ISOLATION AND CHARACTERIZATION OF ANDROGEN RECEPTORS FROM MALE TARGET CELLS

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ISOLATION AND CHARACTERIZATION OF ANDROGEN RECEPTORS FROM MALE TARGET CELLS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
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Promotor:	Prof. Dr. H.J. van der Molen
Co-referenten:	Prof. Dr. M. Gruber Prof. Dr. F.H. Schröder

De in dit proefschrift beschreven experimenten werden uitgevoerd op de afdeling Biochemie II (Chemische Endocrinologie) van de Fakulteit der Geneeskunde der Erasmus Universiteit Rotterdam.

Aan mijn ouders
Aan Marja

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VOORWOORD

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Chapter 1

SCOPE OF THIS THESIS

Androgens exert their action after binding to cytoplasmic receptors resulting in the formation of androgen-receptor complexes. This initial event is followed by activation, translocation to the nucleus and interaction with chromatin acceptor sites of the androgen-receptor complexes. Bound to chromatin, the androgen-receptor complex stimulates many biochemical events resulting in gene expression and starting with RNA-synthesis. Further detailed understanding of the complex processes requires a purified receptor preparation. Due to the low amount of receptors present in androgen target organs, large scale isolation of these receptors requires a suitable source. Thusfar it seems that androgen receptors from different sources have similar characteristics. In this respect seminal vesicles of the ram contain an androgen receptor comparable to the receptor present in rat prostate (chapter 4.3 and appendix paper II). Studies were performed to purify the receptor present in ram seminal vesicles, and an almost two thousand fold purified receptor preparation has been obtained (chapter 4.4 and appendix paper III). Nuclear localization and acceptor sites of androgen-receptor complexes on the chromatin in target cells have hardly been studied. To gain more insight in the mechanism of interaction of androgen-receptor complexes with chromatin acceptor sites, the usefulness of purified androgen receptors was investigated. In preliminary studies high affinity interaction of androgen-receptor complexes with isolated chromatin was observed (chapter 5) and the possibilities for further investigation are discussed (chapters 6.3 and 6.4).

There is now ample evidence that human breast cancer can be treated with more success with endocrine therapy if the tumor tissue contains specific receptor proteins for the steroid hormone estradiol. For human prostatic cancer which

can be influenced by androgens, it is still not known whether a correlation exists between androgen receptor content and the response to endocrine treatment. A reliable androgen receptor assay is therefore a necessity. Measurement of cytoplasmic receptors in human prostatic tissue is associated with many problems (chapter 3.1.2). In addition, controversial results have been reported in the literature concerning the relationship between cytoplasmic androgen receptor content and the benefit from endocrine management of prostatic diseases. For this reason, a nuclear receptor assay was developed for human prostatic tissue (chapter 3.2 and appendix paper I). This method can be applied to the evalution of a possible correlation between receptor content of prostatic tumors and clinical response to endocrine therapy.

Chapter 2

GENERAL INTRODUCTION ON THE MECHANISM OF ACTION OF STEROIDS

This chapter describes the known and unknown biochemical processes which can play a role in the effects of steroid hormones.

2.1 Definition of steroid receptors

Steroid receptors are intracellular proteins which interact with steroids through specific binding sites to form hormone-receptor complexes. Once the steroid-receptor complex has been formed an integrated sequence of molecular events is started (figure 2.1). Such steroid receptors are characterized by steroid specificity, target cell specificity, high affinity for their specific steroids and low concentration (Gorski et al., 1968; Baulieu et al., 1971).

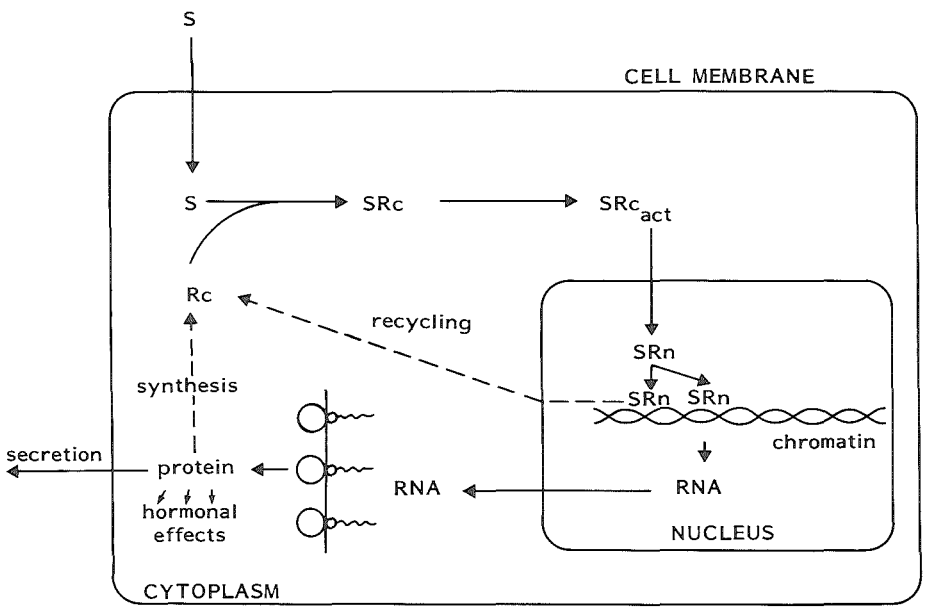

Figure 2.1: Simplified scheme for the action of steroids on their target cells.
Free steroids (S) enter the cell and bind to the cytoplasmic receptor (Rc) to form a steroid-receptor complex (SRc). Activation of SRc results in SRc_{act} which migrates to the nucleus (SRn). The nuclear receptor (SRn, probably identical to SRc_{act}) interacts with the chromatin which ultimately results in the hormonal effects.

2.2 Processes involved from entry of steroid into the cell to gene expression

2.2.1 Entry of steroid into the cell

Steroids can interact with different proteins in blood. This binding has been reviewed in detail by Westphal (1971, 1978). The affinity of these proteins for steroids varies from very low to very high and they are frequently present in high concentrations. Serum albumin, present in large amounts in vascular and extracellular spaces, has a relatively low affinity for several steroids, such as testosterone, estradiol and progesterone. More specific steroid binding proteins, which are present in smaller amounts (less than 1%), have higher affinities for their specific steroid hormones. Sex Hormone Binding Globulin (SHBG), present in man, binds testosterone and estradiol with affinity constants of 1.2 and 0.5 x 10^9 M^{-1}, respectively. Other steroid binding proteins, such as Corticosteroid Binding Protein (CBG) and Progesterone Binding Protein (PBG), bind their steroids with affinities of the same order of magnitude.

Steroids are biologically inactive as long as they are associated with serum proteins (Westphal, 1978; Rao, 1981). The amount of these steroid binders may change with changing levels of hormones. Because binding proteins can influence the amount of free hormone available for receptor binding inside the cell, they can be important in the control of steroid hormone action.

One of the least understood aspects of steroid hormones is their mode of entry into target cells. Early studies of steroid uptake have involved in vivo or in vitro exposure of target and non-target tissues to labelled steroid (Jensen & Jacobson, 1962; Gorski et al., 1968). Such studies indicated that a saturable component is involved in steroid uptake, and might reflect the retention of steroid after interaction with binding proteins inside the cell, rather than a limited rate of entry. The suggestion that estrogens partition between the blood and the tissue in a non-specific, passive manner, has also been reported (Peck et al., 1973). The precise mode of entry of steroids into target cells is still unknown and conflicting results have been described as reviewed by King & Mainwaring (1974), Mainwaring (1977) and Rao (1981). Most results support the idea that steroid uptake occurs by passive diffusion (Müller et al., 1979). This might also be expected because of the lipophilic properties of steroids, which facilitate easy permeation of steroids through the plasma membrane which is rich in lipids.

2.2.2 Binding of steroid to cytoplasmic receptors

Following their entry into target cells, steroids may both bind to various high capacity low affinity binding sites within the cytoplasmic compartment and to the cytoplasmic receptor for that specific steroid to form a steroid-receptor complex. Specific receptors, which are present in limited amounts, have been found for each of five physiologically well-defined steroid hormones: estrogen receptors, early described in rat uterus (Jensen & Jacobson, 1962); androgen receptors in rat ventral prostate (Fang et al., 1969; Mainwaring, 1969); progesterone receptors in guinea pig uterus (Milgrom et al., 1970) and in chick oviduct (O'Malley et al., 1970); glucocorticoid receptors in thymus cells (Munck & Wira, 1971) and mineralocorticoid receptor in toad bladder (Sharp et al., 1966) and also studied in rat kidney (Swaneck et al., 1970). Specific steroid-

receptor interaction is characterized by a high affinity (K_a: 10^9 - 10^{10} M^{-1}). The receptors are present in low amounts (values varying between 10^3 and 5×10^4 molecules per cell).

Cytoplasmic steroid receptors, isolated in hypotonic media from a variety of tissues, sediment on sucrose gradients as an 7-9 S entity, as described in early reports for estrogen receptors (Toft & Gorski, 1966; Erdos, 1968; Rochefort & Baulieu, 1968), for progesterone receptors (Milgrom et al., 1970; Sherman et al., 1970; McGuire & DeDella, 1971), for glucocorticoid receptors (Baxter & Tomkins, 1971 ; Beato & Feigelson, 1972) and for mineralocorticoid receptors (Herman et al., 1968). All these cytoplasmic steroid receptors dissociate into smaller components at high salt concentrations (0.4 M KCl).

For the cytoplasmic androgen receptors, isolated in hypotonic media from rat prostate, sedimention coefficients between 7 S and 12 S have been reported, as reviewed by Hiipakka et al. (1980). These large forms can be transformed to the slower sedimenting 3-5 S forms by incubations at 20-30°C or by raising the salt concentration to 0.4 M KCl (Baulieu et al., 1971). Under some conditions the smaller forms can also be converted to larger forms (Liao et al., 1975; Hu & Wang, 1978; Colvard & Wilson, 1981). However, aggregation as well as the formation of the 8 S form, which can be provoked by changes in pH (Liao et al., 1975), do not occur with partially purified androgen-receptor complexes (Liao & Liang, 1974). Liao and associates, therefore, suggested that the formation of the 8 S form obviously involves other cellular materials (Tymoczko et al., 1978). This suggestion was also made by Colvard & Wilson (1981) who postulated an "8 S androgen receptor-promoting factor" which converts the 4.5 S form of the receptor to the 8 S form. This factor, present in serum of mature male rats and in all tissues of male rats known to contain androgen receptors, appeared to be produced by androgen responsive cells.

Whether the large differences in receptor size, as discussed above, simply reflect preparative methods is not

clear. In this respect, studies of Wilson & French (1979) using the protease inhibitor diisopropyl fluorophosphate (DFP) and other protease inhibitors, suggest that receptor degradation leading to the 3.6 S and 3.0 S forms is an *in vitro* phenomenon as a result of proteolytic degradation. Recently in our laboratory, only a small increase in sedimentation coefficient of rat prostate androgen receptor (3.0 to 3.5 S) has been observed after homogenization of the tissue in the presence of DFP (Mulder et al., 1981). It was shown that also several other substances, e.g., pyridoxal 5'-phosphate, heparin and Cibacron blue effect the sedimentation coefficient.

Apart from the commonly studied sedimentation behaviour, several other criteria have been used for the characterization of steroid receptors. For androgen receptors, the 8 S form appears to have a molecular weight of 276,000, an Einstein-Stokes radius of 84 Å, a frictional ratio (f/fo) of 1.96 and it requires SH-groups for stability (Mainwaring, 1969). The dissociation constant (K_d) of the 5α-dihydrotestosterone-receptor complex is $2.4\text{-}4.0 \times 10^{-9}$ M (Ritzén et al., 1971; Mainwaring, 1977) and it has an isoelectric point (pI) of 5.8 (Mainwaring & Irving, 1973; Tindall et al., 1975). The receptor is extremely heat labile (Baulieu & Jung, 1970) and has a high affinity only for androgenic steroids (King & Mainwaring, 1974).

2.2.3 Transformation and activation of receptor before translocation to the nucleus

After binding of the steroid to cytoplasmic receptors several not completely understood processes occur before the steroid-receptor complex translocates to the nucleus. The steroid-receptor complex rapidly changes its properties and in some way becomes activated. "Activation" is a term that is generally used to define the process whereby steroid receptors are converted to a form that can bind to either nuclei, chromatin, DNA or DNA-like substances. Experiments

under cell-free conditions were initiated by Jensen and coworkers (Jensen et al., 1969; 1972) and they have shown that estrogen receptors in uterine cytosol, after incubation at low temperatures for short times with estradiol, were unable to bind to nuclei at 0 °C, whereas the same estradiol-receptor complexes temporarily heated to 25-30°C would bind to nuclei at 0°C. This activation process is associated with a shift in the dissociation rate constant of the steroid from its receptor. A two-component exponential dissociation of estradiol from the receptor has been demonstrated (Weichman & Notides, 1977). The first, rapid estradiol dissociating component is a property of the non-activated receptor, while the second, slower dissociating component is a property of the activated receptor. Parallel to this activation, heating of the estradiol-receptor complexes also caused a change in their sedimentation from 4 S to 5 S on sucrose gradients at high ionic strength (Notides et al., 1975). This change in receptor sedimentation coefficient is defined as receptor "transformation" (Bailley et al., 1980) and this shift to a faster sedimenting form has been observed only for estradiol-receptor complexes. The molecular details of receptor activation and transformation are not completely understood, but the conversion of the "native" 4 S estradiol-receptor complex to the nuclear 5 S form appears to involve more than a simple conformational change. The activated or transformed complex (MW: 130,000-140,000) has a higher molecular weight than the native form (MW: 70,000-80,000) (Yamamoto & Alberts, 1972; Little et al., 1973; Notides & Nielsen, 1974) and the conversion reaction follows second order kinetics (Notides et al., 1975; Little et al., 1975; Notides, 1978). It has been suggested that the 5 S sedimenting from is a result of dimerization (Notides, 1978).

Apart from heating, steroid receptor activation (defined as binding to DNA-cellulose or nuclei) can also be provoked by other procedures, like: increasing ionic strength (Jensen & DeSombre, 1972; Mainwaring & Irving, 1973), ammonium sulphate precipitation and gel filtration on sephadex

G-25 (Liao et al., 1980), dilution (Bailley et al., 1977) or dialysis against buffer (Sato et al., 1980).

In contrast to estrogen receptors, activation and/or transformation of the dihydrotestosterone-receptor complex is associated with a decrease in sedimentation rate to 3 S (Liao et al., 1975; this thesis: chapter 4.3). Similar decreases have been reported for the progesterone-receptor complexes of hamster (Chen & Leavitt, 1979) as well as guinea pig and rabbit uterus (Saffran et al., 1976) while no difference was observed in the sedimentation rates of native and activated progesterone-receptor complexes of chick oviduct (Buller et al., 1975[a]) and for glucocorticoid-receptor complexes of rat liver (Kalimi et al., 1975).

Enzymatic processes in receptor activation

Much of the available information is in support of considering activation as a temperature or salt induced conformational change in receptor molecules, but there is also evidence that activation may be an enzymatic process. Puca and coworkers have isolated and partially characterized from calf uterine cytosol a calcium-dependent protease, named "Receptor Transforming Factor", which converts the cytoplasmic estrogen receptor to a nuclear binding form (Puca et al., 1972; Sica et al., 1973). The biological significance of these studies, however, remains to be established. Also in human uterine cytosol a protein has been described, which is different from the calf uterine "Receptor Transforming Factor" and which may also be involved in estrogen receptor activation (Notides et al., 1973). In addition it has been shown that exogeneous and endogeneous proteases *in vitro* can cleave the rat hepatic glucocorticoid receptor in smaller components with a greater binding capacity for nuclei and DNA-cellulose (Wrange & Gustafsson, 1978).

High molecular weight components in receptor activation

In addition to the possible enzyme regulated activation of steroid-receptors, the presence of a receptor "activator" protein in rat uterine cytosol (Thampan & Clark, 1981) as well as of a macromolecular "inhibitor" protein of glucocorticoid receptor activation (Simons et al., 1976), have been reported. In rat prostate, protein factors have been described to inhibit binding of the androgen-receptor complex to cell nuclei and chromatin (Fang & Liao, 1971; Shyr & Liao, 1978). Apart from proteins, other high molecular weight components (RNA) have been shown to interfere in interaction of androgen-receptor complexes with DNA-like substances (Liao et al., 1980; Lin & Ohno, 1981).

Low molecular weight components in receptor activation

Irrespective of high molecular weight components, the possible involvement of low molecular weight components, which can inhibit the activation process of several steroid receptors, have been described (Cake et al., 1976; Bailly et al., 1977; Goidl et al., 1977; Sato et al., 1978, 1979, 1980). The low molecular weight inhibitor of glucocorticoid receptor activation was defined as "modulator" by Litwack and associates (Cake et al., 1976; Sekula et al., 1981). In subsequent studies was shown that increased intracellular levels of pyridoxal 5'-phosphate produced antiglucocorticoid effects whereas a reduction in pyridoxal 5'-phosphate content increased the sensitivity of hepatoma cells in culture to glucocorticoids (DiSorbo & Litwack, 1981). It has been concluded from these data that pyridoxal 5'-phosphate is an *in vivo* modulator of the glucocorticoid receptor. The inhibitory role of pyridoxal 5'-phosphate in the binding of several steroid receptors to nuclei and DNA has been reported also by other laboratories, e.g., for glucocorticoid receptor of rat liver (Kalimi & Love, 1980), for the avian progesterone receptor (Nishigori & Toft, 1979), for androgen receptor of rat prostate (Hiipakka & Liao, 1980; Mulder et

al., 1980) and for the uterine estrogen receptor (Muldoon & Cidlowski, 1980; Müller et al., 1980; Traish et al., 1980).

Other low molecular weight factors, ATP and Ca^{++}, can promote activation of steroid-receptor complexes (Sherman et al., 1974, 1978; Kalimi, 1980; Moudgil & Eessalu, 1980; Moudgil & John, 1980).

Phosphorylation-dephosphorylation in receptor activation

Recently several studies have been reported on the effect of molybdate on receptor activation. It appeared that molybdate, known as a phosphatase inhibitor, blocks or inhibits the activation of several steroid receptors (Leach et al., 1979; Nishigori & Toft, 1980; Shyamala & Leonard, 1980). The inhibition of activation by molybdate might suggest that a dephosphorylation step is involved in receptor activation. Other reports have shown, however, that molybdate protects steroid receptors against degradation (Leach et al., 1979; Gaubert et al., 1980; Chen et al., 1981; Miller et al., 1981), which might imply that a phosphorylated receptor is more stabile or that some proteolytic enzyme is inactive in its phosphorylated form. Hence, a phosphorylation-dephosphorylation mechanism involving a phosphatase may effect stability and activation of steroid receptors. There is no definitive information, however, as to whether molybdate acts directly on the steroid-receptor molecule or whether its effect on receptors is indirect, due to its interaction with other components in the cytosol.

Conclusions

In conclusion, the precise nature of the activation process which transforms steroid receptors to its chromatin binding form is still not solved. It remains unclear whether some protein factor in the cytoplasm or some low molecular weight component or a phosphorylation-dephosphorylation process is involved in the activation of steroid receptors. In light of the possibility that dephosphorylation of receptors may

occur prior to recycling of receptors from the nucleus to the cytoplasm, a hyphothetical scheme involving activation, translocation and recycling of receptors is presented in chapter 2.2.6.

2.2.4 Translocation of steroid-receptor complexes to the nucleus and initiation of transcription

Very little is known about the translocation of steroid receptors and other proteins from the cytoplasm to the nucleus. From the effect of temperature on transport rates of receptors (Buller et al., 1975[a,b]) and from studies on binding of receptors to isolated nuclear envelopes (Conn et al., 1977) it is not clear whether a specific carrier mechanism exists. However, studies of the involvement of ATP and inhibitors of nuclear uptake (Lohman & Toft, 1975) suggest the involvement of an energy-dependent or enzymatic step in the uptake of receptors by nuclei. The nuclear concentration of steroid receptors may also simply reflect the hydrophobic nature of the steroid-receptor complex (Williams & Gorski, 1974; Sheridan et al., 1979, 1981). Whatever the mechanism of translocation may be, it seems likely that steroid hormones exert their primary effects at the level of transcription. After binding of the activated steroid-receptor complexes to "acceptor-sites" on nuclear chromatin, activation of the transcription process at chromatin "effector-sites" induces the appearance of specific new RNA-sequences. Early experiments have been performed with several steroid hormone systems, including estrogens (Jensen et al., 1968; O'Malley et al., 1969; King & Gordon, 1972), progestins (O'Malley & McGuire, 1968; Schwartz et al., 1976), glucocorticoids (Kenny & Kull, 1963; Baxter et al., 1972) and androgens (Fang & Liao, 1971; Mainwaring & Peterken, 1971).

The sequence of events, which result in the alteration of gene expression elicited by the steroid hormone, has been extensively studied in the chick oviduct system and has been

reviewed by Thrall et al. (1978). Within 1 min after injection, labelled hormone can be detected within target and non-target cells. In target cells, the steroid is bound to its receptor within 1-2 min and the steroid-receptor complexes are detected bound to chromatin within 2-4 min after injection of the steroid. Within 5 min of injection, the steroid in target cells is predominantly bound to the nucleus whereas in non-target cells the steroid diffuses back into circulation. The synthesis of different gene product does not always occur as a primary event. For example, the egg-white protein, conalbumin, begins to acculmulate very soon after estrogen administration, while this occurs only after 3 h for ovalbumin (Palmiter et al., 1976). In a recent study (Palmiter et al., 1981) it has been suggested that a single binding site for the receptor is involved in conalbumin gene regulation and multiple sites are involved in ovalbumin gene regulation.

The amount of receptors necessary for full physiological response is not precisely known. Total concentrations of cellular steroid receptors have been reported for many different tissues in many different species (King & Mainwaring, 1974). A reasonable average amount for the total number of estrogen receptor molucules per uterine cell is approximately 20,000 (Clark & Peck, 1979; Clark et al., 1980), whereas only 1,000-2,000 sites are involved in full uterotrophic response (Clark & Peck, 1979). The remaining receptors, according to Clark, are present mainly to ensure that the steroid is effectively accumulated in the target cell. These findings are supported by Mulvihil & Palmiter (1980) who have shown that 800-1,500 nuclear acceptor sites per tubular gland cell of the chick oviduct must be occupied with progesterone-receptor complex for 8 h in order to get full induction of conalbumin mRNA and ovalbumin mRNA.

2.2.5 Nature and localization of nuclear acceptor sites

Nature and saturability of acceptor sites on the chromatin

Nuclear DNA is organized into nucleosomes which consist of a core particle containing an octomer of histones H2A, H2B, H3 and H4 (two each), surrounded by 140 nucleotide pairs of DNA (Felsenfeld, 1978), and an internucleosomal or linker region which is composed of about 60 nucleotide pairs of DNA and histon H1 (Kornberg, 1974; Noll, 1974). These nucleosomes are surrounded by non-histone proteins. The repeating subunit structure of the chromatin is shown in figure 2.2. The mechanism of transfer of the biochemical information, contained in steroid-receptor complexes, to the transcriptional apparatus is still a central problem in steroid hormone action. Many laboratories are involved in investigations on the precise nature and subnuclear distribution of the acceptor sites for the activated steroid-receptor complexes and

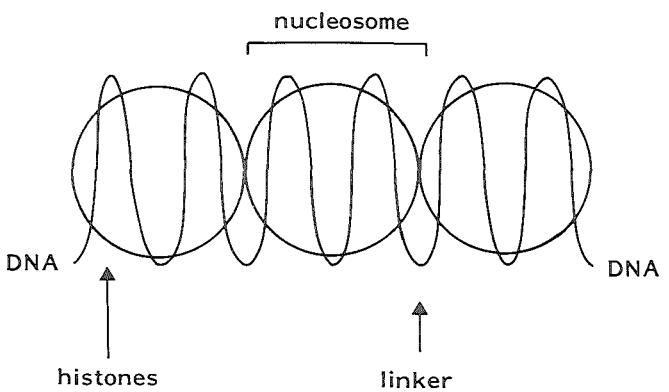

Figure 2.2: Repeating subunit structure of the chromatin.

on the biochemical effects of this interaction for the regulation of biochemical events, in particular RNA synthesis. Most of the steroid-receptor complexes are associated with the chromatin (Spelsberg, 1974). In addition, the binding of isolated steroid-receptor complexes to the chromatin has been shown to be a saturable process (Kon et al., 1980; Thrall & Spelsberg, 1980; Tsai et al., 1980). Many components of the nucleus have been proposed as the acceptor. Estradiol-receptor complexes have been found in association with, or have shown an affinity for respectively, histones (Kallos et al., 1981), non-histones (Spelsberg et al., 1979; Ruh et al., 1981), DNA (Majumdar & Frankel, 1978) and nuclear matrix, which consists of residual elements of the nuclear envelope and pore complexes, remnants of an internal fibrogranular network and residual nucleoli (Barrack et al., 1977; Barrack & Coffey, 1980). For androgen-receptor complexes interaction has been suggested with non-histones (Klyzsejko-Stefanowicz et al., 1976; Wang, 1978; Hiremath et al., 1981) which were further characterized as non-histone proteins with a basic overall charge (Mainwaring et al., 1976). Glucocorticoid-receptor complexes have shown an affinity for DNA (Bugany & Beato, 1977), and the progesterone receptor can interact with non-histones (Thrall & Spelsberg, 1980). In addition, the purified A-subunit of the progesterone receptor showed interaction with DNA (Hughes et al., 1981). The observations of Thrall & Spelsberg (1980), suggesting that DNA alone is not the acceptor site, do not exclude the possible existence of a very limited number of DNA-sites with specific sequences as acceptor sites, as postulated by Yamamoto & Alberts (1975). In this respect the saturable binding to DNA containing limited nicks, which becomes non-saturable as nicks were increased (Thrall & Spelsberg, 1980; Hughes et al., 1981), may indicate that experiments showing non-saturable binding to DNA may reflect increased binding to DNA damaged during isolation (Buller & O'Malley, 1976; Simons et al., 1976). Moreover it has been

suggested that certain specific sequences in eukaryotic DNA preferentially bind the estradiol-receptor complex (Majumdar & Frankel, 1978) and the glucocorticoid-receptor complex (Payvar et al., 1981). In addition, androgen-receptor complexes have been shown to recognize specific RNA having appropriate nucleotide sequences (Liao et al., 1980). The preferential binding of the progesterone receptor to single-stranded DNA (Hughes et al., 1981; Schrader et al., 1981) and the definition of Champoux (1978) that a helix-destabilizing protein displays preferential binding to single-stranded DNA, led to the speculation (Hughes et al., 1981) that the receptor can act as a helix destabilizing protein. However, due to the artificial binding of receptors this speculation remains premature. Deterioration of the integrity of chick oviduct chromatin increased its capacity to bind the progesterone-receptor complex, possibly via exposure of previously "masked" acceptor sites (Webster & Spelsberg, 1979). In combination with observations that two or more different acceptor sites are present (Clark et al., 1976; Spelsberg, 1976; Markaverich & Clark, 1979) it is tempting to speculate that steroid-receptor complexes act on the nucleus by initial binding to exposed regions of DNA with possible specific sequences causing helix-destabilization, followed by increasing RNA-polymerase activity, either directly or after some other activation process initiated by steroid-receptor complexes.

