism of translocation is unknown, studies of the estrogen receptor have shown various metabolic inhibitors have no effect on the process. Therefore, it was believed that the process did not involve an energy utilizing system (55). Further, there was no

evidence that the steroid was transferred from one receptor to another. Instead, it appeared that the steroid remained bound to the same protein in both the cytoplasmic and nuclear compartments (56).

It is now evident that hormone receptors are primarily nuclear proteins whether or not they are occupied by steroid hormones (45). This conclusion is based on data derived from two different types of studies. First, utilizing enucleation procedures to separate the nucleus from the cytoplasm, estrogen receptors were found only in the nuclear fractions (46). Second, using immunocytochemistry with a monoclonal antibody specific for the estrogen receptor (47) or the androgen receptor (48,49,57), the receptors again appeared to be localized in the nuclei.

These findings have led some investigators to propose two new models to explain the presence of steroid receptors in the cytosol. In one model, receptors which are not occupied by steroids are located in the nucleus, but they are extracted into the cytosol by the hypotonic buffers used during tissue homogenization. Steroid-occupied receptors are in an activated form which has a higher affinity for nuclear acceptor sites. Therefore, these activated receptors require a high salt concentration to extract them from the nuclei (58).

In the second model, investigators have proposed that

unoccupied nuclear and cytoplasmic receptors are in equilibrium with each other, and the final distribution is dependent on the volumes of the nuclear and cytoplasmic compartments (59,60). In the intact cell where the nuclear and cytoplasmic volumes are constant, the majority of the unoccupied receptors are located in the nucleus. Upon homogenization of the cell in aqueous buffer, the cytoplasmic volume increases dramatically while the nuclear volume remains constant. The dilution of the cytoplasm decreases the cytosolic receptor concentration, and the nuclear receptors redistribute to the cytoplasm until the equilibrium is reestablished. Therefore, as the homogenization volume increases, more receptor is extracted from the nucleus into the cytosol (60). In addition to these studies, several investigators have reported that androgen receptors which are unoccupied by androgens are present in the nuclei of the rat and human prostate (61-63).

Activation of the androgen-receptor complex.

Regardless of the location of the androgen receptor, binding of androgen to the receptor produces alterations in the tertiary and quaternary structure of the receptor molecule. These alterations, which are believed to be irreversible, produce an "activated" androgen-receptor complex in which the receptor is in a 4.5S, 58 Å form with a

molecular weight of 117,000 (64,65). This form of the receptor has an increased propensity to bind to nuclear acceptor sites (35).

During in vitro studies, the 4.5S receptor appears to exist in equilibrium with an 8 - 9S, 85 - 106 Å receptor which has a molecular weight in the range of 280,000 to 365,000 (64). Whether or not this form of the receptor exists in vivo is not known. It is possible that the 4.5S receptors aggregate in the presence of non-physiologic buffers to form the 8S receptor. It is known that the 8S form will not bind to nuclear acceptor sites when added to preparations of purified nuclei or nuclear matrices (66). This observation has led investigators to postulate that the 8S receptor may actually exist in vivo and serve to modulate androgen actions.

The 8S and 4.5S forms of the androgen receptor can be interconverted in vitro by a variety of processes. In KCl concentrations greater than 0.1M, the receptor is predominantly in the 4.5S form while in low salt solutions the 8S form predominates (64). Other studies have shown the presence of a protein in serum and target tissues which is capable of converting the 4.5S form to the 8S form. This protein, known as the 8S androgen receptor promoting factor, appears to be produced by androgen-responsive cells. It is postulated that it may serve to modulate the actions of

androgens by converting the receptor to a form incapable of binding in the nucleus (67). Further, warming the 8S receptor to 30°C converts it to the 4.5S form (39,68).

A number of divalent cations produce an interconversion between the 8S and 4.5S forms. Calcium and magnesium promote formation of the 4.5S receptor while zinc and cadmium favor the 8S form (69). In addition to the effects of cations, one anion is known to influence the androgen receptor form. The molybdate ion stabilizes the receptor in the 8S form (69-73). However, it will not effect a conversion from 4.5S to 8S. It merely prevents alteration of receptors that already exist as 8S (69).

Recently, two separate laboratories succeeded in cloning the gene for the human androgen receptor (74,75). The protein product from the transcription of the gene was specific for androgens and bound androgens with a high affinity. The molecular weight of the putative androgen receptor was reported to be 76,000 by Chang, et al (74) and approximately 99,000 by Tilley, et al (75). This latter group also reported a sedimentation coefficient of 8.3S in low salt sucrose gradients. While the reported molecular weights more closely approximate the weight of the 4.5S form of the receptor, the sucrose density gradient data match the 8S form. This discrepancy may be related to the assay conditions under which each of these determinations was made

(i.e., denaturing gel electrophoresis for the determination of the molecular weights versus low salt gradients for the sedimentation coefficient).

Binding of androgen-receptor complexes to nuclear acceptor sites

Once the steroid-receptor complexes are "activated," they bind with high affinity to specific acceptor sites in the nucleus. Numerous investigators have attempted to characterize the exact nature of these sites in a variety of different steroid-responsive tissues (65,68,76-93). These studies have resulted in identifying virtually every component of the nucleus as the acceptor region including crude chromatin (82,83), DNA (68,84-86,93), nuclear proteins (87,88,93), ribonuclearprotein particles (89,90), nuclear membranes (91,92), and the nuclear matrix (76,80,81). Of these sites, it is currently believed that the androgen-receptor complexes bind to both chromatin and the nuclear matrix.

It has been known for some time that nuclei in steroid responsive cells from non-castrated animals contain two populations of receptors (78,79). The first population can be extracted from the nucleus by high ionic strength buffers (0.6 M KCl) and are referred to as nuclear salt-soluble receptors, salt-extractable receptors, or, merely, nuclear

receptors. It is believed that these receptors are associated with the chromatin domains. More recent studies indicate that they may be associated with the nuclear matrix, but they are not occupied by steroid and, therefore, are not active (94). The second population of receptors, which is resistant to high salt extraction, is believed to be tightly associated with the nuclear matrix due to their activation by hormone (78-81,95).

RNA and DNA synthesis

While the exact role of the different acceptor sites has not been determined, it is known that binding of the androgen-receptor complexes in the nucleus stimulates RNA synthesis (96-100) and, in androgen-dependent tissues, DNA synthesis (96,101-104). These processes have been studied extensively in the ventral prostate from rats 7 days post-castration. After injection of testosterone, ribosomal RNA synthesis begins within 30 minutes. This event is followed by messenger RNA synthesis (1 - 2 hours post-injection) and, after 2 - 3 days, DNA synthesis (35).

Current evidence indicates that steroid hormones function by direct binding of the hormone-receptor complex to a regulatory element on the DNA known as the hormone responsive element (HRE). The HRE is within or flanks the gene it controls, and the HRE will function independently of

orientation or position. In the presence of the steroid-hormone complex, the HRE acts as an enhancer element to stimulate transcription initiation (105-107).

The molecular structure of the androgen receptor has only recently been determined by gene cloning studies (74,75). Like all of the other steroid hormone receptors, the androgen receptor contains a cysteine-rich region near the middle of the protein. This region is the putative DNA-binding region, and the cysteines in the area are believed to interact with metal ions, particularly zinc, to form and stabilize DNA binding "fingers" which project out from the receptor and interact with DNA. The steroid binding site is located on the carboxy terminus of the receptor and, like the DNA binding region, has an identical amino acid sequence for both the human and rat androgen receptor. In addition, there is a high degree of homology in these regions when compared with other steroid hormone receptors (74).

O'Malley and other investigators have proposed that the tissue-specific effects of androgens are probably related to the availability of inducible genes that are contained in structurally distinct domains that allow them to respond to steroid receptor stimulation (108,109). The availability of these domains is the result of molecular differentiation. The regions of chromatin containing actively expressed genes are firmly attached to the nuclear matrix and, perhaps, more

easily transcribed (108).

In addition to the direct regulation of gene transcription, steroids have been shown to modify post-transcriptional gene expression. Several investigators have shown that steroid hormones can affect mRNA stability (110), protein processing (111), and protein turnover (112,113).

The mechanisms by which androgen-receptor complexes mediate DNA synthesis are poorly understood. However, it is believed that the initial androgen stimulation of RNA synthesis is involved (35). A number of different proteins are required for DNA synthesis and cell division, and androgens are known to enhance the activity of many of these proteins. Most notable of these proteins is the 9S form of DNA polymerase which is the active form of the polymerase necessary for DNA synthesis (96,114), the DNA binding proteins which regulate the entry of cells into mitosis (115-117), and the DNA unwinding proteins which relax the DNA helix and allow DNA polymerase access to single strands for replication (114,118). Whether one of these proteins or a yet unelucidated protein has the ultimate control over regulation of DNA synthesis has not been determined.

Breakdown of the androgen-receptor complex

The fate of the receptor complex after its interaction with the nucleus is unknown. Although some investigators

have suggested that the receptor is recycled to the cytoplasm (90,119,120), the data are not conclusive. Further, no studies have yet explained how DHT itself is eliminated from the nucleus (35).

The Nuclear Matrix

The nuclear matrix (matrix) is the salt-insensitive and chromatin-depleted scaffolding of the nucleus. It consists of a residual nuclear envelope and lamina, a highly condensed nucleolus, and a granular and interchromatinic network (81,121).

The matrix is an operationally defined structure and is prepared by sequentially treating purified nuclei with Triton X-100, DNAse I, hypotonic buffer, and hypertonic (high salt) buffer (80,122) [Table 4]. Triton X-100 is a

Table 4
Structure and composition of the nucleus and the nuclear matrix.

Constituent	Nucleus %	% Removed	Matrix %
Protein	69.0	85	88.6
DNA	23.9	99	2.1
RNA	3.4	70	8.7
Phospholipid	3.7	98	0.6

(Compiled from reference 122).

non-ionic detergent which extracts phospholipids. The DNAse I enzyme cleaves the nucleic acids (both DNA and RNA) which are subsequently extracted by the hypotonic and hypertonic buffers. The hypotonic buffer removes approximately 75% of the DNA, 20% of the RNA, and 50% of the nuclear protein. The high salt (1.6 M NaCl) buffer extracts the majority of the remaining DNA. In addition, the hypertonic extraction removes an additional 50% of the initial RNA and 35% of the initial nuclear proteins (122). Consequently, the resulting matrix structure is composed mostly of salt-resistant proteins (89%) and RNA (9%). The matrix proteins consist of three major acidic, non-histone proteins with molecular weights in the range of 60,000 to 70,000 and several minor proteins with molecular weights in the range of 100,000 to 200,000 (81,123).

A number of specific biological functions have been attributed to the matrix (Table 5) including DNA organization (124-127) and replication (127-130), gene

Table 5
Functional characteristics of the nuclear matrix.

Differential protein phosphorylation during cell cycle
Reversible contraction induced by cations
DNA replication and organization
Association of newly transcribed RNA
Binding sites for steroid receptors

transcription (131-134), processing and transporting of RNA (135,136), protein phosphorylation (137,138), and changes in nuclear morphology (139). In addition, cells in which little DNA or RNA synthesis takes place, such as adult chicken erythrocytes, are found to lack the internal portion of the nuclear matrix. Instead, they consist of empty shells made of the nuclear envelope and lamina (140).

Sex steroid binding sites have been demonstrated in association with the matrix in rat prostate (80), rat uterus (141,142), and chicken liver (80). Since steroid-receptor complexes are known to stimulate DNA and RNA synthesis, it has been postulated that the matrix is the site of modulation of these functions. It is significant to note that although the matrix contains only 15% of the original nuclear proteins, it contains 45 - 60% of the nuclear steroid receptors, indicating an enrichment of receptors in the matrix fraction (143). Further, investigations have shown that treatment of the matrix with RNAse will remove the majority of the matrix-associated receptors. Therefore, it is believed that these receptors are associated with residual or newly transcribed RNA in the matrix (81).

In addition to the data which show that steroid hormones bind to receptors on the matrix, several studies have shown that steroid-receptor complexes will bind to nuclear matrix acceptor sites under cell free conditions in

vitro (94,144,145). These data have been interpreted to support the concept that the ultimate site of steroid hormone action is the nuclear matrix. Additional data from recent studies indicate that steroid receptors are capable of binding to the nuclear matrix in the absence of hormone (94). However, these receptors are unable to function as gene regulatory elements until they bind steroid hormones (146), and, unlike steroid-occupied matrix receptors, they are sensitive to extraction in 0.6M KCl (94).

Pathophysiological Significance of Androgens

Androgens are responsible for a number of pathophysiological conditions including benign prostatic hyperplasia (BPH), prostatic carcinoma, acne, and hirsutism (females). Neither BPH nor prostatic cancer develop in castrated males (147,148). In an unselected series of autopsies, BPH was evident in 50 - 60% of men between 40 and 59 years of age and in 95% of men over 70 years of age (149). Although BPH was clinically evident in only 5 - 10% of these individuals, it is, nevertheless, a prevalent disorder associated with aging for which there currently are only limited non-invasive techniques of therapy. However, inhibitors of 5α-reductase activity are actively being studied for their ability to reduce levels of prostatic DHT (150). This treatment can reduce prostatic volume in BPH by

20 - 30%, and it is hoped that this will prove to be an effective, non-surgical form of management.

Eighty percent of all prostate cancers are initially androgen dependent (151). Although the exact statistics are not yet compiled, it is estimated that in 1989 there will have been 103,000 new cases of prostatic cancer and that 29,000 men will have died of the disease. In addition, as of 1990, prostate cancer exhibits the highest incidence rate of all cancers in males in the United States, and it is the third leading cause of cancer deaths in males (152). Current management of prostate cancer consists of radical prostatectomy, radiotherapy, or hormonal therapy (estrogens, orchiectomy, GnRH analogs, and antiandrogens) (153). These forms of treatment have a number of adverse side effects, including, most notably, impotence and, with estrogen therapy, cardiovascular complications (154).

A number of investigators have attempted to correlate the quantity and subcellular distribution (i.e., cytosolic, nuclear salt-soluble, matrix-associated) of androgen receptors with the clinical response to therapy, prognosis, or etiology of BPH and prostate cancer (143,155-165). The results of these studies indicate that the cytosolic androgen receptor concentrations in BPH and prostate cancer do not differ significantly from the concentrations in normal prostates. However, several investigators have

reported that both the nuclear salt-soluble androgen receptors and the nuclear matrix-associated androgen receptors are significantly higher in patients with BPH than in normal prostates (143,157,161). In contrast, in studies of prostates from patients with prostate cancer, the mean salt-soluble receptor concentration is elevated, but the matrix-associated receptor level remains comparable to normal (143). It should be noted, however, that there is great variation between cancer specimens. While the mean salt-soluble level is significantly higher than normal, the individual levels range from sub- to supra-normal (162). Further, the actual quantity of salt-soluble androgen receptors appears to be an important indicator of prognosis. Patients with the highest salt-soluble receptor concentrations have a longer duration of response and survival following hormonal therapy than those with relatively low levels (160,162).

While quantitation of the subcellular distribution of androgen receptors appears to be important in predicting the prognosis of a patient, the exact role of each receptor type in the etiology of BPH and prostate cancer has not yet been determined.

Objectives

In summary, investigators currently believe that most,

if not all, androgen receptors are associated with the nuclei of target cells. In addition, there is increasing evidence that the nuclear matrix plays an important role in the synthesis of RNA and DNA. The data indicate that in pathophysiological states such as BPH and prostate cancer there is a relative increase in the concentration of nuclear androgen receptors. These findings underlie the need to focus on the cell nucleus in an attempt to ascertain the specific role of the salt-soluble and the matrix-associated androgen receptors in regulating normal and abnormal functions of androgen-responsive tissues. While the differential actions of androgens in androgen-dependent and androgen-sensitive tissues are well recognized, no studies have compared these biological responses with the subcellular localization of androgen receptors in these two tissue types. It is postulated that a differential partitioning or localization of androgen-receptor complexes between the nuclear matrix and the salt-soluble receptor fractions is a fundamental aspect of the process by which androgens produce distinct actions in androgen-sensitive and androgen-dependent tissues. The purpose of the present studies was:

1. to delineate a differential growth response to DHT in an androgen-sensitive tissue (kidney) versus an androgen-dependent tissue (ventral prostate) in the

rat model.

2. to characterize and compare the subcellular distribution of androgen receptors between the prostate and the kidney.
3. to characterize the effects of an anabolic steroid (stanozolol) on the prostate and the kidney and relate these actions to the subcellular distribution of androgen receptors in these tissues.

