ON THE SUBCELLULAR DISTRIBUTION OF OESTRADIOL RECEPTORS IN RAT TESTIS AND UTERUS

Proefschrift
ter verkrijging van de graad van doctor in de geneeskunde aan de Erasmus Universiteit te Rotterdam op gezag van de rector magnificus Prof.Dr. B. Leijnse en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaats vinden op vrijdag 10 juni 1977 des namiddags te 3.00 uur.

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Dit proefschrift werd bewerkt in het instituut Biochemie II (Chemische Endocrinologie) van de Faculteit der Geneeskunde, Erasmus Universiteit te Rotterdam.

Het onderzoek werd mede mogelijk gemaakt door steun van de stichting voor Medisch Wetenschappelijk Onderzoek FUNGO.

Foar Heit en Mêm

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Paper I

Effects of oestradiol, hypophysectomy and age on cytoplasmic oestradiol receptor sites in rat testis interstitial tissue
Willem de Boer, Eppo Mulder and Henk J. van der Molen
J. Endocr. 70 (1976) 397-407.

Paper II

Comparative study of nuclear binding sites for oestradiol in rat testicular and uterine tissue. Determination of low amounts of specific binding sites by an ^{3}H-oestradiol-exchange method
Willem de Boer, Joan de Vries, Eppo Mulder and Henk J. van der Molen
Biochem. J. 162 (1977) 331-339.

Paper III

Kinetics of in vitro binding of oestradiol in subcellular fractions of testicular and uterine tissue. Characterization of oestradiol binding in testicular nuclei
Willem de Boer, Joan de Vries, Eppo Mulder and Henk J. van der Molen
J. Steroid Biochem. (1977) in press.

Introduction and scope of the thesis

1.1 Steroid receptors and steroid hormone action

There is good evidence that the concentration of nuclear steroid-receptor complexes shows a direct relationship with the effect of steroids on cells. Anderson et al. (1) showed that uterotrophic responses correlated well with nuclear oestradiol receptor levels. Correlations between concentrations of nuclear receptors and cell specific effects have also been observed for glucocorticoids in eukaryotic cells (2), oestrogens in chicken liver (3,4) and for oestradiol and progesterone in chick oviduct (5-10).

In the search for effects of steroid hormones on cellular metabolism it has been observed that oestradiol can affect several important parameters that may influence gene expression, such as synthesis of histone and non-histone proteins in uterus (11-14), the rate of peptide elongation of uterine ribosomes (15) and the rate of methylation of ribosomal and tRNA's (16,17)

The involvement of RNA synthesis during the early effects of steroids has also been well established for several tissues. Within 0.5-4 h after administration of oestradiol to mature or ovariectomized rats, uterine nuclear RNA-polymerase activity is stimulated (18,19,20), predominantly at nucleolar sites (21,22). The oestrogen induced stimulation of RNA synthesis could be prevented if puromycine or cycloheximide was administered prior to injection of the hormone (18,23), indicating the necessity of a continuous protein synthesis for the expression of the oestrogen effect. In fact, a group of proteins, including one acidic protein, called IP (induced protein), is formed in response to oestrogen administration (24,25,26). The synthesis of this protein, which appears within 15 min after oestradiol injection, can be blocked by actinomycin D (27,28) and cordycepin (29).However, the biological significance of this IP, which recently has been purified and characterized as a polypeptide of molecular weight 45,000 (30), and its possible relation to the stimulation of

nucleolar RNA-polymerase remains to be elucidated.

In more recent studies the effect of steroid hormones on template activity of nuclear chromatin of target tissues has been investigated. It was found that both nucleolar (I) and nucleoplasmic (II) RNA-polymerase were stimulated but at different times after oestradiol administration (31,32,33, 34).

More detailed studies concerning the possible role of hormone-receptor complexes were carried out by O'Malley and coworkers for the effects of progesterone and oestradiol in the chick oviduct (35,36). They could demonstrate that the accumulation of steroid-receptor complexes in nuclei or on the chromatin caused an increase in the number of initiation sites for RNA-polymerase molecules prior to the increase in ovalbumin synthesis, which is the cell's response to hormone administration (10,37,38).

Based on the kind of information described above, it is now generally believed, that effects of steroid hormones in target cells are mediated through the binding of steroids to specific receptor molecules and the subsequent interaction of steroid-receptor complexes with the chromatin. The quantity of nuclear receptor molecules appears to be important for the magnitude of a cell's response. The interaction between the steroid-receptor complexes and the genome causes activation or derepression of transcription or post-transcriptional regulation of RNA synthesis. The products of mRNA and rRNA dictate the synthesis of specific proteins, which ultimately determine the morphogenetic and physiological responses to the hormone.

1.2 Scope of this thesis

It has been shown previously by Brinkmann et al. (39), that the interstitial compartment of the rat testis contains a limited number of specific oestradiol receptor sites with a high affinity for oestrogens (K_a for oestradiol is $10^{10} M^{-1}$). This receptor, which in the presence of oestradiol

will be translocated into the nuclear fraction (40), shows a steroid specificity comparable to that of the uterine receptor for oestradiol (41).

The physiological significance of the uptake of oestradiol and its subsequent binding to the oestradiol receptor in the testis, is not yet understood. In studies of de Jong et al. (42,43) it was found that oestradiol concentrations in rat testis interstitial tissue ($0.5-1 \times 10^{-9}$M) are higher than those in seminiferous tubules.

Actions of oestradiol in the testis on DNA, RNA and protein synthesis have been reported for Balb/c mice (44). It has also been suggested that after long-term treatment with oestrogens, the observed decrease of testosterone levels in rat plasma and testicular tissue would occur without interference of the LH secretion (45,46,47). Other studies (48), however, indicated that the observed oestradiol effect could be fully explained through a feedback action of administered oestrogens on pituitary LH secretion. The lack of a distinct and well-defined effect of oestradiol in the testis made it important to investigate whether oestradiol and oestradiol binding sites in both the cytoplasmic and nuclear compartments of the testicular tissue would show the same behaviour as in an established oestradiol target tissue. In some of our studies therefore the nuclear translocation of the oestradiol receptor in uterine tissue was used for comparison, because this tissue contains an oestradiol receptor which is known to be related to the effects of oestradiol on the uterus.

The results of experiments on the regulation of the cytoplasmic oestradiol receptor and the nuclear oestradiol receptor are discussed in chapter 6 and appendix papers I and II. Also in chapter 6 and appendix paper III the processes of translocation and nuclear binding of oestradiol-receptor complexes in testicular and uterine tissue are compared.

It is now well accepted that steroid hormones exert their actions in target tissues via the binding of steroid-receptor complexes to chromatin constituents in the nuclear fraction. There is hardly any information, however, about the nature

and the localization of the so-called 'acceptor sites' on the chromatin, which bind the steroid-receptor complexes. We have studied this aspect of steroid hormone action in uterine nuclei and preliminary results are discussed in chapter 5.

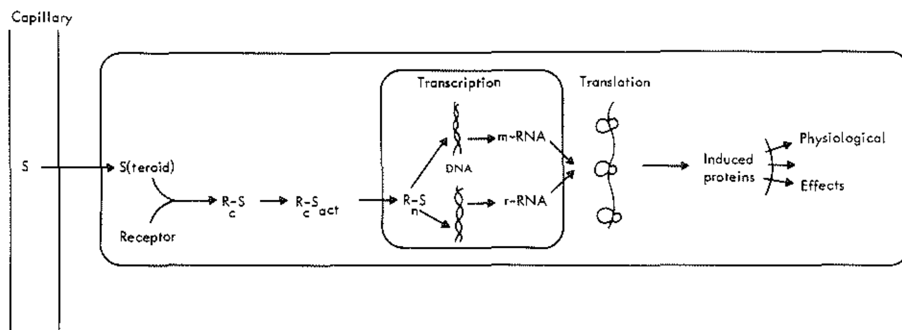


Figure 1 Interaction of steroid hormones with a target cell

Summary of the literature on steroid interactions with target cells

2.1 Interaction of steroid hormones with target cells

It is generally accepted that receptors play an important role in the expression of steroid hormone effects in target tissues (35,49-53) and binding of steroids to specific receptors appears to be related to and precedes the physiological effects of steroids. However, the precise sequence of events which occur between the entry of the steroid in the cell and the expression of the hormone effect is still unknown. Present knowledge about the fate of steroid hormones in a target cell can be summarized as depicted in Fig. 1. The steroid hormone enters a cell, binds to specific receptor proteins located in the cytoplasm, followed by translocation of the hormone-receptor complex into the nucleus where a presumed specific interaction of the complex with chromatin constituents ultimately leads to specific changes in the cell metabolism ascribed to that specific steroid hormone.

A brief summary about the following aspects, as indicated in Fig. 1 will be given in the following paragraphs:

a) entry of steroid hormones into the target cell.
b) nature of cytoplasmic receptors and the interaction of steroids with the receptor.
c) steroid induced changes of the cytoplasmic receptor.
d) translocation of the steroid-receptor complex from the cytoplasm into the nucleus.
e) interaction of steroid-receptor complexes with nuclear components.
f) dissociation of steroids from receptor and/or acceptor sites.
g) regulation of the amount of steroid hormone receptors.
h) receptors and actions of steroid hormones.

This discussion will mainly concern receptors for oestrogens and progestins.

2.2 Entry of steroid hormones into a target cell

Steroid hormones outside target cells are generally bound to (plasma) proteins which, in comparison to the intracellular receptor proteins, show a moderate binding specificity and a rather low affinity for steroid hormones ($K_{association} = 10^5 - 10^8 M^{-1}$). For example serum albumin, the most abundant protein constituent of plasma, binds oestradiol, progesterone, testosterone and oestrone with a K_a in the order of $10^5 - 10^6$ (54). Although the more specific steroid binding globulins (CBG, SBG, DBG, PBG, EBG) are present in smaller amounts, they still will strongly bind the larger part of steroids in the blood because of their higher affinity ($K_a = 10^7 - 10^9 M^{-1}$) even if total blood steroid levels are low(55-59).

The biological function of the plasma steroid binding proteins is not clear. They may protect steroids from degradation in the liver, but there is no strong evidence that blood proteins can indeed act as a reservoir from which steroids can be fed gradually to target organs. In fact, there are some indications that the biological activity of steroids is probably related to the unbound fraction of steroids in plasma (60,61), which is only a small percentage of the total amount.

Before the free steroid can interact with receptor proteins inside the cell, it has to pass the cell membrane. It has been generally assumed that the cell membrane provides little or no barrier to the diffusion of steroids into cells, because of their lipophilic properties. It has been shown by Jensen and Jacobson (62) that oestradiol and oestrone are taken up rapidly by most tissues of the rat, suggesting a simple but rapid diffusion process. A protein mediated process was proposed for the entry of oestradiol in uterine cells by Milgrom et al. (63), who found that the rate of entry of oestradiol was reaching a maximum in the range of

physiological hormone concentrations and that it could be inhibited by SH-blocking agents. This saturable protein is probably not the oestrogen receptor since the uptake of diethylstilboestrol, which also binds to the receptor, could not be blocked significantly. However using N-ethylmaleimide as a SH-blocking agent Peck et al (64) found no support for a saturable transport mechanism for oestradiol in uterine tissue.Similar results obtained with thymus cells indicated that the membrane of thymus cells is also freely permeable for corticoids. It can be concluded therefore that steroids can enter the cell by simple diffusion and there is little evidence to support facilitated transport mechanisms for steroid hormones.