Separation of active and inactive regions of the chromatin

As reviewed by De Boer (1977) there is considerable evidence that chromatin consists of a condensed fraction (heterochromatin) and a less condensed fraction (euchromatin). Autoradiography on electron microscopic level of $[^3H]$Uridine incorporation into calf thymus nuclei has revealed that only the diffuse less condensed region of the chromatin is active

in RNA synthesis (Littau et al., 1964). Many attempts have been made to fractionate the template into active and inactive portions. The main approach in this fractionation has been to cleave the DNA or the chromatin by mechanical shearing or sonication. The methods, however, transform the chromatin into a form which has little resemblance to the genome structure *in vivo* (Noll et al., 1975). For maintenance of the structural integrity of the chromatin *in vitro*, the use of nucleases has been found to be superior over shearing by physical methods (Doenecke & McCarthy, 1976; Nicolini et al., 1976). Digestion of the chromatin by nucleases therefore might better preserve the structural differences between the template active and template inactive components of the chromatin. Three different nucleases, DNase I, DNase II and micrococcal nuclease, have mainly been used for these studies. DNase I digests preferentially active chromatin in a random manner and can be used for studying transcriptionally active chromatin rather than as a method for the separation of template active and template inactive fractions (Weintraub, 1975). After digestion of the DNA of the chromatin to the extent that 10-20% of the DNA is acid-soluble (very small chromatin fragments dissolve in 0.7 M perchloric acid at $0^{\circ}C$ for 30 min), specifically expressed gene sequences have been released from the remaining DNA. With this procedure non-expressed genes are still present in the undigested chromatin. Ovalbumin gene sequences could be selectively degraded by DNase I digestion of the nuclei isolated from oviducts which actively expressed the ovalbumin gene. In contrast, when chicken liver nuclei in which the ovalbumin gene is not expressed, are digested with DNase I, no loss of albumin gene sequences had been observed (Garel & Axel, 1976).

Separation of transcriptionally active and inactive chromatin has been introduced by Bonner and coworkers (1975). The method involves digestion of the chromatin with DNase II and precipitation of the template inactive chromatin with $MgCl_2$. A brief exposure to this nuclease releases pieces of chromatin which contain approximately 10% of the

DNA, but are enriched 20-fold in nascent RNA-chains, indicating that the released fraction is enriched in template active chromatin (Levy & Baxter, 1976).

More specific cleavage of the DNA of the chromatin by nucleases can be performed with micrococcal nuclease (Kornberg & Thomas, 1974). Mild digestion with this enzyme cleaves chromatin DNA to fragments of various lenghts by attacking the linker DNA which connects the nucleosome cores. Template active chromatin is more accessible to micrococcal nuclease digestion than template inactive chromatin and more extensive digestion results in a chromatin fraction consisting of mononucleosome cores more or less depleted of linker DNA. It is obvious that by controlled digestion, resulting in a series of nucleosomal oligomers and monomers, primarily from active chromatin, this enzyme offers a powerful tool for studying the distribution of steroid-receptor complexes over the chromatin.

Recently, it has been reported that certain "high mobility group" proteins, HMG 14 and 17, bind specifically to the nucleosomes which are active in transcription (Albanese & Weintraub, 1980; Weisbrod et al., 1980). HMG-depleted nucleosomes, orginally active in transcription, have been separated from the bulk of the inactive nucleosomes present in the population with immobilized HMG 14 and 17 (Weisbrod & Weintraub, 1981). It remains to be established, however, whether this procedure, which involves chromatin pretreated with 0.35 M NaCl to remove the HMG 14 and 17, is still suitable for steroid-receptor-acceptor studies.

Location of acceptor sites on the chromatin

Several studies with specific nucleases have shown that the steroid-receptor complex is enriched in the transcriptionally active fraction of the chromatin. This has been found for the estradiol-receptor complex (Hemminki, 1977; Alberga et al., 1979; Scott & Frankel, 1980; Schoenberg & Clark, 1981) for the glucocorticoid-receptor complex (André et al., 1980) and for androgen-receptor complexes (Davies et al.,

1980; Weinberger & Veneziale, 1980; Hiremath et al., 1981). There are some reports suggesting that the steroid-receptor complex can be located also on the nucleosomes (Massol et al., 1978; Senior & Frankel, 1978; Scott & Frankel, 1980), but location on the internucleosomal regions (Senior & Frankel, 1978; Rennie, 1979; Davies et al., 1980; Rochefort et al., 1980; Scott & Frankel, 1980) appears more likely.

2.2.6 Nuclear retention and replenishment of steroid receptors

The mechanism for replenishment of steroid receptors in the cytoplasm, once they have acted on the genome as steroid-receptor complex, has been studied mainly in the estradiol receptor system. In these studies antagonists of estradiol action have widely been used. Anti-estrogens are compounds which prevent estrogens from expressing their full effects on estrogen target tissues. While they antagonize estrogen-stimulated tissue growth, they can be considered as partial agonists (Katzenellebogen et al., 1978). This partial agonist action is a result of the translocation of the anti-estrogen-receptor complex to the nucleus and subsequent interaction with the genome. Following initial stimulation of tissue growth, e.g. by the non-steroidal anti-estrogens nafoxidine and tamoxifen, replenishment of cytoplasmic receptors is much slower than observed with agonists like estradiol (Clark et al., 1973; Capony & Rochefort, 1975; Horwitz & McGuire, 1978). Therefore, multiple cycles of nuclear binding and stimulation are largely retarded (Clark et al., 1978). With agonists, like 17β-estradiol, receptors in the cytoplasm are gradually replenished, resulting in control receptor levels after 11-16 h (Sarff & Gorski, 1971; Anderson et al., 1974). It has been shown that replenishment after administration of some "short-acting" estrogens is very rapid. After a single injection of 2-hydroxy-estradiol, replenishment is complete within 3 h (Martucci & Fishman, 1979) and after injection of 16α -

estradiol within 4 h (Kassis & Gorski, 1981). These latter authors concluded that estrogen receptor replenishment is entirely due to receptor recycling rather than that replenishment is partly the result of resynthesis of receptors (Mêster & Baulieu, 1975; Jungblut et al., 1979). Injection of 17β-estradiol into immature rats causes a decrease in total uterine receptor content (Zava et al., 1976; Kassis & Gorski, 1981) and in studies with MCF-7 human breast cancer cells, treated with 17β-estradiol, a rapid loss of total cell receptor content has been observed after receptor accumulation in the nucleus (Horwitz & McGuire, 1978, 1980). This approximately 70% loss (after 3-5 h) of total receptor content without reappearance in the cytoplasm has been termed "processing" (Horwitz & McGuire, 1978). After 3-5 h incubation nafoxidine-bound nuclear receptors appear not to have been processed at all and with tamoxifen only 30% of nuclear receptors are lost before the level of nuclear receptors remains constant (Horwitz & McGuire, 1978). This apparent loss of receptors could be due to a change in the receptor molecule and as a result the receptor can not bind steroid and therefore not be measured anymore. This hypothesis is supported by recent observations of Auricchio and associates (Auricchio & Migliaccio, 1980; Auricchio et al., 1981[a,b,c]) suggesting that the long half-life or nuclear retention of the anti-estrogen-receptor complex versus the short half-life of the estradiol-receptor complex in uterine nuclei in vitro is the result of the ineffectivity of a nuclear factor to inactivate the anti-estrogen-receptor complex. These authors showed that this nuclear factor is probably a phosphatase, which is not present in non-target tissue nuclei, and changes part of the estrogen receptor to a form which can not bind estradiol anymore. Experiments performed with the partially purified phosphatase and isolated calf uterine estrogen receptor suggest that the receptor itself might have been dephosphorylated (Auricchio et al., 1981[b,c]). The phenomenon of processing as described by Horwitz & McGuire (1978, 1980) can be explained by these assumptions. After recycling to the cytoplasm the dephos-

phorylated receptor protein is ready to be phosphorylated again to fulfil a further cycle of activation, translocation and interaction with the genome. Additional confirmation of these hypothesis might be drawn from (a) experiments with purified progesterone receptors, which appeared to be good substrates for phosphorylation (Weigel et al., 1981), and from (b) studies with the glucocorticoid receptor which requires phosphorylation of the receptor before interaction with the steroid occurs (Nielsen et al., 1977[a,b]; Litwack et al., 1980).

From the results in the literature, described in this and preceeding chapters, it is tempting to speculate that the possible events involved in the mechanism of action of steroids follow the hypothetical scheme presented in figure 2.3, with as a major characteristic the involvement of a phosphorylation-dephosphorylation process of the receptor. At the moment, however, there is no evidence available that this process is applicable to any of the different steroid hormone receptors. Especially, with respect to androgen receptors, little is known at the moment about the processes occuring before, during, and after binding of the androgen-receptor complexes to nuclear acceptor sites.

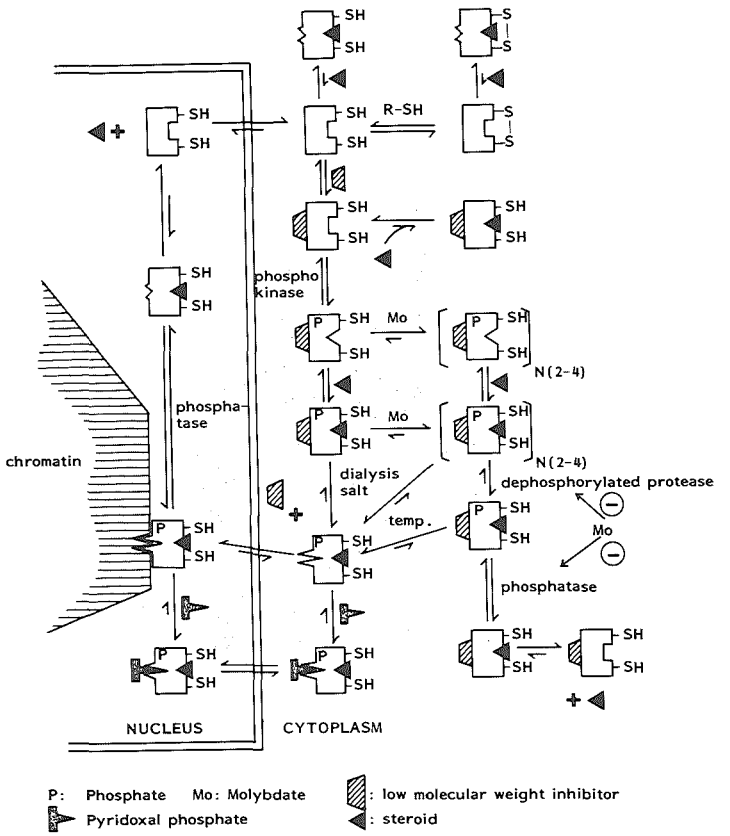

Figure 2.3: Possible events involved in the mechanism of action of steroid hormones.
After recycling from the nucleus the dephosphorylated unoccupied receptor interacts with a low molecular weight inhibitor of activation in the cytoplasm. This complex can be phosphorylated by a kinase resulting in a phosphorylated receptor which is able to bind steroid. In vitro, the non-activated steroid-receptor complexes are stabilized by molybdate, either directly or via inhibiting a phosphatase in the cytoplasm. This phosphatase either dephosphorylates the receptor protein which results in rapid dissociation of the labile dephosphorylated steroid-receptor complex, or this phosphatase dephosphorylates a protease which is active in dissociating aggregrated steroid-receptor complexes if the protease is in its dephosphorylated

form. The non-activated steroid-receptor complexes are activated by dissociation of the low molecular weight inhibitor from the phosphorylated steroid-receptor complexes by e.g., dialysis, increase in temperatures or high salt concentrations (in vitro). The activated steroid-receptor complexes translocate to the nucleus where they interact with the chromatin. The steroid-receptor complexes are released by indirect action of a phosphatase which is present in the nucleus and dephosphorylates the receptors. This dephosphorylation results in rapid dissociation of the steroid-receptor complexes, resulting in unoccupied receptors which are unable to bind to the chromatin. The unoccupied receptors recycle to the cytoplasm where they are ready to fulfil a further cycle as has been described above. Alternatively, the activated steroid-receptor complexes can be transformed to a non-chromatin binding form by pyridoxal 5'-phosphate which blocks the chromatin binding site.

2.3 References

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Chapter 3

ANDROGEN RECEPTORS IN HUMAN PROSTATIC TISSUE

This chapter is a review on the measurement of androgen receptors in human prostatic tissues. A nuclear androgen receptor assay has been developed and the prognostic value of a nuclear androgen receptor assay for clinical applications will be discussed.

3.1 Summary of the literature

3.1.1 Introduction

A correlation between the content of steroid receptors in mammary tissue and the response to endocrine therapy in advanced breast cancer was first described by Jensen and coworkers (1971). As reviewed by McGuire et al. (1975), endocrine therapy is essentially useless in patients who were "receptor negative" while 60% of "receptor positive" patients responded to such therapy. Receptor positive patients appear to respond poorly to chemotherapy (Lippman et al., 1978). On basis of these results on mammary cancer it is reasonable to investigate whether a similar correlation might exist for human prostatic carcinoma, the second most frequently occurring malignant disease in males in the western world. To investigate this possibility it would be necessary to estimate receptors in human prostatic tissue.

The growth and function of the prostate are primarily dependent on androgenic stimulus. The major circulating androgen, testosterone, is almost completely of testicular origin (Lipsett, 1970). After castration the prostate atrophies rapidly, but retains its normal function following androgen stimulation (Bruchovsky et al., 1975).

The human prostate can be divided into a central or periurethral part and a dorsal or peripheral part (McNeal, 1972). Benign prostatic hyperplasia (BPH) is extremely rare

in the peripheral parts of the gland (Moore, 1943). Prostatic carcinoma, however, starts predominantly (97-98%) in the peripheral parts of the prostate (Dube et al., 1973).

Clinical response of BPH to anti-androgen therapy has been reported (Scott, 1971) and 75-80% of all prostatic carcinomas appear to respond to endocrine management (Fergusson, 1972). In this respect is was of importance to investigate the possibility if the estimation of androgen receptors could assist in selecting patients, with non-operable tumors, who will benefit from endocrine treatment. Receptor negative patients might then be treated immediately with other therapies, like irradiation with ^{125}I (Whitmore 1972). Also a combination of therapies should be considered (Sinha et al., 1977; Chisholm, 1981).

3.1.2 Problems associated with the estimation of androgen receptors in human prostatic tissues

Several aspects of estimating steroid receptors have been discussed. The principal intracellular androgenic hormone in rat prostate has been identified as 5α-dihydrotestosterone (DHT) (Anderson & Liao, 1968; Bruchovsky & Wilson, 1968). From this finding, the first attempts to characterize human prostatic androgen receptors employed radioactive DHT (Hansson et al., 1971; Mainwaring & Milroy, 1973; Geller et al., 1975). Difficulties arose, however, when it was found that cytosols of human prostates contain Sex Hormone Binding Globulin (SHBG), probably as a result of plasma contamination, which binds DHT with equal affinity as the androgen receptor (Steins et al., 1974; Cowan et al., 1975). Hence, using DHT as ligand, the amount of cytoplasmic androgen receptor may be overestimated due to the additional binding to SHBG. A second problem has been the high endogeneous content of DHT in human prostatic tissue (Siiteri & Wilson, 1970), expressed in ng DHT/g tissue for normal, BPH and carcinoma tissue: 2.3, 3.9 and 5.0, respectively (Geller et al., 1979). With such high

concentrations of steroids (ratio DHT/receptor higher than 10/1), it might be expected that most androgen receptors are occupied and as a result are predominantly located in the nucleus (Mainwaring, 1977). An additional problem in measuring the remaining occupied receptors in the cytoplasm is the time required to exchange added labelled steroid for endogeneously bound DHT at low temperatures. This large incubation time of cytosol samples causes degradation of receptors by the presence of significant amounts of proteolytic enzymes in these cytosols. With DHT as exchange ligand, such long incubation times of cytosol samples also result in metabolism of DHT, which occurs rapidly (Attramadal et al., 1975; Shida et al., 1975), even at low temperatures (Snochowski et al., 1977). Most of the problems are associated with the measurement of cytoplasmic receptors, rather than of nuclear receptors, because in the nuclear extracts no SHBG and no metabolism of DHT have been found, whereas proteolytic degradation is negligible (J.A. Foekens & J. Bolt-de Vries, unpublished observations). However, when the synthetic androgen methyltrienolone (R1881) is used for labelling of receptors, many of these obstacles can be overcome by measuring cytoplasmic receptors. R1881 has the great advantage of binding to intracellular receptors but not to SHBG and it is not metabolized (Bonne & Raynaud, 1975, 1976; Snochowski et al., 1977; Menon et al., 1978). A complication of using R1881 is its ability to bind to progesterone receptors (Zava et al., 1979), which have been reported to be present in human prostate cytosol (Cowan et al., 1977; Tilley et al., 1980) and also, but to a lesser extent in nuclear extracts (Sirett & Grant, 1978). Therefore, precautions have to be taken and samples should be labelled in the presence of a 500-fold excess of the glucocorticoid triamcinolone acetonide which appears to block completely the progesterone receptor for R1881 binding without interfering with the binding of R1881 to the androgen receptor.

In conclusion, the estimation of androgen receptors in cytosols of human prostatic tissue is associated with

several problems which, in addition to the expected predominantly nuclear localization of the receptor, render the measurement of nuclear androgen receptors more promising.

3.1.3 Cytoplasmic and nuclear androgen receptors

The literature up to 1977 concerning androgen receptors in human prostatic tissue, has been reviewed extensively by Menon and coworkers (1977[a]). Generally used assay procedures involve gel filtration, charcoal separation, sucrose gradient centrifugation, ion exchange chromatography, electrophoresis, protamine sulphate precipitation or equilibrium dialysis. The large variation of receptor values in cytosol samples of BPH tissue (0-4,000 fmol/g tissue) can be attributed completely to the complicating factors associated with estimating these receptors, as has been discussed in chapter 3.1.2. The amount of nuclear androgen receptors, first reported by Menon et al. (1977[b]), appeared to be 10-60 fmol/g tissue. In more recent studies several laboratories have also measured nuclear androgen receptors. Recently reported results on the amounts of androgen receptor in BPH tissue are listed in table 3.1 for cytosol receptors and in table 3.2 for nuclear receptors. Only the results obtained with methods which are moderately reliable (using experimental conditions not likely to introduce artifacts) have been considered in the tables. The results indicate, that most receptors are located in the nucleus (e.g. Sirett & Grant, 1978[b]: 404 fmol/g tissue in nuclear extracts compared with 141 fmol/g tissue in cytosols). It is difficult to express all the results in the same way, e.g. as sites/cell, because the different groups used different procedures in preparing cytosols and nuclear extracts, which may have influenced the estimated amounts of protein and DNA, which were used to express the amount of receptors. Although the ratio of nuclear versus cytoplasmic receptor is approximately 3:1, it still has to be evaluated whether this is the actual distribution. In a recent report (Trachtenberg et al., 1981) it has been esta-

Literature	Assay	incubation conditions time/temp. (Ligand)	fmol receptor/g tissue	fmol receptor/mg protein	fmol receptor/mg DNA	dissociation constant (nM)
Sirett & Grant (1978b)	LH-20	20h/15°C (R1881)	141±38			0.85±0.26
Sirett et al. (1980)	LH-20	20h/15°C (R1881)		s: 27±5 e: 30±6	368±77 110±11	1.10±0.19 0.48±0.13
Krieg et al. (1977)	Agar	24h/0°C		12.3		
Hicks & Walsh (1979)	DCC	20h/4°C (R1881)		48±22		1.3±0.6

LH-20, Sephadex gel filtration
Agar, agar gel electrophoresis
DCC, dextran coated charcoal

s: stromal cells
e: epithelial cells

Table 3.1: Cytoplasmic androgen receptors in human BPH-tissue.

Literature	Assay	incubation conditions time/temp. (Ligand)	fmol receptor/g tissue	fmol receptor/mg protein	fmol receptor/mg DNA	receptor sites/ cell	dissociation constant (nM)
Sirett & Grant (1978b)	LH-20	24h/15°C (DHT)	404±43				3.97±0.73
Sirett et al. (1980)	LH-20	24h/15°C (DHT)			s: 556±76 e: 697±182		2.14±0.29 1.75±0.29
Menon et al. (1977b)	PSP	20h/4°C (DHT)	10-60				3.4
Menon et al. (1978)	DCC	20h/0°C (R1881)		67.5			2.6
Hicks & Walsh (1979)	DCC	20h/4°C (R1881)		104			2.8±0.8
Lieskovsky & Bruchovsky (1978)	DCGF	18h/4°C (DHT)				1400	4.5
Shain et al. (1978)	DCC	24h/15°C (R1881)					0.31±0.4

LH-20, Sephadex gel filtration
PSP, protamine sulphate precipitation
DCC, dextran coated charcoal adsorption
DCGF, dual column (G 25-G 200) gel feltration

s: stromal cells
e: epithelial cells

Table 3.2: Nuclear androgen receptors in human BPH-tissue.

blished that the amount of estimated cytoplasmic receptors can be increased almost four fold by addition of molybdate and a protease inhibitor, phenylmethylsulphonylfluoride (PMSF), prior to the incubation. This suggests that also the figures listed in tabel 3.1 could well be underestimations of the actual receptor values. For this and several already mentioned reasons it might be more meaningful to estimate in human prostatic tissues nuclear rather than cytoplasmic androgen receptors, with the ultimate goal to study whether a possible relation exists between the amount of receptors and the response to endocrine management.

3.1.4 Prognostic value of estimation of androgen receptors in treatment of prostatic diseases

Endocrine treatment, introduced by Huggins & Hodges (1941), has been a main therapy for prostatic carcinoma and 75-80% of all prostatic carcinomas respond to estrogen therapy (Fergusson, 1972). A complication of estrogen therapy is the high rate of cardiovascular complications (reported by the Veterans Administrative Cooperative Urological Research Group, 1967). Consequently, castration has become more widely used as a primary form of therapy in prostatic carcinoma and this treatment appears to be at least as effective as estrogen treatment in controlling the growth of prostatic carcinoma (Fergusson, 1972). Other forms of endocrine manipulations in patients with prostatic carcinoma are addition of anti-androgens like, cyproterone acetate (Isuguri et al., 1980) megestrol acetate and medroxyprogesterone (Rafla & Johnson, 1974; Johnson et al., 1975), and flutamide (Sogami & Whitmore, 1979). Also corticoids have been used with some success (Miller & Hinman, 1954).

It remains possible, in analogy with the endocrine management of breast tumors, that the 20-25% non-responders to endocrine therapy of prostatic carcinoma are "androgen receptor negative". Some attempts have been made to find a correlation between androgen receptor content and the res-

ponse to endocrine management. Wagner & Schultz (1978) concluded from their series of patients that no correlation existed between androgen receptor content and the response to endocrine therapy. In contrary, Ekman and associates (1979), reported a good correlation between cytoplasmic androgen receptor levels and the response of the tumors to endocrine management. These conflicting results might be due to the inacuracy of the measurements of cytoplasmic receptors described in the previous section. Therefore, a reliable androgen receptor assay (preferably for nuclear receptors) could become increasingly important because the estimation of androgen receptors in prostatic carcinoma tissue may assist in selecting the appropriate therapy for patients suffering from this disease.

3.2 Nuclear androgen receptors in human prostatic tissue. Extraction with heparin and estimation of the number of binding sites with different methods.

The conditions for assay of nuclear androgen receptors in human prostatic tissue has been evaluated with BPH tissue, which was available in sufficient amounts, and could be stored at -80 °C.

A comparison was made between the estimated amounts of receptors using protamine sulphate precipitation, sephadex LH-20 gel filtration and agar gel electrophoresis as techniques for specific isolation of receptors. Ratios of the amount of receptors found with these three different techniques were 100:88:61. Hence, protamine sulphate precipitation was accepted as most suitable for routine assay. Extraction of androgen receptors from a nuclear pellet with a heparin (1 mg/ml) containing buffer appeared to be twice as efficient as the commonly used 0.4 M KCl extraction (82 ± 7 and 49 ± 2 fmol/mg protein respectively, which corre-

sponds with 2333 ± 192 and 1005 ± 31 molecules/nucleus; means ± S.D., n=5).

For clinical application a method has been evaluated which involves extraction of nuclear pellets with a heparin containing buffer (1 mg/ml), exchange labelling of nuclear extracts for 20 h at 10°C and estimation of receptor-bound labelled DHT after protamine sulphate precipitation. Detailed procedures of this assay are described in appendix paper I.

The applicability of the reported nuclear androgen receptor assay (appendix paper I) for biopsy-size specimens of BPH tissue has been investigated in a subsequent report (Blankenstein et al., 1982). It appeared that it is technically possible to estimate nuclear androgen receptors in as little as 25 mg of prostatic tissue. However, the inhomogeneous distribution of androgen receptors in the tissue, excludes the possibility to obtain a meaningful receptor value on a single biopsy of BPH tissue which contains both epithelial and stromal cells.

It remains to be established whether biopsy-size specimens of prostatic carcinoma, which consists almost completely of epithelial cells, gives a more homogeneous distribution of the androgen receptor over the tissue.

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Chapter 4

CHARACTERIZATION AND PURIFICATION OF ANDROGEN RECEPTORS

4.1 Purification of steroid receptors

The lack of purified androgen-receptor complexes is one of the most serious handicaps for the progress of research on the mechanism of action of androgens. The availability of purified androgen-receptor complexes is of extreme importance for a.o. studies on receptor site-ligand interactions, for the determination of the structure of the hormone binding site and for investigating receptor proteins as metabolic regulators, especially of genetic transcription. Unless highly purified preparations of androgen-receptor complexes are used, the interpretation of experiments *in vitro* are always open to criticism and to difficulties in interpretation. For example, nucleases or unspecified activators and inhibitors could create serious experimental artifacts when studying interaction of androgen-receptor complexes with chromatin in cell-free conditions. For further study of the mechanism of action of androgens and for clinical applications the availability of antibodies against the receptor is desirable. Fortunately, the recently developed monoclonal antibody technique does not require a 100% pure receptor preparation to isolate specific antibodies. Hence, it may be possible that with an approximately 1-10% pure receptor preparation an antibody against androgen receptors may become available in the near future.

The purification of androgen-receptor complexes is associated with serious difficulties. The receptor proteins are notoriously labile (Mainwaring, 1969) and tissues containing androgen receptors contain very high proteolytic activity which resists commonly used inhibitors, such as PMSF (Mainwaring, 1978). The receptors are present in only minute quantities in androgen target cells (approximately 40 µg of receptor protein is present in a kilogram of rat

prostatic tissue; Mainwaring & Irving, 1973). The only reliable means for detecting the receptor protein is its association with a labelled ligand and dissociation of the bound radioactive ligand must be prevented. Therefore, fractionation methods must be rapid to prevent dissociation and denaturation of the labile androgen-receptor complex. In addition, the resolution and specificity of conventional methods of protein fractionation have been found insufficient, sofar, for androgen receptor purification. Apart from a single study (Mainwaring & Irving, 1973) in which, in an isolation procedure on a small scale, a 5,000 fold purification of androgen receptors from rat prostates was reached mainly on basis of the high separation power of a preparative iso-electric focussing system, no futher reports on the purification of androgen receptor have been published.