MATERIALS AND METHODS

Animals, Tissues, and Steroid Treatment

Adult, male Sprague-Dawley rats (80-120 days old) were obtained from Bantin & Kingman (Fremont, CA). Animals were utilized either intact (i.e. non-castrated) or were castrated via the trans-scrotal route using ether anesthesia. All animal use was consistent with the Public Health Service Policy and Guide for the Care and Use of Laboratory Animals and was approved by the Oregon Health Sciences University Animal Care Committee (protocols 86-133G and 89-150).

Dihydrotestosterone or stanozolol (Sigma Chemical Co.,St. Louis, MO) was administered either by subcutaneous injection (in corn oil or dimethylsulfoxide [DMSO]) or by subcutaneous implants (placed using ether anesthesia). Implants were prepared using 3.0 cm lengths of Silastic® tubing (Dow Corning #602-305; I.D.=0.078 in; O.D.=0.125 in) packed with dry steroid and the ends sealed with Silastic® medical adhesive (Dow-Corning #891). The final length of steroid in the tubing was 2.5 cm. An implant of this size produces physiologic levels of androgen and is sufficient to regenerate DNA in a regressed prostate in castrated rats to within 90% of intact levels (166).

Animals were killed by stunning followed by cervical

dislocation. Tissues (ventral prostate, seminal vesicles, kidney, and/or liver) were removed and placed immediately into ice cold 0.25 M sucrose in TM buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.4).

In Vitro Incorporation of ^{3}H-Thymidine into Accessory Sex Organs, Kidney and Liver DNA

Castrated rats (7 days) were injected with dihydrotestosterone (1.0 mg/kg) or received a DHT implant as described above for 1, 2, 3, 5, or 10 days. Intact control rats received daily injections of corn oil or an empty implant. At 24 hours after the final hormone injection, the animals were killed, and the ventral prostate, seminal vesicles (empty), liver, and kidneys were rapidly dissected. In a second study, intact and castrated rats were utilized without treatment. Animals were killed and tissues collected as described above. Excised tissues were rinsed thoroughly in ice-cold saline and blotted. Tissue slice preparations (0.5 mm) were prepared using a McIlwain tissue chopper (Brinkmann Instruments, Westbury, N.Y.). Approximately 100 mg of ventral prostate, seminal vesicle, liver, and kidney were used to study the incorporation of ^{3}H-thymidine (thymidine-methyl-H^3, New England Nuclear, Boston, Mass.; specific activity = 48.95 Ci/mmole) into DNA.

The tissue slice preparations were incubated in 1.0 ml

of Krebs-Ringer bicarbonate buffer (pH = 7.4) which contained glucose (100 mg%) and ^{3}H-thymidine (4 μCi/ml). A Dubnoff metabolic incubator was utilized for the incubation procedures which were carried out at 37°C for 60 minutes under a continuous atmosphere of 95% O_2 and 5% CO_2. Incubations were terminated by the addition of 1.0 ml of ice-cold perchloric acid (0.8 M) and the incubation tubes were immediately placed in an ice bath. The slice preparations were subsequently homogenized in 2.0 ml of ice-cold perchloric acid (0.4 M) and centrifuged at 1000 x g for 5 minutes. After centrifugation, the supernatants were discarded and the pellets were washed twice with 3.0 ml of ice-cold perchloric acid (0.4 M). The final pellets were resuspended in 2.0 ml of ice-cold perchloric acid (0.4 M). The DNA was extracted by acid hydrolysis (85°C, 30 min), and a 1.0 ml aliquot was removed from each sample for determination of DNA according to the method of Burton (167) using calf thymus DNA as a standard. In addition, a 0.5 ml aliquot was removed for quantitation of ^{3}H-thymidine incorporated into DNA. Samples were counted using a Beckman LS7500 liquid scintillation spectrophotometer. Counts per minute were converted to dpm by use of a quench curve prepared from a set of quenched tritium standards (Beckman Instruments, Irvine, CA). The radioactivity incorporated into the ventral prostate, seminal vesicle, liver, and

kidney DNA was expressed as dpm/ug DNA.

Tissue Homogenization

All tissues utilized for quantitation of the subcellular distribution of androgen receptors were freshly excised. Preliminary data revealed that freezing the tissues (-60°C) resulted in a marked redistribution of androgen receptors between the different subcellular fractions (see appendix, Figure A-1). Tissues were maintained at 0 - 4°C at all times unless specified otherwise.

Tissues were homogenized in 5 volumes (5:1 vol:wt based on starting tissue wt) of 0.25 M sucrose/TM buffer. All buffers contained 1 mM phenylmethylsulfonylfluoride (PMSF) added immediately prior to use. PMSF is a serine protease inhibitor shown to protect steroid hormone receptors from degradation (80). Tissues utilized for preparation of cytosolic or nuclear salt soluble fractions were homogenized with a Polytron® (Brinkmann Instruments, Westbury, N.Y.; 2 x 10 sec bursts with 10 sec cooling between). After homogenization, samples were centrifuged (800 x g, 15 min) to yield crude cytosolic (supernatant) and nuclear fractions (pellet).

Tissues utilized for preparation of nuclear matrices were homogenized using a Potter-Elvehjem Teflon-glass homogenizer (1100 RPM, 15 strokes). After homogenization,

samples were centrifuged (800 x g, 15 min) and the supernatant was aspirated to leave the crude nuclear pellet.

Preparation of Cytosols

Crude cytosols from prostate or kidney were treated with buffer concentrate to yield a final concentration of 50 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.2 M sucrose, and 0.8 mM $MgCl_2$ ± 20 mM Na_2MoO_4, pH 7.4. Sodium molybdate was shown to significantly increase the amount of androgen receptor measured in prostate cytosols from castrated rats (see appendix, Table A-1) and was, therefore, added to all cytosols being utilized to quantitate cytosolic AR concentration. In cytosols utilized for cell-free binding of cytosolic AR to nuclear acceptor sites, sodium molybdate was omitted because previous studies have indicated that the molybdate ion inhibits binding of receptors to nuclear acceptor sites (144). The crude cytosols (± Na_2MoO_4) were incubated (15 min with mixing every 5 min) with the pellet from an equal volume of dextran-coated charcoal (0.05% dextran + 0.5% charcoal in TEDG buffer) in order to remove endogenous steroid. Final cytosols were obtained by ultracentrifugation (100,000 x g, 1 h). Cytosolic protein concentration was determined by the method of Lowry et al (168) using bovine serum albumin (BSA) as a standard. The cytosolic protein concentration was

adjusted to 2.5 mg/ml using TEDG buffer ± 20 mM Na_2MoO_4 (50 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, ± 20 mM Na_2MoO_4, pH 7.4).

Preparation of Nuclear Salt Extracts

Crude nuclear pellets were washed twice with 5 volumes 0.25M sucrose/TM buffer with centrifugation (800 x g, 15 min) and aspiration of wash buffer after each wash. Pellets were extracted (30 min) with 4 volumes 0.6 M KCl in TEDG buffer then centrifuged (40,000 x g, 30 min) to separate the salt extract from the extracted nuclear pellet. The salt extract was diluted with an equal volume of TEDG buffer containing 40 mM Na_2MoO_4 to produce a final buffer of TEDG, 20 mM Na_2MoO_4, and 0.3 M KCl. The protein concentration of the salt extract was determined by the method of Lowry et al (168) using BSA as a standard. The concentration was adjusted to 0.25 mg/ml by diluting with TEDG buffer + 20 mM Na_2MoO_4.

The nuclear pellet was extracted with 0.6 M perchloric acid (85°C, 30 min), centrifuged (1500 x g, 10 min), and the supernatant analyzed for DNA content by the diphenylamine method (167) using calf thymus DNA as a standard.

Preparation of Purified Nuclei

Purified nuclei were prepared by treating crude

prostatic or renal nuclei with 10 volumes 1% Triton X-100 in 0.25 M sucrose/TM buffer (15 min), centrifugation (800 x g, 15 min), and sedimentation through 2.2 M sucrose/TM buffer (40,000 x g, 1 h). The purified nuclei were resuspended in 0.25 M sucrose/TM buffer using a Dounce homogenizer and an aliquot was analyzed for DNA content by the diphenylamine method (167) using calf thymus DNA as a standard.

Preparation of Nuclear Matrices

Nuclear matrices (matrices) were prepared using the procedure of Barrack and Coffey (80). Briefly, purified nuclei were sequentially treated with 5 volumes DNAse I (20 μg/ml) [from bovine pancreas, Sigma Chemical Co. #DN-EP] in 10 mM Tris-HCl (pH 7.4)/5 mM $MgCl_2$ (15 min), 2.5 volumes low magnesium (LM) buffer containing 10 mM Tris-HCl (pH 7.4) and 0.2 mM $MgCl_2$ (15 min), and finally with 5 volumes 1.6 M NaCl in LM buffer (30 min). The matrices were then washed and resuspended with TE buffer (10 mM Tris-HCl, 1.5 mM EDTA, pH 7.4) in a Dounce homogenizer. Centrifugation of samples was carried out at 800 x g for 15 min following DNAse treatment and 1500 x g for 30 min. following all subsequent treatments and washes.

Electron Photomicroscopy

Nuclei and matrices were prepared as described above

and were pelleted by centrifugation (1500 x g, 30 min). Pellets were fixed in 2% cacolydate-buffered glutaraldehyde containing 1% formaldehyde. The nuclear spheres and matrix spheres were resuspended in 2% agar, post-fixed in 2% osmium, dehydrated in gradated ethanol solutions followed by toluene, and imbedded in Polybed 812. One micron thick sections and 700 Å thin sections were prepared using a Sorval MT2 ultramicrotome. The resultant sections were viewed and photographed with a Phillips EMU-200 transmission electron microscope.

Quantitation of Cytosolic Androgen Receptors

Aliquots of cytosol were incubated (0-4°C, 16 h) with ^{3}H-R1881 (methyltrienolone; 17β-hydroxy-17α-methylestra-4,9,11-trien-3-one; [17α-methyl-^{3}H]R1881, 86 Ci/mmol, New England Nuclear, Boston, MA) at 0.25 - 5.0 nM (prostate) or 0.25 - 2.0 nM (kidney) (169). Each aliquot also contained 0.5 μM triamcinolone acetonide (Sigma, St. Louis, MO) to prevent association of ^{3}H-R1881 with progesterone receptors (170). Specificity of binding was established by incubating ^{3}H-R1881 in the presence of 100-fold excess unlabeled R1881. The optimal incubation time and temperature were ascertained from preliminary experiments (see appendix, Table A-2).

Bound and free radiolabel were separated using either dextran-coated charcoal [DCC] (0.05% dextran + 0.5% charcoal

in TEDG buffer) or hydroxylapatite [HAP] (60 - 65% in TEDG buffer). The HAP (Bio-gel HTP hydroxylapatite, DNA grade, Bio-Rad Laboratories, Richmond, CA) was prepared by washing twice in three volumes TK buffer (50 mM Tris-HCl, 10 mM KH_2PO_4, pH 7.2).

An equal volume of DCC or HAP suspension was added to the labeled cytosol aliquot. The DCC was incubated (5 min), and centrifuged (1500 x g, 10 min). The HAP was incubated (0°C, 30 min) with mixing every ten minutes and centrifuged (800 x g, 5 min). The HAP-precipitated ^{3}H-R1881-androgen receptor complex was washed with TEDG buffer (3 x 2 ml) then extracted with 1.0 ml 95% ethanol (25°C, 1 h).

The DCC supernatants or the HAP ethanol extracts were transferred to plastic vials, solubilized with 5 ml Ready-Solv HP/b (Beckman, Fullerton, CA), and radioactivity was quantified by scintillation counting. Counts per minute were converted to dpm by use of a quench curve as described above. In general, specific radioligand binding was 300 - 4000 dpm above background for the ligand concentration range of the titration analysis. The data were analyzed using the method of Scatchard (171). Both DCC and HAP produce comparable results in the quantitation of cytosolic AR (see appendix, Table A-3).

Quantitation of Androgen Receptors in Nuclear Salt Extracts

Aliquots of salt extract were incubated (12°C, 20 h) with 0.5 μM triamcinolone acetonide and ^{3}H-R1881 at 0.25 - 5.0 nM (prostate) or 0.25 - 2.0 nM (kidney). Specificity of binding was established by incubating ^{3}H-R1881 in the presence of 100-fold excess unlabeled R1881. Incubation time and temperature required to establish equilibrium were determined in preliminary experiments (see appendix, Figure A-2). Bound and free radiolabel were separated using HAP as described above. The ethanol extract was transferred to plastic vials, solubilized with 5 ml Ready-Solv HP/b, and radioactivity was quantified by scintillation counting. Counts per minute were converted to dpm by use of a quench curve as described above. In general, specific radioligand binding was 600 - 3000 dpm above background for the ligand concentration range of the titration analysis. The data were analyzed using the method of Scatchard (171).

Quantitation of Matrix-associated Androgen Receptors

Aliquots of matrix suspension were incubated (0-4°C, 20 h) with 0.5 μM triamcinolone acetonide and ^{3}H-R1881 at 0.5 - 5.0 nM (prostate) or 0.25 - 2.0 nM (kidney). Specificity of binding was established by incubating ^{3}H-R1881 in the presence of 100-fold excess unlabeled R1881 (competitor).

Incubation of matrices with radiolabeled steroid was

initially conducted at both 0°C and 12°C to ascertain optimal binding conditions. While incubation at 12°C produced a higher binding capacity than at 0°C (2658 versus 1462 fmol/mg DNA, respectively) the higher temperature also decreased the binding affinity (K_D = 3.50 nM at 12°C, 1.48 at 0°C). Since matrix androgen receptors generally exhibit K_D values in the 1 - 2 nM range (80), further studies were conducted at 0°C.

After incubation, each matrix aliquot was diluted with 1 ml TE buffer and centrifuged (1500 x g, 30 min). The matrix pellet was washed with 2 ml additional TE buffer, centrifuged (1500 x g, 30 min), and extracted with 95% ethanol (25°C, 1 h). The ethanol extract was transferred to plastic vials, solubilized with 5 ml Ready-Solv HP/b, and radioactivity was quantified by scintillation counting. Counts per minute were converted to dpm by use of a quench curve as described above. In general, specific radioligand binding was 600 - 8000 dpm above background for the ligand concentration range of the titration analysis. The data were analyzed using the method of Scatchard (171).

Cell-free Binding of Cytosolic Androgen Receptors to Nuclear Acceptor Sites

Binding of cytosolic AR to purified nuclei was carried out using a modification of the method described by Barrack

(144). Briefly, kidney and prostate cytosols from rats castrated 24 h previously were prepared in the absence of molybdate and incubated (0-4°C, 16 h) with a single saturating concentration of ^{3}H-R1881 (2.0 nM for kidney; 5.0 nM for prostate). Triamcinolone acetonide ± unlabeled R1881 (competitor) was added as described above. After incubation, aliquots of labeled cytosol (± competitor) were added in duplicate to a pellet of purified nuclei freshly prepared from prostate or kidney from rats castrated 24 h earlier. The cytosol/nuclear suspensions were incubated (0-4°C, 2 h) on a rotary shaker (150 rpm) then centrifuged (1500 x g, 30 min) to separate the cytosol from the nuclear pellet. Nuclei were washed, centrifuged, and quantified as described above for matrix pellets. Total cytosolic AR available for binding to nuclei was quantified by treating aliquots of labeled cytosol (± competitor) without nuclei with DCC or HAP. The percent of total AR which bound to the nuclei was calculated as follows:

(nuclear bound AR/total AR) x 100.

Statistical Analyses

The statistical significance of differences between group mean values was established using paired or unpaired t-tests. Statistical differences between three or more group mean values were determined by analysis of variance and

subsequent application of the Student-Newman-Keuls test. A probability value of 0.05 or less was considered significant. Statistical calculations were performed using "Primer of Biostatistics: The Program" and the associated text to supplement the computer program (172).

RESULTS

I. Androgenic Steroids and Growth

Dihydrotestosterone injections

In the long term (7 day) castrated rat, daily injections (for seven days) with dihydrotestosterone (DHT) at either 1 or 5 mg/kg body weight resulted in significant increases in prostatic weight and DNA content compared to controls (Table 6). In contrast, while both doses of DHT also produced an increase in renal weights, there was a lack of concomitant increase in DNA content compared to controls. When 7 day castrated rats were treated for 7 days with daily injections of vehicle only, there was no further change in the DNA content of either the prostates or the kidneys. However, while there was no change in the weight of the kidneys, the weight of the prostates decreased significantly.