2.3 Nature of the cytoplasmic receptor and the interaction of the steroid with the receptor

After entering a cell steroid hormones can be bound to many proteins. In most cases this binding is 'nonspecific', but the binding of the steroid to the receptor is the exception. This steroid receptor interaction is characterized by a high affinity (K_a=$2x10^{10}$-$1x10^{9}M^{-1}$), a limited number of receptor molecules per cell (3000-40,000 receptors/cell) and competition with small amounts of compounds which are chemically similar to the specifically bound hormones. Little is known about the physicochemical nature of the strong interaction between the steroid and the receptor molecule. It has been suggested that the bulkiness and the flatness of the steroid plays a more important role in receptor binding than the detailed electronic structure of the steroid nucleus (65). This would imply that the site of interaction is located inside the receptor molecule rather than on the surface of the protein. A localization of the steroid binding sites inside the receptor proteins could also be responsible for the very high affinity constant for receptor binding of steroids, the extremely slow rates of association and dissociation of steroids at low temperatures, the acceleration

of rates of exchange of unbound steroids with bound steroids by freezing and thawing and the inability of ethanol (30%) and detergents (Triton-X-100 or deoxycholate) to dissociate steroids from receptors at low temperatures (66). It has also been suggested (67) that the binding sites could be located in a hydrophobic pocket in the receptor protein and that it is necessary for the receptor to 'envelop' the steroid molecule.

Treatment of steroid hormone receptors with proteolytic enzymes generally destroys the steroid binding capacity. Other enzymes like DNAse and RNAse do not effect the binding properties indicating that nucleic acids do not play a significant role in the interaction between receptors and steroids.

The state of receptor molecules under physiological conditions is still unknown. On sucrose gradients sedimentation values between 3S and 12S have been observed depending on the protein concentration and the ionic strength of the medium (68). In hypotonic media receptors mostly appear as an 8S sedimenting entity. Increasing salt concentrations reduce the size of the molecule to a 4S sedimenting molecule at 0.4 M KCl. In 0.15 M KCl (isotonic media) receptor molecules with sedimentation values of 4S or 6S have been observed (69,70,71). After homogenization of uterine tissue without adding buffer only a 6S form of the oestradiol receptor could be detected in the cytosol (72). These observations indicate, but do not prove, the possibility that the 6S form is the predominant receptor form in the target cell cytoplasm. During the past years several attempts have been made to estimate the molecular weight of steroid hormone receptor molecules as they are present in the cell. For the uterine oestradiol receptor with a sedimentation value of 4S, Notides and Nielsen estimated a molecular weight of about 80,000 (73). Puca et al. consider the 8.6S sedimenting molecule of the oestradiol receptor to be a dimer of the 5.3S form, which may have a molecular weight of about 118,000 (74,75).

The progesterone receptor in chick oviduct is probably considerably larger than the uterine oestradiol receptor. The 8S form is thought to be a tetramer of the 4S form, which has a molecular weight of 90,000 (76). More recent studies showed that the 6S form of the progesterone receptor, probably the native form, consisted of two 4.2S subunits, with molecular weights of 110,000 and 117,000 respectively. The subunits differ in binding characteristics as will be discussed later (77).

It can be concluded that steroid receptors are proteins and show a very specific high affinity interaction with their respective ligand. The nature of the interaction between the steroid and the receptor molecule is not yet understood. Steroid hormone receptors in cell fractions have thusfar been detected only after binding of a radioactive steroid. Therefore these studies may not give reliable information about the receptor as it exists in the cytoplasm of a cell prior to the interaction with the steroid.

2.4 Steroid induced changes in the cytoplasmic receptor

Steroid-receptor complexes are found predominantly in the nuclear fraction, while free receptors remain in the cytoplasm. The nature of the changes in the structure of the receptor protein during or after binding of the steroid is still unknown. It has been shown (78,79,80) that free oestradiol receptors are more susceptible to changes in temperature than the oestrogen-receptor complex. Other studies (81,82,83) have shown that the presence of the steroid is essential for induction of specific changes in the structure of the receptor protein at a temperature of 20°C or higher, although opposite results have also been reported (83). After interaction of the steroid with its receptor site in vivo or in vitro a change in the sedimentation value of the complex has been observed: the 4S form of the uterine oestradiol receptor is converted to a 5S form (84). This change in receptor conformation probably occurs in the cytoplasmic compartment of the cell, because no oestradiol binding proteins with a se-

dimentation value of 5S can be observed if nuclei are incubated with oestradiol in the absence of cytoplasm (82,85). Also, if uterine nuclei are incubated with isolated oestradiol-receptor complexes at 25°C or at 0°C with cytosol which has been preincubated at 25°C, a considerable accumulation of nuclear receptor can be observed (85). The 4S → 5S receptor transformation takes place only slowly in the cold, proceeds rapidly at 25°C to 37°C and is accelerated with increasing pH over the range 6.5-8.5. The presence of salt, EDTA, Ca^{2+}, Mg^{2+} and Mn^{2+} retards the transformation(82).

A somewhat different view has been presented by Puca and coworkers (75,86), who believe that oestradiol binds to a 5.3S cytosol receptor and that this complex is cleaved by a proteolytic factor (the 'receptor transforming factor') to a 4.5S complex which is retained by the nuclei.

On basis of these results it could be proposed that the cytoplasmic receptor binds the steroid, thereby transforming the steroid-receptor complex to a 5S form which then translocates into the nucleus. Some data, however, do not support this. Siiteri et al. (87) reported the presence of 4S oestradiol binding macromolecules in carefully washed nuclei, and they suggested that the 4S form could have been converted into a 5S form inside the nucleus. In addition observations of Yamamoto (88) indicate that the presence of DNA could increase the conversion rate of the 4S form into the 5S form. Some yet unknown steps may therefore be involved in the transformation of cytoplasmic receptor molecules and the subsequent transfer of the complexes into the nucleus.

In contrast to the transformation theory of steroid receptors, it has been suggested by Notides and Nielsen (73), that the 4S and 5S sedimenting entities are chemically different molecules. They suggested that the 4S protein, with a mol. weight of 80,000 forms a complex with a second cytoplasmic protein, thus creating the 5S receptor molecule with a mol. weight of 130,000. This view is supported by studies of Yamamoto (88) which indicated in addition, that the unknown factor was present in both nontarget and target cells.
In summary,therefore,it can be concluded that steroid hormo-

nes interact with and bind to receptor proteins, in vivo as well as in vitro, but almost nothing is known about the nature of the free receptor in vivo, the exact subcellular localization of the receptor transformation process and the mechanisms which ultimately result in a receptor protein suitable for the nuclear translocation. Future studies with purified receptors (89,90,91) might provide the information necessary for a better understanding of the processes of steroid receptor interaction and receptor transformation.

2.5 The translocation of the steroid-receptor complex into the nucleus

Early observations from Jensen (92,93) indicated that after incubation of uterine tissue in vitro with radioactive oestradiol the major part of the hormone was located in the nuclear fraction. Subsequently Jensen and Gorski (92,94,95, 96) found that the nuclear accumulation of uterine hormone-receptor complexes was accompanied by a concomitant decrease of the steroid bound in the cytoplasmic cell fraction. Similar observations were made using in vivo studies (97,98). The process of translocation in uterus is not under the control of protein synthesis and RNA synthesis (95) and cannot be influenced by inhibitors which either affect the energy utilization of a cell (95) or interfere with microtubules or microfilaments, such as cytochalasin or vinblastin (99). However recent observations on chick liver indicate that a polypeptide with a high turnover might be involved in the interaction of steroid receptor molecules with the acceptor site (100). This could be one of the subunits of the receptor itself or a polypeptide involved in binding the hormone-receptor complex to the chromatin. Addition of SH-blocking agents (iodoacetamide or p-chloromercuribenzoate) gave a considerable inhibition of the process, probably via an interaction of the agents with SH-groups on the receptor surface (83,95).

In contrast to the results obtained for chicken liver it has been reported that the rate of translocation of uterine

receptor molecules was directly proportional to the concentration of the oestrogen-receptor complex in the cytoplasm at the start of the experiment (101). Therefore it can be assumed that translocation in uterus occurs without any interference of other cellular functions and appears to be the consequence of the inherent properties of the receptor as modulated by the binding of the steroid. The movement of the steroid-receptor complexes might take place via a simple diffusion process. For oocyte cytoplasm it has been shown that various materials could be transported from the cytoplasm into the nuclei via the nuclear pores (102,103), and that proteins with a molecular weight of about 70,000 enter nuclei only at a very slow rate. It remains to be proven whether receptor proteins with even larger molecular weights (80,000-130,000) can simply diffuse through the nuclear pores of a cell. From the calculation of the axial ratio of the receptor proteins (ratio 7-14) it was concluded that receptors are rod shaped (84,90,104) and this might be in favour of the transport of receptors into the nuclei.

Only after interaction of the receptor with its steroid hormone the hormone-receptor complex can translocate into the nucleus. Free receptor molecules are located in the cytoplasm. There are two possible explanations for this phenomenon: 1) the free receptor is bound to some cytoplasmic constituents and 2) free receptor molecules cannot pass the nuclear membrane. The observations of Little et al. (105), Robel et al. (106) and Hirsch et al. (107) are in favour of the first explanation. These groups did observe microsomal and lysosomal forms of oestradiol and androgen receptors respectively. Observations on the glucocorticoid receptor are in favour of the second explanation. Munck et al. (67) and Bell and Munck (108) showed that prewarmed (25°C) cytosol receptor could translocate into nuclei at a temperature of 3°C. This observation could reflect that in the presence of steroid at elevated temperatures an inactive form of the steroid receptor is transformed into an active form, which can pass the nuclear membrane. In all translocation

studies of steroid-receptor complexes very little attention has been paid to a possible role of the nuclear chromatin, the substance which ultimately binds the complexes. It has been observed, however, that progesterone treatment (3 days) of ovariectomized rabbits did increase the number of nuclear acceptor sites and the binding affinity for the retention of oestradiol-receptor complexes in uterine tissue (109).

From the summarized studies it is evident that the translocation of steroid receptor molecules is preceded by a still unknown change in the steroid-receptor complex itself. Studies presented in the literature make it very likely that this change is mainly due to the interaction of a second protein with the steroid-receptor complex (73,88,110). Binding of the transformed steroid-receptor complexes to high affinity nuclear acceptor sites will result in a shift of the equilibrium, which is supposed to exist between the number of binding sites in the cytoplasmic and nuclear fraction, towards the nuclei.

2.6 Interaction of steroid receptors with nuclear components

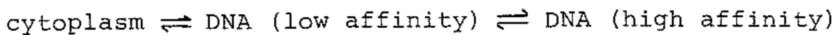
A 'translocation model' for steroid-receptor complexes has been derived from earlier studies about the lac-operon in E.coli. In this prokaryote, it was observed that the repressor of the lac-operon, an allosteric protein, binds to specific DNA regions and that this binding can be modulated by small molecular weight substances. After the initial observation that steroid-receptor complexes could be translocated into the nuclear fraction (92,94) an intensive search was started for a so-called nuclear acceptor, a component which had to account for both the accumulation of receptor in the nucleus and the nuclear response to the hormone.

Several studies have shown, that steroid receptors could associate with crude chromatin (111-116), as well as with DNA (either from eukaryotes or prokaryotes) (88,117-120), specific acidic (116,121) or basic proteins (122,123,124), ribonucleoprotein particles (125,126) and the nuclear membrane (127). Recently it was shown by Müller et al. (128)

that the oestradiol receptor in quail oviduct could associate with RNA-polymerase I, a finding which is consistent with the stimulation of RNA-polymerase activities observed in uterus after oestradiol administration. Thus RNA-polymerase may be another nuclear site of oestrogen receptor action and binding.