In early studies, partial purification of estrogen and progesterone receptors by ammonium sulphate precipitation and DEAE-sephadex chromatography has been achieved (DeSombre & Gorell, 1975; Puca et al., 1975; Schrader, 1975). In addition, purification of the native estradiol-receptor complex with heparin-sepharose has been very successful (Molinari et al., 1977). Steroid receptors, however, possess both a steroid binding-site and a DNA binding-site. Therefore, more recently, advantage has been taken of the property of steroid receptors to interact with DNA and DNA-like structures (e.g. phosphocellulose) once the receptors are in their activated form (as discussed in chapter 2.2.3). With these kind of gels, considerable success has been obtained in the purification of receptors for estradiol (Eisen & Glinsmann, 1978), for glucocorticoids (Wrange et al., 1979) and for progesterone (Schrader et al., 1977; Coty et al., 1979; Weigel et al., 1981).

Purification of some steroid receptors to homogeneity has been accomplished with affinity chromatography. Advantage has been taken of the availability of an unoccupied steroid binding-site of the receptor, and in these studies, matrices with immobilized steroids were used. Notwithstanding the successes obtained with affinity chromatography

of several steroid receptors (Coffer et al., 1977; Kuhn et al., 1977; Govindan & Sekeris, 1978; Sica & Bresciani, 1979; Govindan, 1980; Greene et al., 1980; Smith et al., 1981), this method, which requires that the receptor has to be presented to the columns in its unoccupied form, has not been successful thus far for the isolation of androgen receptors (Mainwaring, 1978; Mulder & Vrij, 1979; Mainwaring & Johnson, 1980).

In this thesis, experiments are described concerning purification procedures, other than affinity chromatography, and characterization of androgen receptors from ram seminal vesicles. The characteristics of these receptors are compared with the characteristics of androgen receptors from rat prostate.

4.2 Materials and methods

Tissue: Seminal vesicle tissue from adult rams were removed as soon as possible after killing the animals and was immediately frozen at -20°C. After transportation from the slaughterhouse to the laboratory the tissue was stored at -80°C. When indicated fresh tissue was used after transportation on ice to the laboratory. Rat prostate tissue was used 1 day after castration.

Purification columns: DEAE-sephadex (A-50), 2',5'-ADP-sepharose, poly U-sepharose, carboxymethyl-sepharose, and sephadex LH-20 were obtained from Pharmacia, Sweden. DNA-cellulose was prepared according to Alberts & Herrick (1971) and heparin-sepharose by the procedure of Cuatrecasas (1970). Bio-Gel P-6DG was purchased from Bio-Rad Laboratories, Richmond, California, USA, phosphocellulose from Whatman Inc., U.K., and Ultrogel ACA-44 from LKB Instruments Ltd., U.K.

Cytoplasmic and nuclear receptors: All procedures were performed at 0-6°C. For preparation of cytosols, containing cytoplasmic receptors, ram seminal vesicles stored at -80°C or fresh ventral prostates of rats one day after castration were used. Minced tissue was homo-

genized in 2-3 volumes of TEDG buffer (10 mM Tris-HCl, 1.5 mM EDTA, 1.5 mM dithiothreitol with 10% glycerol; buffer A, pH 7.4) either with a Waring blendor for 3 x 20 s or with 3 x 10 s strokes of an Ultraturrax tissue-homogenizer (in some experiments, as indicated, sodium molybdate was added to the homogenization buffer). The homogenate was centrifuged for 45 min at 96,000 x g_{av} in a Beckman SW-27 rotor and the supernatant was designed as "cytosol". For labelling of cytoplasmic receptors, cytosols from rat prostates were incubated for 2 h at 0 °C with 10 nM |^{3}H|R1881 (spec. act. 87 Ci/mmol) or with 10 nM |^{3}H|DHT (spec. act. 103 Ci/mmol) in the absence and presence of a 100-fold excess non-radioactive steroid to correct for non-specific binding. Cytosols from seminal vesicles of rams were incubated for at least 20 h at 6-10 °C with concentrations of radioactive steroids as indicated. For labelling of nuclear receptors, tissue minces were incubated for 1 h at 37 °C in Eagle's minimal essential medium with 20 nM |^{3}H|testosterone (spec. act. 93 Ci/mmol) in the absence and presence of a 100-fold excess non-radioactive steroid to correct for non-specific binding. The tissue was homogenized, as described, in buffer B (buffer A without glycerol) and the 700 x g nuclear pellet was prepared. The pellet was washed with buffer B containing 0.2% Triton X-100 and subsequently twice with buffer B. A nuclear extract was prepared by extracting the washed nuclear pellet with 0.4 M KCl in buffer B (pH 8.4) for 1 h at 0 °C and centrifugation for 15 min at 10,000 x g.

Sucrose gradient centrifugation: An aliquot of 250 µl was centriguged at 1 °C in 4.8 ml of linear 10-30% (w/v) sucrose gradients for 210 min at 370,000 x g_{av} in a Beckman VTi-65 rotor, resulting in a good separation between 3 S and 9 S sedimenting entities. Alternatively, 200 µl samples were centrifuged at 1 °C in 4.0 ml of linear 5-20% (w/v) sucrose gradients (in buffers without glycerol) for 18 h at 310,000 x g_{av} in a Beckman SW-60 rotor for characterization of the 3-5 S region and (in buffers with glycerol) for 16 h

at 150,000 x g_{av} for characterization of the 7-10 S region of the gradients.

Agar gel electrophoresis: Agar gel electrophoresis was perfromed as described by Wagner (1972). Free steroid migrates to the cathodic region of the agar gel whereas receptor-bound steroid migrates to the anodic region of the gel during electrophoresis of 50 µl samples for 90 min at 130 mA at 0°C.

Sephadex LH-20 gel filtration: Sephadex LH-20 gel filtration was perfromed essentially as described by Ginsberg et al. (1974). Aliquots of 50 or 100 µl were applied on small columns of Sephadex LH-20 (Pasteur pipettes) and protein-bound steroids were eluted in the void volume fractions, whereas free steroid was separated by gel exclusion chromatography.

Protamine sulphate precipitation: This method was performed as described by Chamness et al. (1975). A protamine sulphate concentration of 1 mg/ml was used to precipitate the protein-bound steroids for 10 min at 0°C. For partially purified receptor preparations the modified method as described by Mulder et al. (1981) was used. The modification involves the addition of 10 mM pyridoxal 5'-phosphate prior to the precipitation assay. After centrifugation and washing of the precipitates the radioactivity in the pellets was counted after dissolving the precipitates in 0.5 ml Soluene for 10 min at 60°C.

Column chromatography: Column chromatography was performed as described by Mulder et al. (1979) with the notable exception that in some cases a gradient of increasing KCl concentrations was used for elution of receptors from the column. When indicated, also the time of incubation with the column material was varied.

Protein determination: Protein was estimated according to Bradford (1976).

Liquid scintillation counting: Samples for counting of radioactivity were mixed with 10 ml of Insta-Gel (Packard Instrument) as scintillation cocktail. Protamine sulphate precipitates were dissolved in 0.5 ml Soluene for 10 min at 60 °C and counted in 10 ml of Insta-Gel after addition of 1% (v/v) acetic acid and 0.1% butylated hydroxytoluene.

4.3 Results on characterization of androgen receptors from ram seminal vesicles

As has been discussed in chapter 4.1.1, the availability of a purified androgen receptor preparation appears necessary for further studies on the mechanism of action of androgens. The limited amounts of androgen receptor present and the small size of androgen target tissues render the rat prostate an unattractive source for androgen receptor purification. Cytoplasmic androgen receptors are present in seminal vesicles of rat and mouse (Mainwaring & Mangan, 1973) and in ram testis (Monet-Küntz et al., 1979). We have investigated the presence of androgen receptors in ram seminal vesicles as a possible source for large scale receptor purification.

Nuclear androgen receptors

Nuclear androgen receptors from ram seminal vesicles were identified by sucrose gradient centrifugation and by agar gel electrophoresis. A specific androgen-binding protein sedimented at 3 S on sucrose gradients (figure 4.1A) similar to the nuclear androgen receptors from rat prostate (figure 4.1B). Agar gel electrophoresis at pH 8.4 showed an electrophoretic mobility of bound radioactivity towards the anodic region of the agar gel (figure 4.1C). Similar electrophoretic behaviour has been observed for androgen receptors extracted from nuclei of rat prostates (figure 4.1D).

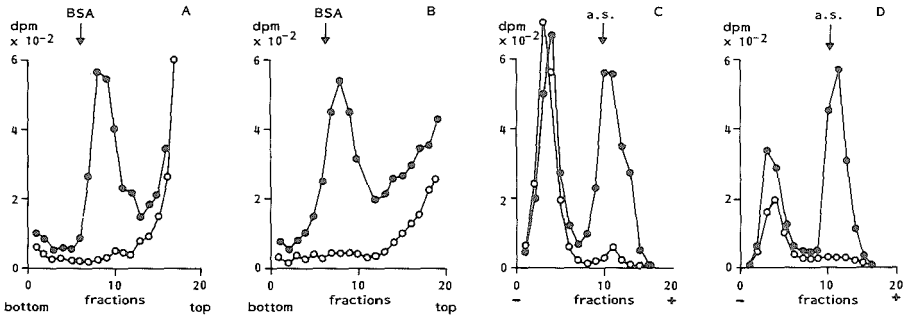

Figure 4.1: Sucrose gradient centrifugation and agar gel electrophoresis of nuclear androgen receptors from rat prostates and ram seminal vesicles.

Labelled nuclear extracts of rat prostates and ram seminal vesicles were analysed with sucrose gradient centrifugation (A and B, respectively) or agar gel electrophoresis (C and D, respectively).

●——● , total binding; ○——○ , non-specific binding; BSA, position of sedimentation marker, bovine serum albumin (4.6 S) after centrifugation; a.s., "application site", sample applied at the start of electrophoresis; + and - , anodic region, cathodic region of the agar gel respectively; free steroid was present in fraction 2-6 and protein-bound steroid was present in fraction 9-13.

Cytoplasmic androgen receptors

Seminal vesicles were obtained from a group of non-castrated rams. Approximately 700 pg androgen has been measured*). For this reason binding-studies required long incubation times to allow the added radioactive steroid to be exchanged with the endogeneously receptor-bound non-radioactive steroid. Under these conditions androgen receptors are very labile and especially a 7-12 S sedimenting form of the receptor from ram seminal visicles, on sucrose gradients, could never be demonstrated when no

*) Analysis performed by Dr. F.H. de Jong.

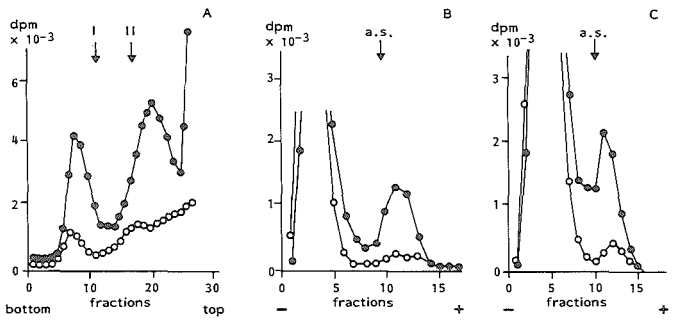

Figure 4.2: Sucrose gradient centrifugation and agar gel electrophoresis of cytoplasmic androgen receptors.
Labelled cytosols of ram seminal vesicles, prepared in the presence of 50 mM molybdate, were analysed with sucrose gradient centrifugation (A) and agar gel electrophoresis (B). Labelled cytosol of rat prostates was analysed with agar gel electrophoresis (C). I and II, positions of sedimentation markers γ-globulin (7.2 S) and BSA (4.6 S), respectively. For further details see the legend to figure 4.1.

precautions were taken. After addition of molybdate, which can stabilize the androgen receptor from rat prostate (Gaubert et al., 1980) as well as other steroid receptors (Chen et al., 1981; Miller et al., 1981), two distinct [^{3}H] R1881-binding proteins in cytosols from ram seminal vesicles, one sedimenting at approximately 9 S and one at about 3 S, could be demonstrated by sucrose gradient centrifugation (figure 4.2A). Apart from a small shift in sedimentation values, similar profiles have been obtained for sucrose gradients of cytoplasmic androgen receptors from rat prostate (Mulder et al., 1980). Analysis by agar gel electrophoresis shows an electrophoretic mobility towards the anodic region of the gel, both for cytoplasmic receptor from ram seminal vesicle and from rat prostate (figure 4.2B and 4.2C, respectively).

Activation of androgen-receptor complexes

A general characteristic of steroid receptors is their ability to bind to DNA-like matrices once the recep-

tors are in their activated state (Fleischman & Beato, 1979; Litwack et al., 1980; Moudgil & John, 1980). It was investigated whether this also holds for the androgen receptor from seminal vesicles of rams. Cytosols containing two distinct $|^3H|$R1881-binding proteins, as analysed by sucrose gradient centrifugation, were incubated with 2',5'-ADP-sepharose or phosphocellulose. Only the slower sedimenting 3 S form appeared to be retained by 2',5'-ADP-sepharose (appendix paper II) and phosphocellulose (figure 4.3A/B), probably representing the activated steroid-receptor complex. The fraction which did not bind to phos-

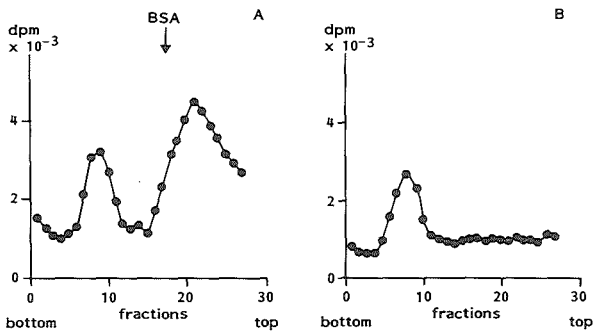

Figure 4.3: Binding of androgen receptors to phosphocellulose: Analysis with sucrose gradient centrifugation.
Labelled cytosol, prepared in the presence of 50 mM molybdate from fresh ram seminal vesicles, was analysed with sucrose gradient centrifugation (A). The same cytosol was allowed to incubate with phosphocellulose for 2 h at 6°C and the fraction which did not bind to phosphocellulose was analysed with sucrose gradient centrifugation (B). Both samples (250 µl) were layered on 10-30% linear sucrose gradients after treatment of the sample with charcoal. Non-specific binding was negligible (not shown). Centrifugation was performed for 210 min at 1°C in a Beckman VTi-65 rotor at 370,000 x g_{av}.

phocellulose (figure 4.3B) was stored at -20°C and after thawing several procedures known to activate steroid-receptor complexes were performed with this fraction containing only the 9 S sedimenting form of the receptor.

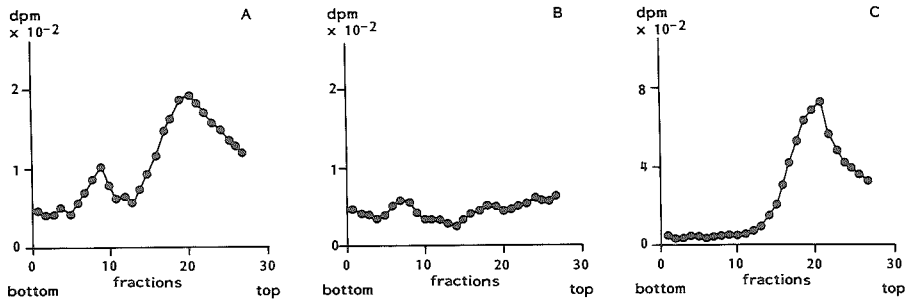

Figure 4.4: Activation of androgen receptors: analysis with sucrose gradient centrifugation.
The fraction of the cytosol which did not bind to phosphocellulose, as analysed in figure 4.3B, was subjected to several procedures known to activate steroid receptors. This "activated" preparation was analysed with sucrose gradient centrifugation (A). By subsequent incubation of this "activated" sample with phosphocellulose (2 h at 6°C), the fraction which did not bind to phosphocellulose and the fraction which could be eluted from phosphocellulose were analysed with sucrose gradient centrifugation (B and C, respectively).

After incubation at 0.4 M KCl, sephadex G-25 gel filtration and dilution, the 9 S sedimenting form was almost completely converted to a 3 S sedimenting form (figure 4.4A compared to figure 4.4B). This fraction containing activated androgen-receptor complexes, originating from the non-activated 9 S form, was incubated with phosphocellulose. This resulted in a complete loss of the 3 S sedimenting entity when the non-bound fraction was analysed on sucrose gradients (figure 4.4B), whereas still a small residue of the already little 9 S peak (figure 4.4A) could be demonstrated (figure 4.4B). The part of the activated androgen-receptor complexes which actually was retained by phosphocellulose (figure 4.4A minus figure 4.4B) was eluted with KCl and sedimented in the 3 S region of sucrose gradients (figure 4.4C). From these results it can be concluded that the non-activated 9 S sedi-

menting form of the androgen-receptor complex from ram seminal vesicles, can be converted into the activated 3 S sedimenting form.

Characteristics of androgen-receptor complexes

The receptor from cytosols from ram seminal vesicles, sedimenting at 3 S on sucrose gradients, could be destroyed by heating for 30 min at 50°C. The complex with [^{3}H]R1881 dissociated very slowly at low temperatures. The apparent equilibrium-dissociation constant (K_d) was 3.8 x 10^{-10} M. The receptor showed comparable relative affinities for androgenic steroids, whereas other steroids like estradiol, R5020, progesterone, diethylstilbestrol and triamcinolone acetonide only competed at very high concentrations. For further details see: appendix papers II and III.

Conclusions

The androgen-binding protein present in seminal vesicles of rams has many characteristics of the rat prostate androgen receptor. Ram seminal vesicles contain approximately 1 pmol androgen receptors per gram of tissue. Because of the availability of large amounts of tissue, ram seminal vesicles appear to be a suitable source for large scale purification of androgen receptors.

4.4 Results on purification of androgen receptors

4.4.1 Partial purification of cytoplasmic and nuclear androgen receptors from rat prostate

The lability of androgen receptors, probably due to proteolytic breakdown by enzymes in the crude cytosol fraction, makes it necessary to introduce a fast and simple first purification step. Ammonium sulphate precipitation and

substituted matrices	recovery (%) of receptors		purification factor of receptors	
	from cytoplasma	from nuclei	from cytoplasma	from nuclei
heparin-sepharose	80	70	10	1
DNA-cellulose	50	10	15	-
poly U-sepharose	70	95	15-30	5
2'5'-ADP-sepharose	80	90	25-50	8
carboxymethyl-sepharose	55	ND	10	ND
DEAE-sephadex	45	ND	3	ND

Table 4.1: Purification of cytoplasmic and nuclear androgen receptors from rat prostates. (ND, not determined).

protamine sulphate precipitation with a 70% recovery and 3-20 fold purification and 90% recovery and 10 fold purification, respectively, were not successful despite the relatively high recoveries and purification factors. The limited success of these procedures as first purification steps was due to the high losses (approximately 85%) in the subsequently required steps (dissolving the pellet and desalting) prior to further purification procedures. These losses might be due partly to coprecipitation of proteolytic enzymes. Therefore, we have investigated the usefulness of DNA-cellulose and a variety of substituted sepharose columns in initial purification of androgen-receptor complexes from rat prostate. Results of recovery and purification of cytoplasmic and nuclear receptors are listed in table 4.1, showing that with these column materials, nuclear receptors are more difficult to purify than cytoplasmic receptors. This is probably due to the more similar charges of the proteins which are present in the nuclear extracts, whereas in the cytosols proteins are present with different overall charges. In general, receptor

samples were used after storage at -80°C, and storage at this temperature resulted in aggregation of receptor proteins. Heparin-, 2',5'-ADP-, and poly U-sepharose appeared to be effective in deaggregating complexes for nuclear androgen receptors. Results of studies with aggregated nuclear receptors, as analysed with sucrose gradient centrifugation, are shown in figure 4.5A (receptors at the bottom of the gradient) and analysed with agar gel electrophoresis, are

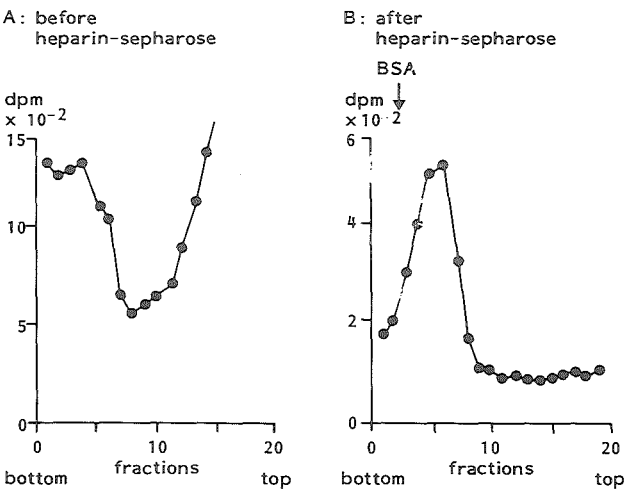

Figure 4.5: Sucrose gradient centrifugation of nuclear androgen receptors from rat prostates.
Nuclear androgen receptors, stored at -80°C, were analysed with sucrose gradient centrifugation, before (A) and after (B) interaction with heparin-sepharose. Centrifugation was performed in 5-20% linear sucrose gradients, containing 0.4 M KCl, for 18 h at 310,000 x g_{av} in a Beckman SW-60 rotor at 1°C.

shown in figure 4.6A (receptors at the application site of the gel after electrophoresis). Interactions of receptors with heparin - sepharose or poly U-sepharose appeared to result in deaggregation of the receptors as demonstrated by a shift in sedimentation coefficient (figure 4.5B) and electrophoretic behaviour (figure 4.6B), respectively.

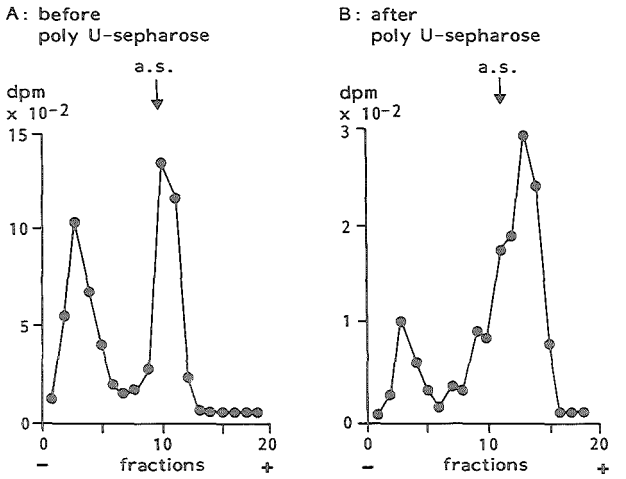

Figure 4.6: Agar gel electrophoresis of nuclear androgen receptors from rat prostates.
Nuclear androgen receptors, stored at -80°C, were analysed with agar gel electrophoresis, before (A) and after (B) interaction with poly U-sepharose.

2',5'-ADP-sepharose appeared to be most suïtable for initial purification of cytoplasmic androgen receptors from rat prostate (table 4.1). In other experiments (Mulder et al., 1980) it was shown that phosphocellulose chromatography also gave promising results (80% recovery and 23 fold purification).

4.4.2 Partial purification of cytoplasmic androgen receptors from ram seminal vesicles

Based on results obtained with partial purification of the androgen receptors from rat prostate, experiments were performed to investigate whether the procedures were also applicable for large scale isolation of androgen receptors from seminal vesicles of rams. Comparable to results obtained with androgen receptors from rat prostate, DEAE-sephadex and DNA-cellulose were found to be unsuitable matrices for initial purification. Phosphocellulose chroma-

tography resulted in recoveries of 90.0 ± 5.0% (± S.D., n=4) and a purification factor of 10.1 ± 4.4% (± S.D., n=4). With 2',5'-ADP-sepharose somewhat lower recoveries (70%) with higher purification factors (30-50 fold) were found. For this reason 2',5'-ADP-sepharose chromatography was adapted as first step in our purification procedures. To reduce the high costs of large scale receptor purification, initial procedures had to be performed with non-radioactively labelled steroid-receptor preparations. These procedures involve tissue processing followed by activation of receptors and 2',5'-ADP-sepharose binding. After binding of the receptors to 2',5'-ADP-sepharose, which reduced the volume of the receptor preparations significantly, labelling of receptors has been performed on the gel by exchange of added radioactive ligand for bound non-radioactive steroid. The procedure as summarized in the scheme of figure 4.7,

1. CYTOSOL (prepared in buffer A, containing 50mM molybdate and 10nM testosterone)
2. first activation step (incubation for 30min at 6°C after addition of 1/5 vol. buffer A, containing 2M NaCl and 20nM testosterone)
3. second activation step (dilution with 4vol. buffer A, containing 50nM testosterone)
4. 2'5'-ADP-sepharose binding (for 20h at 6°C with 30ml gel)
5. washing procedures (with buffer A)
6. labelling of receptors on 2'5'-ADP-sepharose (for 60h at 6°C in buffer A, containing 10mM molybdate)
7. washing and elution of the gel (see: figure 4.8)

Figure 4.7: 2',5'-ADP-sepharose chromatography of androgen receptors from ram seminal vesicles.
Tissue was homogenized in 1 vol buffer with a Waring blendor for 3 x 20 s. Washing procedures (step 5) were performed on a glass filter, followed by more extensive washing in a Bio-Rad column. Labelling of receptors (step 6) was performed with 20 nM |^{3}H|DHT. Washing and elution of the gel (step 7) was performed as described in the legend to figure 4.8.

was found to be very successful. The procedure described involves stabilization of the androgen receptor by molybdate and excess non-radioactive testosterone. High concentrations of molybdate partly results in the formation of the aggregated non-activated form of the receptor (figure 4.2A and 4.3A) which does not bind to 2',5'-ADP-sepharose (appendix paper II) and phoshocellulose (figure 4.3B). Therefore, additional activation steps, as described in step 2 and step 3 of the scheme presented in figure 4.7. are required to achieve maximal binding of receptors to 2',5'-ADP-sepharose. The extensive washing procedures (step 5) are necessary to remove excess non-labelled testosterone and non-bound protein prior to incubation with [^{3}H] DHT (step 6).