Dihydrotestosterone implants

Prostates from rats 10 days post-castration revealed significant decreases in both weight and DNA content compared to intact (i.e., non-castrated) controls (Figure 3, intact versus day 0). Treatment with DHT Silastic® implants produced significant increases in prostatic weight and DNA content when assayed 3 days after implanting. While the

Table 6
Effect of dihydrotestosterone (DHT) injections on prostate and kidney weights and DNA content in rats seven days post-castration.

Treatment	Duration of Treatment (days)	Prostate		Kidney	
		Weight (mg)	DNA (μg)	Weight (g)	DNA (mg)
7 day castrate	0	123 ± 16	430 ± 33	1.22 ± 0.07	3.36 ± 0.11
DMSO Vehicle	7	74 ± 4**	407 ± 22	1.25 ± 0.06	3.53 ± 0.10
DHT (1 mg/kg)	7	360 ± 26*	613 ± 21*	1.43 ± 0.05*	3.44 ± 0.12
DHT (5 mg/kg)	7	431 ± 35*	694 ± 49*	1.51 ± 0.02*	3.53 ± 0.06

Data are expressed as mean ± SEM (n=4)

*$p \leq 0.05$ compared to controls

**$p \leq 0.05$ compared to 7 day castrate controls

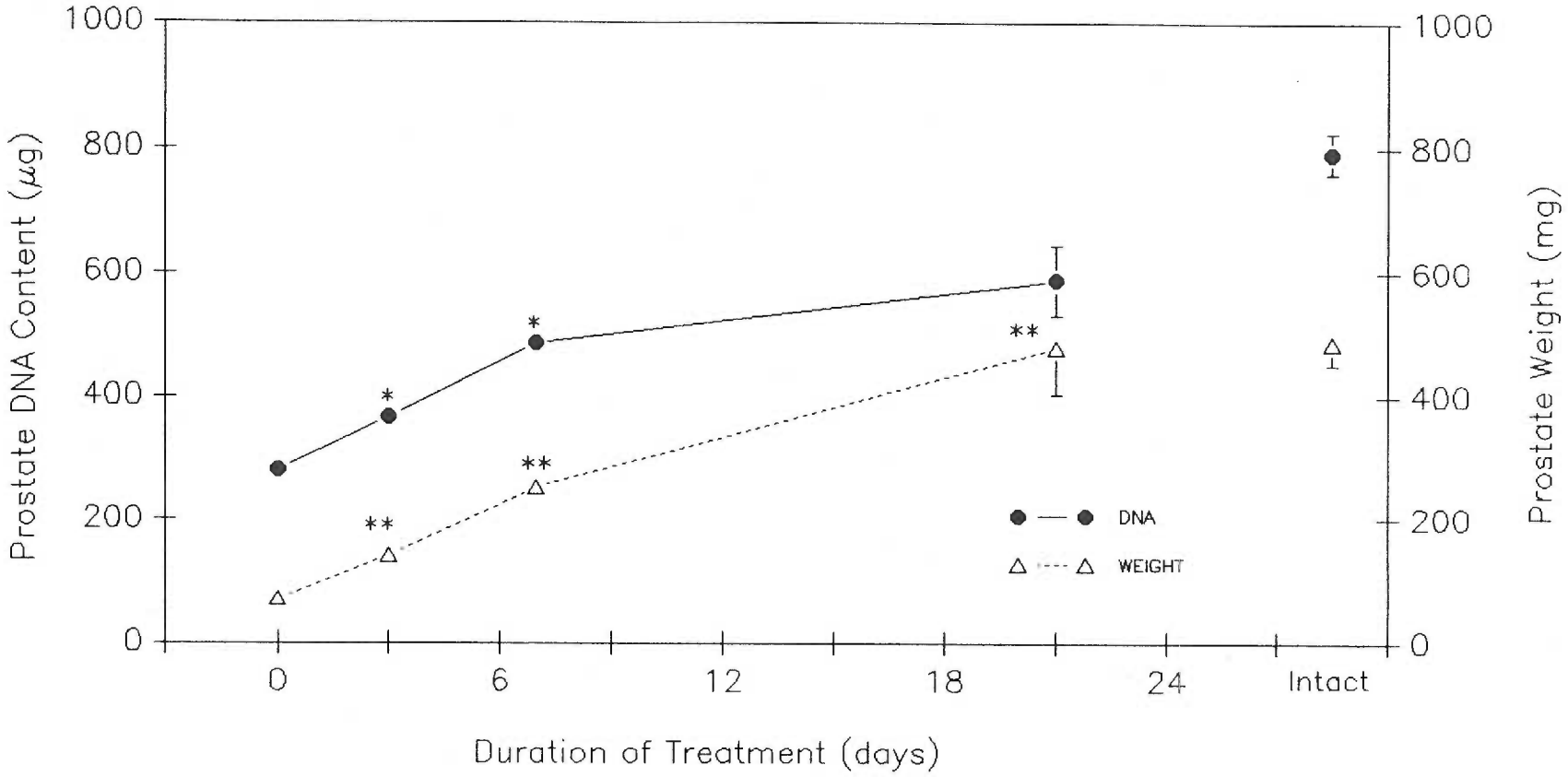


Figure 3: Effect of dihydrotestosterone (DHT) implants on prostate weight and DNA content in rats. Ten days post-castration implants were placed subcutaneously. Data are expressed as mean ± SEM (n=3). *p<0.05.

weight continued to increase throughout 21 days of treatment, there was no significant change in DNA content between days 7 and days 21 of treatment. In addition, while the prostatic weights after 21 days of DHT treatment were comparable to weights in intact rats, the DNA content was approximately 75% of intact values.

In kidneys from 10 day castrated rats treated with DHT implants, there was no significant change in either weight or DNA content throughout the 21 day treatment period (Figure 4). The data in Figure 4 also reveal that, unlike the prostate, the kidneys showed no loss of weight or DNA content during the post-castration period (intact rats versus day 0 rats).

^{3}H-thymidine incorporation into DNA

- intact and castrated rats

Seven days after castration, the <u>in vitro</u> incorporation of ^{3}H-thymidine into the DNA of prostates and seminal vesicles was significantly decreased compared to intact controls (Table 7). This castration-induced decrease in ^{3}H-thymidine incorporation was not observed in the kidney or liver.

- *dihydrotestosterone injections*

During the first two days of treatment, injections of DHT (1 mg/kg/day) into rats seven days post-castration

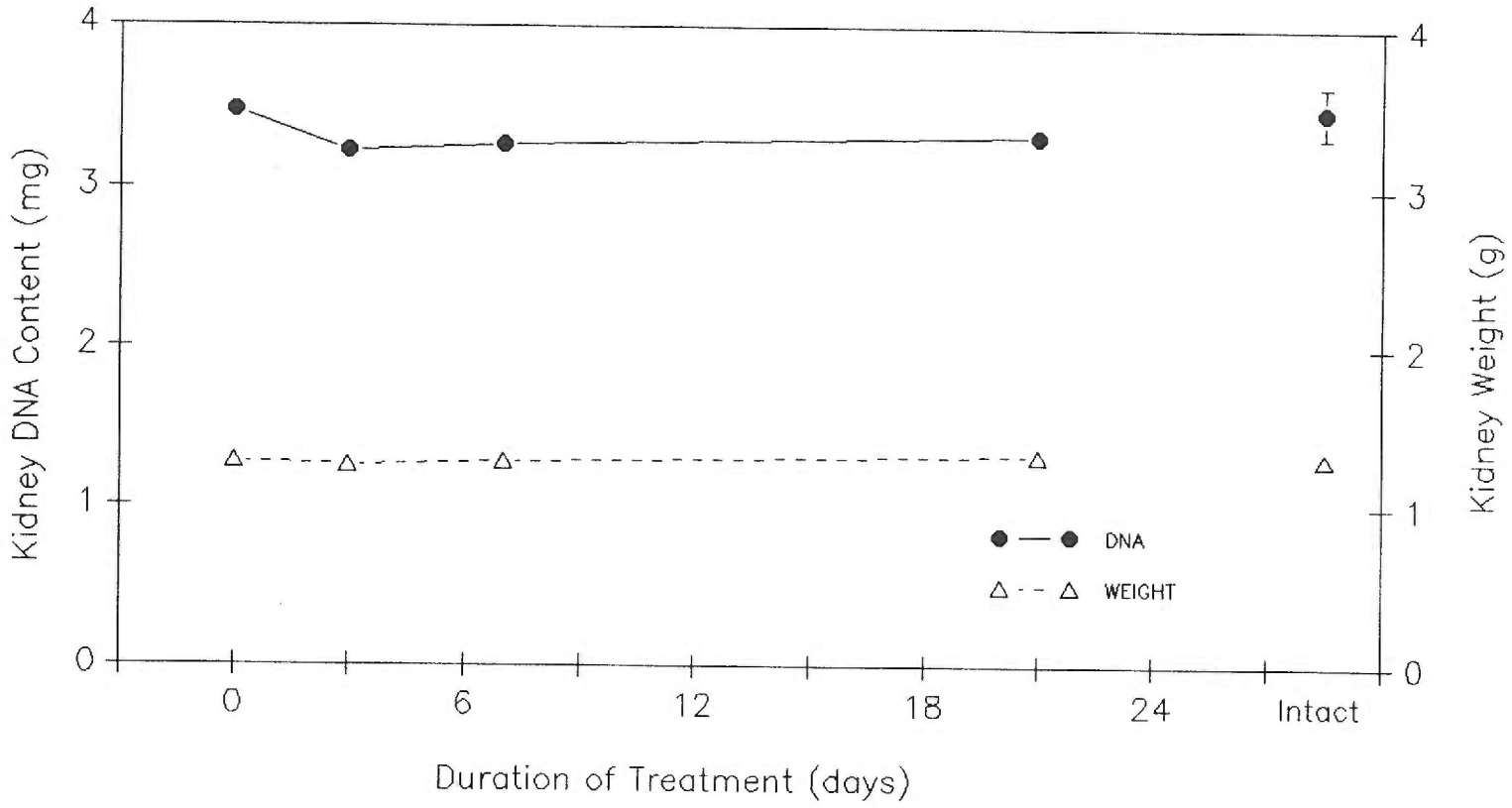


Figure 4: Effect of dihydrotestosterone (DHT) implants on kidney weight and DNA content in rats. Ten days post-castration implants were placed subcutaneously. Data are expressed as mean ± SEM (n=3).

Table 7
In vitro ^{3}H-thymidine incorporation into DNA of tissues from intact rats and rats seven days post-castration.

Group	^{3}H-thymidine Incorporated (dpm/μg DNA)			
	Prostate	Seminal Vesicles	Kidney	Liver
Intact	23 ± 1	44 ± 3	23 ± 1	39 ± 2
Castrated	11 ± 1*	24 ± 2*	21 ± 1	43 ± 3

Data are expressed as mean ± SEM (n=8)

*$p \leq 0.01$ compared to intact

produced no change in the in vitro incorporation of ^{3}H-thymidine into DNA from the prostate or the kidney (Figure 5). In the kidney, this incorporation of ^{3}H-thymidine remained unaltered through 10 days of treatment. In contrast, the incorporation of ^{3}H-thymidine into prostatic DNA was significantly increased at day 3 of treatment. This increase in rate of ^{3}H-thymidine incorporation was transient, and thymidine incorporation returned to intact levels by day 5 of treatment.

- *dihydrotestosterone implants*

Results similar to those for DHT injections were observed in prostates from 7 day castrated rats treated with DHT implants (Figure 6). However, when DHT implants were used, the stimulation of ^{3}H-thymidine incorporation occurred earlier (day 2). Therefore, this incorporation occurs prior to the increase in DNA content observed at day 3 (Figure 3). The levels of ^{3}H-thymidine incorporation returned to intact levels by the fifth day after implantation and remained unchanged through day 10 of treatment.

II. Subcellular Distribution of Androgen Receptors in Androgen-Responsive Tissues

Radioligand binding to prostate cytosolic androgen receptor

The data in Table 8 show a comparison of the binding

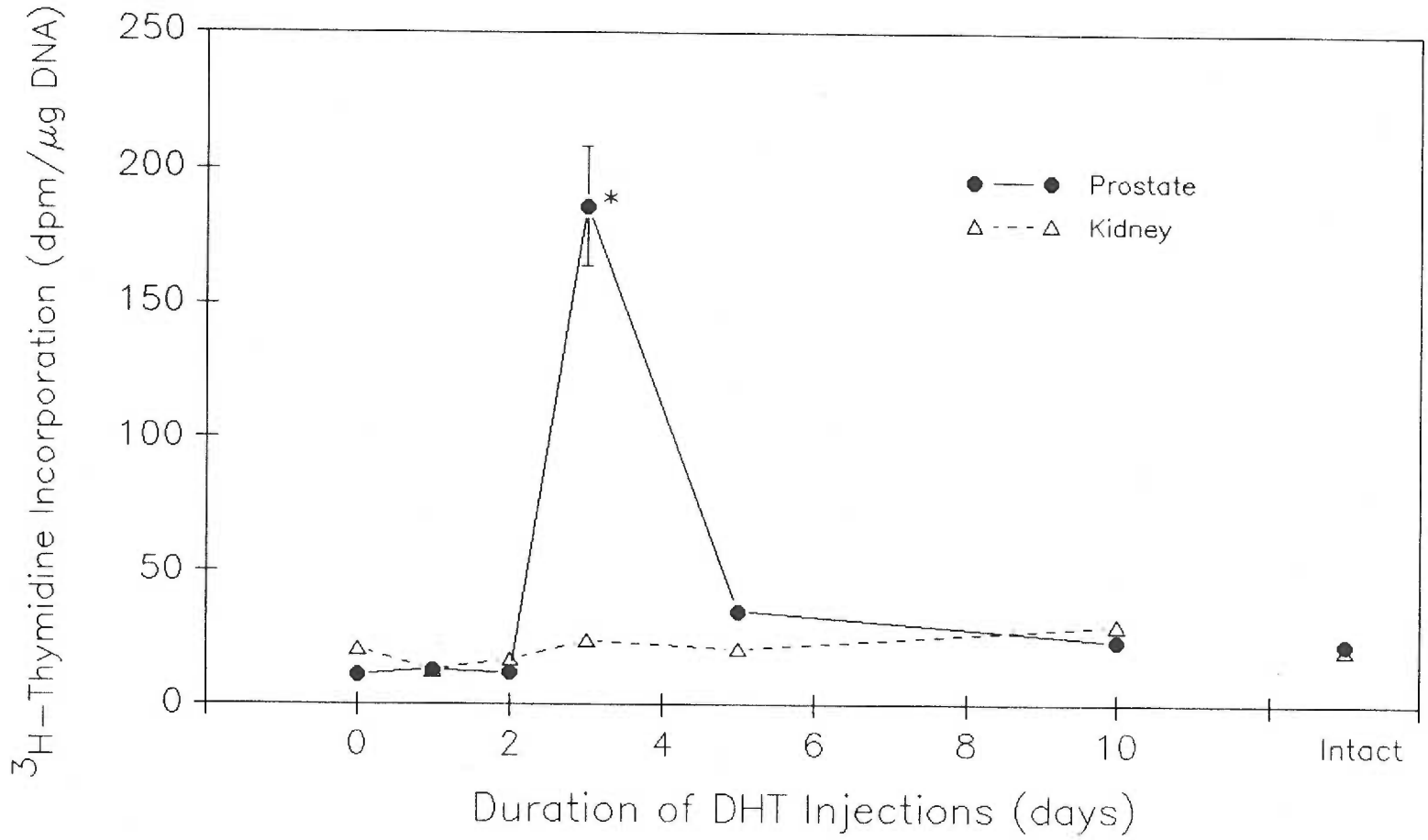


Figure 5: Effect of dihydrotestosterone (DHT) injections (1 mg/kg/day) on the <u>in vitro</u> incorporation of ^{3}H-thymidine into prostatic and renal DNA in rats. Seven days post-castration animals were injected subcutaneously once daily for 1 to 10 days. Data are expressed as mean ± SEM (n=8). *p<0.01.