Steroid-receptor complexes bound to target cell nuclei are commonly demonstrated using extraction of nuclei with high salt, a method which releases part of the nuclear bound receptors. With in vitro cell-free studies it has been possible to demonstrate a saturable, tissue specific interaction between receptor-hormone complexes and nuclei (115,129,130, 131). However in these studies variations in the ionic strength of the incubation medium caused an overestimation of the concentration of specific acceptor sites (132). Recent studies have raised questions concerning the use of a cell-free system as a model for translocation studies (133,134, 135), because it was demonstrated that nuclear binding could be inhibited by a cytoplasmic factor, different from the receptor. In addition nuclei preloaded with hormone-receptor complexes and nuclei without receptors showed the same kinetics for uptake of steroid-receptor complexes. Williams and Gorski (101) observed that a fixed percentage of the total number of receptors that bound oestradiol, were translocated into the nucleus at any oestradiol concentration and they concluded that an equilibrium exists between the oestrogen receptors in the cytoplasm and in the nuclei. This observation supports the view that the translocation is independent on high affinity binding sites in the nucleus. Based on these and other experiments (88,120) Yamamoto and Alberts have proposed that in the nucleus a very large number of low affinity binding sites in combination with only a very few high affinity binding sites are responsible for known hormone effects. In their studies they found that the oestrogen-receptor complex binds to DNA with a low affinity ($K_a=10^4M^{-1}$) but due to the larger number of these binding sites, high affinity binding sites might be masked (136). This model of

Yamamoto and Alberts is similar to the model explaining the control of the lac-operon in prokaryotes (137), which involves regulatory proteins interacting with nonspecific binding sites on the DNA but which in addition have a very high affinity for some specific regions of the DNA. Thus an equilibrium develops between binding to a large number of nonspecific low affinity sites and to the limited number of specific higher affinity sites. In this respect the oestrogen receptor can be thought of as being in an equilibrium between three sites:


cytoplasm ⇌ DNA (low affinity) ⇌ DNA (high affinity)

Results obtained for a variety of tissues are in favour of this model. Alberga et al. (138) have presented evidence that uterine nuclei contain an oestradiol binding protein with a very high affinity ($K_a = 10^{14} M^{-1}$). In addition Anderson et al. (139) have suggested that the continuing presence of oestrogen-receptor complexes in the nuclei of uterus is required for the responses to oestrogen. It was concluded that particularly the late responses are dependent on a pool of oestrogen that shows delayed disappearance from the target cell and which is defined as the nuclear fraction which resists extraction from nuclei with 0.4 M KCl (140). The demonstration of similar non-KCl extractable nuclear receptors in other tissues (31,141-144) might indicate that this particular type of nuclear receptor is indeed important in the mechanism of action of steroid hormones.

The interaction of a steroid hormone-receptor complex with possible nuclear acceptor sites has been most extensively studied by O'Malley et al. for the progesterone receptor in the chick oviduct. The progesterone-receptor complex binds to chromatin isolated from the oviduct to a greater extent than to chromatin isolated from nontarget tissues (116). With chromatin reconstituted from dehistonized chromatin and nonhistone proteins, it was shown that the binding specificity resides in the acidic protein fraction. Thus if nonhistone proteins from a nontarget tissue such as spleen, were used, binding of the progesterone-receptor complex did

not occur (116). Fractionation of the acidic proteins revealed one group of the nonhistone proteins, called AP_3, as the most effective in the observed binding on the chromatin. In addition they could demonstrate that steroid-receptor complex accumulation in nuclei or on the chromatin did increase the number of initiation sites for RNA-polymerase molecules prior to the increase in ovalbumin synthesis which is the cell response to hormone administration (10,37,38,145). Only the complete set of subunits of the progesterone receptor (subunit A and B) could increase the number of initiation sites, whereas the B subunit, which binds to chromatin but not to DNA (146), did not have this effect. The A subunit, which only binds to DNA (146), could only enhance the number of initiation sites on the chromatin if present in a tenfold higher concentration than needed for the intact 6S dimer (147). On basis of their studies the following model was proposed: The 6S cytoplasmic steroid-receptor complex enters the nucleus and binds to chromatin acceptor sites with moderate affinity through the presence of the B subunit. As a result of this association the A subunit is released and searches along the adjacent genome for specific effector sites with a high affinity constant for the subunit. The ultimate binding of this A subunit to such an effector site would then promote a destabilization of the DNA duplex and create new binding sites for RNA-polymerase molecules and initiation sites for RNA synthesis (35,36). Most of the described studies were carried out in vitro with isolated chromatin and purified E-coli RNA-polymerase. Therefore the question remains whether endogenous RNA-polymerase molecules act on similar initiation sites as has been observed for the prokaryotic enzyme and whether the initiation sites used in vitro are identical to the actual sites in vivo.

In conclusion it appears, that interaction of steroid-receptor complexes with still poorly defined constituents of the chromatin leads to an activation of specific genes. About the steps between the initial interaction of the hormone-receptor complex with the chromatin and the transcrip-

tion of specific gene sequences only speculations can be made (148). It seems very likely that as a result of the interaction of steroid-receptor complexes with the chromatin ultimately new initiation sites for the transcription machinery are created.

2.7 Dissociation of steroids from receptor sites and intracellular recycling of receptors

One of the main questions, still unanswered, concerns the disappearance of the steroid-receptor complex from the nucleus. It was shown after in vivo administration of radioactive oestradiol that larger doses of the hormone disappeared more rapidly from uterine tissue than low doses. This difference in release could be explained through the presence of larger amounts of nonspecific low affinity binding sites at high doses of oestrogen (149). In other studies it was shown that,after injection of oestradiol,receptor sites could not immediately reassociate with oestradiol in vitro after releasing the ligand. Only 16 h after the injection of the hormone the amount of cytosol receptor had returned to control levels and this process of replenishment could be inhibited by cycloheximide and actinomycin D, indicating that probably both RNA and protein synthesis were required (98). There are also indications that nuclear RNP-particles may be involved in steroid receptor recycling in target cells and that such a recycling may be functionally related to gene expression (150). RNA sequences, transcribed after the entry of steroid-receptor complexes in the nucleus might combine with the steroid-receptor complexes to form RNP-particles. After the maturation, RNP-particles leave the nucleus and may alter the protein synthesizing capacity in the target cell cytoplasm.

For glucocorticoid receptors in cultured thymus cells a correlation between ATP levels and the magnitude of specific cortisol binding has been observed (151). It has been suggested that ATP is necessary for the activation of the receptor for binding of the steroid (108). In other studies it has been shown that the release of glucocorticoid receptor from fibroblast nuclei and its generation to an active form was regulated by an energy dependent step (152,153). For the pro-

gesterone receptor and the androgen receptor a specific interaction with mononucleotides has been observed (154,155), indicating again that other factors in addition to the steroid are involved in the mechanism of action of steroid hormones.

From these studies it is clear that more investigations are required to solve the remaining questions concerning the dissociation of the steroid-receptor complexes in the nucleus and the fate of the free receptor. A steroid receptor antibody technique could be very useful in this respect.

2.8 Regulation of the amount of steroid hormone receptors

Several studies have reported on the regulation of cytoplasmic and nuclear steroid receptor concentrations. In the rat uterus, a tissue in which most cells appear to be a target for oestrogens, oestradiol receptors have been detected immediately after birth of the animal (156,157,158). A receptor for oestradiol could already be demonstrated in the Müllerian duct cells (159,160). In the immature animal the receptor concentration in the cytoplasm first rises to a maximum of 20,000-60,000 sites/cell at the age of about 10 days, declining subsequently to a level of 15,000-20,000 sites/cell at 20-22 days. After puberty the values vary between a minimum just after oestrus (2000 sites/cell) and a maximum at pro-oestrus (20,000 sites/cell) (98,161,162,163). In pregnancy the maximum cytosol receptor concentrations coincide with the implantation of embryos and reaches 40,000 sites/cell in the endometrium (163). In studies concerning the measurement of cytoplasmic receptor levels it is uncertain whether the data obtained are a true reflection of the total available or of the number of non-occupied receptor sites.

The nuclear levels of the oestrogen receptor as measured by an ^{3}H-oestradiol exchange assay (164) vary also during the oestrus cycle, closely following the variation in oestrogen secretion (165,166). The maximum at pro-oestrus was about 5000 sites/cell, the minimum value at metoestrus

900 sites/cell. The total available number of receptor sites, measured after administration of a saturating dose of oestradiol, was similar for rats either in metoestrus or in prooestrus. This indicates that the cyclic fluctuation in nuclear receptor sites during the oestrus cycle reflects the change in the distribution of oestradiol receptors between the cytoplasm and the nuclei. From these results and the observation that cytoplasmic receptor levels decrease after castration (161,167), it is clear that oestrogens do influence the receptor concentration. However, it is not known whether or not the development of receptor from birth to puberty is influenced by oestrogen production.

Administration of oestradiol to immature rats (20-23 days) caused a 50% increase of the initial receptor content in uteri 24 h after injection of the animal (98). The process of replenishment of cytoplasmic receptor molecules, which occurred between 4 and 24 h after oestradiol administration was inhibited by cycloheximide, indicating the involvement of protein synthesis (98,144,168). However in other studies part of the replenishment process could not be inhibited by cycloheximide (92,144). Also in some studies it was observed that the decrease in cytosol receptor after administration of oestradiol in vivo could not be accounted for by the complexes measured in the nuclei (92,169). Only 50% of the cytoplasmic receptor molecules could be recovered in the nuclear fraction. A process of receptor inactivation was postulated to explain this phenomenon. For uterus it has been demonstrated that progesterone could influence the cytoplasmic and nuclear oestradiol receptor levels (170), an effect which may be important in the regulation of oestradiol receptor sites in female rats (171). Observations that hypophysectomy did not affect the receptor levels in the uterus indicate that pituitary hormones are not necessary for the maintenance of cytoplasmic receptor levels (168).

The highest amount of the progesterone receptor in the

Methods used for studying steroid receptor interactions

3.1 Measurement of specific steroid binding sites

If intact tissue or isolated cell fractions containing oestradiol receptors are incubated with tracer amounts of radioactive oestradiol (range 10^{-10}-10^{-8}M), the steroid becomes bound by both nonspecific binding sites of low affinity (K_a=10^{6}-$10^{8}M^{-1}$) and specific binding sites of high affinity (K_a=10^{9}-$10^{10}M^{-1}$).

One can distinguish between steroid bound to nonspecific sites and specific binding sites, by taking advantage of the fact, that, in addition to their differences in affinity, the specific binding sites are present only in very limited amounts. Thus if samples are incubated either with radioactive oestradiol or with radioactive oestradiol in the presence of a 100-200 fold excess of non-radioactive oestradiol, the difference between the bound radioactivity recovered in each sample represents the amount of specifically bound oestradiol. This procedure can only be used if the nonspecific binding component behaves as a linear function of the steroid concentration with the employed concentration range.

All commonly used methods for measuring steroid binding sites are based on the separation of macromolecular bound steroid and free steroid at temperatures between 0-4°C. At these temperatures no appreciable dissociation of steroid occurs from the formed steroid-receptor complexes. The number of specific binding sites can be measured as described above. In this thesis four different methods have been used:

1. Sephadex gel chromatography
2. Sucrose density gradient centrifugation
3. Agar-gel electrophoresis
4. Hydroxylapatite column chromatography

3.2 Sephadex gel chromatography

This method originally developed by Williams and Gorski (180) has been used in the present studies only for cytosols. Small portions of cytosol (50 µl) after incubation with radioactive steroid were layered on Sephadex G-25 columns (8x0.5 cm). Macromolecular bound steroid (recovered in the void volume) and free steroid were separated by eluting the column with buffer as illustrated in Figure 1. The shaded area indicates the number of specific oestradiol binding sites which was obtained as described under 3.1.