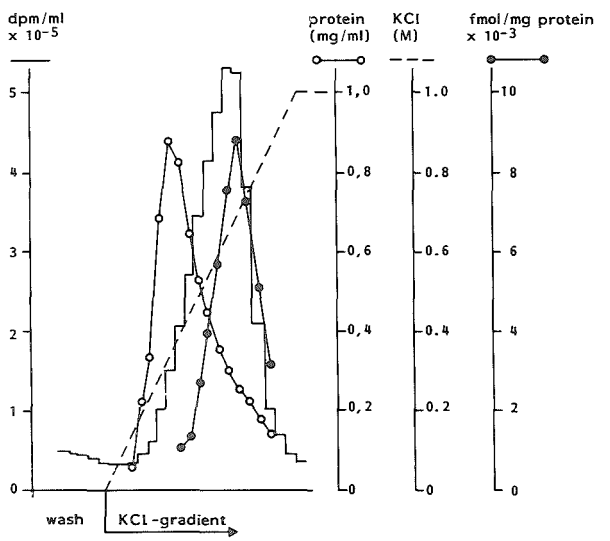

Figure 4.8: Purification of androgen receptors from ram seminal vesicles by 2',5'-ADP-sepharose chromatography after activation of the androgen-receptor complexes.
Activated [^{3}H]DHT-receptor complexes, prepared from 110 g of tissue as described in figure 4.7, were eluted from 30 ml 2',5'-ADP-sepharose with a KCl-gradient, after extensive washing with buffer A. Proteins were eluted with a gradient of 100 ml of buffer A and 100 ml of buffer A containing 1 M KCl. 10 ml fractions were collected from a Pharmacia column (size, 1.6 x 20 cm) and were counted for radioactivity.
——— , radioactivity; o——o , protein; ●——● ,specific steroid binding activity; ----, KCl concentration.

Results of the final washing and elution procedures are shown in figure 4.8. Separate peaks of protein and radioactivity were observed. The pooled eluate fraction (indicated as ●——● in figure 4.8) contained 144 pmol [^{3}H] DHT-receptor complex with a specific activity of 4630 fmol/mg protein. Based on this initial procedure, further purification procedures involving purification and concentration with ammonium sulphate precipitation and subsequent Ultrogel ACA-44 gel chromatography have been established. By these procedures an almost 2,000 fold purification with a recovery of 33% could be obtained (appendix paper III). The labelled receptor was characterized by electrophoresis and sucrose gradient centrifugation (sedimentation at approximately 3 S). The purified receptor has a DNA binding-site and is specific for androgenic steroids (appendix paper III).

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Chapter 5

INTERACTION OF PARTIALLY PURIFIED ANDROGEN-RECEPTOR COMPLEXES WITH CHROMATIN

5.1 Introduction

The nature of the interaction and the location of androgen-receptor complexes on target cell chromatin, resulting in ultimate gene expression, are still not known (chapter 2.2.5). From experiments with rat prostatic tissue there are indications that the acceptor sites are located on internucleosomal chromatin regions (Rennie, 1979; Davies et al., 1980). These studies involved digestion of the chromatin with nuclease after labelling the chromatin with radioactive androgen-receptor complexes, either by incubating the chromatin of non-castrated rat prostates with $|^3H|$dihydrotestosterone *in vitro* or by labelling *in vivo* after castration and subsequent injection with $|^3H|$testosterone. Other studies, after *in vivo* labelling of the chromatin followed by nuclease digestion, suggest that the chromatin acceptor sites are located in the transcriptionally active fraction and consist of non-histone proteins (Hiremath et al., 1981). A different approach to study acceptor sites on the chromatin involves reconstitution experiments with purified androgen-receptor complexes and isolated chromatin or nuclei, followed by digestion with specific nucleases and analysis of the fractionated chromatin by gradient centrifugation. In these reconstitution experiments, incubations have to be performed at temperatures close to 0°C. In addition, purified receptor preparations have to be used. These are important considerations, since proteolysis of receptors may result in decreased amounts of measurable acceptor sites and on the other hand, degradation of the chromatin may expose additional acceptor sites not rou-

tinely expressed in vivo (Spelsberg et al., 1977). By using purified receptor preparations, the amounts of non-specific binding proteins that might mask acceptor sites will also be limited. Interaction of partially purified androgen-receptor complexes with Sertoli cell chromatin of rat testis and seminal vesicle nuclei of guinea pigs has been reported (Tsai et al., 1980; Weinberger & Veneziale, 1980). Care has to be taken with respect to the salt concentration used during incubation. In the presence of 0.15 M KCl, binding of progesterone-receptor complexes was limited to the highest affinity class of acceptors in chick oviduct chromatin (Pikler et al., 1976). Binding of |^{3}H|DHT-receptor complexes to nuclei in the presence of 0.15 M KCl has been observed to be saturable and limited to a single class of acceptor sites with high affinity and limited amounts (Weinberger & Veneziale, 1980). Because no reports were available on reconstitution experiments of isolated androgen-receptor complexes and chromatin, followed by nuclease digestion and analysis of the labelled chromatin by gradient centrifugation, we have studied the possible interaction between partially purified labelled androgen-receptor complexes of ram seminal vesicles and rat prostates with chromatin of either rat prostate-, ram seminal vesicle or non-target tissue.

5.2 Material and methods

All preparative procedures were performed at 0-4°C.

Purification of nuclei

Procedure A : procedure performed essentially as described by Magliozzi et al. (1971) with modifications.
Ram seminal vesicles stored at -80°C were allowed to thaw on ice, and were subsequently minced with scissors and homogenized with a Waring Blendor in two volumes of 10 mM Tris-HCl (pH: 6.7), containing 0.25 M sucrose and 1.5 mM $CaCl_2$. After filtering the homogenate over two layers of

aseptic gauze, further homogenization was performed manually in a Dounce apparatus with 35 strokes of a loose-fitting pestle. The homogenate was centrifuged for 15 min at 2,000 x g and the crude nuclear pellet was resuspended in a solution containing, 0.44 M sucrose, 0.2 mM $Pb(Ac)_2$ and 0.3% Triton X-100 (pH: 6.5-7.0) and rehomogenized with 20 strokes in a Dounce apparatus. Following a further cycle of centrifugation, homogenization and centrifugation in a solution containing, 0.44 M sucrose, 1 mM $MgCl_2$ and 0.3% Triton X-100 (pH: 6.5-7.0), the nuclear pellet was resuspended in 10 mM Tris-HCl buffer (pH: 6.7), containing 0.88 M sucrose and 1.5 mM $CaCl_2$ and layered on top of a discontinuous sucrose gradient, containing 2.2 and 1.8 M sucrose respectively, in 10 mM Tris-HCl with 0.5 mM $CaCl_2$ (pH: 6.7). After centrifugation for 90 min at 95,000 x g_{av} in a Beckman SW-27 rotor, the resulting nuclear pellet was washed with 10 mM Tris-HCl buffer (pH: 7.0), containing 0.25 M sucrose and 1 mM $MgCl_2$. After centrifugation for 15 min at 2,000 x g the purified nuclei were stored at -20°C until use.

Procedure B : procedure performed essentially as described by De Pomerai et al. (1974) with some modifications. Rat prostate or spleen and ram seminal vesicle or spleen tissue (stored at -80°C) was homogenized in two volumes of 10 mM Tris-HCl buffer (pH: 7.0), containing 0.25 M sucrose and 1 mM $CaCl_2$, either with a Waring blendor for 2 x 30 s, or with an Ultraturrax tissue homogenizer for 3 x 10 s, with intermediate cooling. The homogenate was filtered over two layers aseptic gauze and centrifugation was performed for 10 min at 2,000 x g. The crude nuclear pellets were washed twice with 10 mM Tris-HCl buffer (pH: 7.0), containing 0.25 M sucrose and 1 mM $MgCl_2$ and 0.2% Triton X-100, followed by filtration over 63 µ gauze. The pelletted nuclei were subsequently resuspended in 10 mM Tris-HCl buffer (pH: 7.0), containing 0.7 M sucrose, 1 mM $MgCl_2$ and 0.2% Triton X-100 and were layered on a cushion of the same buffer with 2.2 M sucrose. Centrifugation was performed for 90 min at 95,000 x g_{av} in a Beckman SW-27 rotor, and the

purified nuclei were washed with 10 mM Tris-HCl buffer (pH: 7.0), containing 0.25 M sucrose, 1 mM $MgCl_2$. The pellet obtained after centrifugation for 10 min at 2,000 x g was either stored at -20 °C or used directly.

Isolation of chromatin

Purified nuclei were washed with 10 mM Tris-HCl buffer (pH: 7.0) and after centrifugation for 10 min at 2000 x g the nuclear pellet was resuspended in 1 mM Tris-HCl; pH: 6.0. Following incubation for 1 h at 0 °C, the suspension was briefly sonicated for 3 x 3 s, with an Ultrasonic disintegrator (MSE, 150 watt, setting low 3), to ensure disruption of the swollen nuclei without gross damage to the chomatin structure. After centrifugation for 10 min at 2,000 x g the supernatant contained the chromatin.

Incubation of chromatin with micrococcal nuclease

Chromatin (1.5 mg/ml DNA as measured by light adsorption at A_{260} with the assumption that 22 A_{260} units correspond with 1 mg DNA) was incubated with micrococcal nuclease (Sigma) at 0-4 and 20 °C. The reaction mixture contained 5 mM Tris-HCl (pH: 8.0 at 20 °C), 0.1 mM $CaCl_2$ and 0.15 units micrococcal nuclease/A_{260} unit. The reaction was stopped by the addition of EDTA (5mM final concentration).

Isolation of chromatin fragments

Large chromatin fragments, after digestion with micrococcal nuclease, were removed by centrifugation for 5 min at 15,000 x g. The supernatant (small soluble chromatin fragments) was centrifuged at 2 °C for 16 h at 160,000 x g_{av} in a Beckman SW-27 rotor through a 5-25% linear sucrose gradient, containing 10 mM Tris-HCl (pH: 7.0) and 2 mM EDTA. Fractions of 7 drops were obtained by piercing the bottom of the tube and the absorbance at 260 nm was monitored.

Partial purification of androgen-receptor complexes

Androgen-receptor complexes, partially purified with 2',5'-ADP-sepharose and ammonium sulphate precipitation, were used. Purification procedures were used as described in chapter 4.4.1.

Interaction of androgen-receptor complexes with chromatin

Interaction of androgen-receptor complexes with chromatin was performed in buffer A (10 mM Tris-HCl, 1.5 mM EDTA, 1.5 mM dithiothreitol and 10% glycerol; pH: 7.4) with 0.15 NaCl. Experimental procedures are described in the legends to the figures.

Protamine sulphate precipitation

For estimation of the amount of androgen-receptor complexes, protamine sulphate precipitation as described by Mulder et al. (1981), was used. This method involves incubation of receptor preparations with 10 mM pyridoxal phosphate for 10 min at 0 °C prior to the assay.

Streptomycin sulphate precipitation

Streptomycin sulphate precipitation of complexes between chromatin and the steroid-receptor complex was performed essentially as described by Webster et al. (1976). After incubation for 30 min at 0 °C with streptomycin sulphate centrifugation was performed for 5 min at 5,000 x g followed by washing of the precipitates five times with 1 ml 0.02% streptomycin sulphate.

5.3 Results

In studies with rat prostate chromatin after digestion with micrococcal nuclease to the extent of 5% acid solubility of the DNA, Rennie (1979) could detect only a

trace amount of labelled receptors not bound to chromatin. In that study, when nuclease digestion was continued until 15% of the DNA was acid-soluble, the amount of receptor released from the chromatin was increased. This implies that retention of androgen-receptor complexes by chromatin is sensitive to micrococcal nuclease digestion (Rennie, 1979). Therefore, in studies on the interaction of androgen-receptor complexes with the chromatin, mild digestion with micrococcal nuclease, not extending 5% acid-solubility of the DNA (very small DNA fragments are soluble in 0.7 M perchloric acid at 10°C), is a prerequisite. Samples of chromatin from ram seminal vesicle, digested to approximately 3-5% acid-solubility of the DNA (micrococcal nuclease digestion for 1 and 3 min at 20°C and 10, 20 and 30 min at 0-4°C) were analysed with sucrose gradient centrifugation. Only the fractions containing small DNA fragments (mainly consis-

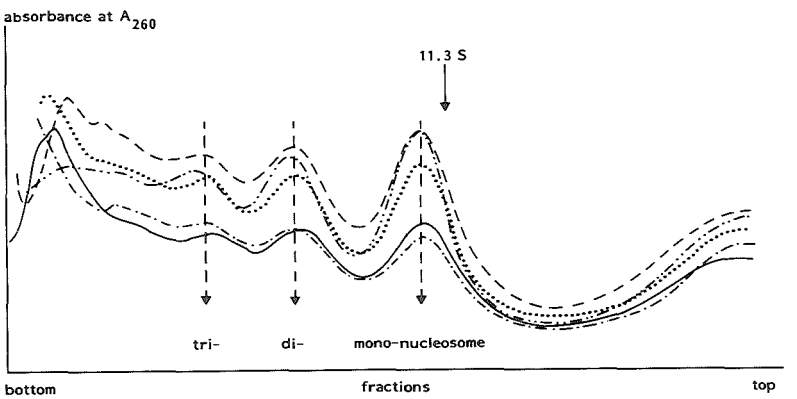

Figure 5.1: Sucrose gradient centrifugation of ram seminal vesicle chromatin after digestion with micrococcal nuclease.

After digestion with micrococcal nuclease (0.15 units/A_{260} unit) and centrifugation for 5 min at 15,000 x g, 500 µl samples were layered on 5-25% linear sucrose gradients and centrifugation was performed for 16 h at 160,000 x g_{av} in a Beckman SW-40 rotor. Arrow indicate position of sedimentation marker catalase (11.3 S). Digestion with micrococcal nuclease for, 1 min at 20°C: ——— ; 3 min at 20°C: ----- ; 10 min at 0-4°C: -.-.- ; 20 min at 0-4°C: ····· ; 30 min at 0-4°C: -··-··

ting of fragments smaller than tetra-nucleosomes representing approximately 10-40% of the total DNA), were analysed and the resulting profiles are shown in figure 5.1. Absorbence at the top of the gradient represents the very small DNA fragments which are soluble in perchoric acid. Comparable results have been obtained with rat prostate chromatin after micrococcal nuclease digestion (D. Breedveld, unpublished observations).

In reconstitution experiments with isolated steroid-receptor complexes and chromatin, possibly containing endogenous nucleases, incubations have to be performed at temperatures as close as possible to $0^{o}C$ to maintain the intactness of the chromatin structure. In addition, androgen-receptor complexes are more stable at low temperatures. The conditions generally used for the various steroid-receptor systems in reconstitution experiments are 1-2 h at $0^{o}C$ (Mainwaring & Peterken, 1971; Klyzsejko-Stefanowicz et al., 1976; Socher et al., 1976; Wang, 1978). The stability of $|^{3}H|$R1881-receptor complexes for 2 h at $0-4^{o}C$ was studied and no significant dissociation or denaturation of

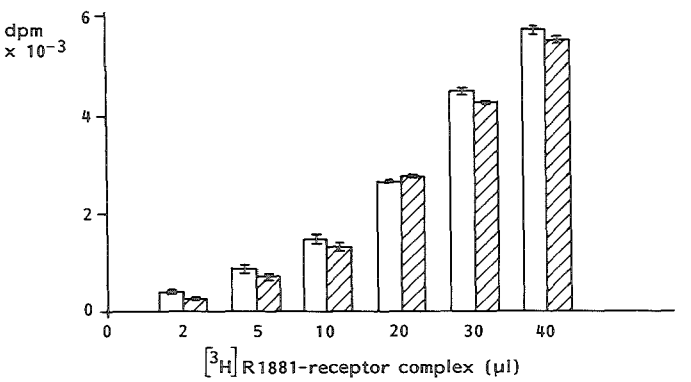

Figure 5.2: The effects of time and dilution on the stability of androgen-receptor complexes from ram seminal vesicles.

$|^{3}H|$R1881-receptor complexes (0.97 fmol/µl) partially purified with 2',5'-ADP-sepharose chromatography and ammonium sulphate precipitation (50% saturation), followed by desalting with Bio-Gel P-6DG, were used. $|^{3}H|$R1881-receptor complexes were estimated with protamine sulphate precipitation before (open bars) and after incubation for 2 h at $0^{o}C$ (shaded bars) in a final volume of 1 ml of buffer A. Values are means ± range, n=3.

the [^{3}H]R1881-receptor complex was observed (figure 5.2). To investigate whether in reconstitution experiments androgen-receptor complexes show any interaction with isolated chromatin (or fragments), a quantitative assay is required to separate free and chromatin-bound androgen-receptor complexes. One procedure involved precipitation of the chromatin fragments together with bound steroid-receptor complexes by streptomycin sulphate (Webster et al., 1976). In studies with chick oviduct chromatin, no significant coprecipitation of progesterone-receptor complexes, not bound to chromatin, has been observed (Webster et al., 1976). Experiments with increasing amounts of partially purified [^{3}H]R1881-receptor complexes from ram seminal vesicles, however, show that even at low concentrations of streptomycin sulphate (0.1 mg/ml as compared with 1.0 mg/ml used by Webster et al., 1976), a significant amount of [^{3}H]R1881-receptor complexes is precipitated (figure 5.3).

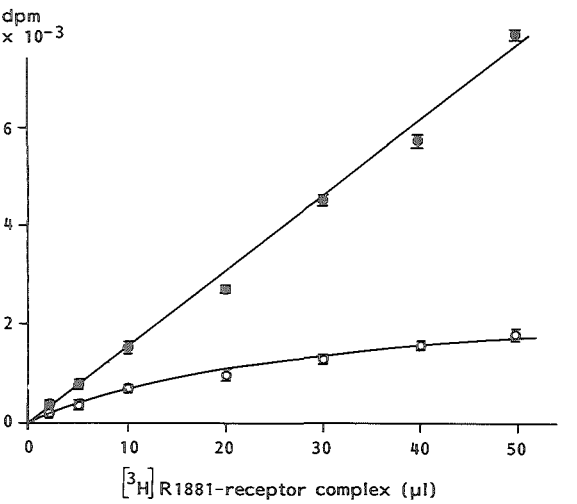

Figure 5.3: Protamine sulphate precipitation and streptomycin sulphate precipitation of androgen receptors from ram seminal vesicles.
[^{3}H]R1881-receptor complexes, prepared as described in the legend to figure 5.2, were used.
●——●, protamine sulphate precipitation; ○——○, streptomycin sulphate precipitation. Values are means ± range, n=3.

At low concentrations of receptor, almost 50% of the receptor precipitated with streptomycin sulphate in the absence of chromatin (figure 5.3). Due to the significant coprecipitation of androgen-receptor complexes with streptomycin sulphate, another procedure to separate free and chromatin-bound steroid-receptor complexes has to be used. It was found to be possible to separate free and chromatin-bound receptors by centrifugation at high speed. The assay was evaluated with partially purified $|^3H|$DHT-receptor complexes and chromatin isolated from prostates of rats one day

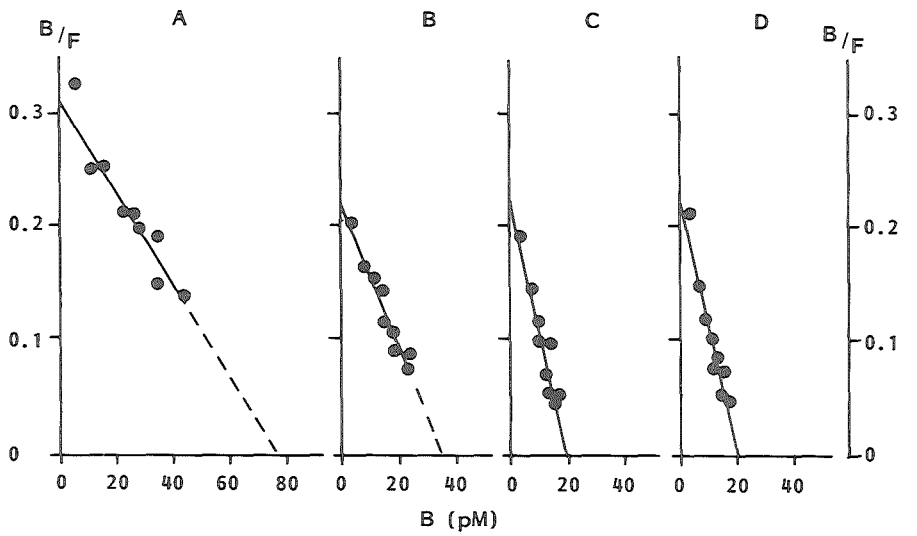

Figure 5.4: Scatchard analysis of the interaction between rat prostate androgen-receptor complexes and chromatin. Increasing amounts of $|^3H|$DHT-receptor complexes (65-720 fmol, partially purified as described in the legend to figure 5.2, were incubated for 90 min at 0-4°C with a fixed amount of chromatin (2.2 A_{260} units/incubation) in 1 ml buffer A with 0.15 M NaCl. After incubation the amount of total receptors were estimated with protamine sulphate precipitation. The amount of $|^3H|$DHT-receptor complexes bound to chromatin was estimated by centrifugation for 5 min at 40,000 x g and counting of the pellet after one (A), two (B), three (C) or four (D) washing steps with 1 ml buffer A followed by centrifugation for 5 min at 40,000 x g.

C: B_m : 19 pM; K_d : 0.88 x 10^{-10}M.
D: B_m : 20 pM; K_d : 0.86 x 10^{-10}M.

after castration. Although it seems that there is a single class of acceptor sites after one and two washing steps (figure 5.4 A/B), this probably reflects experimental artifacts due to non-specific binding of free $|^3H|$DHT-receptor complexes to chromatin or to the wall of the assay tube. Due to the limited range of $|^3H|$DHT-receptor complexes, used these low affinity binding sites, however, could not be demonstrated in the figures 5.4 A/B. After the third and fourth washing step, the non-specifically bound $|^3H|$DHT receptor complexes are released because the dissociation constant (K_d: 0.88 and 0.86 x 10^{-10} M, respectively) and the amount of acceptor sites (0.19 and 0.20 pmol/mg DNA, respec-

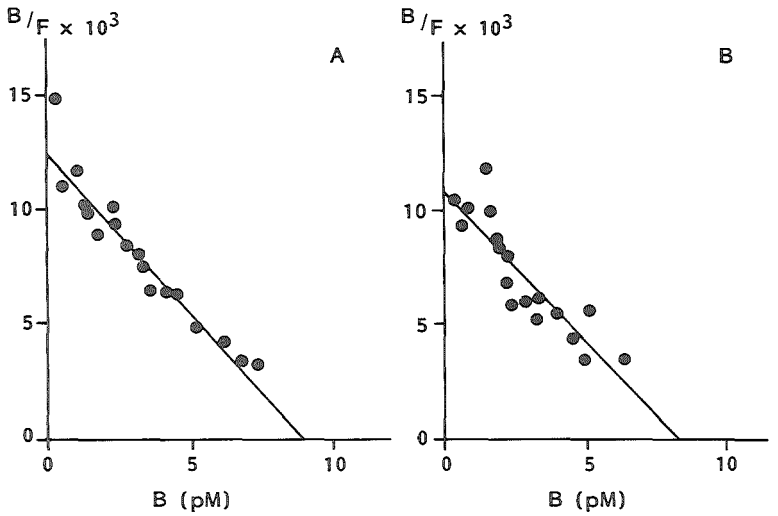

Figure 5.5: Scatchard analysis of the interaction between androgen-receptor complexes from ram seminal vesicles and chromatin of prostate (A) and spleen (B) of castrated rats.
Increasing amounts of $|^3H|$DHT-receptor complexes (15-810 fmol) were incubated for 90 min at 0-4°C with a fixed amount of chromatin (1.6 A_{260} units/incubation) in 1 ml buffer A with 0.15 M NaCl. The amount of total and chromatin-bound the androgen-receptor complexes were estimated after four washing steps as described in the legend to figure 5.4.

A: B_m : 9.1 pM; K_d : 0.73 x 10^{-9}M.
B: B_m : 8.3 pM; K_d : 0.77 x 10^{-9}M.

tively) remained apparently similar (figure 5.4 C/D). The estimated dissociation constant appears to be essentially the same as has been found for the interaction between androgen-receptor complexes and chromatin from guinea pig seminal visicles (K_d: 0.83 x 10^{-10} M; Weinberger & Veneziale, 1980). In heterologous reconstitution experiments with partially purified androgen-receptor complexes from ram seminal vesicles and chromatin from prostate and spleen of rats one day after castration (figure 5.5 A/B), dissociation constants were found to be a factor 10 higher (K_d: 0.73 x 10^{-9} M and K_d: 0,77 x 10^{-9} M, respectively), than observed for the homologous reconstitution experiments as described in figure 5.4. The estimated amounts of chromatin acceptor sites were of the same order of magnitude (0.13 and 0.12 pmol/mg DNA for prostate and spleen respectively, as compared to 0.20 pmol/mg DNA in the experiments described in figure 5.4.).

5.4 Discussion and conclusion

For the assay of androgen-receptor complexes bound to chromatin, high speed centrifugation appeared to be superior over streptomycin sulphate precipitation.

In preliminary reconstitution experiments is was demonstrated that isolated androgen-receptor complexes show high affinity interaction with isolated chromatin. In homologous reconstitution experiments with receptor and chromatin from rat prostate, the estimated dissociation constant appeared to be a factor 10 lower than in heterologous reconstitution experiments with androgen-receptor complexes isolated from ram seminal vesicles and chromatin of prostate and spleen of castrated rats. No tissue specifity was observed. These results may reflect binding to regions of the DNA, which become exposed as a result of some disruption of the chromatin by the mild sonication procedure, necessary for lysis of the nuclei. The limited amounts (1,000-1,500 sites/nucleus) of high affinity sites (K_d: 0.9 x 10^{-10} M), however, which remain constant after three washing steps may

be indications of a specific process (figure 5.4). In addition, the observed saturable binding of the progesterone receptor to DNA containing limited nicks which becomes non-saturable as nicks were increased (Hughes et al., 1981), are in favor with the assumption that there was no gross damage of the chromatin, as a result of the isolation procedure or the presence of nucleases, in our experiments. It remains to be established, however, whether the interaction observed in heterologous reconstitution experiments (figure 5.5 A/B) really reflects binding of the androgen-receptor complexes to chromatin acceptor sites. In this respect control experiments involving interaction of isolated androgen-receptor complexes with purified nuclei, probably containing intact chromatin, have to be performed.

Homologous reconstitution experiments with ram seminal vesicle receptor and chromatin were not successful sofar, due to the lack of availability of chromatin with unoccupied acceptor sites. Therefore, ram seminal vesicles have to be isolated from castrated rams to investigate whether dissociation constants and binding capacities are comparable to the rat prostate system. In such experiments it should also be possible to evaluate the chromatin interaction of androgen-receptor complexes with chromatin in heterologous reconstitution experiments, which apparently show no tissue-specificity.

In conclusion, the preliminary experiments described appear promising, because the results have shown that the purified androgen-receptor complexes still possess their chromatin-binding sites. In future reconstitution experiments the location of chromatin-acceptor sites, when shown to be specific, should be studied with controlled nuclease digestion and sucrose gradient centrifugation analysis of the released fragments.