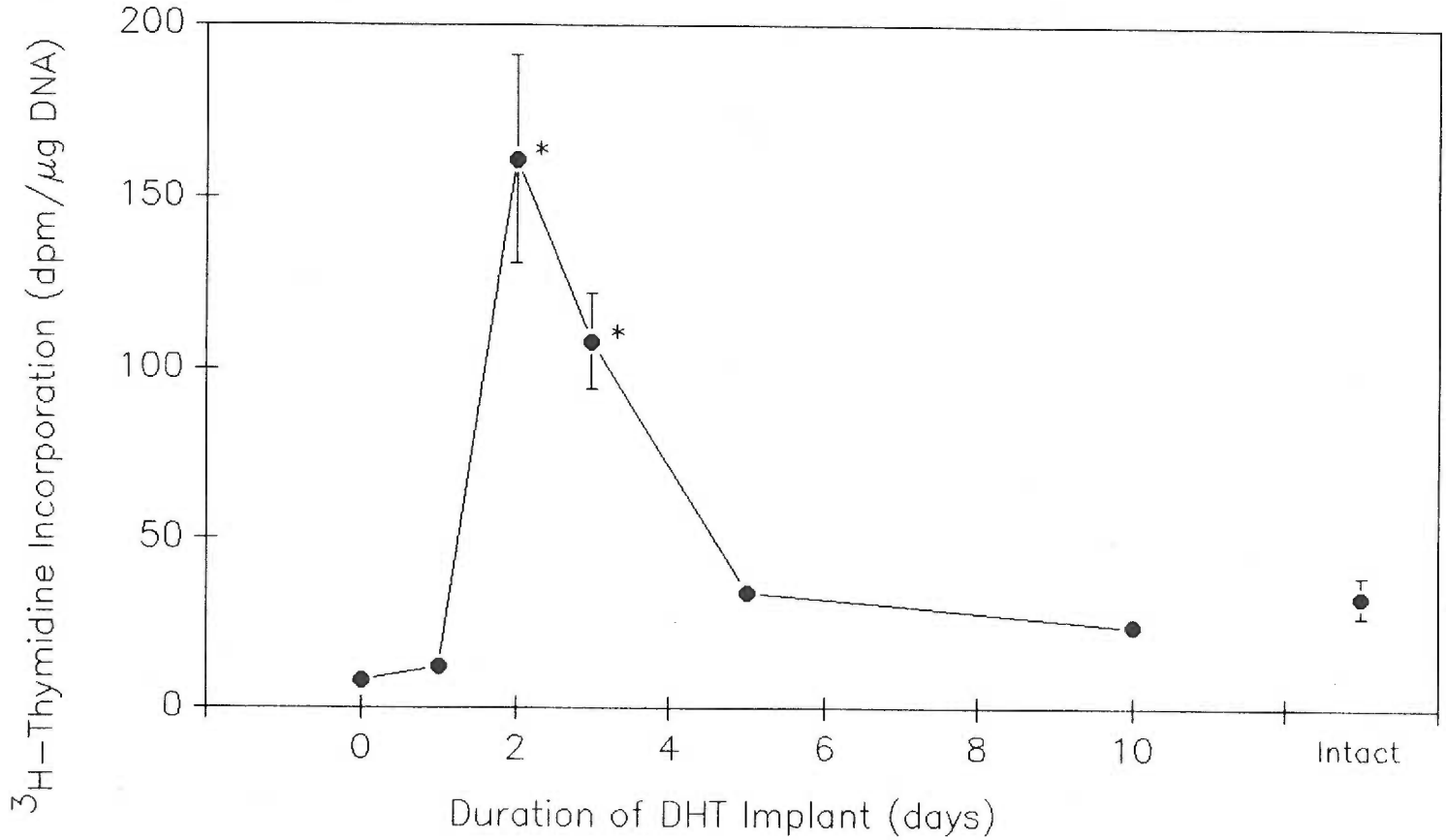


Figure 6: Effect of dihydrotestosterone (DHT) implants on the <u>in vitro</u> incorporation of ^{3}H-thymidine into prostatic DNA. Seven days after castration, implants were placed subcutaneously. Data are expressed as mean ± SEM (n=8). *p<0.01.

Table 8
Comparison of radioligand binding to prostatic cytosolic androgen receptors from intact rats.

Radioligand	Cytosolic Androgen Receptor	
	K_D (nM)	Capacity (fmol/mg DNA)
^{3}H-R1881	0.48 ± 0.03	1244 ± 49
^{3}H-DHT	0.78 ± 0.20	835 ± 135
^{3}H-Testosterone	1.85 ± 0.32*	1446 ± 364

Data are expressed as mean ± SEM (n=4-10)

*$p \leq 0.05$ compared to ^{3}H-R1881 or ^{3}H-DHT

affinities (as reflected by the equilibrium dissociation constant [K_D]) and the estimated capacity of the prostate cytosolic androgen receptor (AR) measured using three different radioligands (^{3}H-R1881, ^{3}H-DHT, or ^{3}H-testosterone). Representative Scatchard plots of the binding data for each of these radioligands are shown in Figure 7. While there was no significant difference between the amount (capacity) of cytosolic AR measured by the three different ligands, ^{3}H-testosterone bound with a significantly lower affinity (higher K_D) than did ^{3}H-R1881 or ^{3}H-DHT (1.85 ± 0.32 nM versus 0.48 ± 0.03 and 0.78 ± 0.20 nM, respectively). In contrast, ^{3}H-R1881 and ^{3}H-DHT bound to AR with similar affinities.

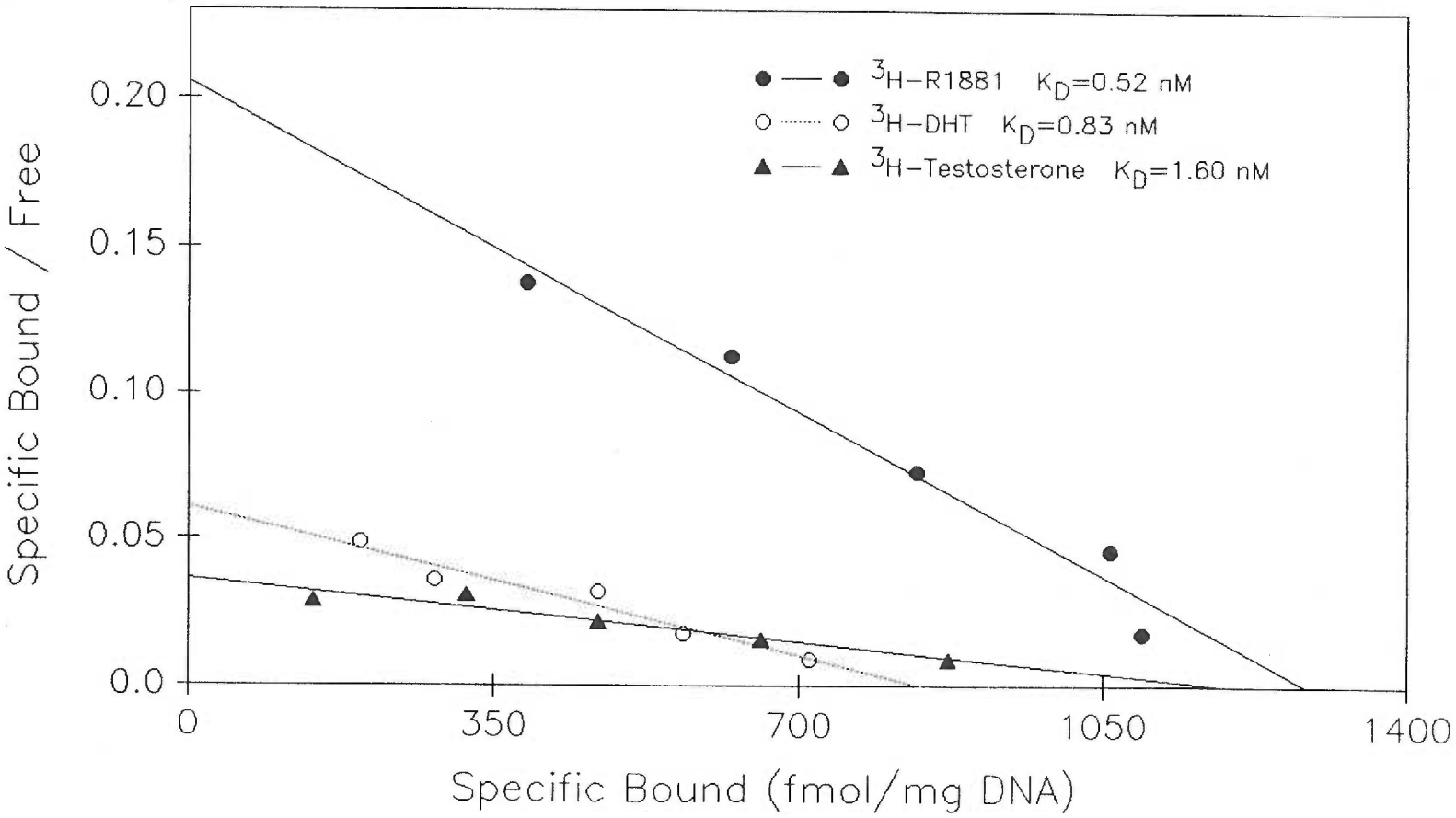


Figure 7: **Representative Scatchard plots comparing the binding of various radioligands to prostate cytosolic androgen receptors.**

Cytosolic and nuclear androgen receptors in the prostate and the kidney

The data in Table 9 indicate that prostates and kidneys obtained from intact rats both contained high affinity cytosolic and nuclear AR. The prostate, however, contained significantly higher titers of receptor (cytosolic + nuclear AR ≈ 4000 fmol/mg DNA in the prostate versus ≈ 450 fmol/mg DNA in the kidney). In addition, while the capacity of nuclear AR in the prostate was approximately two times higher than the capacity of cytosolic AR, the reverse is seen in the kidney (capacity of cytosolic AR was twice that of nuclear AR).

Twenty-four hours after castration, the subcellular distribution of AR in the prostate was significantly altered. Castration produced approximately a three-fold increase in the quantity of AR measured in the cytosolic fraction (3324 ± 181 versus 1244 ± 49 in the intact), and concomitantly, there was a significant decrease in prostatic nuclear AR (119 ± 7 versus 2989 ± 262 fmol/mg DNA in the intact). Further, the measured binding affinities for ^{3}H-R1881 of both the nuclear and cytosolic AR increased after castration. In the kidney, there was no significant effect of castration observed in the measured capacity of cytosolic AR (298 ± 81 versus 362 ± 63 fmol/mg DNA pre- and post-castration, respectively), but an increase in binding

Table 9
Specific ^{3}H-R1881 binding to cytosolic and nuclear androgen receptors in prostates and kidneys from intact rats and rats 24 hours post-castration.

Tissue	Cytosolic AR		Nuclear AR	
	K_D (nM)	Capacity (fmol/mg DNA)	K_D (nM)	Capacity (fmol/mg DNA)
Prostate - intact	0.48 ± 0.03	1244 ± 49	0.62 ± 0.06	2989 ± 262
- castrated	0.24 ± 0.02	3324 ± 181*	0.35 ± 0.05	119 ± 36*
Kidney - intact	0.32 ± 0.03	298 ± 81	0.39 ± 0.04	149 ± 7
- castrated	0.18 ± 0.05	362 ± 63	-	<10

Data are expressed as mean ± SEM (n=3-15)

*p≤0.01 versus intact

affinity was observed. In contrast, there was no detectable nuclear AR binding in the kidney 24 hours after castration.

Specificity of cytosolic and nuclear androgen receptors

The ability of increasing concentrations of unlabeled steroid to displace specific binding of ^{3}H-R1881 from prostate cytosolic AR is shown in Figure 8. These data indicate that the competitor:^{3}H-R1881 concentration ratios at fifty percent inhibition of binding (IC_{50}) were 0.8, 1.3, and 4.6 for R1881, DHT, and testosterone, respectively. Estradiol-17β produced only 20 - 30% inhibition of ^{3}H-R1881 binding at 100-fold molar excess, and extrapolation of the curve estimates an IC_{50} of approximately 180.

The IC_{50} values are an indirect measurement of steroid binding affinity, and the values obtained from Figure 8 were in agreement with the direct measurements of K_D shown in Table 8. Relating the K_D and IC_{50} values of DHT and testosterone to R1881, the calculated DHT:R1881 ratio is 1.63 whether measured directly or estimated from the displacement curves. The testosterone:R1881 ratios are 3.85 and 5.75 when measured directly or calculated from the IC_{50} values, respectively.

Based on the displacement curves in Figure 8, it was determined that a 100-fold molar excess of an androgen produced maximal displacement of ^{3}H-R1881 binding while a

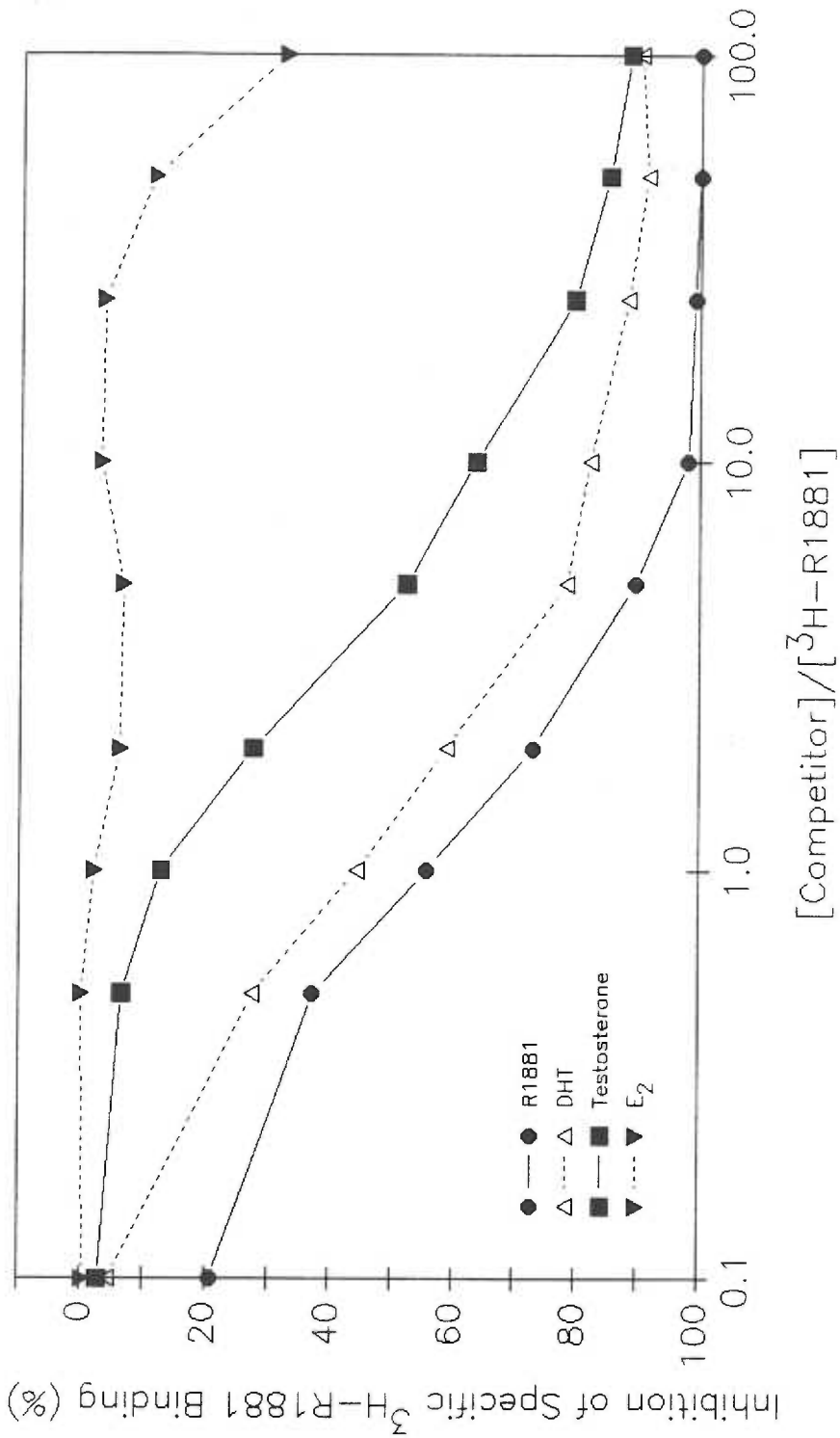


Figure 8: Specificity of ^{3}H-R1881 binding to prostate cytosolic androgen receptor.

non-androgen (estradiol-17ß) produced only minimal competition. In order to confirm that both cytosolic and nuclear AR from the prostate and the kidney recognize and are specific for androgens, a screening specificity study was conducted using only 100-fold molar excesses of unlabeled R1881, DHT, testosterone, and estradiol (Table 10). These data indicate that cytosolic and nuclear AR obtained from either the prostate or the kidney selectively recognized and bound all three androgens. In contrast, all of these AR preparations showed only minimal displacement of ^{3}H-R1881 in the presence of estradiol-17ß.