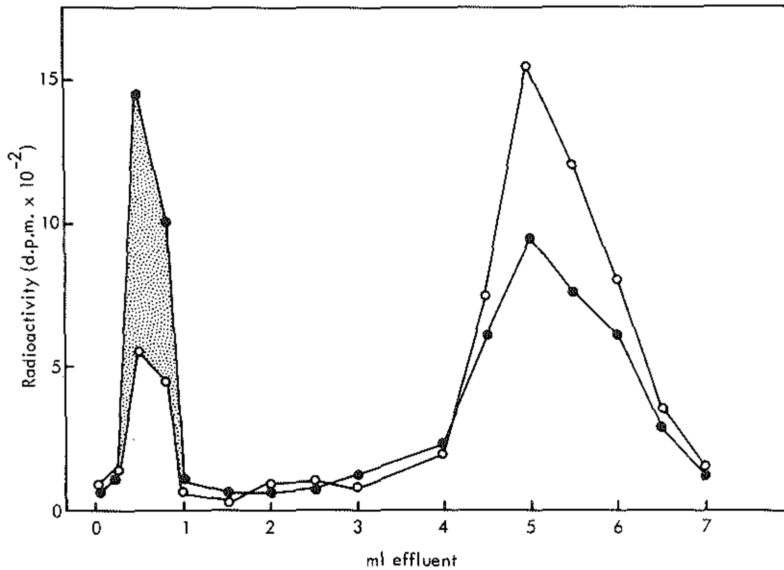


Figure 1 Sephadex gel chromatography of testicular cytosol labelled with ^{3}H-oestradiol.

● - ● cytosol incubated with ^{3}H-oestradiol

o - o cytosol incubated with ^{3}H-oestradiol plus a 200-fold excess non-radioactive oestradiol.

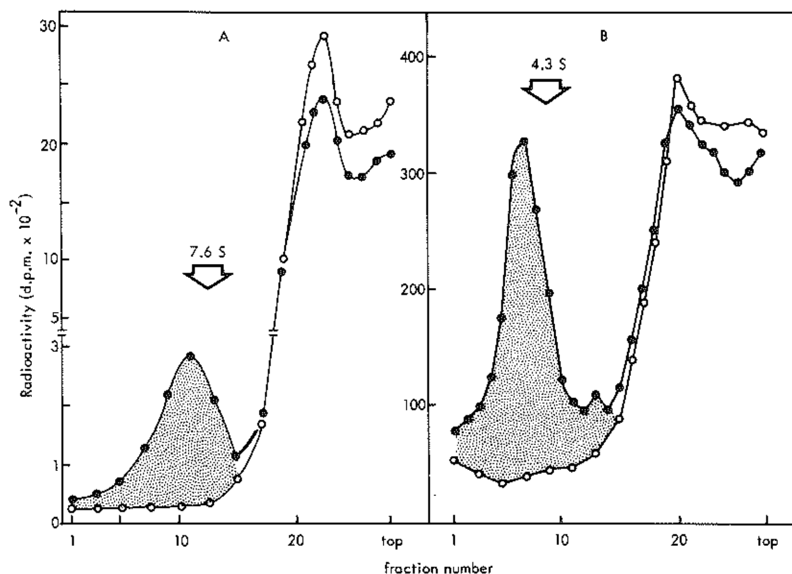


Figure 2 Gradient centrifugation of ^{3}H-oestradiol-receptor complexes present in cytosol (Fig. 2A) and nuclear extract (Fig. 2B).

● - ● incubation with ^{3}H-oestradiol

o - o incubation with ^{3}H-oestradiol plus a 200-fold excess non-radioactive oestradiol.

The arrows indicate the positions of bovine serum albumin (4.3S) and alcohol dehydrogenase (7.6S).

3.3 Sucrose density gradient centrifugation

This method first introduced by Toft and Gorski (181) was used in the present studies for the measurement of both cytosol receptors and nuclear receptors which were extracted from nuclei with 0.4M KCl. An aliquot of 200 μl of incubated cytosol was layered on a linear 5-20% (w/v) sucrose gradient and was centrifuged for 16 h at 150,000 g_{av} to achieve separation of macromolecular bound steroid (8S) and free steroid (Fig. 2A). Nuclear receptors, extracted with 0.4M KCl, were

separated on linear 5-20% (w/v) sucrose gradients, containing 0.4M KCl. Gradients were centrifuged for 18 h at 260,000 g_{av}, which gave a clear separation of macromolecular bound steroid (5S) and free steroid (Fig. 2B). The shaded areas represent the amount of specifically bound oestradiol measured as described under 3.1.

3.4 Agar-gel electrophoresis

This method was introduced by Wagner (182) and was used in the present studies only for the measurement of cytoplasmic receptor sites. A 50 µl aliquot of incubated cytosol was layered on an agar plate kept at 0°C. After electrophoresis for 90 min at 130 mA per plate a good separation could be obtained between bound oestradiol and free oestradiol, which moves to the cathode as a result of electroendosmosis (Fig. 3). The shaded area of Figure 4 represents the amount of specifically bound oestradiol.

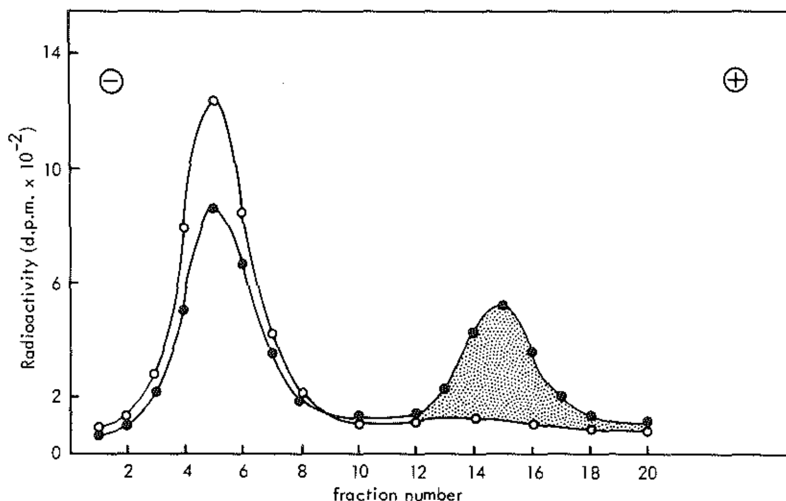


Figure 3 Agar-gel electrophoresis of testicular cytosol labelled with ^{3}H-oestradiol.

● - ● cytosol incubated with ^{3}H-oestradiol

o - o cytosol incubated with ^{3}H-oestradiol plus a 200-fold excess non-radioactive oestradiol

3.5 Hydroxylapatite column chromatography

This method introduced by Gschwendt (183) for measurement of the amount of nuclear steroid-receptor complexes is based on hydroxylapatite column chromatography in the presence of 5M urea and 2M KCl. Nuclei, nuclear extracts or subnuclear fractions in urea and KCl are applied to a hydroxylapatite column (1.4x6.0 cm) in the presence of 1 mM phosphate buffer (pH=6.8). Under these conditions nonhistone proteins and nucleic acids bind preferentially to the column, whereas histone proteins are not retained at all. Using a stepwise elution system with 20 mM, 200 mM and 500 mM phosphate buffer respectively, containing urea and KCl, two nonhistone protein

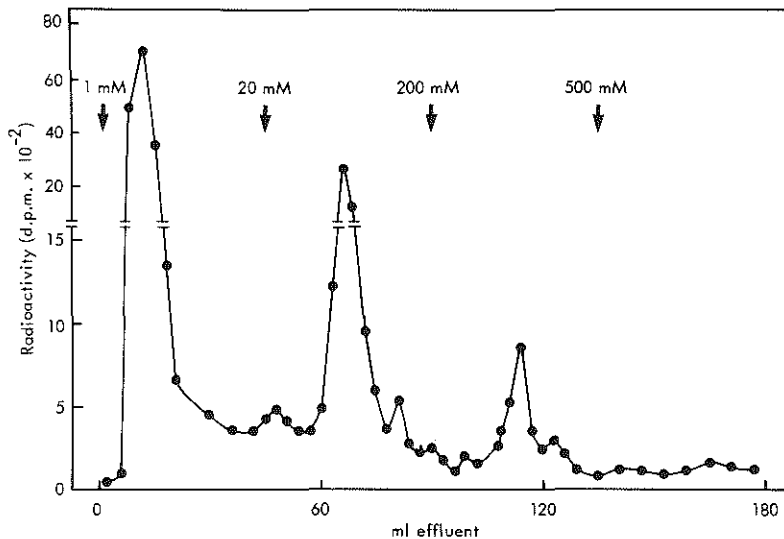


Figure 4 Hydroxylapatite column chromatography of uterine nuclei, containing ^{3}H-oestradiol-receptor complexes, after solubilization in 5M urea/2M KCl.
The column was eluted with phosphate buffers of increasing ionic strength containing 5M urea/2M KCl.

fractions and a fraction containing DNA can be obtained as described by McGillivray (184). The elution pattern in Figure 4 was obtained from uterine nuclei, loaded with ^{3}H-oestradiol. This technique was used for the present investigations concerning the localization of subnuclear oestradiol-receptor complexes in uterus.

3.6 Determination of nuclear receptor sites in the presence of endogenous steroids

In tissues, which contain rather high amounts of steroids, either from endogenous production or through exogenous administration, a certain fraction of the receptor population moves into the nuclear fraction. For a quantitative measurement of the number of nuclear receptor sites it is necessary to use a method that can distinguish between the total available amount of receptor sites and the number of receptor sites occupied by steroid. In the exchange assay, developed by Anderson et al. (164) isolated nuclei are incubated with radioactive oestradiol at elevated temperatures in order to achieve an exchange of non-radioactive oestradiol for radioactive oestradiol. After removal of excess free steroid by charcoal treatment, the amount of radioactive steroid bound to receptor sites was measured directly or after extraction of nuclei with 0.4M KCl.

Introduction and discussion of experimental work

4.1 Introduction

Receptors for steroid hormones in target cells play an important role in the mechanism of action of steroid hormones (49-53), but it is not (yet) clear whether the reverse is also true, i.e. whether the presence of a steroid receptor does reflect that a tissue can be considered as a target tissue for the steroid.

The interstitial tissue is a steroid producing tissue which is influenced by luteinizing hormone (LH) and secretes testosterone into the blood stream (43). Processes involved in spermatogenesis are located in the other tissue compartment of the testis, the seminiferous tubules. Spermatogenesis requires both testosterone and follicle stimulating hormone (FSH) (185,186). Recent experiments have shown that Sertoli cells in the seminiferous tubules may be considered as target cells for FSH (187,188) and that these cells can produce oestradiol from testosterone under the influence of FSH (189). Considering these results one might speculate about a mutual interaction between the two testicular compartments. Oestradiol produced in the Sertoli cell might influence the metabolism of steroids in the Leydig cell via the hormone receptor interaction and the subsequent translocation of the oestradiol-receptor complexes into the nuclei. The Leydig cell in turn might influence the Sertoli cell via changes in the testosterone production. If oestradiol affects the Leydig cell via receptor steroid interactions, changes in the amount and in the subcellular distribution of the receptor might provide useful information concerning the function of oestradiol and its receptor. Therefore we have attempted to investigate the regulation of the number of cytoplasmic receptor sites for oestradiol as a reflection of the total available number of receptor sites. Changes in the number of total available receptor sites might be directly correlated with the number of receptor sites which can ultimately

be bound in the nuclear fraction. It is most likely that the ultimate effect of steroid hormones will occur via an interaction of hormone-receptor complexes and the chromatin. For other tissues it has been shown that hormone effects are directly related to the number of oestradiol-receptor complexes found in the nucleus (1-10) and prolonged effects of steroid hormone effects appear to be dependent on the retention of steroid-receptor complexes in the nuclear fraction. This process could be correlated with the depletion and replenishment of cytoplasmic receptor sites (98,143,168).