5.5 References

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Chapter 6

GENERAL DISCUSSION

6.1 Usefulness of nuclear androgen receptor assay for clinical evaluation in endocrine treatment of prostatic diseases

A possitive correlation exists between estrogen receptor content and the response to endocrine therapy of human breast cancers (Jensen, 1981). It appeared reasonable to suggest that in analogy with breast cancer a correlation could exist between the androgen receptor content and the benefit form endocrine management of human prostatic diseases. In 75-80% of all prostatic carcinomas the growth of the tumor can be controlled by hormonal manipulations (Fergusson, 1972). It is possible that the prostate tissue of the remaining 20-25% of the patients not responding to endocrine therapy have low androgen receptor levels or no receptors et all. For this reason a reliable androgen receptor assay has to be available for clinical purposes. With a reliable assay it might be possible to select patients which could benefit from the appropiate therapy. As has been discussed in chapter 3.1.4, controversial results have been reported concerning the correlation between androgen receptor content and the response to endocrine treatment (Wagner & Schultz, 1978; Ekman et al., 1979). These studies, however, involved the measurement of cytoplasmic receptors and, as has been discussed in chapter 3.1.2, the accurate estimation of androgen receptors in cytosol fractions of human prostatic tissue is associated with many problems. The predominantly nuclear localization of androgen receptors in these tissues (Sirett & Grant, 1978; Hicks & Walsh, 1979) and the fact that androgens exert their major influence within the nucleus of target

cells (chapter 2), suggest that the measurement of nuclear receptors may be more valuable. The applicability of the assay developed (appendix paper I) for nuclear androgen receptors for biopsy-size specimens has been investigated (Blankenstein et al., 1982). With this method, it was found that nuclear androgen receptors can be measured in as little as 25 mg of tissue. At present no reports have appeared in the literature dealing with nuclear androgen receptor levels and the response to endocrine therapy. For this reason it remains to be established whether nuclear androgen receptor levels will show useful correlations with the clinical response to endocrine management. The methodology for this study is now available.

6.2 Ram seminal vesicles as a source for large scale receptor purification

For further study of the mechanism of action of androgens, purification of androgen receptors is of extreme importance (chapter 4.4.1). However, several serious problems are associated with the purification of androgen receptor proteins, e.g. the extreme lability of androgen-receptor complexes and the small amount of androgen receptors present in androgen target tissues. In addition, these tissues contain high amounts of proteolytic enzymes which resist commonly used inhibitors, such as PMSF (Mainwaring, 1978). Another difficulty involves the requirement of radioactive androgen-receptor complexes which is necessary to follow the receptor proteins during the purification procedures. Purification methods should therefore, also considering the extreme lability of androgen receptors, be rapid to prevent dissociation and denaturation of the androgen-receptor complexes. Despite these drawbacks, which have been discussed in more detail in chapter 4.1.1., we have concentrated our efforts on the purification of these androgen receptors, with the goal to provide more infor-

mation on the mechanism of action of androgens. Future prospects of androgen-receptor research with purified androgen-receptor complexes will be discussed in chapter 6.4. The small size of rat prostates makes this tissue unattractive for isolation of androgen receptors at a large scale. It has been demonstrated, however, that seminal vesicles of rams contain both nuclear and cytoplasmic androgen receptors (chapter 4.3 and appendix paper II). These androgen receptors showed characteristics comparable to the androgen receptors of rat prostate. The amount of receptors present (approximately 1 pmol per gram of tissue) and the availability of the tissue, which can be easily obtained from the slaughterhouse, make ram seminal vesicle tissue very attractive as a source for large scale isolation of androgen receptors. For stabilization of the labile androgen receptor proteins, molybdate, which has also been shown to stabilize androgen receptors from rat prostate (Gaubert et al., 1980), had to be included in our experiments (chapter 4.3 and appendix paper II). Purification of these androgen receptors with affinity chromatography is essentially useless, because this method which involves matrices containing immobilized steroids, requires that the receptor has to be presented to the matrix in its onuccupied form. In ram seminal vesicles, obtained from non-castrated animals, however, most receptors are coupled with endogeneous androgens. Moreover, notwithstanding the successes obtained with affinity chromatography in the purification of several steroid receptors, this method has not been successful for the isolation of androgen receptors from castrated rats (discussed in chapter 4.1.2). In addition, in recent studies, affinity chromatography is not used for large scale purification of progesterone and glucocorticoid receptors (Payvar et al., 1981; Weigel et al., 1981). We have investigated several procedures for protein fractionation which involve mainly affinity for the DNA-site of the receptor (chapter 4.4.2). In these studies, advantage was taken by the fact that steroid receptors possess a common feature, i.e. binding to DNA-like matrices when the receptors are in their activated form (chapter 2.2.3 and 4.3). However, molybdate, which appeared to be

essential for stabilization of receptors, also inhibits the transformation of androgen-receptor complexes to their activated form. This problem has been solved by carefully selecting conditions that sufficiently stabilize the receptor followed by procedures which provoke activation of the androgen-receptor complexes (induced by high salt concentrations, figure 4.7). From the gels tested, 2',5'-ADP-sepharose appeared to be most suitable for initial purification of androgen-receptor complexes (chapter 4.4.1 and 4.4.2). Further purification procedures have been investigated and it has been established that an almost two thousand fold purified receptor preparation could be obtained with an overall recovery of approximately 30%. These procedures involved 2',5'-ADP-sepharose chromatography, ammonium sulphate precipitation and gel chromatography on Ultrogel ACA-44. Further details of these procedures have been described in chapter 4.4.2 and appendix paper III. The purified cytoplasmic receptor, which still possesses its DNA binding site, showed characteristics comparable to the androgen receptor isolated from the cytoplasm of rat prostates, as shown by analysis with agar gel electrophoresis and sucrose gradient centrifugation (appendix paper III). Further purification of this partially purified receptor might be accomplished by the procedure as described very recently by Bruchovsky and associates. This method, which has been found to be successful for a 100 fold purification of the nuclear androgen-receptor complex from rat prostates, involves hydrophobic interaction chromatography with ω-alkyl derivatives coupled to agarose (Bruchovsky et al., 1981). In addition, as a first purification step, the procedure described for the progesterone receptor by Weigel et al., (1981) might also be very worthwile. This procedure involved chromatography of the untreated cytosol on a DNA-cellulose column, resulting in a "flow-through" fraction containing proteins, including the non-activated receptor, which do not interact with DNA-cellulose, whereas the DNA-binding proteins were selectively retained by the column. Subsequently the non-activated receptors in the "flow-through" fraction

were chromatographed on a phosphocellulose column after an activation process of the receptor.

6.3. Interaction of purified receptor preparations with isolated chromatin

Experiments described in chapter 5.3 were performed to gain information about nuclear acceptor sites for androgen receptors on the chromatin. The reconstitution experiments with partially purified androgen-receptor complexes and isolated chromatin are still preliminary. The techniques described in chapter 5.3, however, for micrococcal nuclease digestion of the chromatin and high affinity binding of isolated androgen-receptor complexes with chromatin make it now possible to start experiments on tissue specificity via homologous and heterologous reconstitution experiments. After binding of androgen-receptor complexes to the chromatin a variety of experiments can be performed using different nucleases for specific digestion of the chromatin. Following digestion and analysis of chromatin fragments by sucrose gradient centrifugation before and after selective removal of various chromatin components, information may be obtained about the nature and localization of chromatin acceptor sites. With highly purified receptor preparations, experimental artifacts will probably be minimized (chapters 5.1. and 5.4). For these reconstruction experiments, therefore, the use of highly purified receptor preparations, prepared as described in chapter 4.4.2 and appendix paper III, not containing nucleases*), is required.

*) Recently Dr. M. Parker (Imp. Cancer Res. Fund. London), using a very sensitive assay, showed that these highly purified preparations are free of nucleases. The assay involved incubation of the receptor preparations with circular DNA, followed by analysis on gels.

6.4 Future prospects of androgen-receptor research

During the past fifteen years, since radioactively labelled androgens became available as marker for the receptor protein to which it binds, it has been possible to study androgen receptor interactions. The overall pattern of androgen receptor interactions is reasonably clear, but detailed understanding of the processes of receptor-synthesis, -activation, -translocation, -binding to the nucleus and -replenishment is still far from complete. Procedures for characterization and purification of the extremely labile androgen-receptor complex are rather time consuming. Hence, to prevent dissociation of the androgen-receptor complex, it would be desirable to possess a receptor preparation with a labelled steroid covalently linked to the steroid binding site of the receptor. Covalent coupling has been successfully performed with the subunits A and B of the progesterone receptor by photoaffinity labelling in vitro with the synthetic progesterone R5020 (Schrader et al., 1980). The same method tested for coupling of the synthetic androgen R1881 to the androgen receptor has not been successful thus far (Mulder & Vrij, 1981; Rennie, 1981). Recently, however, Mainwaring & Johnson (1980) succeeded in covalent coupling of a steroid to the androgen receptor by affinity labelling with $|^{14}C|$-17α-bromoacetoxy - testosterone. With this affinity labelled receptor from rat prostate and based on the procedure as described for the purification of the estradiol receptor (Molinari et al., 1977), Mainwaring & Johnson (1980) described priliminary results of a 50,000 fold purification of the androgen receptor. It has to be established, however, whether this purified receptor associated with the affinity label can be translocated into nuclei in a reconstituted cell-free system. Until it has been achieved that purified androgen receptor covalently coupled with a radioactive steroid, which also translocate into nuclei in reconstituted cell-free system, are available, reconstitution experiments have to be performed with labile androgen-receptor complexes. Reconstitution experiments might involve the approach which has been discussed

in chapter 6.3, with the ultimate goal to gain more information about the nature and localization of the chromatin acceptor sites. Another approach for studying chromatin acceptor sites might be to try to exchange the steroid of the chromatin bound androgen-receptor complexes for added radioactive androgens, after isolation of nuclei or chromatin from tissue of non-castrated animals. The main disadvantage of this procedure will be the time required for exchanging the steroid. Long incubation times (20 h at 10°C) in vitro, may cause changes of the native conformation of the chromatin which might result in experimental artifacts.

It has been concluded that steroid hormone-receptor complexes increase rapidly and selectively the rate at which specific transcripts are synthesized. In cultured host cells, bearing integrated mouse mammary tumor virus genes (MMTV-genes) introduced by infection, glucocorticoid hormones specifically stimulate the rate of viral gene transcription in a hormonal dependent manner (Ringold et al., 1977; Grove et al., 1980; Lee et al., 1981). Possible specific interaction of steroid-receptor complexes with naked DNA has been dicussed (chapter 2.2.5). In addition, it has been reported very recently that purified glucocorticoid receptors bind selectively in vitro to a cloned DNA fragment (MMTV-DNA) whose transcription is regulated by glucocorticoids in vivo (Payvar et al., 1981). Possible specific interaction of purified androgen-receptor complexes with cloned DNA fragments might give information about the function of androgen receptors in gene regulation. DNA, complementary to three androgen-dependent mRNAs from rat ventral prostate (cDNAs) has been cloned in the bacterial plasmid pAT153 (Parker et al., 1980). These androgen-dependent mRNAs code for the expression of three polypeptides (subunits of prostatic binding protein (PBP)). These polypeptides, representing about 50% of the total protein synthesized in the rat ventral prostate (Heyns et al., 1977; Parker et al., 1978), are secreted by this tissue. The specific recognition site for androgen-receptor complexes will probably be located upstream the gene which contains the information for prostatic binding protein. With the

cloned cDNAs it might be possible to isolate the original genes which code for PBP, together with its flanking genes. With these probes, binding experiments could be performed with highly purified receptors. A specific binding site might be isolated by selective digestion of the DNA by specific restriction enzymes. In addition, studies on gene regulation by androgens could be performed with an androgen-dependent cell-line in an approach similar as has been described for the glucocorticoid receptor system (Coffini, 1981). Shionogi mammary carcinoma cells, e.g., which are subdivided into androgen-dependent and autonomous cells (King et al., 1976; Bruchovsky & Rennie, 1978) and which originally not synthesize PBP, might be used. After inserting genes coding for PBP, connected with its flanking genes, in the genome of the Shionogi cells, possible expression of these genes on androgen administration might be observed. By specific alteration of the flanking genes before inserting them in the host genome, valuable information on regulation of gene expression by androgens may become available.

Further investigation of the mechanism of action of androgens at the molecular level requires methods to recognize the receptor protein that do not depend on its binding to labelled androgens. One possible approach is the use of specific antibodies against the receptor itself. Antibodies have been raised against the estradiol receptor of calf uterine nuclei (Greene et al., 1980[a]; Jensen et al., 1980), of human breast cancer cytosol (Greene et al., 1980[b]), against the glucocorticoid receptor of rat liver cytosol (Govindan, 1979; Eisen, 1980) and against the progesterone receptor from rabbit uterine cytosol (Logeat et al., 1981). If such antibodies would also be available for the androgen receptor, this would permit the application of immunochemical techniques for the detection of receptors, and such antibodies would therefore offer great promises as reagents for the elucidation of the mechanism of action of androgens at the molecular level. Another important poten-

tial application of antibodies against the receptor might be immunoradiographical analysis of androgen receptors in prostatic cancer tissue as a guide to therapy. To raise antibodies via the monoclonal antibody technique, purified receptor preparations, prepared as described in appendix paper III, may be used.

In conclusion, fruitful further studies in androgen-receptor research involve the complete purification of androgen receptors, covalent binding of steroid to the receptor, monoclonal antibodies to the receptor and in the end a model system in which the regulation of androgen-dependent genes by androgen receptors can be studied in detail.

6.5 References

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SUMMARY

Interaction of a steroid hormone with specific receptor proteins with high affinity, is one of the first events occuring in target organs of that specific hormone. After various as yet ill-understood processes in the cytoplasm, the steroid-receptor complex migrates to the nucleus, which ultimately results in the observed effects on the target cell.

It has been described that human breast tumors can be treated with about 50% more success with endocrine therapy if the tissue contains the specific receptor protein for the female steroid hormone estradiol. Steroid-receptor complexes exert their action in the nucleus and the high endogeneous levels of androgens in human prostatic tissue result in a predominantly nuclear localization of receptors. Hence, it would appear logical to measure androgen receptors in nuclei of human prostatic tissues as a possible guide to therapy. The experiments described in the first part of this thesis were performed with the goal to develop a reliable assay for nuclear receptors for androgens in the human prostate. In further experiments purification procedures for androgen receptors were studied with the ultimate goal to achieve more detailed information on the mechanism of action of androgens. Particularly to investigate nature and subnuclear localization of the acceptor site(s) on the chromatin ("Scope of this thesis", chapter 1).

The literature, dealing with the known and unknown biochemical processes which can play a role in the effects of steroid hormones, has been discussed in chapter 2.

Chapter 3.1 describes the various assays which have been developed to estimate the amount of androgen receptors in human prostatic tissue together with the results reported in the literature concerning both cytoplasmic and nuclear receptors. Especially drawbacks in estimating cytoplasmic

androgen receptors in human prostatic tissues and the prognostic value in estimation of androgen receptors in endocrine treatment of prostatic diseases have been discussed. On account of the many methodological difficulties in measuring cytoplasmic receptors in human prostatic tissues and because most receptors are expected in the nucleus, an assay for nuclear receptors in benign prostatic hyperplastic tissue was developed. Results are described in appendix paper I. In the method described, the commonly used extraction of nuclei is modified. Instead of extracting with a buffer containing 0.4 M KCl, extraction is performed with a buffer with heparin. As a result, the amount of receptors found was approximately two times higher than the values found after KCl extraction. Additionally, heparin stabilizes the steroid-receptor complex.

To gain more insight in the mechanism of action of steroid hormones it is of importance to have a purified receptor preparation. In chapter 4.1 the purification of steroid receptors in general is discussed on basis of results from the literature. Due to very low amounts of androgen receptors in their target cells (approximately 10 µg in thousand rat prostates) it is not practical to isolate the receptors from rat prostate tissue at a large scale. It was investigated whether seminal vesicles of the ram, which can be obtained from the slaughterhouse in large amounts, would be a suitable source for large scale purification of androgen receptors. In chapters 4.2-4.4 and appendix papers II and III experiments are described concerning characterization and purification of androgen receptors from ram seminal vesicles. It appears that this tissue contained almost the same concentration of androgen receptors as rat prostate tissue. It was concluded that ram seminal vesicles tissue is a very suitable source for isolation of androgen receptors. With sucrose gradient centrifugation two forms on the unpurified receptor were characterized, one sedimenting at 9 S and one sedimenting at 3 S. These forms of the receptor have been found also in cytosols of rat prostates. In chapter 4.4. various purification procedures have been

described, showing that 2', 5'-ADP-sepharose chromatography is most suitable as a first purification step. Further purification procedures which resulted in a two thousand fold purified receptor are described in chapter 4.4.1 and appendix paper III.

Interaction of steroid-receptor complexes with chromatin, as described in the literature, is discussed in chapters 2.2.5 and 5.1. Controversial results have been reported with respect to the amounts and localization of acceptor sites in the chromatin. Preliminary experiments with purified androgen-receptor complexes from rat prostates and ram seminal vesicles and isolated chromatin are described in chapters 5.2-5.4. High affinity interaction of androgen-receptor complexes with chromatin was observed.

A "general discussion" is presented in chapter 6. The usefulness of a nuclear androgen receptor assay for human prostatic tissue together with future prospects of androgen-receptor research are described.

SAMENVATTING

De interaktie van steroid-hormonen met specifieke receptor-eiwitten, welke het hormoon met hoge affiniteit binden, is een van de eerste gebeurtenissen die plaats vinden in de zogenaamde doelwitorganen voor dat specifieke steroid-hormoon. Als gevolg hiervan worden diverse complexe processen beïnvloed, hetgeen uiteindelijk resulteert in een toename van de synthese van specifieke eiwitten.

In de literatuur is beschreven, dat endocriene therapie van humane mamma tumoren een twee maal zo grote kans op succes heeft indien het weefsel het specifieke receptor-eiwit bevat voor het vrouwelijke steroid-hormoon oestradiol. Hieruit blijkt dat het van groot belang kan zijn om het receptor-eiwit in dergelijke maligne tumoren te kunnen meten alvorens tot therapie over te gaan. De laatste jaren wordt intensief onderzocht of naar analogie van de mammatumoren een dergelijke korrelatie ook bestaat bij de humane prostaattumoren wat betreft de aanwezigheid van receptoren voor androgenen (mannelijk steroid-hormoon) en de respons op endocriene therapie. Hiervoor is het van essentiëel belang dat er een betrouwbare receptor bepaling beschikbaar is. Volgens het nu algemeen aanvaarde werkingsmechanisme van steroid-hormonen oefent het mannelijk steroid testosteronen zijn werking uit op doelwitweefsels door binding aan een cytoplasmatisch receptor-eiwit, nadat het steroid is omgezet tot dihydrotestosteron. In aansluiting op diverse nog niet volledig begrepen processen in het cytoplasma wordt het steroid-receptor complex in de celkern gebonden aan acceptor plaatsen op de chromatine, hetgeen uiteindelijk leidt tot het waargenomen effekt van het hormoon op de doelwitcel. De werking van het steroid-receptor complex vindt plaats in de kern en het ligt dan ook voor de hand om de hoeveelheid androgeen receptoren in humane prostaatweefsels te meten in de kern, ook al omdat men de meeste receptoren in de kern kan verwachten vanwege het hoge endogene androgeen gehalte.

De in het eerste gedeelte van dit proefschrift beschreven experimenten werden uitgevoerd met het doel om een goede methode te ontwikkelen voor de bepaling van androgeen

receptoren in de kernfraktie van humane prostaatweefsels. In andere experimenten werden methoden voor de zuivering van androgeen-receptoren onderzocht met het uiteindelijke doel om meer gedetailleerde gegevens te verkrijgen over het werkingsmechanisme van androgenen en vooral ook om met gezuiverde receptor preparaten de aard en lokatie van de acceptorplaats(en) op het chromatine te onderzoeken ("Scope of this thesis", hoofstuk 1).

Een literatuuronderzoek, handelend over de tot nu toe bekend geachte processen welke een rol spelen in hormonale effekten vanaf de interaktie met zijn cytoplasmatische receptor tot de uiteindelijke gen-expressie, is weergegeven in hoofdstuk 2.

In hoofdstuk 3.1 is een literatuuroverzicht gegeven van de diverse bepalingsmethoden voor androgeen receptoren in humane prostaatweefsels alsmede van de resultaten die verkregen zijn voor zowel de cytoplasmatische als voor de kern receptoren. Ingegaan wordt op de vele bezwaren die er zijn tegen het meten van deze receptoren in het cytoplasma. De prognotische betekenis van het meten van androgeen receptoren bij de endocriene behandeling van ziekten van de prostaat wordt besproken. Op grond van de methodologische bezwaren tegen het bepalen in humane prostaatweefsels van cytoplasmatische androgeen-receptoren en vanwege de verwachting dat de meeste receptoren in de kern gelokaliseerd zijn, werd een methode ontwikkeld voor de bepaling van receptoren in kernen van benigne hyperplastisch prostaatweefsel. De resultaten zijn beschreven in appendix - publikatie I. In de methode die wordt beschreven is onder andere de tot nu toe gebruikte extraktie van kernen gemodificeerd; in plaats van met 0.4 M KCl wordt geëxtraheerd met een buffer welke heparine bevat. Dit heeft als resultaat dat de gemeten receptor concentraties ongeveer twee maal hoger zijn dan na de extractie met KCl. Bovendien werkt het heparine stabiliserend op het steroid-receptor complex.

Om een beter inzicht te verkrijgen in het werkingsmechanisme van steroid-hormonen is het van belang om over

een gezuiverd receptor preparaat te beschikken. In hoofdstuk 4.1 is een literatuuroverzicht gegeven van de tot op heden beschreven resultaten welke behaald zijn bij de zuivering van steroid receptoren in het algemeen. Aangezien androgeen receptoren in zeer geringe hoeveelheden voorkomen in hun doelwitorganen (ca. 10 microgram in 1000 ratte-prostaten), is het niet goed mogelijk deze receptoren op grote schaal te isoleren uit ratteprostaat. Onderzocht werd of zaadblazen van de ram, welke in grote hoeveelheden via het slachthuis verkrijgbaar zijn, een geschikt weefsel vormen voor zuivering van deze receptoren op grote schaal. In de hoofdstukken 4.2-4.4 en appendix-publikaties II en III zijn experimenten beschreven over de karakterisering en gedeeltelijke zuivering van de androgeen receptor van de zaadblaas van de ram. Het bleek dat de concentraties van de androgeen-receptoren in dit weefsel ongeveer even hoog waren als in de ratteprostaat en er is gekonkludeerd dat dit weefsel een zeer geschikte bron is voor isolatie van de receptor. Op sucrose gradiënten worden in het cytoplasma van de zaadblaas twee vormen van de ongezuiverde androgeen receptor gevonden, n.l. één die sedimenteert als een 9 S- en één als een 3 S-verbinding. Deze vormen van de cytoplasmatische receptor worden ook gevonden in het cytoplasma van de prostaat van de rat. In hoofdstuk 4.4 zijn diverse zuiveringsmethoden beschreven waaruit blijkt dat het gebruik van 2',5'-ADP-sepharose vooralsnog het meest geschikt lijkt te zijn als eerste stap bij de zuivering van de androgeen receptor. De daaropvolgende zuiveringsstappen om te komen tot een tweeduizendmaal gezuiverd preparaat zijn beschreven in hoofdstuk 4.4.1 en appendix publikatie III.

In de hoofdstukken 2.2.5 en 5.1 is een literatuuroverzicht gegeven van studies welke handelen over de interaktie van steroid-hormoon-receptor complexen met chromatine. De verschenen publikaties zijn zeer tegenstrijdig wat betreft het aantal en de lokatie van de acceptor plaatsen op het chromatine. Om meer duidelijkheid te verschaffen over de werkelijke hoeveelheden en lokatie van de chromatine bindingsplaatsen zijn experimenten uitgevoerd met gedeeltelijk

gezuiverde androgeen-receptor complexen van ratteprostaat en zaadblazen van de ram met hun respektievelijke geïsoleerde chromatines (hoofdstukken 5.2-5.4). Er werden interakties met hoge affiniteit waargenomen tussen de androgeen-receptor complexen en het chromatine (voorlopige experimenten).

In de "General discussion" (hoofdstuk 6) zijn de verkregen resultaten besproken aan de hand van gegevens welke in de literatuur vermeld zijn. De toepassing van de ontwikkelde methode voor de bepaling van kern receptoren bij de endocriene behandeling van ziekten van de prostaat kan van belang zijn en wordt besproken in hoofdstuk 6.1. De resultaten, verkregen bij de zuivering en karakterisering van de androgeen-receptor uit de zaadblaas van de ram en de studies die uitgevoerd zijn met deze gedeeltelijk gezuiverde receptor en geïsoleerde chromatine, worden besproken in het licht van de mogelijkheden voor verder onderzoek.

NON STANDARD ABBREVIATIONS AND TRIVIAL NAMES

BPH	- benign prostatic hyperplasia
dihydrotestosterone	- 17β-hydroxy-5α-androstane-3-one (DHT)
estradiol	- 1,3,5-(10)-estratriene-3,17β-diol
methyltrienolone	- 17β-hydroxy-17α-methyl-4,9,11-estratriene-3-one (R1881)
PMSF	- phenylmelthylsulphonylfluoride
progesterone	- 4-pregnane-3,20-dione
R5020	- 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione
SHBG	- sex hormone binding globulin
testosterone	- 17β-hydroxy-4-androsten-3-one
triamcinolone acetonide	- 9α-fluoro-11β,21-dihydroxy-16α,17-iso-propylidenedioxy-1,4-pregnadiene-3,20-dione

CURRICULUM VITAE

Op 25 februari 1950 ben ik te Rotterdam geboren. In 1969 behaalde ik het HBS-B diploma aan het Caland Lyceum te Rotterdam. Na het vervullen van mijn militaire dienstplicht begon ik in 1971 de scheikunde studie aan de Rijks Universiteit te Leiden. In 1974 behaalde ik het kandidaatsexamen en in 1977 het doctoraalexamen met als hoofdvak biochemie en als bijvakken moleculaire genetica en fysische chemie. Van augustus 1977 tot augustus 1981 ben ik als wetenschappelijk ambtenaar verbonden geweest aan de afdeling Biochemie II (Chemische Endocrinologie) van de Erasmus Universiteit Rotterdam.

Appendix Paper I
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NUCLEAR ANDROGEN RECEPTORS IN HUMAN PROSTATIC TISSUE. EXTRACTION WITH HEPARIN AND ESTIMATION OF THE NUMBER OF BINDING SITES WITH DIFFERENT METHODS

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Summary

A procedure for the estimation of nuclear androgen receptors in benign prostatic hyperplastic tissue is described, which employs extraction of receptors from nuclei with buffers containing heparin. Extraction of a nuclear pellet with a heparin-containing (1 g/l) buffer appeared to have definite advantages over 0.4 mol/l KCl extraction. Heparin appeared to be twice as efficient in extracting androgen receptors. In addition aggregated receptor proteins, formed after storage at −80°C, were partly deaggregated by heparin. Specific isolation of the androgen receptor was performed using either agar gel electrophoresis, protamine sulphate precipitation or LH-20 gel filtration. A comparison was made between the amounts of estimated receptors with these different techniques. Protamine sulphate precipitation resulted in the highest estimates of receptor-bound 5α-[^{3}H]dihydrotestosterone (^{3}H-DHT). Treatment of the labelled nuclear extracts with a charcoal suspension prior to the receptor assay resulted in lower amounts of estimated androgen receptors. A method for routine evaluation of nuclear androgen receptors in prostatic tissue has been evaluated, which involves extraction of nuclear pellets with a heparin-containing (1 g/l) buffer, exchange labelling of the nuclear extracts for 20 h at 10°C and quantification of the receptors with protamine sulphate precipitation.