Nuclear matrix

- *preparation*

Analysis of the nuclear matrix preparation revealed a residual DNA content equal to 3.6% of the starting DNA and a residual protein content of 20%. Based on hemocytometric analysis, the yield of matrix spheres from starting purified nuclei was approximately 50 - 60% (data not shown). These data for composition and recovery of matrix spheres are consistent with previously published values (80).

The quantitation of matrix AR is normalized relative to the DNA content of the starting purified nuclei (since most nuclear proteins and DNA are removed in preparation). By convention, no correction is made to compensate for the

Table 10
Specificity of ^{3}H-R1881 binding to prostate and kidney cytosolic and nuclear androgen receptors (AR).

Competitor[A]	Inhibition of Specific ^{3}H-R1881 Binding (%)			
	Prostate		Kidney	
	Cytosolic AR	Nuclear AR	Cytosolic AR	Nuclear AR
R1881	92 ± 3	99 ± 0	96 ± 4	99 ± 0
DHT	90 ± 4	99 ± 1	86 ± 4	99 ± 0
Testosterone	83 ± 5	92 ± 0	88 ± 7	92 ± 2
Estradiol	13 ± 5[B]	32 ± 1[B]	9 ± 7[B]	23 ± 5[B]

Data are expressed as mean ± SEM (n=3-7)

[A]Steroid competitor present at 100-fold molar excess

[B]$p \leq 0.05$ compared to androgens

nuclear spheres lost during preparation.

Figure 9 shows transmission electron photomicrographs of purified prostatic nuclei (a) and nuclear matrices (b) prepared in this laboratory.

- *matrix-associated androgen receptors*

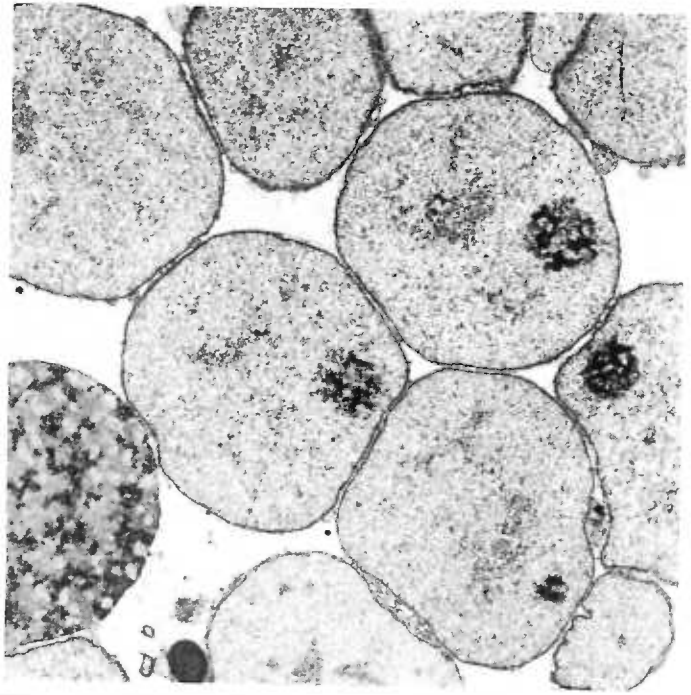
The prostate from intact rats contained significant levels of high affinity (K_D= 1 nM) matrix AR (Table 11). Twenty-four hours post-castration, no matrix AR was detectable in the prostate. In the kidney, no matrix AR was detectable in either intact or castrated rats.

The data in Table 12 indicate that R1881, DHT, and testosterone were all able to displace ^{3}H-R1881 binding to matrix AR in prostates from intact rats. In contrast, estradiol-17ß inhibited ^{3}H-R1881 binding only minimally. These data are in agreement with those in Table 10 which indicate that cytosolic and nuclear AR are also specific for androgens.

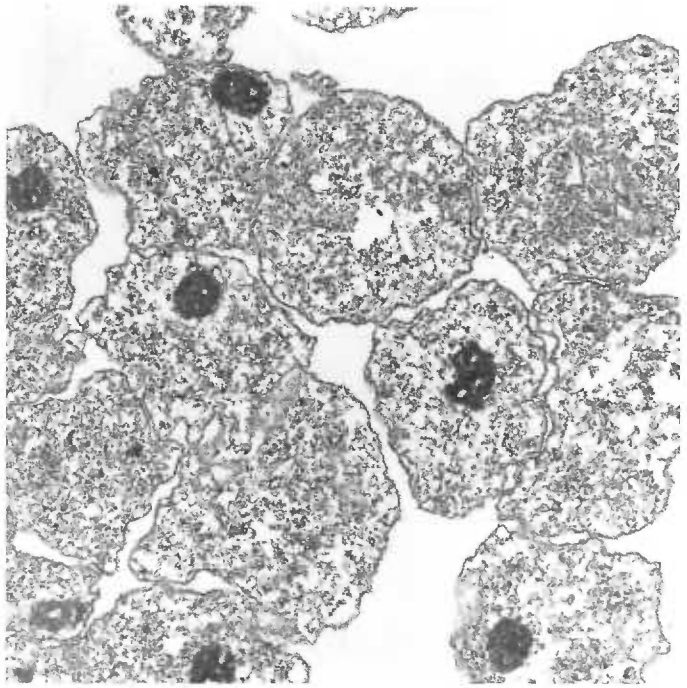
Subcellular localization of androgen receptors

For comparison purposes, Figure 10 summarizes the data contained in Tables 9 and 11. Although the data appear to indicate that the nuclear AR was the predominant form of receptor in prostates from intact rats, it must be emphasized that 40 - 50% of the nuclear spheres were lost

Figure 9
Electron photomicrographs of purified prostate nuclei(a), and prostate nuclear matrices(b). X6,250



(a)



(b)

Table 11
Specific ^{3}H-R1881 binding to nuclear matrix androgen receptors in prostates and kidneys from intact rats and rats 24 hours post-castration.

Group	Nuclear Matrix Androgen Receptor			
	Prostate		Kidney	
	K_D (nM)	Capacity (fmol/mg DNA)	K_D (nM)	Capacity (fmol/mg DNA)
Intact	1.02 ± 0.09	1671 ± 110	-	<10
Castrated	-	<10	-	<10

Data are expressed as mean ± SEM (n=4-9)

Table 12
Specificity of ^{3}H-R1881 binding to prostate matrix androgen receptors (AR).

Competitor[A]	Inhibition of Specific ^{3}H-R1881 Binding (%)
	Matrix AR
R1881	100 ± 0
DHT	96 ± 0
Testosterone	91 ± 0
Estradiol	38 ± 4[B]

Data are expressed as mean ± SEM (n=3)

[A]Steroid competitor present at 100-fold molar excess

[B]$p \leq 0.05$ compared to androgens

during preparation (i.e., 50 - 60% yield of nuclear matrices from starting nuclei). Correcting for this loss suggests that the matrix and nuclear forms of the receptor were present in approximately equal concentrations. Together, nuclear and matrix AR constitute 85 - 95% of the total AR in the prostates from intact rats. In prostates from castrated animals, over 95% of the total AR was present in the cytosolic fraction, and no matrix AR was detectable. In addition, during the 24 hour post-castration interval, there was a loss of approximately 50% of the total prostatic AR compared to the concentrations measured in intact animals (approximately 7000 fmol/mg DNA in intact rats after

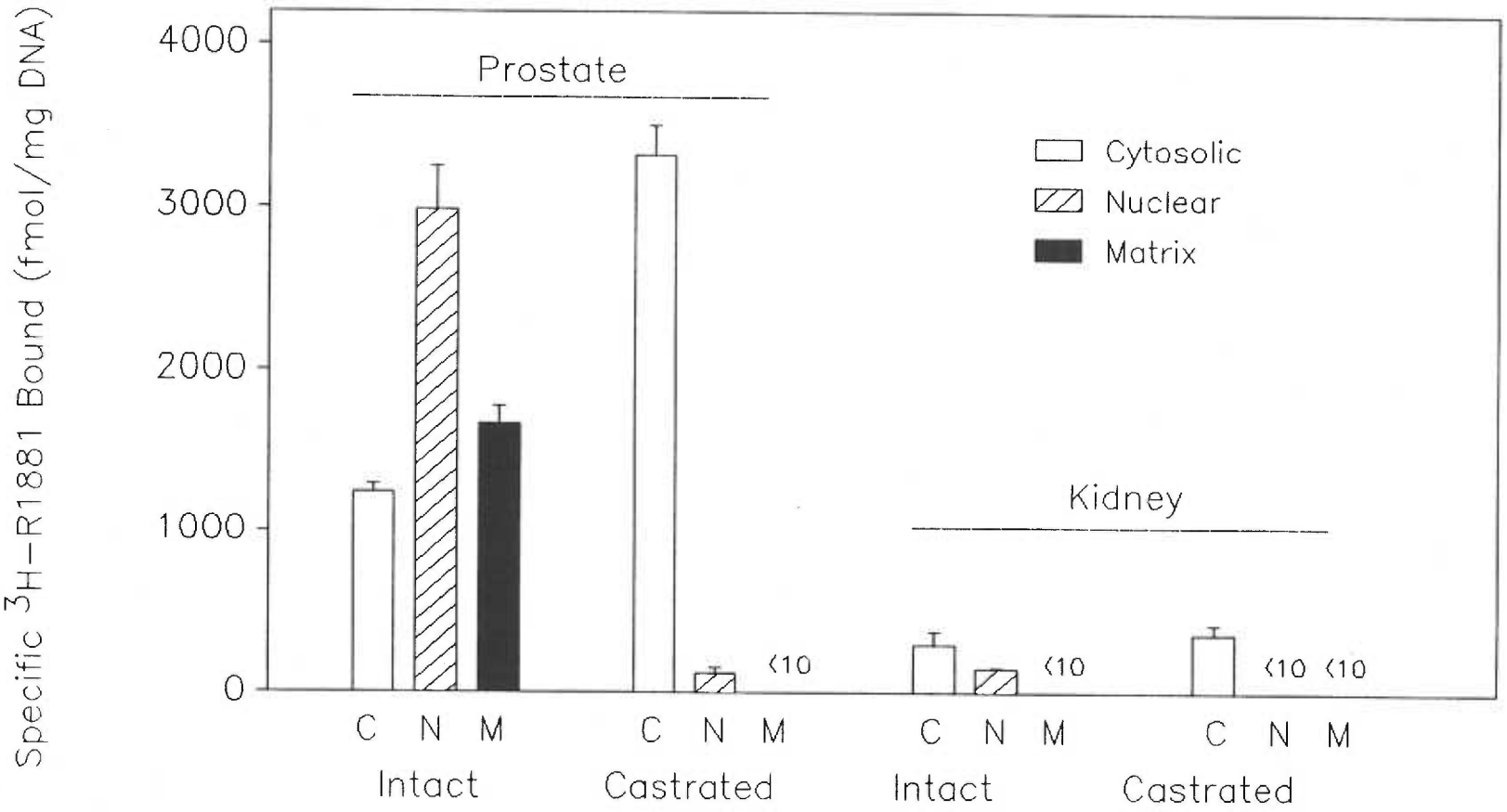


Figure 10: Subcellular distribution of androgen receptors (AR) in prostates and kidneys from intact rats and rats 24 hours post-castration. C=cytosolic AR, N=nuclear AR, M=matrix AR. Data are expressed as mean ± SEM (n=4-15).

correction for matrix loss versus 3500 fmol/mg DNA in castrated rats).

In kidneys, two-thirds of the AR in intact rats was localized in the cytosolic fraction and only one-third was nuclear AR. No matrix-associated AR was detectable. Twenty-four hours post-castration, all of the renal AR was located in the cytosolic fraction, and no significant change in the total amount of AR was noted.

In an attempt to localize AR to the matrix of the kidney, rats 24 hours post-castration were injected with DHT (5 mg/kg) and the subcellular distribution of AR in the prostate and the kidney was examined two hours after the injection (Table 13). This dose of DHT was established based on the previously observed biological responses of the prostate and the resultant increases in weight and DNA to intact levels (Table 6 and Figure 3). The data indicate that even in the presence of a replacement dose of DHT, no matrix AR was detectable in the kidney. This dose did, however, localize AR to the prostate matrix at concentrations comparable to those seen in intact rats. The levels of cytosolic and nuclear AR in the prostate after a single DHT injection were lower than those seen in intact animals (Table 9 and Figure 10), but the total AR titer was equivalent to the total AR in 24 hour castrated animals. A preferential localization of the AR to the matrix was

Table 13
Effect of dihydrotestosterone (DHT) on the nuclear localization of androgen receptors (AR) in prostates and kidneys*.

Subcellular Fraction	Specific ^{3}H-R1881 Binding (fmol/mg DNA)	
	Prostate	Kidney
Cytosolic AR	350 ± 53	185 ± 29
Nuclear AR	1542 ± 55	300 ± 19
Matrix AR	1964 ± 238	<10

Data are expressed as mean ± SEM (n=3)

*DHT (5 mg/kg) was injected subcutaneously to rats castrated 24 hours previously. Tissues were collected 2 hours post-injection.

observed 2 hours after injection in animals castrated 24 hours previously.

In the kidney, DHT injection did not alter the total amount of AR compared to intact rats (≈450 fmol/mg DNA), but DHT did produce a predominance of nuclear AR. Whereas in the kidneys from intact rats cytosolic and nuclear AR titers were 300 fmol/mg DNA and 150 fmol/mg DNA, respectively, the DHT injected animals had the opposite subcellular distribution of renal AR (185 fmol/mg DNA cytosolic AR versus 300 fmol/mg DNA nuclear AR).

Cell-free binding of cytosolic androgen receptors to nuclei

In order to ascertain if renal nuclei lacked matrix AR because of the absence of nuclear matrix acceptor sites for the androgen-receptor complex, a cell-free binding system was developed using a modification of the approach outlined by Barrack (144). In view of the difficulties in preparing nuclear matrices, it was decided to initially develop the binding system using purified nuclei. In this system, cytosolic AR from castrated rats was mixed with purified prostatic or renal nuclei. Castrated rats were utilized because they have the highest concentration of prostatic cytosolic AR and nuclei should lack receptor occupancy of the nuclear acceptor sites.

While the initial studies indicated that the majority

of prostate cytosolic AR added bound to nuclei from either prostates or kidneys (55 and 67%, respectively), it was subsequently determined that most of this "bound" AR (70 - 85%) was actually precipitated AR which could be recovered as an insoluble pellet in the absence of nuclei (Table 14). This precipitation phenomenon was not as prevalent for AR in the kidney cytosol (21 - 25% of the cytosolic AR appeared to bind to nuclei; 7% was recovered as a pellet in the absence of nuclei).

Subsequently it was ascertained that the sedimentation of cytosolic AR was attributable to precipitation induced by the presence of ethanol in the assay system (Table 15). Radiolabeled and unlabeled steroids were added to the receptor binding assay in ethanol solution and this produced a final ethanol concentration of 5.7%. The data indicate that the quantity of receptor that precipitated increased as the ethanol concentration increased. At low concentrations of ethanol ($\leq$ 6%), this precipitation phenomenon was blocked by the presence of 20 mM sodium molybdate in the buffer (Table 15). At higher ethanol concentrations, the ability of the molybdate ion to prevent AR precipitation was diminished.

When the ethanol was removed and the steroids were suspended in aqueous buffer prior to addition to the binding assay, the results of the cell-free nuclear binding studies

Table 14
Precipitation of cytosolic androgen receptors in a cell-free nuclear binding system.

Cytosolic AR	Percent AR Bound to Nuclei		
	Prostate	Kidney	Control
	Nuclei	Nuclei	No Nuclei
Prostate	55 ± 6	67 ± 5*	48 ± 10
Kidney	21 ± 3*	25 ± 4*	7 ± 1

Data are expressed as mean ± SEM (n=10-12)

*$p \leq 0.05$ compared to control

Table 15
Effect of ethanol and 20 mM sodium molybdate on the in vitro precipitation of prostate cytosolic androgen receptors (AR) from rats 24 hours post-castration.