Steroidogenesis in testicular Leydig cells is dependent on the presence of LH. Until now it is not known whether other Leydig cell parameters, which are not directly involved in the steroidogenesis, are also under the control of gonadotrophins. Therefore it was decided to investigate the effect of hypophysectomy on testicular oestradiol receptor concentrations. During the development of an organism a variety of biochemical changes occurs in different cell types; these changes ultimately result in the formation of fully differentiated cells. For some steroid hormone receptors it has been shown that they are present in their target cells immediately after birth of the animal or even in the foetus (159,160,190,191). However most of such data have been expressed in a qualitative way. One might speculate about the ontogeny of steroid hormone receptors. It is not clear whether these receptors are synthesized during the very early stages of the embryonic development or whether they are induced in the foetus or in the immature animal by some unknown factor. If the receptors are induced only at later stages during development, one might suppose that changes either in the number of receptor molecules or in the binding properties of the protein are related to steroid hormone effects, which are only needed at certain stages of development.

We have attempted to study the following aspects of the regulation of cytoplasmic receptor sites in testicular tissue:

1) Effect of in vivo administration of oestradiol on the depletion and replenishment of cytoplasmic receptor sites.
2) Effect of hypophysectomy on cytoplasmic receptor levels.
3) Ontogeny of cytoplasmic receptor sites.

4.2 Effects of oestradiol, hypophysectomy and age on cytoplasmic receptor levels

The results in appendix paper I show that in both mature (3 months old) and immature (23 days old) rats administration of oestradiol resulted in a rapid depletion (within 1 h) of cytoplasmic receptor sites. However 5 h after the hormone administration control levels were restored and remained constant for at least an additional period of 20 h.

In mature rats, 10-15 days after hypophysectomy, no dramatic effects could be observed on cytoplasmic receptor levels, suggesting that neither steroid hormones nor gonadotrophins, which both disappear from testicular tissue as a result of hypophysectomy (42,192), are important in maintaining cytoplasmic receptor levels.

In studies concerning the ontogeny of cytoplasmic receptor sites for oestradiol, a plasma protein in the testicular cytosol preparations, which binds oestradiol with a rather high affinity ($K_a=10^8 M^{-1}$) and which is called α-foetoprotein, interfered with accurate measurements of cytoplasmic receptors. Therefore in the testicular tissue of rats younger than 14 days of age cytoplasmic receptor sites could not be detected. It was shown for rats from 4 days of age onwards, however, that receptors accumulated in the nuclear fraction, after incubation of whole testicular tissue with oestradiol.

These results on the regulation of the oestradiol receptor in testicular tissue showed that oestradiol translocates cytoplasmic receptor sites very effectively, but the hormone itself was not important in maintaining a constant level of total receptor sites. But if hormones exert their effects via a hormone-receptor complex in the nuclei, information on

the regulation of nuclear receptor complexes appears to be essential. Results on this aspect of receptor regulation are described for both in vivo and in vitro studies in appendix papers II and III respectively.

4.3 Effects of oestradiol, hypophysectomy and choriogonadotropin on nuclear receptor sites

For a quantitative measurement of nuclear receptor sites in intact rats it was necessary to use a method that can distinguish between free receptor sites and receptor sites that have been occupied in vivo with endogenous hormone. Therefore a ^{3}H-oestradiol exchange method was developed for testicular tissue of immature rats. Using this method it was possible to obtain quantitative information on the number of oestradiol receptor sites in the KCl extractable nuclear fraction. In appendix paper II it has been demonstrated that hypophysectomy of immature rats did result in a considerable decrease of both the total number of receptor sites and the number of receptor sites occupied in vivo with endogenous oestradiol. In intact rats 20% of the total number of available receptor sites was present in the nuclear fraction, suggesting that the presence of this small but significant amount may be important in maintaining some unknown Leydig cell function.

In immature rats treated with human choriogonadotropin for 5 days it was observed that the number of available receptor sites per testis increased threefold, while 73% of this amount was present in its nuclear form.

4.4 Kinetics of in vitro binding of oestradiol in subcellular fractions of testicular and uterine tissue

Like other tissues the testicular tissue contains two types of nuclear receptor sites, which differ in their extractibility with 0.4M KCl. For uterine tissue it has been described that the number of nuclear receptor sites, which resist KCl extraction, shows a good correlation with the

tissue's response to oestradiol (140). With the ^{3}H-oestradiol exchange method described in appendix paper II only the class of receptor sites which can be extracted from nuclei with KCl could be measured quantitatively. Therefore it was decided to use an in vitro system in order to study the translocation and the subsequent binding of oestradiol-receptor complexes in the KCl extractable and in the non-KCl extractable nuclear fraction in testicular and uterine tissue under similar experimental conditions. In appendix paper III the following aspects have been investigated:

1) Mechanism and rate of translocation of cytoplasmic receptor molecules into the nuclear fractions of testis and uterus.
2) The nature of the nuclear acceptor sites in the testicular tissue.
3) The effect of energy deprivation (following addition of KCN) on the subnuclear distribution of oestradiol receptor sites in testicular and uterine tissue.

The latter aspect concerning the role of energy in the subnuclear distribution of receptor sites was studied because it has been reported that cellular ATP might be involved in the action of glucocorticoids, progesterone and dihydrotestosterone (151,153,154,155).

The results obtained on the comparison between receptors in testis and uterus in appendix paper III showed that the interaction of oestradiol-receptor complexes with nuclear acceptor sites in testicular and uterine nuclei followed different kinetics. In both tissues the number of binding sites in the KCl extractable and non-KCl extractable fraction of the nuclei showed an increase observed during the first 30 min of incubation. Thereafter the concentration of oestradiol receptor sites in the nuclear extract of uterine tissue showed a decrease, but reached a steady state level (50% of the maximum value) 60 min after the start of the incubation. During this same period no changes could be

observed for the number of oestradiol receptor sites in the KCl extract of testicular nuclei. The difference between both tissues in the time dependent uptake of binding sites in the non-KCl extractable nuclear fraction was even more striking. In uterine tissue the concentration of binding sites decreased to levels 50% of the maximum value and this level was maintained for at least an additional period of 60 min. In testicular nuclei a similar decrease was observed, but 60 min after the start of the incubation almost all binding sites had dissapeared from the non-KCl extractable fraction. This suggests that a difference exists between the dissociation rates of the steroid-receptor complexes from the acceptor sites in testicular and uterine nuclei.

The effect of energy deprivation, as a result of the addition of KCN, on the number of binding sites was similar for testicular and uterine tissue. A significant increase was observed for the number of binding sites in the non-KCl extractable nuclear fractions, whereas the number of KCl extractable nuclear receptor sites remained unaffected.

For testicular tissue some attempts have been made to solubilize the non-KCl extractable receptor sites. However, of all the methods used only mild trypsin treatment could release a limited number of receptor sites in a soluble form. After trypsin treatment the additional receptor sites had a sedimentation value of 4S on sucrose gradients, instead of a sedimentation value of 5S. Attempts with DNAse and deoxycholate were unsuccessful: only an increase in the amount of unbound oestradiol could be obtained.

4.5 Conclusions

In summary it can be concluded that the distribution of testicular oestradiol receptor molecules between the cytoplasmic and the nuclear fractions is influenced by the oestradiol concentration in testicular tissue. After being translocated into the nucleus,oestradiol-receptor complexes

are bound by at least two different types of chromatin acceptor sites which differ in affinity for the oestradiol-receptor complex as determined by KCl extraction. In contrast to the oestradiol receptor in uterus testicular receptors are retained by the high affinity acceptor sites inside the nucleus only for short periods. This might reflect that, although both tissues contain oestradiol receptors in comparable amounts, different mechanisms are involved in the binding of oestradiol-receptor complexes to and the subsequent release from the acceptor sites inside the nucleus.

The experiments described thusfar were performed in order to obtain more information about the regulation and subcellular localization of testicular oestradiol-receptor complexes. It appeared that the kinetics of the interaction of the oestradiol-receptor complex with nuclear constituents from testis and uterus were different. However these results do not permit definitive conclusions concerning the molecular mechanisms underlying these differences. This would require more information about the nature of the interaction between the oestradiol-receptor complexes and the chromatin. In this respect results of experiments on the interaction of the oestradiol-receptor complex and uterine chromatin are presented in chapter 5. Uterine tissue was chosen for this purpose because in this tissue a possible interaction of steroid-receptor complexes with a specific nuclear fraction i.e. DNA or chromatin proteins, might be correlated with the tissue response to oestradiol. A comparison of these mechanisms in a well-known responsive tissue, like the uterus and in a tissue with a less defined response like testis, might indicate whether the presence of steroid receptors defines a tissue as a 'hormone target tissue', even if distinct actions of the steroids are not (yet) known.

Distribution of oestradiol-receptor complexes in subnuclear fractions of uterine tissue after administration of oestradiol in vivo and in vitro

5.1 Introduction

An important step in the response of a tissue to a steroid hormone is the interaction of the steroid with its receptor in the cytosol of the target tissue. The complex formed between hormone and receptor migrates into the nucleus and binds to acceptor sites on the chromatin and this binding ultimately causes a response of the tissue to the hormone via changes in RNA and protein synthesis (193-196).

The location of the oestradiol binding protein or the newly synthesized RNA in chromatin is not known. The present study was started in an attempt to obtain information on the subnuclear localization of the steroid-receptor complex.

There is considerable morphological (197,198,199) and physical (200,201) evidence that chromatin consists of a biological 'active' form (euchromatin) and a transcriptionally repressed form (heterochromatin). In the literature some methods have appeared on the fractionation of chromatin in an active and an inactive form (202-207), but these methods have the disadvantage that the chromatin is fractionated after its isolation from purified cell nuclei. During the isolation procedure RNA-polymerase molecules and other chromatin proteins (histones and nonhistones) might be released or become inactivated. In addition it seems very likely that cleavage of the chromatin, by DNAse digestion or by shearing of the chromatin, produces either artificial initiation sites or damaged template DNA in the fragments. Tata and Baker (208) and Chesterton et al. (209) have described a method for fractionation of isolated eukaryotic nuclei in 8 different subnuclear fractions in a single step. In this method, introduced by Frenster et al. (210) the nuclei are gently sonicated in an isotonic buffer and sepa-

rated in nuclear sap, euchromatin, heterochromatin, nucleoli and nuclear membranes by virtue of their different sedimentation rates in a discontinuous sucrose gradient. Individual fractions are considerably enriched in either one type of the chromatin, one type of the RNA-polymerases or enriched in poly A-rich Hn-RNA (211,212).

In the present study it was tried to apply this method to the subnuclear distribution of oestradiol receptors in nuclei, isolated from uterus after treatment of either ovariectomized rats or isolated uterine tissue with oestradiol.

5.2 Materials and methods

Animal and tissue treatment

Mature female rats of the Wistar strain, ovariectomized for 3-5 days, were used. Two different methods were used for the preparation of nuclear ^{3}H-oestradiol-receptor complexes:

1) Isolated uterine tissue was incubated for 30 min at 37°C in KRBG-buffer (pH 7.4) either with 10^{-8}M radioactive oestradiol or with 10^{-8}M radioactive oestradiol plus a 1000-fold excess of non-radioactive oestradiol. Thereafter the tissue was rinsed with buffer and nuclei were isolated.
2) Rats were injected subcutaneously with 5 µg of oestradiol and 60 min later uterine nuclei were isolated.