Introduction

The prostate is a target organ for androgens and does not develop in the absence of androgens. Androgens play a role in hyperplasia and cancer of the

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Abbreviation used: methyltrienolone (R 1881), 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one.

human prostate, because after castration no such tumours were found [1]. The well-established relationship between the occurrence of hormone receptors and the hormonal dependence of mammary tumours [2] has stimulated the belief that estimation of the androgen receptor content of the human prostate could contribute to a better understanding of the development of benign prostatic hyperplasia (BPH) and the hormonal dependence of prostatic carcinoma. Several laboratories have estimated cytoplasmic androgen receptors in prostatic tissue, but the receptor values showed a wide variation, probably as result of the heterogeneity of the tissue samples and the different experimental conditions used for receptor estimation [3,4]. The measurement of cytoplasmic androgen receptors in prostatic tissues is complicated by the presence of high proteolytic activity and of high levels of endogenous DHT [5,6]. Additionally the presence of sex hormone binding globulin and a progesterone receptor can give erroneous results.

It can be expected from the high endogenous levels of DHT that most of the cytoplasmic receptor in the prostate is translocated to the nucleus [7]. Hence, it could be meaningful to measure nuclear androgen receptors rather than cytoplasmic receptors, although the reported nuclear receptor content of BPH tissues also shows a rather wide variation [3,8—11]. We have attempted to overcome some of the technical difficulties and in this report we present the results of: (1) a more efficient procedure for extraction of androgen receptors from nuclear pellets of BPH tissue; (2) optimal conditions for exchange of unlabelled endogenous receptor-bound steroid with radioactive added steroids in heparin extracts; (3) a comparison between agar gel electrophoresis, LH-20 gel filtration, protamine sulphate precipitation and sucrose density gradient centrifugation as methods for quantitative estimation of receptors; and (4) the effect of charcoal treatment of labelled nuclear extracts prior to the receptor assay.

On the basis of these results we have combined several of these procedures in a method for routine estimation of nuclear androgen receptors in prostatic tissue. Results obtained with this method were compared with those published previously by others. A preliminary report of our results has been published [12].

Materials and methods

Tissue

Prostatic tissues were obtained from patients with benign prostatic hyperplasia undergoing open prostatectomy. After surgery, tissues were immediately placed on ice, divided in samples of 1—2 g and stored frozen at —80°C for 1—3 months. Before processing the tissue was thawed on ice.

Steroids

The following steroids were used: 5α-dihydrotestosterone (17β-hydroxy-5α-androstane-3-one) (DHT); testosterone (17β-hydroxy-4-androsten-3-one); progesterone (4-pregnene-3,20-dione); oestradiol (1,3,5(10)-estratriene-3,17β-diol); methyltrienolone (17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one, R 1881) and (1,2,4,5,6,7-^{3}H)-DHT (specific activity 114 Ci/mmol).

^{3}H-DHT was purchased from the Radiochemical Centre, Amersham, U.K.

Non-labelled R 1881 was obtained from NEN Chemicals GmbH, F.R.G. Other unlabelled steroids were obtained from Steraloids, Pawling, New York, U.S.A.

Preparation of nuclear extracts

After mincing the thawed BPH tissue partly with scissors, the minces were homogenized between two stainless steel screens (mesh size 80). During homogenization 0.5 g minced tissue was kept in 40 ml buffer A (50 mmol/l Tris-HCl, 2.5 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.55 mol/l sucrose, pH 7.5). After homogenization the suspension was divided into two equal parts and each part was layered onto 25 ml buffer A′ (sucrose concentration in buffer A increased to 0.88 mol/l) and centrifugation was performed with a HB-4 rotor for 10 min/ $2000 \times g$ in a Sorvall RC2B centrifuge. After centrifugation the supernatant was discarded and the nuclear pellets were resuspended each in 750 μl buffer B (10 mmol/l Tris-HCl, 1.5 mmol/l EDTA, 1.5 mmol/l dithiothreitol, 0.05 mol/l NaCl, pH 7.5) and were pooled. In 100 μl of this suspension the number of nuclei were counted as described by Lieskovsky and Bruchovsky [10]. The remainder of the nuclear suspension was centrifuged for 10 min/$800 \times g$ and the nuclear pellet was mixed with 2 mmol/l phosphate buffer (pH 8.0), containing 1 g/l heparin unless stated otherwise, until a final suspension was reached of $1-2 \times 10^7$ nuclei/ml. The resulting suspension was left for 1 h followed by centrifugation for 30 min/$100\,000 \times g$ in a Beckman L5-65 centrifuge with a SW-60 rotor. The supernatant was used as "nuclear extract". All procedures were performed at 0—4°C.

Labelling of nuclear extracts

The nuclear extracts were incubated for different periods of time and at different temperatures with 10—100 nmol/l ^{3}H-DHT. In parallel experiments nuclear extracts were incubated with labelled steroid and a 200-fold excess of non-radioactive DHT. Competition experiments were performed with 10 nmol/l ^{3}H-DHT in the absence and presence of 100 and 1000 nmol/l non-labelled competitors.

Estimation of receptor levels

Charcoal pretreatment of nuclear extracts

To remove excess non-bound steroid and to exclude low affinity binding sites, labelled nuclear extracts were treated with 0.5% charcoal (Dextran 300 coated), prior to the receptor assay. The charcoal treatment was performed for 10 min at 0°C followed by centrifugation for 10 min/$1000 \times g$. The supernatant fraction was analyzed either by agar gel electrophoresis, protamine sulphate precipitation, LH-20 gel filtration or sucrose density gradient centrifugation.

Agar gel electrophoresis

Agar gel electrophoresis was performed for 90 min at 130 mA as described by Wagner [13].

Protamine sulphate precipitation

Protamine sulphate precipitation was performed by the method of Chamness et al. [14] with acid-washed (11.5 × 75 mm) disposable glass tubes. The tubes were incubated at 30°C for 15 min with 0.5 ml buffer B containing 0.1% bovine serum albumin and were then rinsed with 1 ml ice-cold buffer C (10 mmol/l Tris-HCl, 1.5 mmol/l EDTA, 1.5 mmol/l dithiothreitol, 10% glycerol, pH 7.5). Protamine sulphate (Organon, Oss, The Netherlands) was diluted to a final concentration of 1 g/l in buffer C and 450 μl was added to each tube. A protamine sulphate concentration above 1 g/l did not change the amount of precipitable specifically bound radioactive steroid. After treatment with charcoal 50 μl samples were added to the protamine sulphate solution, mixed with a Vortex and left for 5—10 min at 0°C before centrifugation at 2400 × g for 15 min. The firmly coated precipitates were washed five times with 1 ml buffer C without further centrifugation. The pellets were dissolved with 0.5 ml soluene (Packard Instrument) for 10 min at 60°C and counted for radioactivity. All values reported are means of duplicate experiments.

LH-20 gel filtration

LH-20 (Pharmacia) gel filtration was performed by the method of Ginsburg et al. [15]. After treatment with charcoal, 50 μl nuclear extract samples were analyzed with buffer C as elution buffer. The void volume fractions were pooled and counted for radioactivity. All values reported are means of duplicate experiments.

Sucrose density gradient centrifugation

After treatment with charcoal 200 μl of the nuclear extracts were layered on 5—20% linear sucrose gradients prepared with Buffer C containing 0.6 mol/l KCl. Centrifugation was performed in a SW-60 rotor for 24 h at 370 000 × g_{av} in a Beckman L5-65 centrifuge at 1°C. Finally 200 μl fractions were collected by piercing the bottom of the tube and counted for radioactivity.

Liquid scintillation counting

For counting of radioactivity, samples were mixed with 10 ml of Insta-Gel (Packard Instruments) as scintillation cocktail. The pellets obtained after protamine sulphate precipitation were dissolved in soluene and were subsequently mixed with Insta-Gel containing 1% acetic acid and 0.1% butylated hydroxy toluene.

Protein estimation

The amount of protein in nuclear extracts was estimated as described by Peterson [16], which involves a protein precipitation step prior to the assay to eliminate interfering ingredients present in the buffers.

Results

Optimalization of nuclear receptor assay

Estimation of receptor complexes in nuclear pellets

In a first series of experiments the nuclear pellet from BPH tissue was

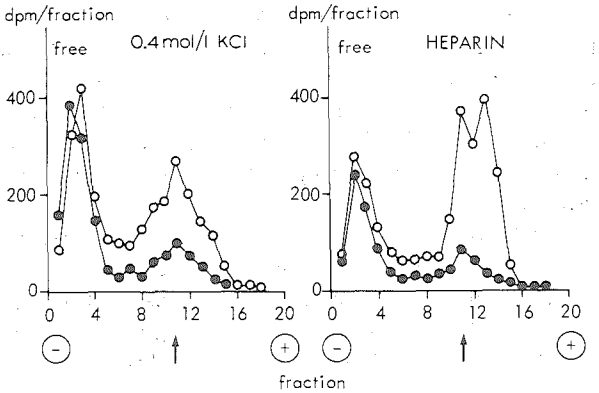

Fig. 1. Agar gel electrophoresis of labelled nuclear extracts of BPH tissue. Extraction of the nuclear pellet was performed for 1 h at 0°C either with 0.4 mol/l KCl (left panel) or with 1 g/l heparin (right panel). Nuclear extracts were incubated for 18 h/10°C with 10 nmol/l ^{3}H-DHT (○———○) or with 10 nmol/l ^{3}H-DHT in the presence of 2 µmol/l non-radioactive DHT (●———●); "free", position of unbound steroid; "arrow", position of sample at start of electrophoresis.

extracted with a buffer containing 0.4 mol/l KCl and the nuclear extract was incubated with ^{3}H-DHT and subsequently subjected to agar gel electrophoresis. Fig. 1 (left panel) shows that the specifically bound radioactive tracer was located at the application site (arrow) and free steroid had migrated to the cathodic region of the gel. This indicates an aggregated form of the receptor protein, which might have been caused by either storage at −80°C or by the homogenization or extraction procedures.

In an earlier report from this laboratory it has been shown that rat prostate nuclear androgen receptor could partly be deaggregated by the use of heparin [17]. After extraction of the nuclear pellet from BPH tissue with a buffer con-

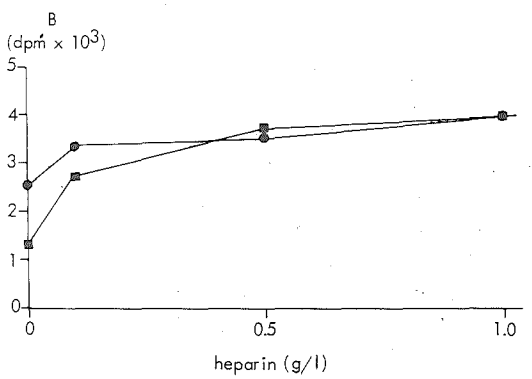

Fig. 2. Effect of heparin concentration on the amount of androgen receptors in nuclear extracts of BPH tissue. Nuclear extracts were incubated for 18 h at 6°C with 10 nmol/l ^{3}H-DHT in the absence and presence of 2 µmol/l non-radioactive DHT. Specifically bound ^{3}H-DHT was estimated after agar gel electrophoresis and plotted; ■———■, without sonication; ●———●, with sonication for 3 X 5 s (with intermediate cooling) with an MSE 100 W Ultrasonic Disintegrator stand height 4. Sonication was performed at the start of the 1 h extraction period.

taining heparin followed by agar gel electrophoresis, the specifically bound ^{3}H-DHT was reflected in a peak towards the anodic region of the agar gel, which indicates deaggregation of the androgen receptor molecules (Fig. 1, right panel). After agar gel electrophoresis of nuclear extracts containing heparin, in some cases a residual peak of specifically bound ^{3}H-DHT was still observed at the application site, which suggests that deaggregation was not complete. In the calculations of the total amount of specifically bound steroid, we included this amount of specifically bound steroid at the application site. Additionally, extraction of nuclear pellets with a buffer containing heparin (1 g/l) appeared to be twice as efficient in extracting androgen receptors as extraction with a buffer containing 0.4 mol/l KCl (82 ± 7 and 49 ± 2 pmol/g protein respectively, which corresponds with 2333 ± 192 and 1005 ± 31 molecules/nucleus; mean ± S.D., $n = 5$).

The effect of sonication and the influence of the heparin concentration on the amount of extractable androgen receptors was investigated. From the results given in Fig. 2 it appeared that addition of 0.5 or 1.0 g/l heparin to the extraction buffer abolished the effect of sonication on the extractable amount of androgen receptors, which was observed in the absence of heparin. Sonication of nuclear pellets, however, caused an increase in non-specific binding. The amounts of specifically bound steroid (as plotted in Fig. 2) were not different. In all further experiments a heparin concentration of 1 g/l was used and sonication of the nuclear pellet was omitted. Extraction was complete after 1 h at 0°C and as a standard procedure $1—2 \times 10^7$ nuclei were extracted with 1 ml extraction buffer.

Labelling of nuclear extracts via the exchange procedure

To evaluate optimal conditions for receptor estimation, exchange was studied at different temperatures and for different times and optimal concentration of added radioactive steroid was determined. The exchange of added radioactive steroid (10 nmol/l ^{3}H-DHT) was studied at different temperatures for 18 h. Optimal exchange of added radioactive ligand for endogenous bound steroid was achieved between 8°C and 20°C with a maximum at approximately 10°C. To determine the optimal concentration for added ^{3}H-DHT, a Scatchard plot was constructed (Fig. 3), showing a single type of binding site with an apparent K_D of 1.7×10^{-9} mol/l. It was calculated from the Scatchard plot that maximal exchange occurred when the ratio free/bound ligand is approximately 80 : 1. For tissue samples with high levels of androgen receptors, a concentration of 50 nmol/l ^{3}H-DHT present during the exchange appeared to be sufficient. To ensure that no underestimation of receptors occurred as a result of the endogenous DHT present, $1—2 \times 10^7$ nuclei were extracted with 1 ml extraction buffer. In the diluted extracts used, the concentration of endogenous (non-labelled) DHT was maximally 0.5 nmol/l ($n = 15$). The added amount of radioactive ligand should be at least 100 times higher than endogenous steroid. Therefore the use of 50 nmol/l ^{3}H-DHT in the presence of such small amounts of endogenous steroid will not lead to underestimations of receptors. Studies with variable exchange times at 10°C with 50 nmol/l ^{3}H-DHT as exchange ligand showed that after 20 h a maximal amount of androgen receptors was labelled, which remained constant up to at least 50 h (Fig. 4).

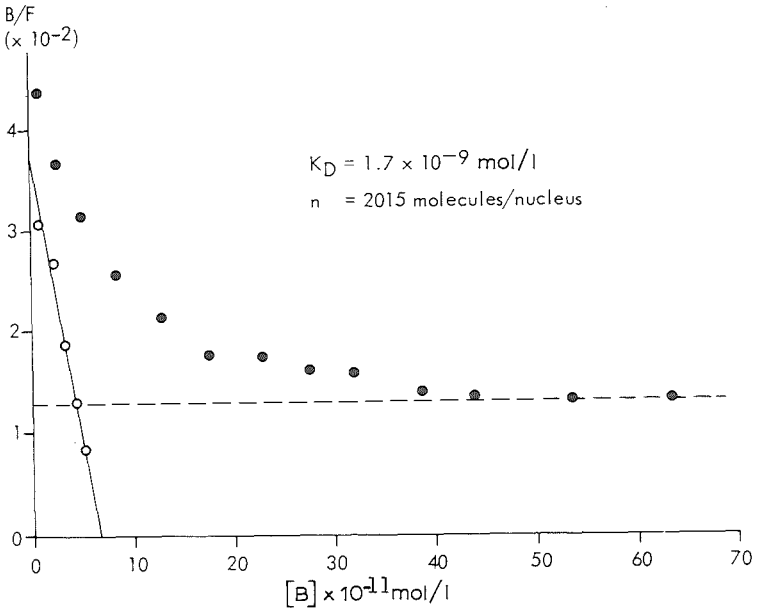

Fig. 3. DHT binding in nuclear extracts of BPH tissue. Nuclear extract samples were incubated for 20 h at 10°C with increasing amounts of ^{3}H-DHT. Bound (B) and free (F) steroid were separated by protamine sulphate precipitation. Recoveries for free steroid were taken directly from the incubation samples. Correction for non-specific binding was made according to the method described by Rosenthal [18]. (●——● before correction; ○——○ after correction). [B], presumably bound.

Comparison of different androgen receptor assays

Results of receptor assays obtained with protamine sulphate precipitation as compared with agar gel electrophoresis or with LH-20 gel filtration are presented in Fig. 5. The amounts of androgen receptor molecules per nucleus are plotted and showed good mutual correlation (correlation coefficients of r = 0.98, n = 8 and r = 0.99, n = 7) respectively. The application of agar gel electro-

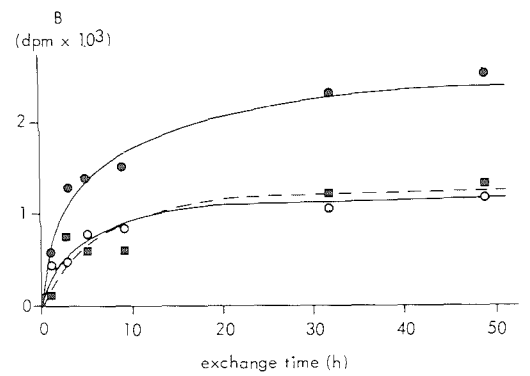

Fig. 4. Effect of incubation time on labelling of androgen receptors in nuclear extracts of BPH tissue. Nuclear extracts were incubated at 10°C with 50 nmol/l ^{3}H-DHT in absence (●——●) and presence of 10 μmol/l non-radioactive DHT (○——○). The amounts of androgen receptors were quantified with protamine sulphate precipitation (■——■ specifically bound ^{3}H-DHT).

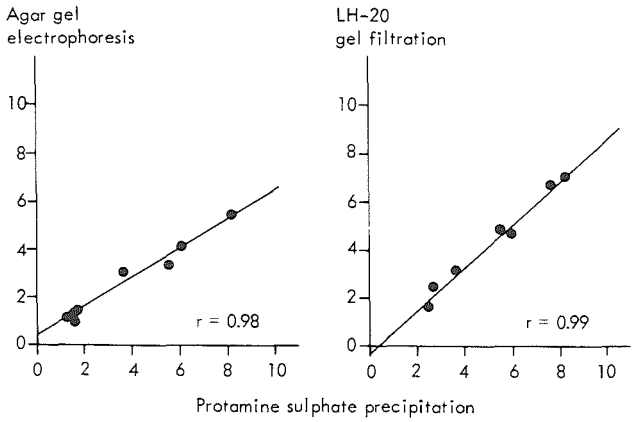

Fig. 5. Comparison of the amount of androgen receptors in nuclear extract of BPH tissue, estimated by either protamine sulphate precipitation or agar gel electrophoresis and LH-20 gel filtration. Nuclear extracts were incubated for 18—20 h at 10°C with 10 nmol/l ^{3}H-DHT in the absence and presence of 2 μmol/l non-radioactive DHT and the number of molecules/nucleus (X10^3) are plotted: $[AR]_{agar} = 0.61\ [AR]_{P.S.} + 314$ ($n = 8$); $[AR]_{LH\text{-}20} = 0.88\ [AR]_{P.S.} - 182$ ($n = 7$)).

phoresis and LH-20 gel filtration as techniques to separate bound from free ligand resulted in lower estimated androgen receptor levels than protamine sulphate precipitation (26 and 16% respectively).

An example of a profile of radioactive DHT obtained after sucrose gradient centrifugation is shown in Fig. 6. The specifically bound ^{3}H-DHT mainly migrated in the 4 S region. The higher amount of radioactivity present at the

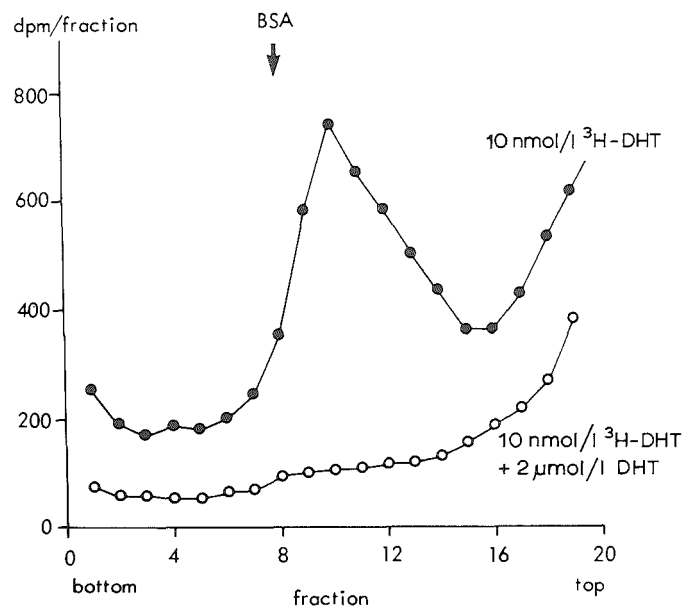

Fig. 6. Sucrose density gradient centrifugation of labelled nuclear extracts of BPH tissue. Nuclear extracts were incubated with 10 nmol/l ^{3}H-DHT in the absence (●———●) and presence of 2 μmol/l non-radioactive DHT (○———○). ("Arrow" indicates position of BSA (4.6 S).)

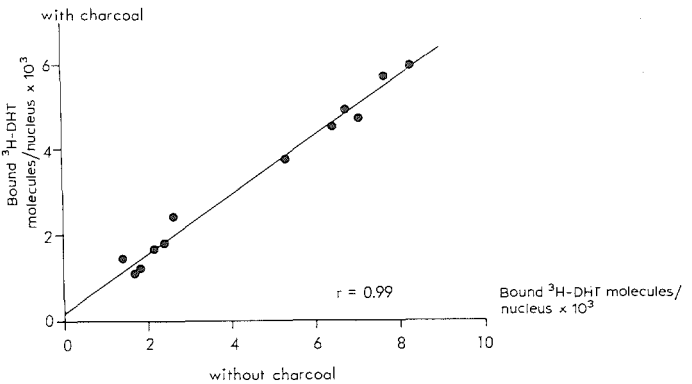

Fig. 7. Effect of charcoal pre-treatment on the estimated amount of androgen receptors in labelled nuclear extracts of BPH tissue. Nuclear extracts were incubated for 20 h at 10°C with 10 nmol/l ^{3}H-DHT in absence and presence of 2 μmol/l non-radioactive DHT. Samples were analyzed by either LH-20 gel filtration or protamine sulphate precipitation and the number of specifically bound ^{3}H-DHT in molecules/nucleus ($X10^3$) are plotted: ($[AR]_{+cc} = 0.69\ [AR]_{-cc} + 176$ ($n = 12$)).

top of the gradient (upper curve Fig. 6) is probably due to partial dissociation of the androgen receptor complex during the run.

The effect of charcoal pre-treatment of the nuclear extracts prior to the receptor assay

The androgen receptor estimations described so far in this report were performed after treatment of the incubated nuclear extracts with a charcoal suspension prior to the receptor assay. This pre-treatment resulted in the removal of excess non-bound steroid and exclusion of low affinity binding sites.

Fig. 7 shows the amount of specifically bound ^{3}H-DHT in molecules/nucleus, estimated after treatment of the labelled nuclear extract with charcoal prior to the receptor assay, versus the amount of estimated receptors when charcoal treatment was omitted. The number of receptors estimated was 26% lower when charcoal pre-treatment was performed.

TABLE I

SPECIFICITY OF ^{3}H-DHT BINDING TO NUCLEAR EXTRACTS OF BPH TISSUE

Nuclear extracts were incubated for 20 h at 10°C with 10 nmol/l ^{3}H-DHT in absence and presence of 100 and 1000 nmol/l non-radioactive competitors. Receptors were estimated by protamine sulphate precipitation.

Competitor	Residual binding (%)	
	100 nmol/l	1000 nmol/l
None	100	100
Dihydrotestosterone	11— 20 *	0
Methyltrienolone	22— 30 *	20 ± 12 **
Testosterone	41— 65 *	20 ± 15 **
Progesterone	112—126 *	100 ± 15 **
Oestradiol	101—103 *	70 ± 6 **

* Duplicate experiments.

** Mean ± S.D. ($n = 4$).

Specificity of DHT binding to nuclear extracts of BPH tissue

The specificity of DHT binding in nuclear extracts of BPH tissue was studied. When ^{3}H-DHT was incubated in the presence of steroid competitors, it was found that radio-inert dihydrotestosterone, R 1881 and testosterone showed the highest competition, whereas oestradiol and progesterone did not show any competition in 10-fold excess and only small competition in 100-fold excess (Table I).

Discussion

The present results show that the use of a buffer containing heparin for extraction of receptors from nuclear pellets of BPH tissue offers several advantages over extraction with a buffer containing KCl. Compared to extraction with 0.4 mol/l KCl the yield of solubilized receptors after heparin extraction was improved. This may reflect that with heparin also the salt-resistant receptor sites [10] are solubilized. An additional advantage of extraction with a buffer containing heparin appeared to be the partial deaggregation of the receptor proteins (Fig. 1). This is in agreement with previous observations that rat prostate nuclear androgen receptors after storage at —80°C could be deaggregated by heparin [17].

The purpose of the present study was to define optimal conditions for the exchange of occupied nuclear receptors and to evaluate some of the discrepancies described in the literature concerning the amount of nuclear androgen receptors in BPH tissue [3,8—11]. The differences reported are probably not only the result of heterogeneity of the tissue samples, but are also caused by storage of tissue and the experimental conditions used for procedures such as homogenization of the tissue, the exchange of bound ligands, and the separation of bound from free ligand.

In the comparison of values for nuclear androgen receptor levels estimated by either protamine sulphate precipitation, LH-20 gel filtration or agar gel electrophoresis, the highest values of specifically bound steroid obtained by single point assays with 10 nmol/l ^{3}H-DHT as exchange ligand were obtained with the protamine sulphate precipitation technique. Decreasing values of bound steroid were obtained in the sequence: protamine sulphate precipitation > LH-20 gel filtration > agar gel electrophoresis (Fig. 5). The number of androgen receptors estimated appeared to be inversely proportional to the time required to perform the assay, probably due to lability and fast dissociation of androgen-receptor complexes. This was also supported by the observation that after sucrose gradient centrifugation (24 h) a significant difference was found in radioactivity at the top of the gradient (Fig. 6).