Ethanol (%)	Percent of Total AR Precipitated	
	Molybdate Absent	Molybdate Present
2.6	10	5
6.0	46	4
12.0	78	48
20.0	83	75

Data are mean of two observations

were inconsistent and uninterpretable (data not shown). Consequently, in view of the logistical problems associated with studying the interaction between AR and the nucleus in a cell-free system, an alternative approach was developed. Specifically, a pharmacological approach was employed to compare anabolic and androgenic steroid-induced nuclear localization of AR.

III. Anabolic Steroids and Growth

Subcellular distribution of AR after stanozolol treatment

When rats 24 hours post-castration were injected with either DHT or stanozolol (STAN) at a dose of 5 mg/kg, there was a significant quantity of AR localized to the prostatic nuclear and matrix fractions 2 hours post-injection (Table 16). While the total AR present in the cells was not

Table 16
Subcellular distribution of androgen receptors (AR) in prostates at 2 and 24 hours after administration of dihydrotestosterone (DHT) or stanozolol (STAN) to rats castrated 24 hours previously.

Subcellular Fraction	Specific ^{3}H-R1881 Binding (fmol/mg DNA)			
	2 Hours Post-Injection		24 Hours Post-Injection	
	DHT[A]	STAN[A]	DHT	STAN
Cytosolic AR	350 ± 53	1585 ± 72[B]	721 ± 22	2533 ± 128[C]
Nuclear AR	1542 ± 55	1307 ± 216	1909 ± 261	954 ± 238[D]
Matrix AR	1964 ± 238	1359 ± 174[B]	1073 ± 87	524 ± 81[C]
Total AR	3856 ± 250	4251 ± 287	3703 ± 291	4011 ± 298

Data are expressed as mean ± SEM (n=3-5)

[A]Steroids injected subcutaneously at a dose of 5 mg/kg

[B]$p \leq 0.05$ versus DHT treatments

[C]$p \leq 0.05$ versus DHT treatments and STAN at 2 hours

[D]$p \leq 0.05$ versus DHT at 24 hours

significantly different between the DHT and STAN treated animals (3856 and 4251 fmol/mg DNA, respectively), the subcellular distribution was different. The DHT treated animals had significantly more matrix AR than the STAN treated animals (1964 ± 238 versus 1359 ± 174 fmol/mg DNA). Correspondingly, the STAN treated animals had significantly higher levels of cytosolic AR (1585 ± 72 versus 350 ± 53 fmol/mg DNA), but the level of nuclear AR was not different between the two groups. The matrix AR represented 51% and the nuclear AR represented 40% of the total AR in the DHT treated animals. In contrast, prostatic AR from the STAN treated animals was almost equally divided between the cytosol, nuclear, and matrix fractions (37, 31, and 32% respectively).

Twenty-four hours after injection, the total amount of prostatic AR in the two treatment groups remained unchanged. However, the prostates from STAN treated animals contained significantly less matrix AR and proportionately more cytosolic AR compared to either of the DHT treated groups or to the STAN treated group at two hours (Table 6). While the nuclear AR in the STAN treated animals was not different at 2 and 24 hours after injection, at 24 hours after treatment it was significantly lower than the levels of AR obtained after DHT treatment. While the DHT treated animals retained the majority of the AR in the nucleus (52% nuclear, 29%

matrix), the STAN treated animals had a markedly lower percentage of receptors associated with the nucleus (24% nuclear, 13% matrix) at 24 hours post-injection.

Specificity of stanozolol for androgen receptors

The displacement curve for the competition of stanozolol for specific ^{3}H-R1881 binding indicates that stanozolol bound to the prostate cytosolic AR but with a lower affinity than either DHT or R1881 (Figure 11). Similar results were seen in displacement curves generated with the prostate nuclear AR (Figure 12). Both studies indicate that stanozolol exhibited about a 15 - 20 fold lower affinity for the AR than did DHT or R1881. At a 100-fold molar excess, the displacement of ^{3}H-R1881 binding to matrix AR was similar to that seen in the cytosolic or nuclear fractions (Table 17). These latter data suggest that stanozolol probably exhibits the same affinity for AR regardless of the subcellular location of the receptors.

Effects of stanozolol on prostate and kidney growth

- short term (24 hour) castrated rats

Rats 24 hours post-castration were injected daily for seven days with either DHT (5 mg/kg), STAN (5 or 25 mg/kg), or DMSO vehicle as a control. In rats treated with DHT, there was a significant increase in the weight of the

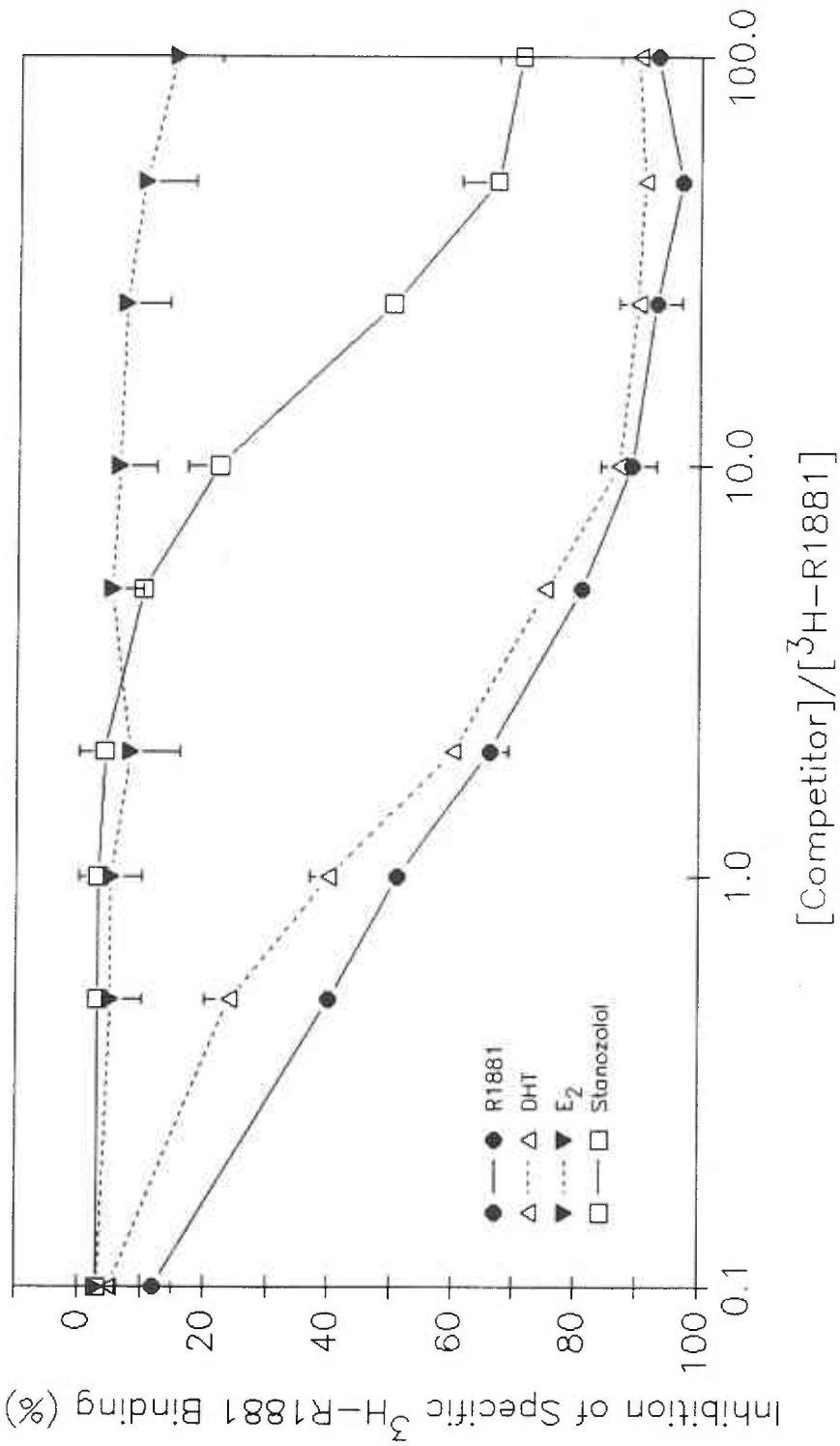


Figure 11: Inhibition of specific ^{3}H-R1881 binding to prostate cytosolic androgen receptors by stanozolol. Data are expressed as mean ± SEM (n=3).

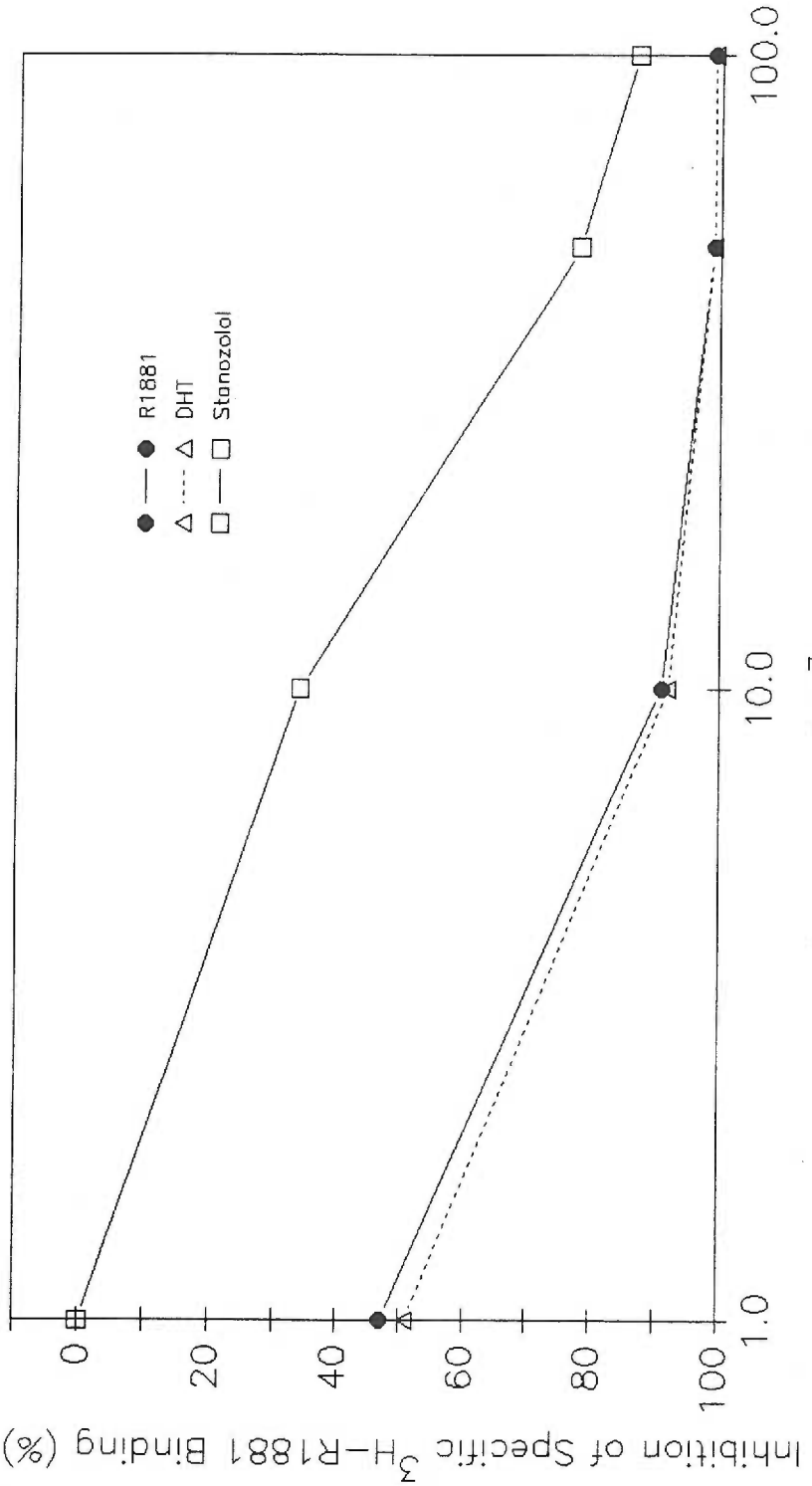


Figure 12: Inhibition of specific ^{3}H-R1881 binding to prostate nuclear androgen receptors by stanozolol. Data are expressed as mean ± SEM (n=3).

Table 17
Inhibition of specific ^{3}H-R1881 binding by stanozolol in the prostate.

Competitor[A]	Inhibition of Specific ^{3}H-R1881 Binding (%)		
	Prostate		
	Cytosolic AR	Nuclear AR	Matrix AR
R1881	90 ± 5	100 ± 0	100 ± 0
DHT	90 ± 10	99 ± 1	96 ± 0
Stanozolol	81 ± 3	89 ± 1	76 ± 7

Data are expressed as mean ± SEM (n=3)

[A]Steroid competitor present 100-fold molar excess

prostates, but there was no change in the DNA content as compared to 24 hour castrate control values (Table 18). STAN treated animals (5 or 25 mg/kg) revealed no change in either the weight or DNA contents in the prostate compared to 24 hour castrate controls. In addition, there was no difference in the prostate DNA content between the STAN and DHT treated animals. DMSO controls had significantly lower prostatic weights and DNA contents compared to either DHT or STAN treated animals.

In the kidney, there were no significant differences in the DNA content between 24 hour castrated rats, DMSO controls, DHT or STAN treated rats. The kidney weights in the DHT treated rats increased significantly over all other

Table 18
Effect of dihydrotestosterone (DHT) and stanozolol (STAN) on prostate and kidney weights and DNA content in rats 24 hours post-castration.

Treatment[A]	Prostate		Kidney	
	Weight (mg)	DNA (μg)	Weight (g)	DNA (mg)
DMSO Vehicle	100 ± 15[C]	399 ± 27[C]	1.29 ± 0.04	3.43 ± 0.05
24 h castrate[B]	447 ± 34	752 ± 31	1.27 ± 0.01	3.22 ± 0.11
DHT (5 mg/kg)	859 ± 36[C]	799 ± 45	1.60 ± 0.03[C]	3.17 ± 0.09
STAN (5 mg/kg)	385 ± 29	809 ± 43	1.38 ± 0.02[C]	3.35 ± 0.14
STAN (25 mg/kg)	455 ± 26	805 ± 37	1.24 ± 0.03	3.20 ± 0.04

Data are expressed as mean ± SEM (n=3-6)

[A]Rats were injected subcutaneously with steroid or DMSO vehicle once daily for 7 days

[B]Tissues collected 24 hours post-castration (no treatment)

[C]$p \leq 0.05$ compared to other treatments and controls

treatments and controls. In addition, STAN at a 5 mg/kg dose, but not at a 25 mg/kg dose, produced a significantly higher kidney weight compared to controls (Table 18).

- long term (7 day) castrated rats

Rats seven days post-castration were injected daily for seven days with varying doses of DHT (1, 5, 10, or 25 mg/kg) or STAN (5, 10, 25, 50, or 100 mg/kg). The ventral prostate and kidneys were then analyzed for weight and DNA content.

The data in Figure 13 indicate that all doses of DHT produced a significant increase in both the weight and DNA content of the prostate compared to 7 day castrated or DMSO vehicle controls. In contrast, regardless of the dose, STAN failed to induce any significant change in the prostatic weights or DNA content compared to the controls.

In the kidneys, neither STAN nor DHT produced any changes in the total DNA content regardless of the dose administered (Figure 14). All doses of STAN also failed to produce a change in the organ weights. While a dose of 1 mg/kg DHT did not significantly affect the weight of the kidneys, higher doses (5, 10, and 25 mg/kg) caused a significant increase in kidney weights.