The nuclear suspensions thus obtained were incubated for 60 min at 20°C with 10^{-8}M ^{3}H-oestradiol in order to obtain complete exchange between receptor bound oestradiol and added ^{3}H-oestradiol (164).

Preparation of nuclei

Uterine tissue (approximately 1 g) was homogenized in an Ultraturrax homogenizer for three periods of 10 sec with intermittant cooling periods of 10 sec in 3 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.32M sucrose, 2.5 mM KCl, 2.5 mM $MgCl_2$ and 25% glycerol (TKMSG-buffer). The homogenate, after dilution with TKMSG-buffer (1:1) and after filtration through two layers of 500 μ gauze, was centrifuged for 10 min at 600 g. After washing the crude nuclear pellet with TKMSG-buffer, containing 0.1% Triton X-100, the nuclei were collected for 10 min at 600 g. After washing with TKMSG-buffer, the nuclear suspension was resuspended and washed three times with 50 mM Tris-HCl, pH 7.5, containing 12.5 mM NaCl, 12.5 mM KCl, 5 mM $MgCl_2$ (TKMNa-buffer). The ultimate nuclear suspension was resuspended with gentle homogenization (2-3 strokes in an hand-operated all-glass Potter-Elvehjem homogenizer) in 2.5 ml TKMNa-buffer. The nuclei thus obtained appeared intact by phase contrast microscopy and had a protein/DNA ratio in the range of 1.7-1.9. The whole procedure is summarized in Figure 1.

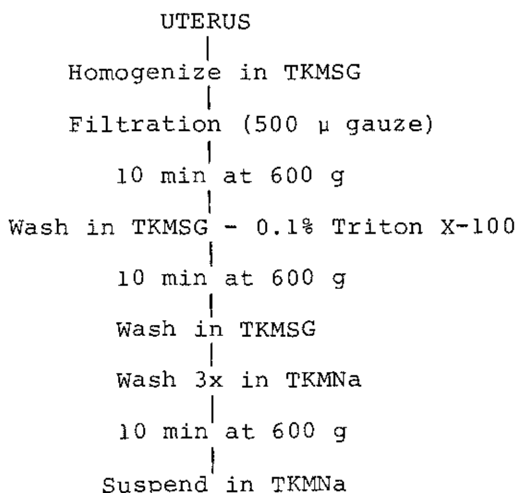


Figure 1 Flow sheet summarizing the procedure for isolation of uterine nuclei

Fractionation of nuclei

Uterine nuclei suspended in 2.5 ml TKMNa-buffer were placed in a 15 ml round-bottom tube and were exposed to 15 sec sonication at 20 kHz and a probe amplitude of 70 microns using an MSE sonicator with the microtip placed 3-5 mm below the surface. This treatment was sufficient for the disruption of 90-95% of the nuclei as was checked by phase contrast microscopy. The lysed nuclei were centrifuged at 7000 g for 30 min and the supernatant (nuclear sap) separated from the pellet. The residual pellet was resuspended in 2.5 ml TKMNa-buffer, containing either 90 mM Na citrate and 90 mM K citrate or 90 mM Na chloride and 90 mM K chloride, followed by homogenization with 5-6 strokes in an all-glass hand-operated homogenizer. The suspension thus

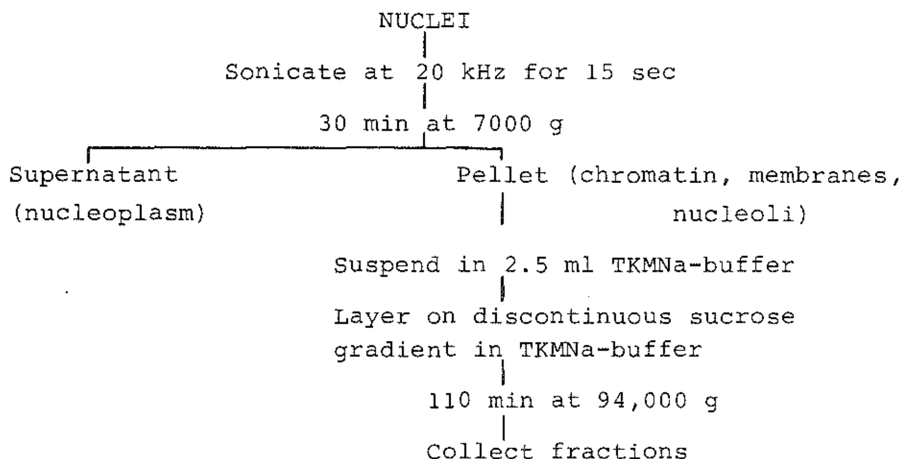


Figure 2 Flow sheet summarizing the procedure for sub-nuclear fractionation of uterine nuclei

obtained was layered on a discontinuous sucrose gradient in TKMNa-buffer containing either 90 mM each of Na and K citrate or 90 mM each of Na and K chloride, in a Beckman SW27 17 ml tube as follows: 2.5 ml 2.0M sucrose, 2.5 ml 1.72M sucrose, 3.0 ml 1.37M sucrose, 3.0 ml 1.17M sucrose and 2.5 ml 0.55M sucrose. The samples were centrifuged at 94,000 g for 110 min at 2°C and the fractions were removed carefully with a Pasteur pipette bent at right angles at the tip. A summary of the fractionation procedure is given in Figure 2.
Figure 3 illustrates the numbering of the fractions obtained after the fractionation is described.

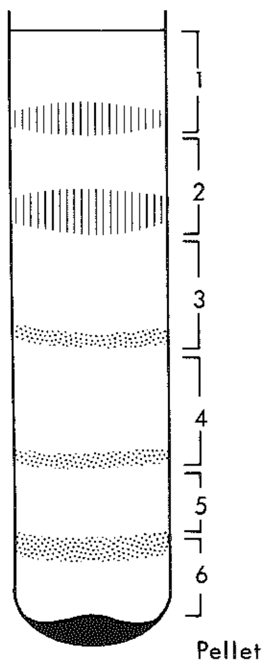


Figure 3 The distribution of subnuclear fractions isolated from nuclei of uterine tissue with discontinuous sucrose density gradient centrifugation

Labelling of RNA in vitro

One uterus, stripped of adhering fat and mesentery, was incubated in 2.0 ml of Krebs Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose and 50 μCi/ml 5-^{3}H-uridine (sp. act. 20 Ci/mmol). Incubation was carried out for 30 min at 37°C in an atmosphere of 95% O_2 : 5% CO_2. Nuclei were prepared from uterine tissue and fractionated by the described procedure in buffers containing K and Na chloride. Nucleic acids in each subnuclear fraction were precipitated with 0.5M $HClO_4$ at 0°C and were washed three times with 0.5M $HClO_4$. RNA in the pellet was dissolved in 1 M KOH at 37°C for 60 min and samples, taken after the addition of $HClO_4$ to precipitate the DNA, were used for the measurement of incorporated radioactivity and RNA content. The pellet was treated with $HClO_4$ at 70°C for 15 min to hydrolyse the DNA and samples were taken to measure the DNA content (see under Measurement of DNA, RNA and protein).

Measurement of specific binding of ^{3}H-oestradiol

Different methods were used for the estimation of ^{3}H-oestradiol binding by receptor proteins:

1) After the isolation of the subnuclear fractions samples were taken for the measurement of total radioactivity present in each fraction. Specific 'binding' in each fraction was determined as previously described by subtracting the nonspecific binding (obtained after incubation of tissue in the presence of excess non-radioactive oestradiol) from the total binding of ^{3}H-oestradiol (144). Binding was expressed as dpm/μg DNA present in each fraction.
2) In some experiments samples of various subnuclear fractions were run on a linear sucrose density gradient (5-20%) in the presence or in the absence of high salt concentrations as described previously (144,213).
3) Binding of ^{3}H-oestradiol by receptor molecules was also demonstrated by hydroxylapatite column chromatography.

The method used was essentially the procedure described by Gschwendt (183), originally developed by McGillivray et al. (184). Hydroxylapatite was washed three times in 5M urea/2M KCl in 1 mM potassiumphosphate buffer pH 6.8 by suspension and decantation. Columns were then packed (1x6 cm), equilibrated and run at $4^{o}C$. Subnuclear fractions containing chromatin were diluted with 5M urea/2M KCl in phosphate buffer (1:1), applied to the column and eluted with 1 mM, 20 mM, 200 mM and 500 mM potassiumphosphate pH 6.8 in 5M urea/2M KCl. The flow rate was maintained at 30 ml/h and 3 ml fractions were collected. In later experiments the columns were prepared in 1 mM potassiumphosphate pH 6.8 containing 0.4M KCl. Subnuclear fractions diluted (1:1) with 0.8M KCl were applied to the column and were eluted with 1 mM, 20 mM, 200 mM and 500 mM potassiumphosphate buffer pH 6.8 respectively, containing 0.4M KCl.

Measurement of DNA, RNA and protein

After accurately measuring the volume of each subnuclear fraction the fractions were diluted 2-3 fold with water. $HClO_4$ was added at $0^{o}C$ to a final concentration of 0.5M and the samples were washed three times with 0.5M $HClO_4$. The pellets were dissolved in 1M KOH at $37^{o}C$ for 60 min. DNA and protein were precipitated after the addition of $HClO_4$ at $0^{o}C$ for 30 min. The supernatant containing the products of RNA hydrolysis was used for RNA estimation. The pellet was treated with $HClO_4$ at $70^{o}C$ for 15 min to hydrolyze the DNA. The residual pellet, containing protein, was dissolved in 1M KOH at $70^{o}C$ for 60 min. DNA in the fractions was measured using the method of Giles and Myer (214), RNA was measured according to Fleck and Munro (215) and the protein content of each fraction was measured by the method of Lowry et al. (216). In some experiments the amounts of DNA in the subnuclear fractions were very small (less than 10 μg/fraction) and in those cases the fluorescence method of Robertson and Tait was used (217).

5.3 Results

Isolation and chemical characterization of subnuclear fractions

In initial experiments it was tried to establish the conditions for fractionation of uterine nuclei. With light microscopy as a parameter it could be observed that sonication for a period of 15 sec was sufficient to disrupt about 90-95% of the uterine nuclei. The pattern of bands obtained after the final ultracentrifugation step in TKMNa-buffer containing K(Na) citrate was similar to that observed for liver nuclei by Tata and Baker (208). After the collection of the various fractions the recoveries of protein, RNA and DNA were measured. Generally approximately 45-50% of the total nuclear DNA was recovered in the pellet fraction. The pellet also contained about 40-50% of the total amount of protein recovered. The very small amount of DNA (in the order of 1% of the total amount) and the relatively large amount of protein in the 7000 g supernatant of the nuclear sonicate were considered to reflect the nucleoplasm in this fraction (Table I).

The results in Table I are representative for the data obtained as percentages of each constituent and as RNA/DNA and protein/DNA ratios. From these data it is evident that the individual fractions differ considerably in composition. However these data cannot be used for the determination of the functional properties of the constituents present in each fraction. Tata and Baker (208,211) showed that subnuclear fractions isolated from rat liver contained different types of chromatin, different types of RNA-polymerase and differed also in the rate of labelling of RNA in vivo. They concluded that the top fractions of the sucrose gradient (fraction 1 and 2) contained the euchromatin, whereas the pellet and fraction 6 contained the heterochromatin. A mixture of both types of chromatin was located in fractions 3, 4 and 5.

Table I Chemical composition of subnuclear fractions of uterine tissue, isolated as given in the flow sheet in Figure 2.
After collection of the different fractions, DNA, RNA and protein were measured in each fraction. Results are given in μg of each component/fraction. NP represents the nucleoplasmic fraction.