Apart from the experiments presented in Fig. 7, in all the androgen receptor assays described in this paper, pre-treatment of the labelled nuclear extracts with a charcoal suspension was used for removal of excess non-bound steroid and for reduction of non-specific binding. When exchange was performed with higher concentrations of ^{3}H-DHT (50 nmol/l) and charcoal pre-treatment was omitted, the values obtained after agar gel electrophoresis and protamine sulphate precipitation were very difficult to interpret. The reason for this difficulty is the very high non-specific binding which occurs after long exchange

periods. Also in the literature very high non-specific binding in nuclear extracts has been reported after exchange for long periods [8,11].

We have concluded from the present results that estimation of nuclear androgen receptors in BPH tissue after storage at −80°C by extraction of the nuclear pellet with a buffer containing heparin offers certain advantages over other methods. Optimal conditions for exchanging added labelled steroids for endogenous bound steroid do not differ much from those reported in the literature [3,8—11], but the protamine sulphate precipitation technique gave the highest numbers of androgen receptors in our estimations. An additional advantage of this technique is the experimental convenience of analyzing several samples at the same time. For routine purposes the best procedure would be the protamine sulphate precipitation technique, because a lot of samples can be processed at the same time. For accurate measurements of receptor levels and dissociation constants, it is advisable to produce Scatchard plots in initial experiments. In subsequent experiments a 2-point assay may give reliable results.

In the literature discrepancies are reported with respect to the amount of cytoplasmic androgen receptors and clinical response in carcinomatous human prostatic tissue [20—22]. However, the measurement of cytoplasmic androgen receptors is complicated by the presence of high proteolytic activity and high levels of endogenous DHT [5,6], which is known to translocate the receptor to the nucleus. Additionally, using R 1881 and DHT as exchange ligands, the presence of a progesterone receptor [23] and sex hormone binding globulin [5] may give erroneous results. In this respect the applicability of a nuclear receptor assay may become increasingly important, because the estimation of nuclear androgen receptors in prostatic carcinoma tissue may assist in selecting the appropriate therapy for patients suffering from this disease.

Acknowledgement

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Appendix paper II
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CHARACTERIZATION AND PARTIAL PURIFICATION OF ANDROGEN RECEPTORS FROM RAM SEMINAL VESICLES

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An androgen receptor has been demonstrated in the cytosol and in the nuclear fraction of ram seminal vesicles.

The cytosol receptor was stabilized by sodium molybdate and 2 distinct [^{3}H]methyltrienolone-binding proteins, one sedimenting at 9S and one sedimenting at 3S, could be demonstrated by sucrose-gradient centrifugation in the presence of 50 mM molybdate. The slower sedimenting form could be partially purified by ADP–sepharose chromatography. The purified receptor still sedimented at 3S after centrifugation on sucrose gradients containing either 0.6 M KCl or 50 mM molybdate. The receptor was destroyed by heating at 50°C for 30 min and its complex with [^{3}H]methyltrienolone dissociated slowly at low temperatures. The apparent equilibrium-dissociation constant (K_D) for the purified receptor was: 3.8×10^{-10} M. The relative affinities for different steroids decreased in the following sequence: 5α-dihydrotestosterone ⩾ methyltrienolone > testosterone ⩾ estradiol > R5020 > progesterone > diethylstilbestrol.

The nuclear androgen receptor sedimented at 3S on sucrose gradients containing 0.6 M KCl. At pH 7.4 it behaved as an acidic protein with an electrophoretic mobility towards the anodic region of the agar gel.

Because of the relatively large content of cytoplasmic and nuclear androgen receptors and the availability of large amounts of tissue the ram seminal vesicles could be a suitable source for large-scale purification of these receptors.

Keywords: androgen receptor; testosterone; protein purification.

From studies on rat prostate it is now generally accepted that androgens initiate their cellular action via steroid–receptor complexes in the cytosol, which after a conformation change are translocated to the nucleus (Liao and Fang, 1969; Mainwaring, 1977). The hypothesis that androgen-hormone receptors associate with chromatin, thus influencing gene transcription, has led to intensive research on the characterization of these receptor molecules and their nuclear-acceptor sites (Rennie, 1979; Davies et al., 1980).

The lack of pure receptors is a major obstacle for further investigation of the mechanism of action. The limited amount of androgen receptor present and the small size of androgen target tissue make the rat prostate unattractive as a source

for androgen receptor purification. The presence of cytoplasmic androgen receptors has been reported also for seminal vesicles of rat and mouse (Mainwaring and Mangan, 1973) and ram testis (Monet-Kuntz et al., 1979). We have investigated ram seminal vesicles as a possible source for large-scale receptor purification.

In this publication we demonstrate the presence of large amounts of an androgen-binding protein with characteristics of an androgen receptor in seminal vesicle tissue of the ram.

MATERIALS AND METHODS

Tissue

Seminal vesicle tissue from adult rams was removed as soon as possible after killing the animals and was directly frozen at –20°C. After transportation from the slaughterhouse, the tissue was stored at –80°C. For measurements of nuclear receptors, fresh tissue was used after transportation on ice.

Steroids

The following steroids were used: 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); testosterone (17β-hydroxy-4-androsten-3-one) (T); methyltrienolone (17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one) (R1881); R5020 (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione); 17β-estradiol (1,3,5-(10)-estratriene-3,17β-diol); progesterone (4-pregnane-3,20-dione); diethylstilbestrol (3,4-bis-(-hydroxy-phenyl)-3-hexene).

[17α-*Me*-^{3}H]R1881 (spec. act. 87 Ci/mmole), unlabelled R5020 and R1881 were purchased from NEN Chemicals, GmbH (West Germany). [1,2,6,7-^{3}H]T (spec. act. 93 Ci/mmole) was obtained from the Radiochemical Centre, Amersham (England).

Other unlabelled steroids were purchased from Steraloids, Pawling, NY (U.S.A.).

Cytoplasmic and nuclear receptors

For preparation of cytosols, seminal vesicles stored at –80°C were used after thawing on ice. Minced tissue was homogenized in 2–3 volumes of TEDG buffer (10 mM Tris–HCl, 1.5 mM EDTA, 1.5 mM dithiothreitol with 10% glycerol; buffer A, pH 7.4) with a Waring Blendor for 1 × 60 s at 4°C (in some experiments, as indicated, sodium molybdate was added to the homogenization buffer). The homogenate was centrifuged for 45 min at 96 000 × g_{av} in a Beckman SW-27 rotor and the supernatant was designated as "cytosol".

For labelling of nuclear receptors, minces of fresh seminal vesicle tissue were incubated for 1 h at 37°C in Eagle's minimal essential medium with 20 nM [^{3}H]testosterone. The tissue was homogenized in 3 volumes of buffer B (buffer A, without glycerol) with 3 × 10 s strokes of an Ultraturrax tissue-homogenizer and the 700 × g nuclear pellet was prepared. The pellet was washed with buffer B contain-

ing 0.2% Triton X-100 and subsequently 2 times with buffer B. A nuclear extract was prepared by extracting the washed nuclear pellet with 0.6 M KCl in buffer B (pH 8.4) for 1 h at 0°C and centrifugation for 15 min at 10 000 × *g*.

Pretreatment with charcoal

Excess unbound steroid was removed in some experiments by adding 2.5 mg dextran-coated charcoal to 1 ml of sample. After mixing, the suspensions were incubated for 15 min at 0°C and charcoal was removed by centrifugation for 15 min at 10 000 × *g*.

Measurement of steroid binding

Separation of free and bound steroid was performed using either Sephadex LH-20 gel filtration, sucrose-gradient centrifugation or agar gel electrophoresis.

Sephadex LH-20 gel filtration was performed according to Ginsburg et al. (1974). After incubation 100-µl samples were analyzed with buffer A as elution buffer. The void volume fractions were pooled and counted for radioactivity.

For sucrose-gradient analysis of cytosol and partially purified receptor preparations, a 250-µl sample was centrifuged at 1°C in linear 10–30% sucrose gradients in buffer A (with or without additions) for 3.5 h at 370 000 × g_{av} in a Beckman VTi-65 rotor. For sucrose-gradient analysis of nuclear extracts, a 200-µl portion of a 0.6 M KCl extract was centrifuged at 1°C in linear 5–20% sucrose gradients in buffer B for 18 h at 310 000 × g_{av} in a Beckman SW-60 rotor.

Agar-gel electrophoresis was performed as described by Wagner (1972).

Protein determination

Protein was estimated according to Bradford et al. (1976).

Liquid-scintillation counting

For counting of radioactivity samples were mixed with 10 ml of Insta-Gel (Packard Instrument) as scintillation cocktail.

RESULTS

Binding of [^{3}H]methyltrienolone to cytosols of ram seminal vesicle tissue

For the study described in this report, seminal vesicles were obtained from a variable population of non-castrated rams. For this reason steroid-binding studies were performed under conditions where exchange of added radioactive steroid with endogenously bound steroid could be expected. Labelling experiments were performed with [^{3}H]methyltrienolone, a synthetic androgen with high affinity for androgen receptors and not liable to attack by 3-hydroxysteroid dehydrogenases (Bonne and Raynaud, 1975). In the absence of molybdate maximal binding of [^{3}H]methyltrienolone to cytosol fractions was obtained between 10 and 23 h with

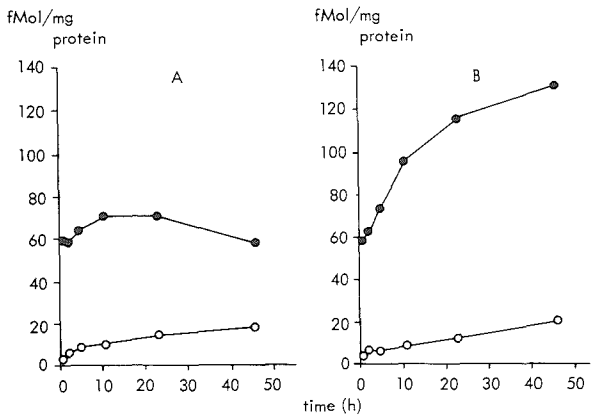


Fig. 1.

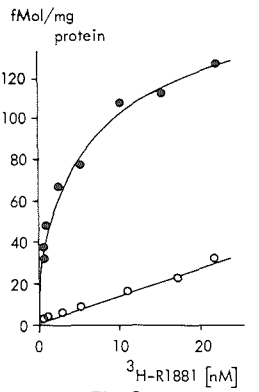


Fig. 2.

Fig. 1. Time course of [^{3}H]methyltrienolone binding to ram seminal vesicle cytosol. Cytosols prepared in absence (A) or presence of 50 mM molybdate (B) were incubated with 10 nM [^{3}H]-methyltrienolone in the absence or presence of 1 μM non-radioactive methyltrienolone at 10°C. After various time intervals a 100-μl aliquot was withdrawn from the incubation mixture to determine total (•) and non-specific binding (○) with Sephadex LH-20 gel filtration.

Fig. 2. [^{3}H]Methyltrienolone binding to ram seminal vesicle cytosol. Cytosol was prepared in the presence of 50 mM molybdate and incubated for 24 h at 10°C with increasing amounts of [^{3}H]methyltrienolone ([^{3}H]R1881) in the absence or presence of a 100-fold excess of non-radioactive methyltrienolone. Samples were analyzed with Sephadex LH-20 gel filtration to determine total (•) and non-specific binding (○).

10 nM [^{3}H]methyltrienolone at 10°C (Fig. 1A). In the presence of 10 mM molybdate maximal binding was achieved after 23 h and remained constant for at least a further 23 h (not shown). In the presence of 50 mM molybdate maximal binding was not yet reached after 23 h (Fig. 1B). In additional experiments with cytosols incubated in the presence of 50 mM molybdate and with increasing amounts of [^{3}H]methyltrienolone, saturation of binding sites appeared to occur at approx. 10 nM [^{3}H]methyltrienolone (Fig. 2). The difference in labelling found between incubations with 10 nM [^{3}H]methyltrienolone in absence or presence of a 100-fold excess of non-labelled methyltrienolone was defined as specific binding.

Specific binding was observed already after incubation for only 45 min at 10°C (Fig. 1A, B), and this suggests the presence of free receptors in the analyzed samples.

2',5'-ADP-sepharose chromatography

Cytosols labelled with [^{3}H]methyltrienolone were partially purified with ADP-sepharose chromatography (Fig. 3). The bulk of proteins bound to the gel was eluted at lower salt concentrations than the bound [^{3}H]methyltrienolone (Fig. 3). The radioactivity eluted from ADP–sepharose was bound to protein, because the

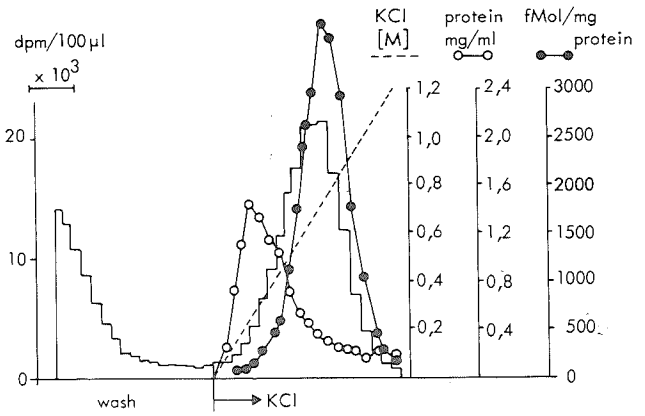


Fig. 3. Partial purification of cytoplasmic androgen receptors from ram seminal vesicles with 2',5'-ADP–sepharose. Cytosol (157 ml) prepared in buffer A containing 25 mM molybdate was diluted with buffer A to a final concentration of 10 mM molybdate. After incubation with continuous shaking for 20 h at 6°C with 2.2 nM [^{3}H]methyltrienolone in the presence of 20 ml swollen ADP–sepharose, the gel suspension was filtered over a glass filter. The remaining gel was packed in a Pharmacia column (size, 1.6 × 20 cm) with buffer A. After washing with buffer A (18 × 20 ml) the gel was eluted with 0–1.2 M KCl in buffer A gradient (20 fractions of 10 ml). The amounts of radioactivity measured in 100 µl of the different samples are plotted (|———|).

radioactivity was eluted in the void volume fraction with Sephadex LH-20 gel filtration, but no protein-bound radioactivity was found after heating the eluates for 30 min at 50°C. This indicates that [^{3}H]methyltrienolone was bound to a saturable heat-labile protein, which is a generally accepted characteristic of a steroid receptor.

Sucrose-gradient analysis of partially purified cytoplasmic androgen receptors

Before and after heating for 30 min at 50°C aliquots of a purified fraction (~3700 fmoles [^{3}H]methyltrienolone/mg protein) were analyzed with sucrose-gradient centrifugation. After centrifugation for 18 h at 1°C no distinct peak of bound [^{3}H]methyltrienolone could be demonstrated and only a tail of radioactivity near the top of the gradient was found. This suggests that the steroid was dissociated from the proteins during centrifugation for 18 h. To overcome this dissociation problem, the time required for the sucrose-gradient centrifugation procedure was shortened to 210 min by centrifugation in a vertical rotor. Fig. 4 shows sucrose-gradient profiles of partially purified cytoplasmic androgen receptors (before and after heating for 30 min at 50°C) after centrifugation with a vertical rotor. On sucrose gradients containing 0.6 M KCl (Fig. 4A) or 50 mM molybdate (Fig. 4B), an androgen receptor sedimenting at 3S was identified. The lower curve in Fig. 4A (heat-denaturated sample) represents free steroid, which was probably released during denaturation of the receptor proteins. When the same fraction was

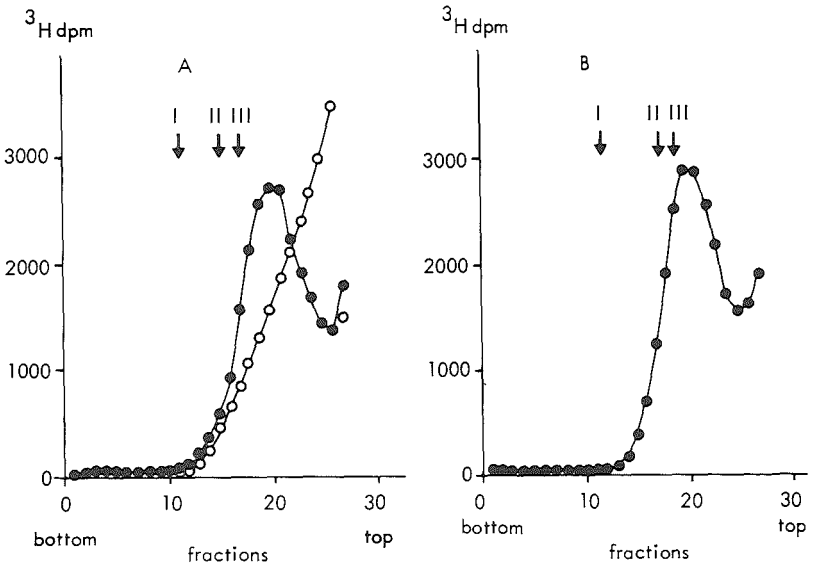


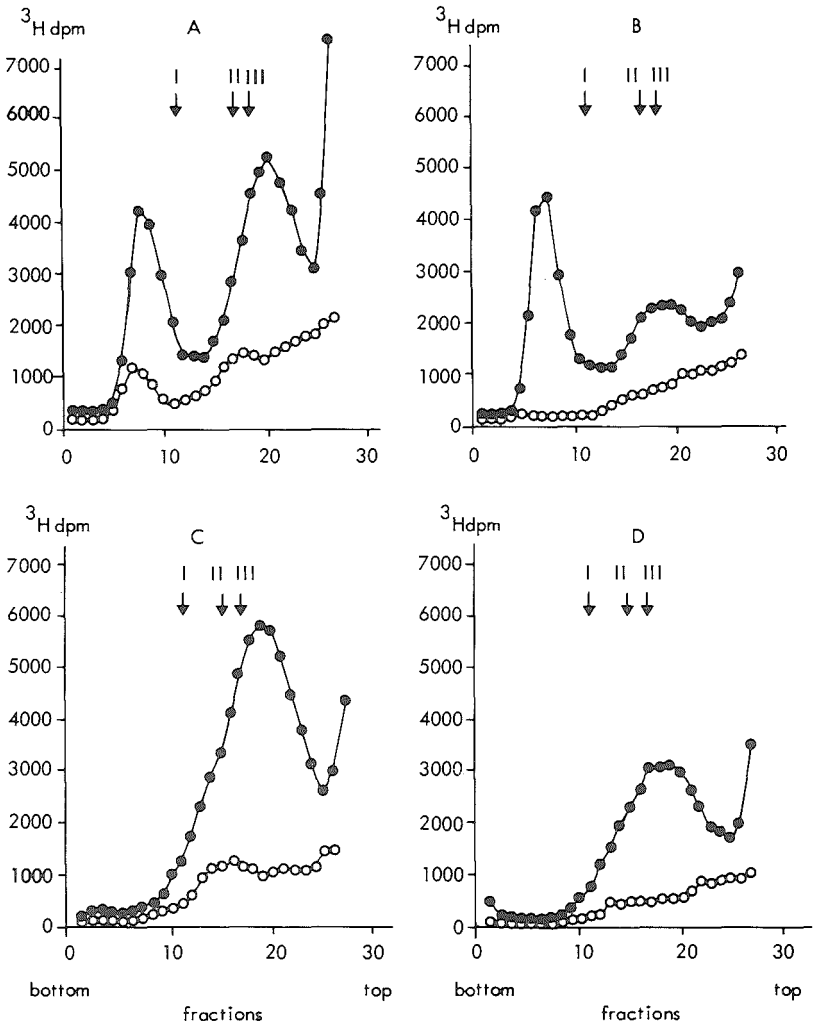
Fig. 4. Sucrose-gradient centrifugation of partially purified androgen receptors from ram seminal vesicles cytoplasm. Eluates from the experiment as described in Fig. 5 were prepared as follows. After incubation of the cytosol with [^{3}H]methyltrienolone and ADP–sepharose, the gel was washed with buffer A (12 × 3 ml) and eluted with buffer A containing 0.6 M KCl (15 × 3 ml). Fractions 5, 6, 7 and 8 were pooled (10500 dpm/100 µl) and 250-µl samples were layered on 10–30% sucrose gradients before (•) and after heating for 30 min at 50°C (○). Centrifugation was performed in a Beckman VTi-65 rotor as described in the legends to Fig. 5. (A) 10–30% sucrose in buffer A containing 0.6 M KCl; (B) 10–30% sucrose in buffer A containing 50 mM molybdate. Arrows indicate positions of sedimentation marker. I, γ-globulin (7.2S); II, bovine serum albumin (4.6S); III, ovalbumin (3.6S).

analyzed with Sephadex LH-20 gel filtration no radioactivity was found in the void volume fractions.

Sucrose-gradient analysis of [^{3}H]methyltrienolone-labelled cytosol

Cytosol prepared in buffer containing 50 mM molybdate was labelled with [^{3}H]methyltrienolone in the absence ("hot") and presence of a 100-fold excess of non-radioactive methyltrienolone ("cold"). Fig. 5A shows 2 distinct [^{3}H]methyltrienolone-binding proteins, one sedimenting at 9S and one sedimenting at 3S on sucrose gradients containing 50 mM molybdate.

Fig. 5. Sucrose-gradient centrifugation of ram seminal vesicle cytoplasmic androgen receptors. Panel A and C ("parallel" incubation): Cytosol prepared in buffer A with 50 mM molybdate was incubated with 10 nM [^{3}H]methyltrienolone in the absence ("hot") or presence of a 100-fold excess of non-radioactive methyltrienolone ("cold") for 20 h at 6°C under continuous agitation. After incubation and treatment with charcoal to remove excess non-bound steroid,



250-μl samples were layered on continuous 10–30% sucrose gradients prepared in buffer A either containing 50 mM molybdate (A) or 0.6 M KCl (C). After centrifugation for 210 min at 1°C in a Beckman VTi-65 rotor at 370 000 × g_{av} in a Beckman L-65-5 centrifuge, 200-μl fractions were collected from the bottom of the tube and counted for radioactivity (●, total; ○, aspecific binding). Panel B and D ("supernatant" fraction): A fraction of the same "hot" cytosol (40 ml) was labelled in the presence of swollen ADP–sepharose under continuous agitation. After incubation for 20 h at 6°C the fraction not bound to ADP–sepharose was analyzed (after charcoal treatment) by sucrose-gradient centrifugation in the same run as described above (B, 50 mM molybdate in buffer A; D, 0.6 M KCl in buffer A). (●, total binding in supernatant fraction; ○, supernatant fraction after heating for 30 min at 50°C in the presence of 1 μM non-radioactive methyltrienolone). I, γ-globulin (7.2S); II, bovine serum albumin (4.6S); III, ovalbumin (3.6S).

Another aliquot of the same "hot" cytosol was mixed with ADP–sepharose at the start of the incubation, allowing the proteins to bind to the gel during the labelling procedure with [^{3}H]methyltrienolone. After incubation and separation of the non-bound ("supernatant") fraction from the gel by low-speed centrifugation, the "supernatant" was analyzed on sucrose gradients containing 50 mM molybdate (Fig. 5B).

The slower sedimenting [^{3}H]methyltrienolone-binding form (3S) was significantly reduced by ADP–sepharose binding, whereas approx. the same amount of the faster sedimenting form (9S) was still present (Fig. 5A compared to Fig. 5B). This implies that only the 3S form of the androgen receptor was bound to the gel. This slower sedimenting form is probably the activated form of the androgen receptor, because activation of steroid receptors is commonly required before binding to nuclei or DNA-like matrices occurs (Spelsberg et al., 1971; Schrader et al., 1972; Weichman and Notides, 1979; Liao et al., 1980; Noma et al., 1980; Sato et al., 1980).

For studying the sedimentation behaviour of the methyltrienolone-binding proteins in high salt, the samples ("hot", "cold", Fig. 5C, and "supernatant" before and after heating for 30 min at 50°C, Fig. 5D) were centrifuged on sucrose gradients containing 0.6 M KCl. A complete shift to the slower sedimenting 3S form was observed, which might reflect activation of the receptor (Fig. 5C compared to Fig. 5A; Fig. 5D compared to Fig. 5B). The fraction which did bind to ADP–sepharose was already analyzed as described in Fig. 4, showing only the 3S form of the androgen receptor either on sucrose gradients containing 0.6 M KCl or 50 mM molybdate.

Denaturation and dissociation of partially purified [^{3}H]methyltrienolone-labelled cytoplasmic androgen receptors

The thermal lability and dissociation kinetics of partially purified [^{3}H]methyltrienolone-labelled androgen receptors were investigated by incubating purified [^{3}H]methyltrienolone-labelled androgen-receptor complexes in the absence and presence of a 100-fold excess of non-radioactive methyltrienolone.

In the absence of excess steroid, incubation of the ^{3}H-labelled androgen receptors for 50 h at 5 or 10°C decreased the binding only for 13 and 18% respectively (Fig. 6A). At higher temperatures dissociation was more pronounced. This suggests that [^{3}H]methyltrienolone was bound to a thermolabile androgen receptor with high affinity. The decrease in binding shown in Fig. 6A presumably is the result of both dissociation of the steroid–receptor complex and denaturation of the receptor.

In the presence of a 100-fold excess of non-radioactive methyltrienolone, the decrease in binding was more pronounced, probably as a result of the dissociation of the [^{3}H]methyltrienolone–receptor complex (Fig. 6B).

Scatchard analysis

For estimation of the affinity constant of partially purified androgen receptors,

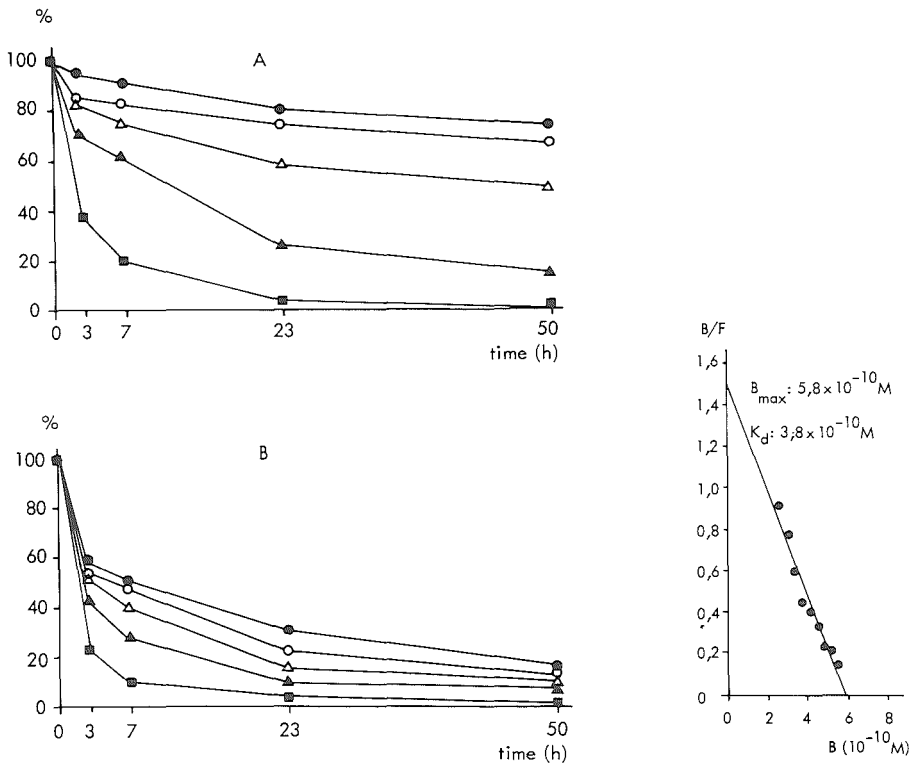


Fig. 6. Denaturation and dissociation kinetics of partially purified [^{3}H]methyltrienolone–receptor complexes. 0.37 nM [^{3}H]methyltrienolone–receptor complex was incubated for different times at different temperatures (●, 5°C; ○, 10°C; ×, 15°C; ▲, 20°C; ■, 25°C). At time intervals as indicated on the abscissa, samples were withdrawn from the incubation mixtures and analyzed for bound radioactivity with Sephadex LH-20 gel filtration. A, without excess methyltrienolone; B, with 3.7 × 10^{-8} M non-radioactive methyltrienolone.