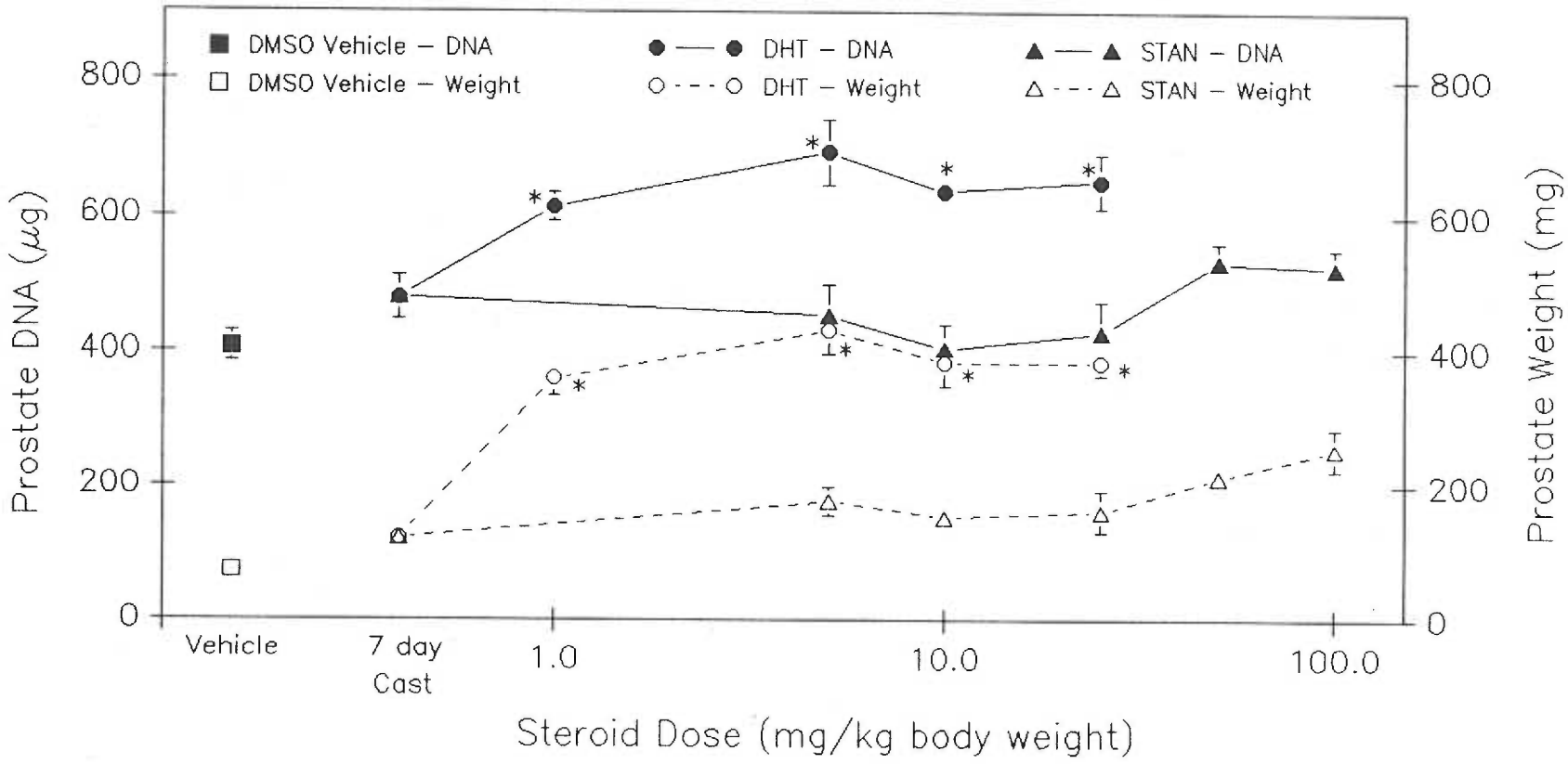


Figure 13: Effect of dihydrotestosterone (DHT) and stanozolol (STAN) on prostate weight and DNA content. Seven days post-castration rats were injected with steroid once daily for 7 days. Data are expressed as mean ± SEM (n=4-5). *p<0.05 compared to castrated control and vehicle control animals.

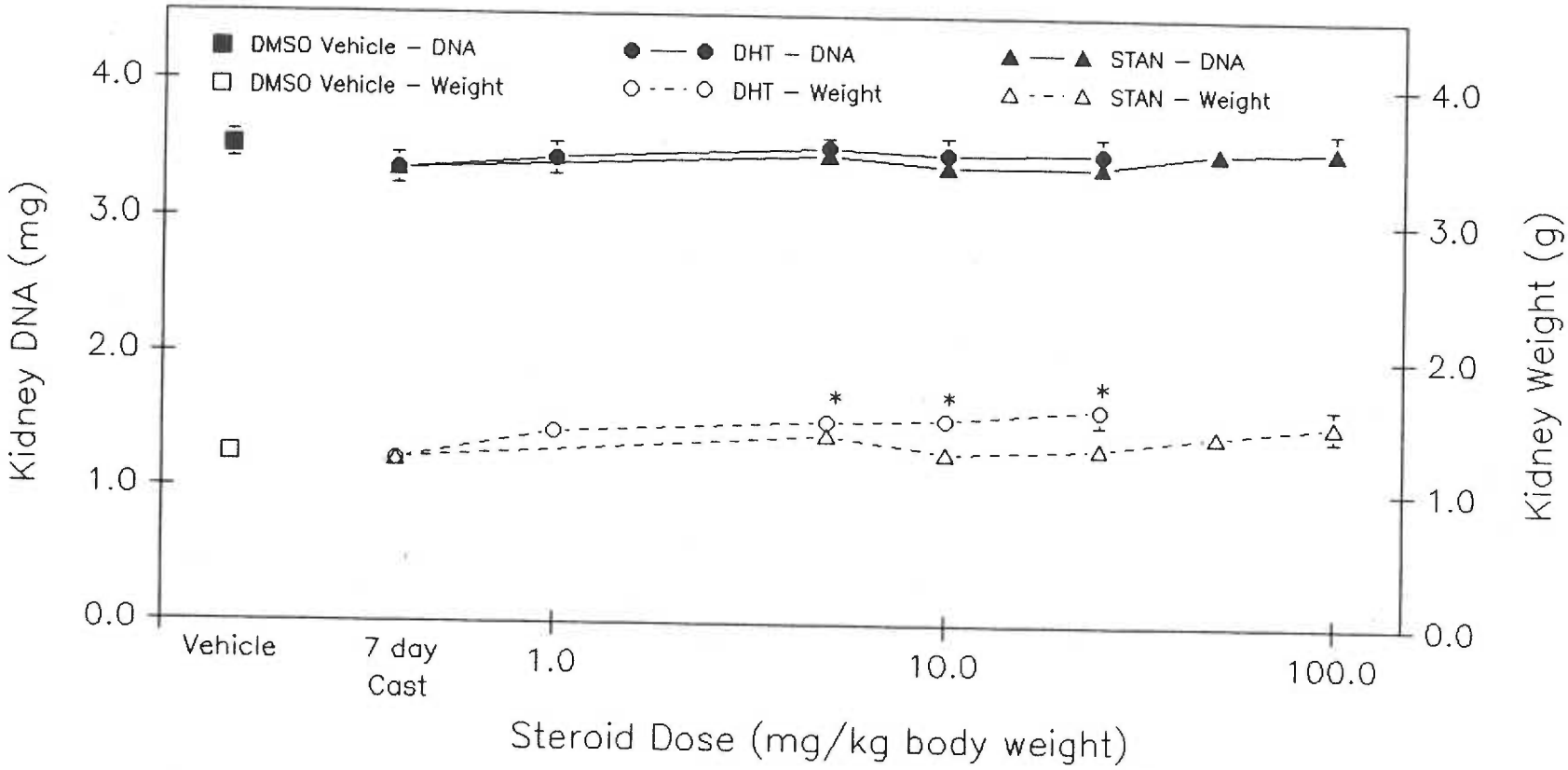


Figure 14: Effect of dihydrotestosterone (DHT) and stanozolol (STAN) on kidney weight and DNA content. Seven days post-castration rats were injected with steroid once daily for 7 days. Data are expressed as mean ± SEM (n=4-5). *p<0.05 compared to castrated control and vehicle control animals.

DISCUSSION

The results of the present study have demonstrated the androgen dependence of the prostate gland. In contrast, the kidney, although sensitive to androgens, does not exhibit dependence on androgens. Additionally, a difference exists between these tissues in the nuclear distribution of androgen receptors (AR). In the prostate, androgen receptors are found in association with the nuclear matrix. In contrast, no androgen receptors are found in association with the renal nuclear matrix.

In the ventral prostate of the rat, long term castration (7 - 10 days) produces decreases in both DNA content and weight of the gland (Figure 3, Table 6). In addition, there is a concomitant reduction in the rate of DNA synthesis (as measured by ^{3}H-thymidine incorporation) in the absence of androgens (Table 7). Androgen replacement therapy in the long term castrated rat produces a hyperplastic response that is characteristic of androgen-dependent tissues, and the prostate responds temporally with an increase in DNA synthesis, DNA content, and weight (Figures 3,5,6). An elevated rate of DNA synthesis occurs prior to the rise in DNA content and rapidly returns to basal intact levels despite continuing hormone treatment.

The DNA content, however, continues to increase for several days. These data regarding prostatic DNA synthesis are in agreement with findings of other investigators (173,174).

It must be noted that the hyperplastic response to DHT only occurs in the castrated rat when the prostate gland is in a regressed state. In the short term castrated rat (24 hours), DHT stimulation produces a hypertrophic effect evidenced by an increase in prostate weight, but it does not affect DNA content (Table 18). This finding is consistent with the fact that, under normal circumstances, once maximal prostate size is attained, DNA synthesis appears to be controlled by a homeostatic mechanism related in some way to cell number and/or to stromal-epithelial interactions (17,175).

Unlike the prostate gland, long term castration does not produce a change in the DNA content or weight of the kidney (Figure 4, Table 6), nor does it produce an effect on the basal rate of DNA synthesis in this organ (Table 7). Androgen replacement in both short term or long term castrated rats produces an increase in kidney weight (hypertrophic response), but it has no affect on the DNA content or rate of DNA synthesis (Figure 5, Table 18). It is unlikely that the absence of a DHT-induced change in renal DNA synthesis or content is due to insufficient androgen stimulation since, in these studies, the presence of

constant DHT stimulation (implant x 21 days) also fails to produce a hyperplastic response in the kidney (Figure 4).

This is the first study to conclusively establish (via ^{3}H-thymidine incorporation studies) that androgens are ineffective in stimulating DNA synthesis in the kidney. In addition, the data further support previous findings which indicate that androgen deprivation or treatment does not significantly alter the DNA content of the kidney, but androgens can increase kidney weight (13,18). The findings of this present study are, however, in contrast to published data which indicate that a decrease in renal weight occurs within 7 days post-castration (13,18). Since these previous studies utilized mice to examine androgen effects on the kidney, this discrepancy may reflect a species variation.

In the short term castrated rat, stanozolol (STAN) treatment maintains the prostate tissue at a non-castrated state, but it fails to stimulate an increase in weight or DNA content. As noted previously, DHT produces a hypertrophic effect evidenced by an increase in organ weight (Table 18) but does not yield a growth response (i.e., increase in DNA). In the long term castrated rat, STAN has no effect on the weight or DNA content of the regressed prostate even when high doses are used to compensate for the reduced affinity of the AR - STAN interaction (Figure 13). In contrast, DHT stimulates an increase in both weight and

DNA even at relatively low doses.

By comparison, in kidneys from long term castrated rats, DHT stimulates an increase in weight but not DNA content while increasing doses of STAN have no affect on kidney weights or DNA content (Figure 14).

In view of the differences in biological responses in the prostate and the kidney produced by anabolic and androgenic steroids, it was of interest to determine if the differences could be related to the subcellular distribution of androgen receptors in these two tissues.

Specific, high affinity, cytosolic and nuclear AR are present in both the prostate and the kidney (Table 9), and they exhibit binding characteristics typical of AR (176). It is interesting to note that in these studies, DHT exhibits approximately a 2 - 2.5 fold higher affinity for AR than does testosterone (Table 8). This difference is most likely the major factor which accounts for the higher potency of DHT in target cells.

Short term castration induces a loss of nuclear AR in both the prostate and the kidney, yielding a predominance of cytosolic AR in both tissues (Table 9). In addition, there is a significant increase in the affinity of the cytosolic AR in these tissues after castration. While this increased affinity may be related to the <u>in vitro</u> conditions used to study AR binding, if a similar change occurs <u>in vivo</u>, it

would promote the association of androgens with the AR when circulating androgen concentrations are low.

While the kidney in the short term castrated rat lacks detectable nuclear AR, there is a low level of residual nuclear AR that remains in the prostate. The concentration of this residual AR appears to be relatively stable as it is present in longer term (7 day) castrated rats in approximately the same concentration (data not shown). The maintenance of this residual nuclear AR suggests that it may also play an important role in the modulation of responses to androgens in the castrated animal. In the absence of androgens, target cells dependent on androgens would remain "primed" with nuclear AR that could subsequently be available for the initiation of androgen-induced cellular responses. This view is consistent with the recent report that unoccupied steroid receptors can interact with the nuclear matrix and that this form of the receptor is extractable with high salt (94). Since the production of AR is autoregulated (176), a possible role for this residual nuclear AR may be to initiate synthesis of new AR as an initial step in enhancing the androgen sensitivity of prostate cells when they are stimulated by androgen.

The kidney and prostate also differ in that only the prostate contains specific, high affinity AR associated with the nuclear matrix (Table 11 and 12). After correcting for

loss of matrix spheres, it is evident that matrix AR and nuclear AR are present in approximately equal concentrations in prostates from intact rats. In addition, collectively nuclear and matrix AR constitute approximately 90% of the total AR in this tissue. While the prostate matrix AR decreases to non-detectable levels within 24 hours after castration, it can be replenished to normal intact levels soon after DHT administration. This replenishment, at least initially, appears to result in a preferential localization of the AR to the matrix (Table 13). These data are in agreement with results previously reported for association of AR with the prostate matrix (80).

In contrast, DHT treatment fails to localize AR to the matrix in the kidney. However, it should be emphasized that the nuclear AR in the kidney is increased by DHT to levels twice that seen in intact rats, implying that adequate, if not supraphysiologic, levels of DHT are impacting the kidney under these conditions. Inasmuch as no matrix AR is detectable in the kidney, these data suggest that renal nuclei lack sites on the nuclear matrix which are capable of binding androgen-receptor complexes. While the possibility exists that proteases in the kidney could have destroyed the matrix acceptor sites for AR, this seems unlikely. First, all buffers contained PMSF, a protease inhibitor which protects prostatic nuclear matrices from degradation.

Secondly, the nuclear matrix consists mainly of structural proteins and no data have shown the presence of matrix-associated proteases.

While no previous studies exist which have examined matrix AR in the kidney, this finding is consistent with the published data which indicate that tissue-specific growth effects of steroids reflect changes in hormonally-regulated genes rather than a difference in the mechanism of hormone action (109). If differentiation of the kidney is such that the genes needed for hyperplastic responses are not available for expression, then the absence of matrix AR sites which would be associated with these genes might be anticipated.

In an attempt to ascertain if the absence of matrix AR in the kidney is due to a lack of acceptor sites, cell-free binding studies were designed to directly examine the interaction between prostate and kidney cytosolic AR and purified nuclei from these tissues. While the initial studies using a cell-free system appeared promising, it was subsequently established that under the conditions of the assay, prostate cytosolic AR precipitates from solution and thus interferes with its in vitro binding to nuclei (Table 14). Further studies ascertained that the precipitation of AR is related to the presence of the ethanol from the steroid solutions used in the assay (Table 15).

Interestingly, the presence of molybdate ion prevents the precipitation of the AR but also prevents binding of AR to nuclear acceptor sites (data not shown). Removal of the ethanol from the assay system prevents precipitation of the AR, but the cell-free AR binding data are highly variable and consequently uninterpretable (data not shown).

These observations cast considerable doubt on the validity of the initial report of cell-free binding of AR to nuclei since there is no indication that the study controlled for the potential precipitation of AR which occurs under these circumstances (144). Since most cytosolic steroid receptors are prepared by ultracentrifugation (100,000 x g), the appearance of a precipitable product at subsequent low speed centrifugations (1500 x g) is not expected.

Several interesting features of the AR precipitation phenomenon should be noted. First, it appears, for the most part, to be a feature of the prostate, but not the kidney, cytosol. Second, only a portion of the AR (≈45 - 50% at 6% ethanol) in the prostate cytosol precipitates. Third, the amount of AR which will precipitate is highest during the first day post-castration with the percentage of AR which precipitates decreasing to <10% by 2 days post-castration (data not shown). These factors suggest that the cytosolic AR interacts with a prostate-specific tissue factor or

factors that contribute to the aggregation and precipitation of the AR. It appears that the presence of this factor decreases with time during the post-castration interval.