Fraction	protein	DNA	RNA	RNA/DNA	protein/DNA	% DNA
Intact nuclei	-	-	-	0.20	1.89	
NP	696	12	53	4.41	58.09	1
1	478	532	50	0.09	0.90	
2	496	484	88	0.18	1.02	43 (fractions 1–3)
3	150	20	28	1.40	7.50	
4	152	16	22	1.37	9.50	
5	260	32	34	1.06	8.13	3 (fractions 4–6)
6	132	20	22	1.10	6.60	
Pellet	1860	832	82	0.10	2.24	53

Distribution of RNA labelled in vitro in the subnuclear fractions

The presence or absence of rapidly labelled RNA can be used to determine the distribution of the different types of chromatin over the isolated subnuclear fractions. Therefore uterine tissue was incubated in the presence of ^{3}H-uridine and subnuclear fractions were isolated in TKMNa-buffer containing K(Na) chloride. The results presented in Table II show that RNA with the highest specific activity (dpm ^{3}H/μg RNA) was recovered in fractions 1, 5 and in the nucleoplasm, indicating that rapidly labelled RNA is predominantly located in these fractions. The ratio of incorporated ^{3}H-uridine per μg DNA can be used as a marker for the number of RNA chains which have been synthesized per unit DNA. Table II shows that the top layers of the fractionation gradient contain DNA which is probably representative for active chromatin in vivo. The high ratio of radioactive RNA/μg DNA

Table II Distribution of ^{3}H-labelled RNA in subnuclear fractions of uterus after incubation of the tissue for 30 min at 37°C in the presence of 5-^{3}H-uridine. After isolation of subnuclear fractions acid precipitable radioactivity, DNA and RNA were measured in each fraction.

Fraction	dpm/µg RNA	dpm/µg DNA
Intact nuclei	543	520
NP	360	14,147
1	637	5,984
2	209	8,156
3	101	3,881
4	120	866
5	303	362
6	256	268
Pellet	209	242

as observed for the nucleoplasmic fraction might be the result of the release of rapidly labelled RNA into the nucleoplasmic fraction of uterine nuclei after its synthesis on the chromatin.

Distribution of ^{3}H-oestradiol in subnuclear fractions of uterine tissue

With a nuclear ^{3}H-oestradiol exchange procedure (144,164) it was tried to demonstrate specific binding of oestradiol in subnuclear uterine fractions isolated in TKMNa-buffer containing K(Na)citrate. In this exchange assay uterine nuclei obtained from rats after injection of large amounts of oestradiol and from (control) rats not treated with the hormone were incubated with equal amounts of ^{3}H-oestradiol. From the data presented in Table III it appears that a relative enrichment of specifically bound ^{3}H-oestradiol (ex-

Table III Localization of specific oestradiol-receptor complexes in subnuclear fractions of uterine tissue. Nuclei obtained from oestradiol treated rats and from control rats were incubated with ^{3}H-oestradiol for 60 min at 20°C. After isolation of subnuclear fractions samples were taken for the measurement of radioactivity and the difference between the values obtained in dpm/µg DNA was taken as a measure for the specific binding of ^{3}H-oestradiol.

Fraction	Oestradiol treated	Control	Specific
Intact nuclei	1,793	1,186	607
NP	129,050	42,444	89,213
1	954	279	675
2	967	494	472
3	8,801	3,567	5,235
4	2,679	4,074	-
5	6,380	3,122	3,258
6	4,775	3,040	1,735
Pellet	2,160	1,512	672

pressed as dpm/µg DNA) occurred in fractions 3, 5 and 6 and also in the nucleoplasm.

These data suggest that these subnuclear fractions might contain the so-called 'nuclear acceptor sites' for oestradiol-receptor complexes. In a control experiment sonic disruption of nuclei was performed in the presence of an amount of ^{3}H-oestradiol (either in the absence or in the presence of excess non-radioactive oestradiol) comparable with the amount used in the ^{3}H-oestradiol exchange procedure. From the difference in the total 'binding' and the non-specific 'binding' in this experiment, it could be concluded that ^{3}H-oestradiol was not retained in the various nuclear fractions during the sonication procedure at a temperature of 0°C.

This argues against the possibility that the distrribution of ^{3}H-oestradiol in the subnuclear fractions (Table III) is the result of the interaction of free ^{3}H-oestradiol with the chromatin during the sonic disruption of the nuclei. Nevertheless it remains uncertain whether the obtained ratios (dpm ^{3}H/µg DNA) are a true reflection of the presence of oestradiol-receptor complexes in the individual fractions.

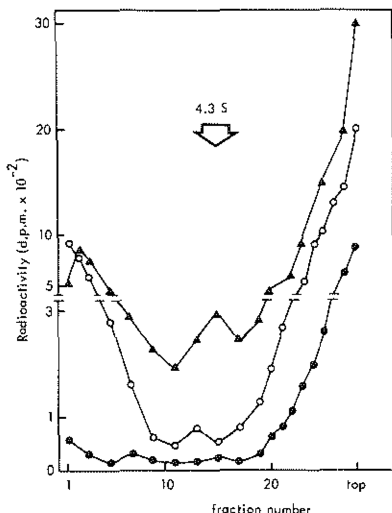


Figure 4 Distribution of ^{3}H-oestradiol in KCl extracts of subnuclear uterine fractions.
Subnuclear fractions isolated from nuclei, loaded with ^{3}H-oestradiol, were diluted with TKMNa-buffer and centrifuged for 60 min at 240,000 g. The pellets thus obtained were extracted with 0.4M KCl and samples were layered on 5-20% sucrose density gradients.
After centrifugation for 18 h at 240,000 g, fractions were collected and assayed for radioactivity.
● - ● Subnuclear fraction 1
o - o Subnuclear fraction 2
▲ - ▲ Subnuclear fraction 3
The arrow indicates the position of bovine serum albumin (4.3S).

In an attempt to demonstrate the binding of oestradiol to macromolecules, subnuclear fractions, after dilution with TKMNa-buffer, were centrifuged for 60 min at 240,000 g. The pellets thus obtained were extracted with 0.4M KCl for 60 min at $0^{o}C$ and the KCl extracts were used for analysis on high salt sucrose gradients. The distribution of ^{3}H-oestradiol in these extracts is shown for subnuclear fractions 1,2 and 3 in Figure 4. A comparable heterogeneous distribution of radioactivity was also obtained for the other subnuclear fractions including the pellet, and this observation made the sucrose density gradient centrifugation technique less suitable for the detection of nuclear receptor sites. Moreover it cannot be excluded that receptor sites were lost during the centrifugation step in diluted buffer. Another disadvantage is that KCl extraction is suitable only for the detection of KCl extractable receptor sites. Considering this, together with the low capacity and the low resolving power of sucrose gradients, it was tried to concentrate the various subnuclear fractions. All methods used, including dialysis and concentration by hollow fibers resulted, however, in considerable losses (about 50%) of both radioactivity and protein and no binding could be observed in the resulting preparations.

Demonstration of nuclear oestradiol receptor sites in uterine nuclei by hydroxylapatite column chromatography in the presence of urea and KCl

It has been demonstrated by Gschwendt (183) for the oestradiol receptor in nuclei from chicken liver that chromatography on hydroxylapatite columns for separation of chromatin proteins as originally developed by McGillivray (184), could be used for the measurement of nuclear oestradiol receptors. This method is based on retention of proteins by the column and the subsequent release by increasing phosphate concentrations. It has also been demonstrated by Gschwendt, that the method could distinguish between KCl extractable

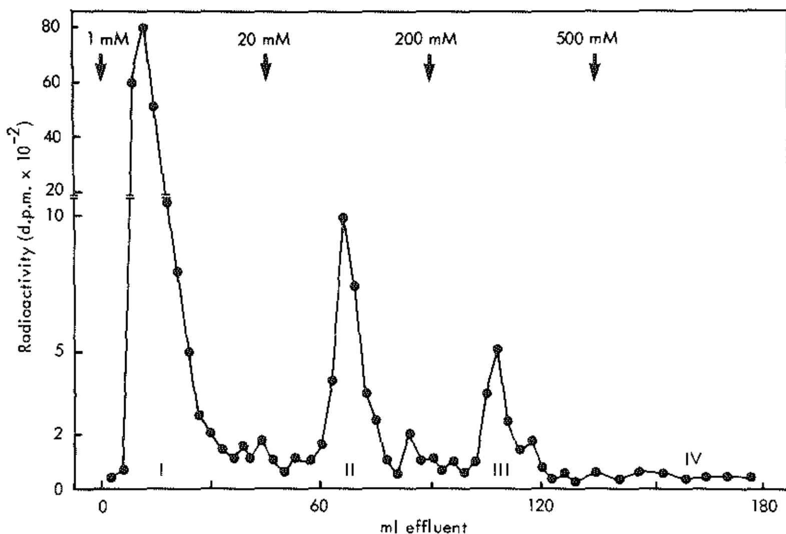


Figure 5 Hydroxylapatite column chromatography of uterine nuclei, loaded with ^{3}H-oestradiol.
Nuclei, solubilized in 1 mM phosphate buffer pH 6.8 containing 5M urea/2M KCl, were applied to the column. The column was eluted with phosphate buffers of increasing ionic strength containing 5M urea/2M KCl. In peak I histone proteins were eluted and non-histone proteins were eluted in peak II and III. The fractions eluted at 500 mM phosphate (IV) contained DNA.

and non-KCl extractable oestradiol receptors on basis of their difference in retention on the column. In addition this technique can be used for the analysis of large volumes of buffer containing high sucrose concentrations.

In initial experiments, we could demonstrate receptor bound oestradiol in isolated nuclei (Figure 5, peak II and III), but not in subnuclear fractions. In control experiments it could be shown, that

1) ureum, which is used to dissociate the chromatin in its constituents, disrupted the binding of oestradiol with its receptor

2) citrate, which is present in the fractionation gradient, extracted considerable amounts of oestradiol-receptor complexes from isolated nuclei and also interfered with the elution of oestradiol-receptor complexes from the hydroxylapatite column.

On basis of these observations it was decided to omit ureum from the elution buffer and to substitute citrate ions for chloride ions in the fractionation gradient. Subnuclear fractions, after addition of 0.8M KCl (1:1), were applied to columns prepared in 1 mM phosphate buffer containing 0.4M KCl. Fractions were collected after elution with 20 mM, 200 mM and 500 mM phosphate buffer, respectively, containing 0.4M KCl. This alternative method appeared to be reliable, because in the order of 80% of the number of receptor sites present in a KCl extract of uterine nuclei could be recovered in fraction II and III after elution of a hydroxylapatite column.

Demonstration of nuclear oestradiol receptor sites in subnuclear fractions of uterine nuclei by hydroxylapatite column chromatography

Subnuclear fractions were isolated in TKMNa-buffer containing KCl and NaCl after incubation of uterine tissue for 60 min at 37°C in the presence of 10^{-8}M ^{3}H-oestradiol. The isolated subnuclear fractions were diluted (1:1) with 0.8M KCl and chromatographed on hydroxylapatite columns. Table IV gives a summary of the results. It was possible to demonstrate binding of ^{3}H-oestradiol in subnuclear fractions 1,2 and 3, in the nucleoplasm (NP) but not in fractions 4,5 and 6. In order to obtain information about the presence of receptor sites in the pellet fraction of the fractionation gradient it was tried to solubilize receptor sites by adding 0.4M KCl for 60 min at 0°C. In a further attempt to demonstrate receptor sites in the pellet fraction the residual (non-KCl extractable) fraction was sonicated for 15 sec in buffer containing 0.4M KCl. The KCl extracts thus obtained

Table IV Separation of oestradiol receptor sites in different subnuclear fractions by hydroxylapatite column chromatography. Data for the amounts eluted in the 3 peaks after chromatography (see figure 5) are given as percentages of the amount of receptor sites which was recovered from the column.