Fig. 7. Scatchard analysis of partially purified [^{3}H]methyltrienolone-labelled androgen receptors from ram seminal vesicles. Part of a labelled fraction (5.5 × 10^{-10} M [^{3}H]methyltrienolone), obtained as described in the legend to Fig. 4, was incubated for 20 h at 10°C with increasing amounts of added [^{3}H]methyltrienolone (up to 10 nM). After incubation the samples were analyzed with Sephadex LH-20 gel chromatography.

the conditions for equilibrium between steroid–receptor complex and free steroid should be known. However, from the results presented in Fig. 6, it is not possible to define the conditions for minimal denaturation of the receptor during equilibration.

For Scatchard analysis, incubation for 20 h at 10°C condition was chosen, as a compromise between minimal denaturation and maximal dissociation. The Scatchard plot is presented in Fig. 7, showing a single type of binding sites with an apparent K_D of 3.8 × 10^{-10} M. The initial sample contained 5.5 × 10^{-10} M bound

[^{3}H]methyltrienolone, as derived from the radioactivity present in the eluate after ADP–sepharose chromatography, which was demonstrated to be receptor-bound with sucrose-gradient centrifugation and with Sephadex LH-20 gel filtration. With Scatchard analysis a B_{max} of 5.8×10^{-10} M was found (Fig. 7).

Specificity of [^{3}H]methyltrienolone binding to partially purified cytoplasmic androgen receptors

The specificity of [^{3}H]methyltrienolone binding to partially purified cytoplasmic androgen receptors was studied by incubations in the absence or presence of 10 and 100 nM non-labelled competitors and subsequent analysis with Sephadex LH-20 gel filtration. Results are expressed as percentage competition and are listed in Table 1. Androgenic steroids showed significant competition, whereas competition with estrogens and progestins was less pronounced.

Nuclear androgen receptors

Nuclear androgen receptors were labelled by incubating tissue minces with [^{3}H]-testosterone and were analyzed with sucrose-gradient centrifugation and agar-gel electrophoresis. A specific androgen-binding protein sedimented at 3S on sucrose gradients (Fig. 8A). Agar-gel electrophoresis at pH 8.4 showed an electrophoretic mobility of bound radioactivity towards the anodic region of the agar gel (Fig. 8B).

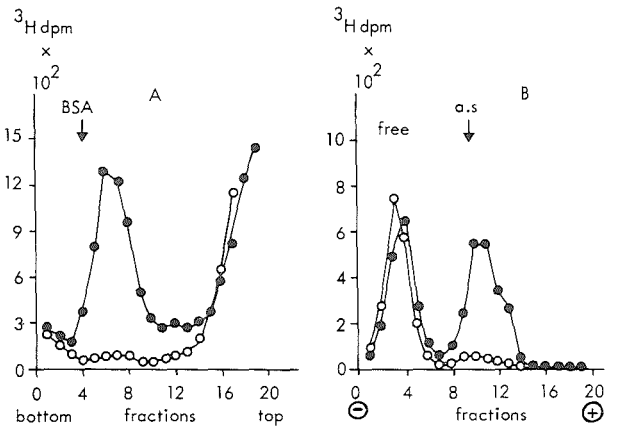


Fig. 8. Sucrose-gradient centrifugation and agar-gel electrophoresis of nuclear androgen receptors from ram seminal vesicles. Labelled nuclear extracts were analyzed with sucrose-gradient centrifugation (A) or agar-gel electrophoresis (B). ●, total binding; ○, non-specific binding; B.S.A., position of sedimentation marker, bovine serum albumin (4.6S); a.s., "application site", sample applied at the start of electrophoresis; ⊕ and ○, anodic region, cathodic region of the agar gel respectively; free, position of free steroid after electrophoresis.

Table 1

Specificity of [^{3}H]methyltrienolone binding to partially purified cytoplasmic androgen receptors from ram seminal vesicles

Partially purified labelled cytosol receptors were incubated for 20 h at 10°C after addition of an extra amount of 1 nM [^{3}H]methyltrienolone (control incubation). In parallel incubations an additional amount of 10 nM and 100 nM competitors was added. After incubation the samples were analyzed for bound radioactivity with Sephadex LH-20 gel filtration. The difference found in bound radioactivity after incubations without and with 100 nM non-radioactive methyltrienolone was arbitrarily chosen as 100% competition.

	% competition	
Competitor	10 nM	100 nM
Methyltrienolone	90	100
Dihydrotestosterone	93	103
Testosterone	59	96
Estradiol	24	50
Progesterone	22	37
R5020	15	40
Diethylstilbestrol	22	30

DISCUSSION

The present results demonstrate that androgen receptors are present in cytosols and nuclear extracts of ram seminal vesicles. Possible binding of steroid to other androgen-binding proteins which might be present in the cytosols was circumvented by the use of methyltrienolone (R1881), a synthetic steroid with a high affinity for androgen receptors (Bonne and Raynaud, 1975). This steroid does not bind to serum proteins (like sex-hormone-binding globulin) or prostatic binding proteins. Binding of [^{3}H]methyltrienolone to a possible progesterone receptor which also binds methyltrienolone with high affinity (Asselin et al., 1979) was insignificant as illustrated by the specificity studies described in Table 1, which show that only androgenic steroids competed with the [^{3}H]methyltrienolone binding for the partially purified androgen receptor. In experiments with unpurified cytosol samples, addition of 500-fold excess triamcinolone acetonide (to block possible progesterone receptors present) did not result in a decrease of specifically bound [^{3}H]methyltrienolone. This implies that also the non-purified [^{3}H]methyltrienolone-binding protein behaves as the androgen receptor.

In initial experiments it was found that with DNA–cellulose chromatography part of the DNA was lost from the cellulose during incubations with cytosol samples, resulting in very low yields of receptor (~10%) after purification. For the experiments described in this paper ADP–sepharose was used, because it is a stable matrix, resistant to DNAases and probably acting in a similar way to DNA–cellu-

lose in receptor binding (Mulder et al., 1980). The form of the receptor which actually binds to nuclei or DNA-like matrices is generally defined as the "activated" receptor. These activated forms can be obtained by several procedures, such as: heating, Sephadex G-25 gel filtration, dialysis, increasing the salt concentration, dilution and ammonium sulphate precipitation. This activation process has been extensively studied for different steroid receptors (De Sombre et al., 1972; Fleischmann et al., 1979; Weichman and Notides, 1979; Bailly et al., 1980; Bloom et al., 1980; Gschwendt, 1980) and recently also for androgen receptors (Noma et al., 1980; Sato et al., 1980). Only the 3S form of the cytoplasmic androgen receptors from ram seminal vesicles was retained by ADP–sepharose, whereas the faster sedimenting (9S, "native") form did not show any interaction with the gel. This suggests that also for cytoplasmic androgen receptor from ram seminal vesicles an activation step is required for binding to ADP–sepharose. Whether this activation process is identical with a conformational change of 9S to 3S, or involves a separate step, cannot be deduced from our results. Molybdate does not completely prevent the presence of the ADP–sepharose binding form in the cytosol (Fig. 5A), which has been reported for several other steroid receptors (Leach et al., 1979; Grody et al., 1980; Maki et al., 1980; Nishigori and Toft, 1980; Noma et al., 1980). The formation of the ADP-binding form in our experiments might have occurred prior to the addition of molybdate. This is in agreement with observations on the avian progesterone receptor, where molybdate no longer inhibits binding to ATP–sepharose once the receptor is present in its DNA-binding form (Toft and Nishigori, 1979), and with glucocorticoid, androgen and estrogen receptors, where molybdate had no effect on nuclear binding of previously activated steroid–receptor complexes (Noma et al., 1980).

Additionally, increasing the salt concentration completely transformed the receptor to its slower sedimentation form (Fig. 5C, D), which is a common feature for steroid receptors (Steggles et al., 1971; De Sombre et al., 1972; Fleischmann and Beato, 1979; Bailly et al., 1980).

The Scatchard plot presented in Fig. 7 only gives an impression of the order of magnitude of the affinity constant. Starting with a partially purified androgen receptor, completely occupied with labelled steroid, the precise equilibrium conditions (minimal denaturation and maximal dissociation) cannot be exactly determined. Under conditions for minimal denaturation and incomplete equilibrium (dissociation too slow), the affinity constant found will be too high.

For the nuclear androgen-receptor, sedimentation profiles after sucrose-gradient centrifugation (Mainwaring, 1977) and electrophoretic mobility during agar gel electrophoresis (Wagner, 1972) are similar to those of rat prostate nuclear androgen receptor.

Receptor proteins are present in only minute quantities in androgen target cells. Approx. 40 μg of receptor protein is present in a kilogram of rat prostate tissue (Mainwaring and Irving, 1973) or expressed otherwise: the ventral prostates of 4000 rats. The ram seminal vesicles used for the studies described in this report con-

tained the same amount of androgen receptors per amount of tissue, and seminal vesicles of approx. 200 rams would contain the same amount of receptor as the ventral prostates of 4000 rats. Therefore, seminal vesicle tissue of rams appears to be a suitable source for large-scale purification of both cytoplasmic and nuclear androgen receptors.

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Appendix paper III

(Bioch. Bioph. Res. Commun., 104 (1982) 1279-1286.

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PURIFICATION OF THE ANDROGEN RECEPTOR OF SHEEP SEMINAL VESICLES

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An androgen receptor was isolated and partially purified from the cytosol fraction of ram seminal vesicles. An almost two thousand fold purification with a recovery of 33% could be obtained by chromatography on 2',5'-ADP-sepharose, ammoniumsulphate precipitation and gel filtration on Ultrogel ACA-44. The labelled receptor was characterized by electrophoresis and sucrose gradient centrifugation (sedimentation at approximately 3S). The purified receptor has a DNA binding-site and is specific for androgenic steroids.

INTRODUCTION

It is now generally accepted that steroid hormone receptors, after binding their specific steroid and subsequent activation, change to a form with a high affinity for DNA and/or nuclear chromatin. The binding of the steroid-receptor complex to nuclear acceptor sites results in steroid specific responses of the cell (1). Purified receptor preparations are of importance for studying steroid-receptor complexes as metabolic regulators of genetic transcription in vitro.

Large scale purification of androgen-receptor complexes has not yet been successful, probably due to the extreme lability of this receptor and the lack of a suitable source for isolation of the androgen receptor complex. The small size of the rat prostate permits purification of the receptor only at a small scale (2). The seminal vesicles of the ram contain an androgen receptor in a similar concentration, but this tissue is available in large

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amounts and appears an attractive source for isolation and purification of larger quantities of receptor (3).

In this report we describe a procedure for purification of the activated (DNA-binding) form of the cytoplasmic androgen receptor of the seminal vesicle of the ram.

MATERIALS AND METHODS

Tissue: Seminal vesicle tissue from adult rams was removed as soon as possible after killing the animals and was directly frozen and stored at -80°C.

Preparation of cytosol: After thawing on ice the tissue (75-100g) was homogenized with a Waring Blendor for 3x20 s at 4°C in 1-1.5 vol. buffer A (50 mM Tris-HCl, 1.5 mM EDTA, 1.5 mM dithiothreitol with 10% glycerol; pH 7.4), containing 50 mM sodiummolybdate and 10 nM testosterone. The homogenate was centrifuged for 60 min at 96,000xg_{av} in a Beckman SW-27 rotor and the supernatant was designed as "cytosol". In some experiments phenylmethylsulphonylfluoride (PMSF, 0.6 mM) and leupeptin (0.25 mM) were added during homogenization.

Purification procedures and labelling of receptors:

2',5'-ADP-sepharose chromatography:

Cytosol (100-150 ml) was diluted five times with buffer A containing 50 nM testosterone and 30-40 ml of 2',5'-ADP-sepharose (2 µmoles/ml; Pharmacia, Sweden) swollen in buffer A was added. After incubation for 20 h at 4°C, the ADP-sepharose was extensively washed with buffer A, to remove unbound proteins and excess non-labelled steroids. The receptors were labelled by incubating the gel in 50 ml buffer A containing 10 mM sodiummolybdate and 20 nM |^{3}H|dihydrotestosterone (S.A. 137 Ci/mmol) for 60 h at 4°C with continuous shaking. Subsequently the gel was packed in a glass column (size: 1.6x20 cm) and after washing with buffer A, the receptors were eluted with a 0-1.0 M KCl gradient in buffer A (30 fractions of 5 ml). For stabilization of receptors 2 nM |^{3}H|-dihydrotestosterone was added to the eluates.

Ammoniumsulphate precipitation:

Selectively pooled eluate fractions, obtained after 2',5'-ADP-sepharose chromatography, were incubated with ammoniumsulphate (50% saturation) for 1 h at 0°C. The precipitate, obtained by centrifugation for 30 min at 20,000xg, was dissolved for 1 h at 0°C in 1 ml of buffer A with 0.4 M KCl. For stabilization of receptors, 2 nM |^{3}H|dihydrotestosterone was added.

Ultrogel ACA-44 chromatography:

Gel filtration of the concentrated receptor preparation on Ultrogel ACA-44 (LKB Instrument GmbH, Germany) was performed in buffer A containing 0.1 M KCl. Elution was performed in a glass column (size: 0.9x60 cm) with an elution rate of 3.0 ml/h and 1 ml fractions were collected. In some experiments the eluate of the Ultrogel ACA-44 column was collected directly on a small (0.2 ml) 2',5'-ADP-sepharose column and eluted from this column with a salt gradient.

Desalting:

Samples of the eluates, obtained from 2',5'-ADP-sepharose columns, were desalted by gel filtration on small columns with Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, U.S.A.).

RESULTS AND DISCUSSION

The cytosol fraction isolated from seminal vesicles of the ram contains an androgen receptor (3). Due to the presence of considerable amounts of androgens in these animals (approximately 690 pg testosterone and dihydrotestosterone per gram of seminal vesicle tissue) a large number of these receptors will be occupied with endogenous steroid. To obtain receptor preparations labelled with radioactive steroids a long incubation at 4-10^{o}C is required in order to exchange endogenously bound steroid with added radioactive ligand. We have previously observed a stabilizing effect of molybdate on the receptor in crude cytosol fractions. In addition it appeared to be of advantage to bind the receptor first to a matrix containing an immobilized nucleotide (2',5'-ADP-sepharose) and to remove contaminating cytosol proteins before the exchange of the non-radioactive endogenous androgens with radioactive dihydrotestosterone. The exchange was optimal after 60 h at 4^{o}C and remained constant for periods up to a week.

The specificity of the receptor bound to 2',5'-ADP-sepharose was evaluated in a competition study with |^{3}H|dihydrotestosterone (Fig. 1). Almost equal competition was obtained for dihydrotestosterone, testosterone and the synthetic androgen methyltrienolone (methyltrienolone does not bind to androgen transport proteins present in plasma (4,5)). Other steroids like estradiol, progesterone and triamcinoloneacetonide only competed at very high concentrations.

The labelled androgen receptor complexes were eluted from 2',5'-ADP-sepharose with a salt gradient. At this stage a 35-50 fold purification with a recovery of 70% was generally obtained. The diluted eluate could be concentrated and further purified by precipitation with ammoniumsulphate at 45-50% saturation. It has been shown that several hormone receptors in crude cytosol frac-

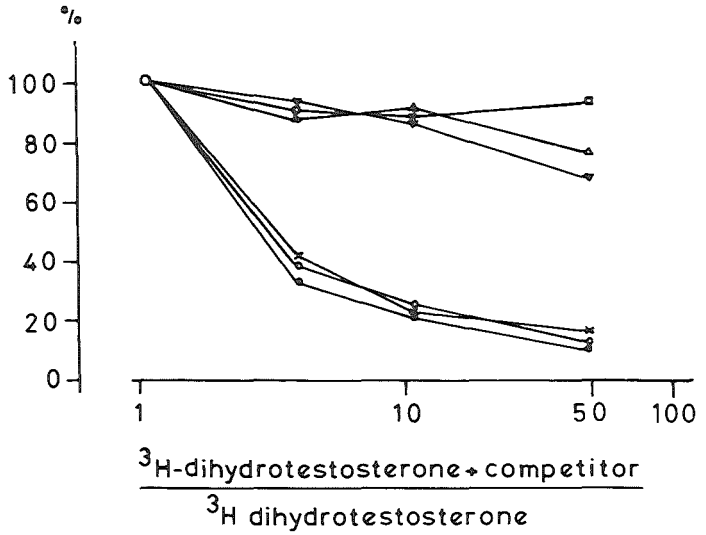

Figure 1: Competition of various steroids for the androgen receptor of ram seminal vesicles. The steroid-receptor complex bound to 0.1 ml 2',5'-ADP-sepharose, prepared as described in the Method section, was incubated for 60 h at 4°C with 20 nM |^{3}H|-dihydrotestosterone in the absence and presence of 60, 200 and 1000 nM non-labelled competitors. After incubation the 2',5'-ADP-sepharose gel-pellets were washed extensively with buffer A and the gels were counted for radioactivity.
●——●: 5α-dihydrotestosterone (17β-hydroxy-5α-androstane-3-one); x——x: testosterone (17β-hydroxy-4-androstene-3-one); ▼——▼: 17β-estradiol (1,3,5-(10)-estratriene-3,17β-diol); o——o: methyltrienolone (17β-hydroxy-17α-methyl-4,9,11-estratriene-3-one); Δ——Δ: progesterone (4-pregnane-3,20-dione); ◘——◘: triamcinoloneacetonide (9α-fluoro-11β,21-dihydroxy-16α,17-isopropylidenedioxy-1,4-pregnadiene-3,20-dione).
"%" = percentage residual binding; the amount of |^{3}H|dihydrotestosterone bound to the receptor incubated in the absence of competitors was chosen as 100%.
"|^{3}H|dihydrotestosterone+competitor/|^{3}H|dihydrotestosterone" = ratio of the sum of the steroid concentration of |^{3}H|dihydrotestosterone (20 nM) + competitor (variable), divided by the concentration of |^{3}H|dihydrotestosterone (20 nM).

tions will precipitate with 30-35% saturated ammoniumsulphate, but the partially purified androgen receptor in the 2',5'-ADP-sepharose eluate could only be precipitated with approximately 10% yield with 35% satured ammoniumsulphate. After increasing the ammoniumsulphate concentration to 50% saturation, more than 80% of the receptors were precipitated. The receptors in the ammoniumsulphate precipitate could be dissolved almost completely in a buffer containing 0.4 M KCl. Subsequent purification of the concentrated receptor sample was performed by gel filtration on Ultrogel ACA-44 (Fig. 2). The receptor is retarded considerably

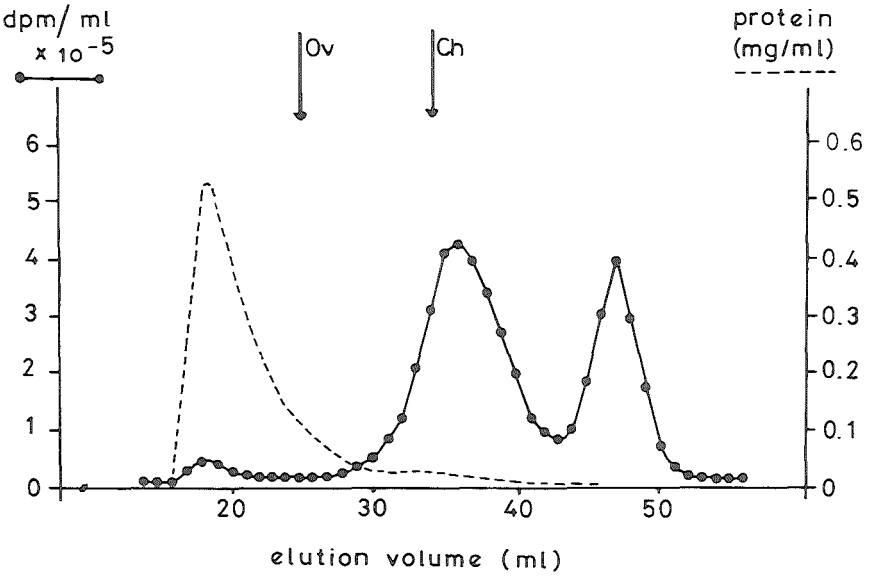

Figure 2: Gel filtration on Ultrogel ACA-44 of androgen receptors from ram seminal vesicles after partial purification with 2',5'-ADP-sepharose chromatography and ammoniumsulphate precipitation. Protein bound radioactive steroid, indicated on the vertical axis as dpm/ml, has mainly eluted between 30 and 43 ml; free steroid between 44 and 51 ml and non-retarded proteins between 16 and 25 ml. Ov and Ch positions of marker proteins: ovalbumin (MW 45,000) and chymotrypsinogen A (MW 25,000).

on this column and if no specific interaction occurs with the gel, this would suggest a Stokes radius for the receptor comparable to globular molecules with a molecular weight between 20,000 and 25,000 Dalton. The receptor eluted from the Ultrogel ACA-44 column could be concentrated on a small column with 2',5'-ADP-sepharose and could be eluted with a salt gradient, resulting in approximately 2000-fold purification with a recovery of 33%. After desalting, this partially purified receptor bound to phosphocellulose and DNA-agarose, reflecting the presence in the receptor molecule of a domain with high affinity for DNA-like structures. The successive purification steps are summarized in Table 1.

The purified receptor could be precipitated with protamine sulphate in the presence of 10 mM pyridoxal phosphate, comparable to observations previously made for the androgen receptor from

Table 1

	volume (ml)	protein (mg)	receptor (pmol)	receptor (pmol/ mg protein)	recovery (%)	purification factor
1. Cytosol	104	1570	90	0.057	100	1
2. ADP-sepharose chromatography	80	27.5	60	2.18	68	38
3. Ammonium sulphate precipitation	1.0	8.3	48	5.7	54	100
4. ACA-44 gel filtration + ADP-sepharose chromatography	3.0	0.27	29	109	33	1.900

Table 1: Purification of androgen receptors from ram seminal vesicles. The total amount of receptor present in the cytosol was estimated as the amount of receptor bound to twice the amount of 2',5'-ADP-sepharose as used for preparative purposes. This amount was defined as 100%. Further increase in the amount of 2',5'-ADP-sepharose did not increase the amount of receptor bound.

prostate nuclei (6). The purified receptor was further characterized with agar gel electrophoresis (Fig. 3A) and sucrose gradient centrifugation (Fig. 3B). The migration towards the anodic region in the agar gel during electrophoresis (Fig. 3A) distinguishes the receptor clearly from dihydrotestosterone binding proteins like SHBG (sex hormone binding globulin), which has an electrophoretic mobility towards the cathodic region of the agar gel (7). In sucrose gradients the purified receptor sedimented at approximately 3S (Fig. 3B), as has been observed for the nuclear form of the receptor present in ram seminal vesicles (3). Isolation of the receptor in the presence of the protease inhibitors PMSF and leupeptin did not influence the elution profiles of the different columns or the sedimentation behaviour. This implies that proteolysis is no major factor in the formation of the "activated" 3S (nuclear-binding) form.

In conclusion, the overall recovery of the purification procedure of the androgen receptor from ram seminal vesicles was 33% and the purification was almost 2000-fold. Assuming one steroid binding site per receptor molecule, the purified preparation has

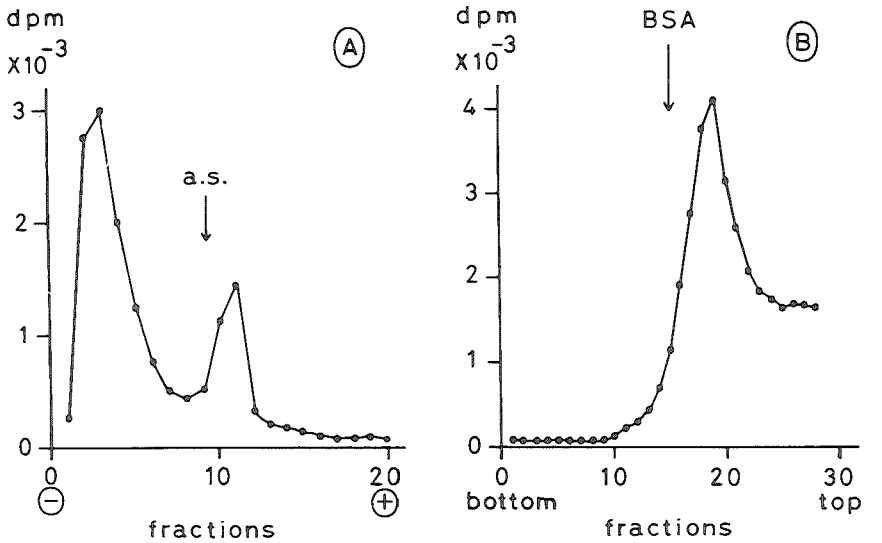

Figure 3: Agar gel electrophoresis and sucrose gradient centrifugation of androgen receptors from ram seminal vesicles after purification as described in Table 1. Experimental procedures as described previously (3).
A: Agar gel electrophoresis after addition of 2 nM [^{3}H]dihydrotestosterone (⊕ : anode; ⊖ : cathode; a.s.: application site of sample at start of electrophoresis. Free steroid was present in fraction 2-6; protein bound steroid was present in fraction 10 and 11).
B: Sucrose gradient centrifugation (BSA indicates position of bovine serum albumin (4.6S) after centrifugation). Centrifugation was performed after addition of 1 mg/ml BSA to the sample. Free steroid remained on top of the gradient.

a purity of 1%. The labile nature of the purified androgen-receptor complex made it thusfar difficult to accomplish further purification. This partially purified receptor preparation will now be used in studies on the interaction of androgen receptors with the genome and for preparation of antibodies against androgen receptors.

The purification procedure described for androgen receptors isolated from tissues containing relatively large amounts of endogenous steroids, might also be useful for the purification of androgen receptors from human prostatic tissues.

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