In addition, the ability of molybdate to prevent precipitation of AR deserves further attention. Molybdate has commonly been utilized in steroid receptor assays because it is observed to increase the quantity of detectable receptors (70-73). The exact mechanism by which molybdate produces this effect is unknown, although several mechanisms have been proposed. An early hypothesis suggested that molybdate inhibits phosphatase activity and consequently prevents dephosphorylation of the receptor (177). Phosphate hydrolysis results in the loss of steroid binding in the receptor protein. Other investigators proposed that molybdate's ability to increase receptor concentration is related to a direct interaction with the receptor (73). More recent evidence indicates that receptor stabilization is the result of the interaction of receptors with heat shock protein-90 (HSP-90). Molybdate is known to promote the interaction of HSP-90 with steroid receptors (178-181). Heat shock proteins are a group of related proteins that are induced by stress and are believed to be involved in maintaining cellular homeostasis (178).

The data from this present study suggest another mechanism by which molybdate may increase cytosolic steroid

receptor titers. Since many investigators add steroids to their assay systems in ethanol solutions, it is likely that some of the receptor precipitates. In assays that utilize dextran-coated charcoal (DCC) to separate unbound (free) radiolabel from radiolabel bound to the AR, centrifugation produces a supernatant which contains the receptor and a DCC pellet which contains free radiolabel. Utilizing this technique, any AR which precipitates will be lost in the DCC pellet. When sodium molybdate is absent from the assay buffer, appreciable quantities of AR could be sedimented with the DCC and escape quantitation (Table 15). Addition of molybdate to the buffer reduces the precipitation of the receptor, thus improving the quantitation of AR. Not surprisingly, such effects could be easily misinterpreted as a stabilization of the AR.

Given the logistical problems associated with the utilization of a cell-free binding system to study the direct interaction of AR with the nuclear matrix, this approach was abandoned. Instead, a pharmacological approach was employed to compare the ability of an androgenic (DHT) and an anabolic (stanozolol) steroid to localize AR within prostatic nuclei. The data from these studies, as previously noted, indicate that STAN is able to maintain the weight and DNA content of the developed prostate gland, but it is unable to stimulate growth of the fully regressed prostate

(Table 18, Figure 13). On the other hand, DHT stimulates hypertrophy of the developed prostate and induces growth (hyperplasia) of the regressed gland.

In the 24 hour castrated rat model, both DHT and STAN stimulate localization of the AR to the matrix, but matrix AR titers are higher following equivalent doses of androgenic steroid (DHT) than following anabolic steroid (STAN) administration. Perhaps more importantly, AR is retained in association with the matrix longer following a single dose of DHT than is seen after STAN (Table 16). Furthermore, the total amount of AR in the nucleus (nuclear AR + matrix AR) 24 hours after injection is two-fold higher in the DHT treated rats compared to animals receiving STAN.

The shorter retention time of AR on the matrix seen after treatment with STAN may be explained by its lower affinity for AR (≈15 - 20 fold). The lower affinity of the receptor for STAN may result in a more rapid dissociation of the STAN from the receptor. Once the receptor is unoccupied, it conceivably could dissociate more readily from the matrix sites as is observed following castration. Alternatively, it is possible that STAN is not capable of inducing the appropriate conformational changes in the androgen receptor required to bind tightly to matrix acceptor sites.

Neither the inability of anabolic steroids to stimulate growth of the regressed prostate nor the effects of anabolic

steroids on the localization of AR to the nuclear matrix have been previously reported. The exact mechanism which differentiates androgenic from anabolic actions remains unknown, but these data suggest that androgenic effects require prolonged retention of the androgen-receptor complex on the nuclear matrix.

This hypothesis is supported by previous work in which two different estrogens, estriol and estradiol-17ß, were examined for their effects on uterine growth (182). The results of this study revealed that the concentration of nuclear estrogen receptor three hours after injection of steroid was the same regardless of which steroid was utilized. At 6 hours post-injection, only basal levels of nuclear receptor were present in the estriol treated animals while the estradiol-17ß treated animals had significantly higher levels of nuclear estrogen receptors. In addition, tissue responses to estradiol and estriol were correlated with nuclear retention. Estradiol-17ß stimulated uterine hypertrophy and hyperplasia while estriol stimulated a variety of biological responses in the uterus (termed uterotropic effects) but failed to produce a hyperplastic response.

The data from the present study support the hypothesis that long term retention of AR on the matrix is necessary for androgenic responses. However, the mechanism by which

anabolic steroids produce their more limited effects is unknown. It is possible that the binding of AR with a lower affinity and/or a shorter duration to matrix sites may result in androgen responses limited to anabolic effects. Conversely, the anabolic-receptor complex may be completely inactive at the nuclear matrix and act at separate nuclear sites.

SUMMARY AND CONCLUSIONS

The results of this study have demonstrated conclusively that in androgen-dependent tissues such as the ventral prostate, androgenic steroids produce both hyperplasia and hypertrophy. In contrast, these hormones produce only hypertrophy in androgen-sensitive tissues such as the kidney.

The major difference in the subcellular localization of AR in these tissues is the absence of nuclear matrix-associated AR in the kidney. It is also evident that the total AR concentration in the prostate is higher. Additionally, while the majority of the AR in the prostate is in the nucleus, cytosolic AR predominates in the kidney based on *in vitro* binding studies. Further, in the castrated rat, residual nuclear AR is present in the prostate but is not detectable in the kidney.

In the developed prostate gland, interaction of an anabolic steroid with AR prevents the regression of the prostate expected after castration. Both DHT and STAN, which are representative of androgenic and anabolic steroids, respectively, localize AR to prostatic nuclei, but DHT produces higher total nuclear AR titers. In addition, AR is retained on the nuclear matrix for a more prolonged period following DHT treatment.

In the regressed prostate gland, anabolic steroids fail to stimulate growth perhaps because anabolic steroids do not produce prolonged retention of the AR in association with the nuclear matrix. It is proposed that adequate retention of the androgen-receptor complex with the nuclear matrix is required to produce full androgenic responses.

In conclusion, androgen sensitive (kidney) and androgen-dependent (prostate) tissues respond distinctly to androgen stimulation. In the prostate, androgen stimulation produces both hypertrophy and hyperplasia. In the kidney, androgen stimulation produces only hypertrophy. A major distinguishing characteristic underlying the differences in androgen responses in these tissues is the presence of nuclear matrix-associated androgen receptors in the prostate and lack of such receptors in the kidney.

The response of androgen dependent tissues to anabolic steroids lacks any proliferative (androgenic) elements. It seems likely that the limited scope of androgenic effects produced by anabolic steroids is related to inadequate localization of AR to nuclear matrix sites. This further supports the view that long term retention of AR on the nuclear matrix is required to produce full androgenic effects in androgen target organs.

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APPENDIX

Table A-1
Effect of 20 mM sodium molybdate on quantitation of cytosolic androgen receptors in rat prostates.

Group	Molybdate Absent		Molybdate Present	
	K_D (nM)	Capacity (fmol/mg DNA)	K_D (nM)	Capacity (fmol/mg DNA)
Intact	0.63 ± 0.04	1204 ± 37	0.48 ± 0.03	1244 ± 49
24 hours post-castration	0.49 ± 0.02	2683 ± 127	0.26 ± 0.02	3524 ± 184*

Data are expressed as mean ± SEM (n=10-19)

*$p \leq 0.05$ compared to capacity in absence of molybdate

Table A-2
Effect of incubation time and temperature on specific ^{3}H-R1881 binding to prostate cytosolic androgen receptor.

Time (hours)	Temp (°C)	Specific ^{3}H-R1881 Bound	
		K_D (nM)	Capacity (fmol/mg DNA)
2	12	0.91 ± 0.39	659 ± 300
4	12	0.88 ± 0.28	1113 ± 196
6	12	0.47 ± 0.06	972 ± 128
12	12	0.39 ± 0.01	1238 ± 161
16	12	0.51 ± 0.08	1426 ± 167
16	0	0.29 ± 0.06	1114 ± 121

Data are expressed as mean ± SEM (n=3)

Table A-3
Comparison of dextran-coated charcoal (DCC) and hydroxylapatite (HAP) in the quantitation of prostate cytosolic androgen receptors (AR) from rats 24 hours post-castration.

Assay Method	Specific ^{3}H-R1881 Bound (fmol/mg DNA)	
	Molybdate Present	Molybdate Absent
DCC	3688 ± 221	2622 ± 224
HAP	3324 ± 181	2636 ± 142

Data are expressed as mean ± SEM (n=15)

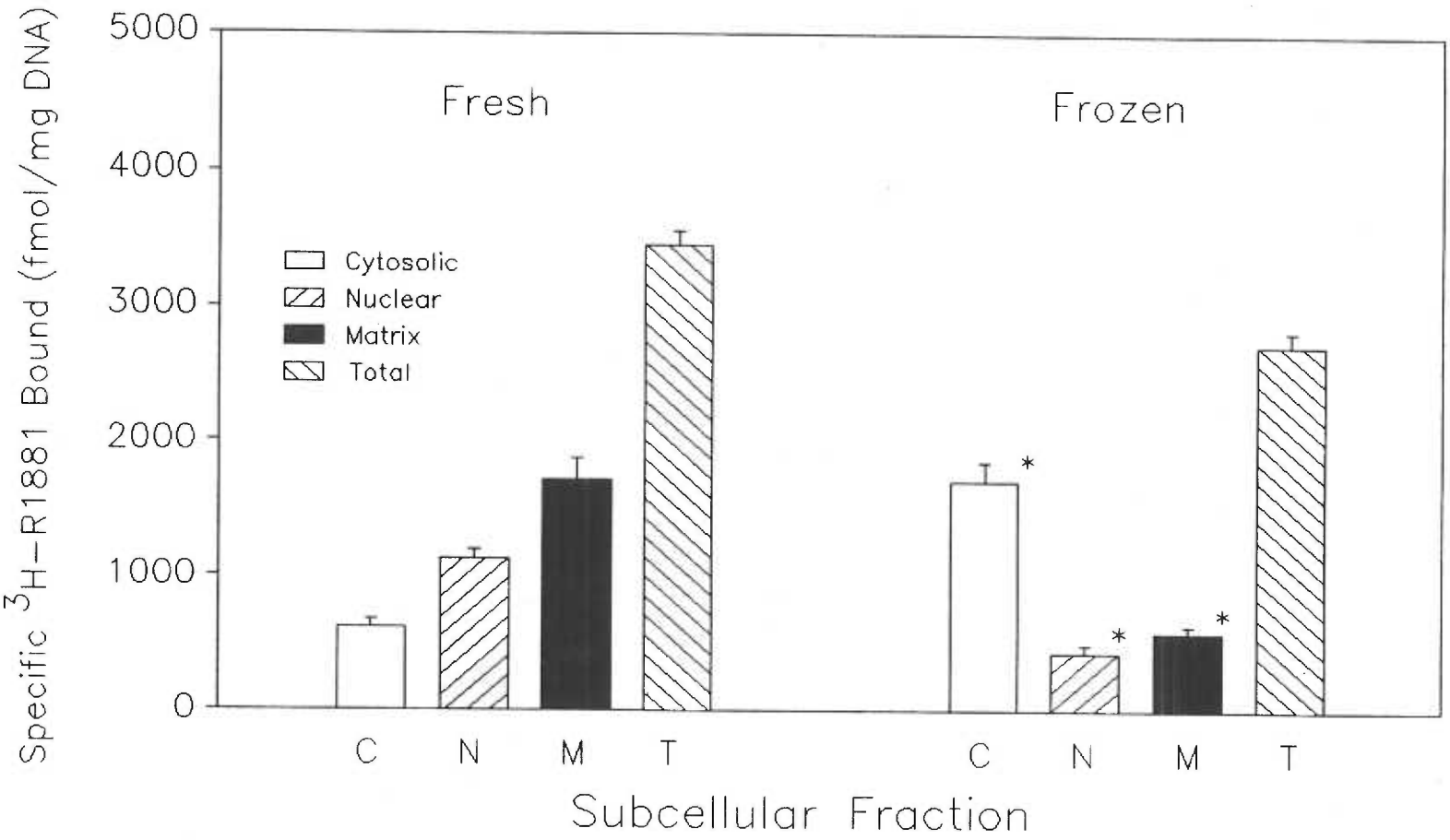

Figure A-1: Effect of tissue freezing on quantitation of androgen receptors (AR) in subcellular fractions of the prostate. C=cytosolic AR, N=nuclear AR, M=matrix AR, T=total AR. Data are expressed as mean ± SEM (n=3-6). *p<0.001 compared to fresh tissue.

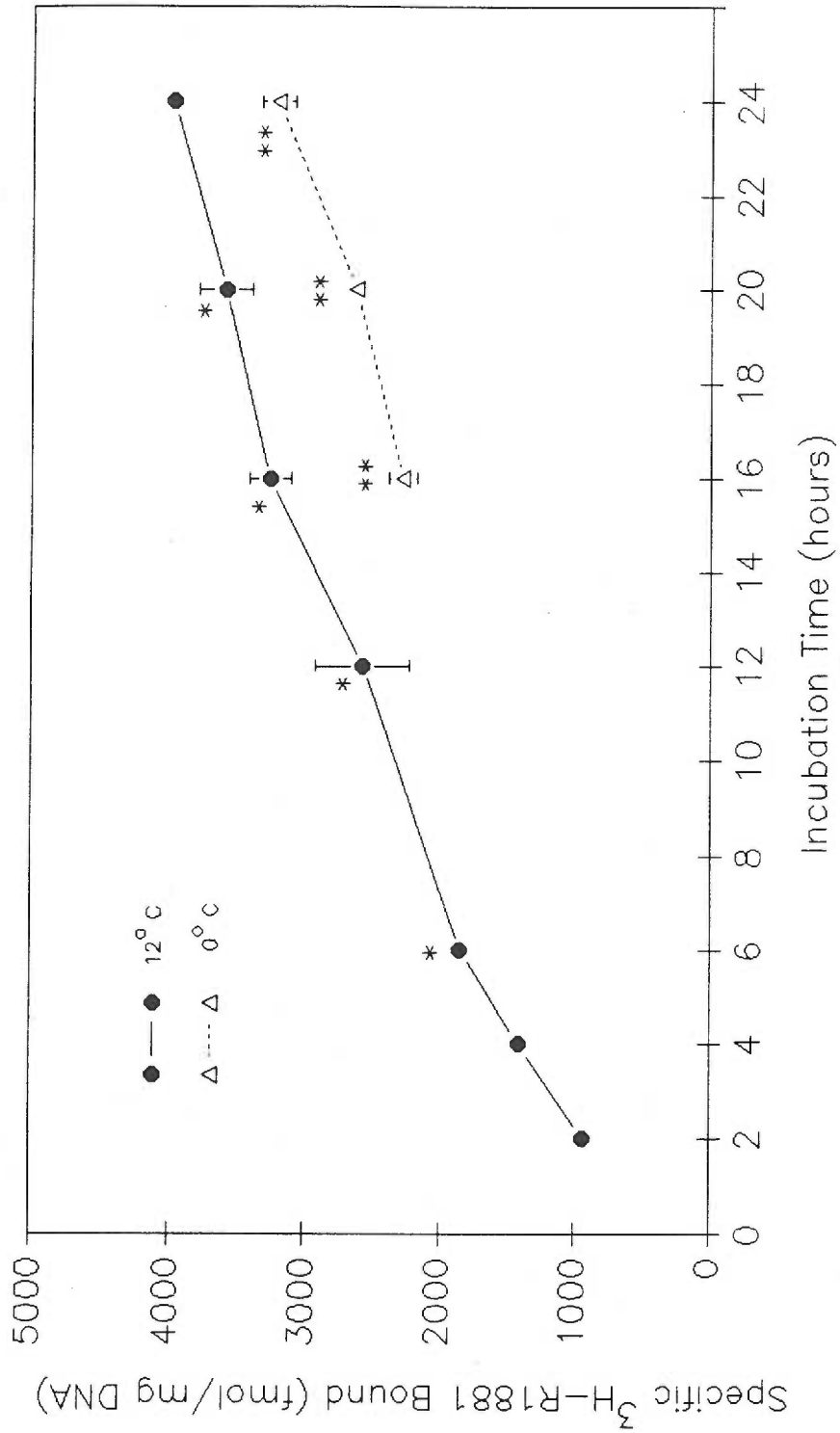

Figure A-2: Effect of incubation time and temperature on ^{3}H-R1881 binding to nuclear androgen receptors. Data are expressed as mean ± SEM (n=3-6). *p<0.05 compared to preceding time period at 12°C; **p<0.05 compared to incubation at 12°C.