Fraction	Elution peak	
	II	III + IV
Nuclear extract in 0.4M KCl	87	13
NP	77	23
1	69	31
2	56	44
3	42	58
Pellet extract in 0.4M KCl	53	47
Pellet after sonication in 0.4M KCl	26	74

were used as such for analysis of receptor sites on hydroxylapatite columns and the results are also presented in Table IV.

Some striking differences can be observed for the amounts of oestradiol receptor sites recovered in the different hydroxylapatite column fractions. In the nucleoplasm as well as in subnuclear fraction 1 most of the binding sites were recovered from the column in elution peak II (70-80%). This distribution of bound ^{3}H-oestradiol on the column is similar to that observed for a KCl extract prepared from intact uterine nuclei. In contrast in subnuclear fractions 2 and 3 in the order of 50% of the number of binding sites was recovered from the column in elution peak II. The relative increase of radioactive oestradiol in elution peak II (as well as in an additional peak IV, which is eluted at 500 mM phosphate buffer) is even more striking for the pellet of the fractionation gradient. In the KCl extractable fraction of this pellet 50% of the number of binding sites was still recovered in elution peak II, whereas from the non-KCl ex-

tractable fraction, applied to the hydroxylapatite column after sonication in 0.4M KCl, only 20-35% was eluted in this peak. A similar treatment, including KCl extraction of nuclei and sonication of the nuclear residual pellet in 0.4M KCl, also resulted in the recovery of non-KCl extractable receptor sites predominantly in elution peaks III and IV.

In summary therefore it can be concluded that non-KCl extractable receptor sites appear to sediment in the heavier sucrose gradient fractions and in the pellet of the fractionation gradient. KCl extractable receptors were recovered predominantly in the nucleoplasm and in the lighter sucrose gradient fractions.

Recovery of oestradiol-receptor complexes in subnuclear fractions of uterus

Considering the quantitative distribution of oestradiol-receptor complexes over the various subnuclear fractions it appears that in the order of 40% of receptor sites was recovered in the top layers of the fractionation gradient (30%) and in the nucleoplasm (10%). The remaining 60% was found in the pellet fraction obtained during the isolation of the subnuclear fractions. From the total amount of nuclear receptor sites present in uterine nuclei before sonication and fractionation, approximately 40% could be recovered. This loss of nuclear receptor sites was not caused by the sonication procedure because after KCl extraction of sonically disrupted nuclei more than 80% of the number of receptor sites present in intact nuclei was recovered as measured by sucrose gradient centrifugation. During the fractionation of uterine nuclei, the sonicated nuclei are considerably diluted during the centrifugation through the discontinuous gradient. This step, which might induce a considerable dissociation of oestradiol-receptor complexes could explain, at least in part, the low recovery of nuclear receptor sites.

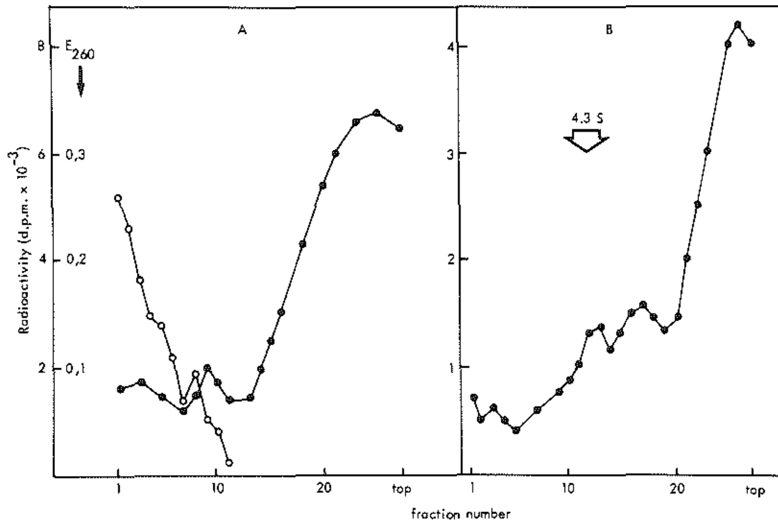


Figure 6 Nature of oestradiol-receptor complexes in uterine subnuclear fractions.

A sample of subnuclear fraction 1 was layered on a linear 5-20% sucrose density gradient prepared in TKMNa-buffer (Fig. 6 A). A sample of the nucleoplasmic fraction was layered on a linear 5-20% sucrose density gradient, prepared in 10 mM Tris-HCl buffer containing 0.4M KCl (Fig. 6 B).

After centrifugation for 18 h at 240,000 g fractions were collected and assayed for radioactivity (● - ●) and DNA (E_{260}) (o - o).

The arrow indicates the position of bovine serum albumin (4.3S).

Nature of oestradiol-receptor complexes in subnuclear fractions

An attempt was made to obtain further information about the state of the oestradiol-receptor complexes in the nucleoplasmic fraction and in subnuclear fraction number 1. A sample of the nucleoplasm was layered on a sucrose density gradient prepared in 0.4M KCl in buffer. After centrifugation and collection of the individual fractions, the amount of radioactivity was determined in each fraction. Figure 6B shows that oestradiol bound to macromolecules in the nucleoplasm sediments heterogeneously in the 3-8S region of the gradient. A sample of subnuclear fraction 1 was layered on a sucrose gradient prepared in TKMNa-buffer in the absence of KCl. Figure 6A shows that subnuclear fraction 1, in addition to oestradiol bound to macromolecules, also contains a heterogeneous fraction of DNA, which was determined by measuring E_{260} in each fraction. This might reflect a possible interaction between the two components, i.e. the oestradiol-receptor complex and the DNA, in vivo.

5.4 Discussion

Chromatin in vivo appears to occur in two forms, heterochromatin, which is inactive in transcription and euchromatin, which includes genes participating in transcription (199). In vitro chromatin can be separated in fractions which have different template activities in incubations with added exogenous RNA-polymerase and different amounts of nascent RNA after labelling in vivo or in vitro (206,209,210,211, 218,219,220,221). Also differences in histone and nonhistone protein content could be observed (221,222,223,224). Although the molecular composition of these chromatin fractions is not known, they may be separated from each other as a consequence of their differences in:

- hydrodynamic properties, which are reflected in shearing or sonication and sedimentation through glycerol or sucrose gradients (208,209,210,219,221).

-electrostatic properties after sonication, as reflected during separation by ECHTAM-cellulose column chromatography (223).
-behaviour during DNAse digestion of chromatin (203,225).

In some of these methods chromatin was isolated from nuclei and fractionated by either of the treatments as described above in order to obtain chromatin fractions. A major disadvantage of this procedure is the fact that isolated chromatin may be different from chromatin in vivo with respect to the location of histones and nonhistones on the chromatin (226). It is also possible that as a result of the isolation and fractionation methods the bulk of RNA-polymerase would be released from its template or become inactivated. Furthermore most procedures used for preparation of chromatin and RNA-polymerases could destroy the nuclear envelope, while, at the same time, methods used for isolating nuclear membranes would not allow a meaningful study of the role of nuclear structures in the regulation of transcription of DNA.

In order to correlate a possible interaction of oestradiol-receptor complexes with their acceptor sites and the ultimate effect of oestradiol inside the nucleus i.e. changes in RNA-polymerase activity and synthesis of new mRNA species, it was tried to disrupt isolated uterine nuclei by sonication and prepare subnuclear fractions on sucrose gradients.

This relatively mild method which was originally introduced by Frenster et al. (210) was applied successfully by Chesterton (209) and Tata and Baker (208) to liver nuclei. However this sonication method had thusfar not been used to study the subnuclear localization of steroid-receptor complexes.

From the present results it appears that administration of oestradiol in vivo resulted in a relative enrichment of oestradiol (expressed as dpm/μg DNA) in the subnuclear fractions of uterus designed fractions 3,5 and 6 and also in the nucleoplasm (Table III). Although it could be demonstrated by a ^{3}H-oestradiol exchange procedure that this relative

enrichment was specific and not due to the sonication procedure at 0°C, it was tried to elucidate the nature of the oestradiol present in each fraction. Sucrose density gradient centrifugation appeared to be less suitable for the analysis of oestradiol-receptor complexes in each fraction. Hydroxylapatite column chromatography was expected to be more suitable for the localization of receptor bound oestradiol, because it had previously been used by Gschwendt (183) for separation of the oestradiol receptor population in chicken liver in KCl extractable and non-KCl extractable receptor sites due to the differences in interaction with the exchange resin. In this procedure the isolated chromatin was first dissociated in histones, nonhistones and DNA by adding 5M urea and 2M KCl.

In the present study it was observed that, in contrast to the oestradiol receptor in chicken liver, steroids are dissociated from the uterine receptor in the presence of urea. In addition it became apparent, that citrate ions in the buffer, which are used for the isolation of subnuclear fractions according to the method of Tata and Baker (208), interfered with both the interaction of oestradiol receptor molecules with the hydroxylapatite column and the interaction of receptor molecules with DNA in intact nuclei. Therefore, in later experiments urea was omitted and chloride ions rather than citrate ions were used in the subnuclear fractionation procedure and in the chromatography of the isolated subnuclear fractions.

Oestradiol receptors present in the individual subnuclear fractions of uterus could be eluted from the hydroxylapatite column at different ionic strengths. KCl extractable receptor sites (eluted predominantly at 20 mM phosphate) were found in the upper region of the fractionation gradient and in the nucleoplasm, whereas non-KCl extractable receptor sites (eluted at 200 mM and 500 mM phosphate) were measured in the pellet fraction. These results indicate that a separation of receptor molecules with different affinities for the chromatin could be achieved. The results might reflect that low affinity acceptor sites were recovered in the light

sucrose fractions and in the nucleoplasm, whereas high affinity acceptor sites were found in the pellet fraction. It has been suggested by others (140), that receptor molecules which interact with high affinity acceptor sites could be important for the response of the uterus to oestradiol. The present observation that these complexes are mainly located in the pellet fraction of the fractionation gradient suggests that this nuclear fraction might be important in the regulation of the activity of steroid hormones.

For the progesterone receptor in chick oviduct it has also been demonstrated that steroid receptor molecules were located in a heavy sucrose gradient fraction, containing the heterochromatin (206). In contrast to these results it has been demonstrated that, using a different technique for fractionation, progesterone receptors (218) and oestradiol receptors (227,228) were located in the lighter chromatin (euchromatin) fraction.

The recovery of receptor sites after the fractionation procedure of uterine nuclei was far from complete (in the order of 40%). A possible explanation may be found in the dilution of the nuclear sonicate during the separation of the subnuclear fractions on the sucrose gradient, which might result in a considerable dissociation of oestradiol-receptor complexes. This explanation is sustained by the observation that a considerable amount of the ^{3}H-oestradiol, which is present in the individual subnuclear fractions, is not bound to macromolecules, whereas in intact uterine nuclei unbound oestradiol was almost absent. In addition it was found that the binding sites were almost equally distributed between the upper two gradient fractions, including the nucleoplasm, and the pellet fraction of the gradient.

No conclusive data were obtained concerning the possible interaction of oestradiol receptor molecules and DNA present in the subnuclear fractions. On low salt sucrose density gradients oestradiol-receptor complexes, present in fraction 1, were shown to sediment heterogeneously in a region where small-size DNA species were also located (Figure 6A).

From the detailed studies of Yamamoto (120) it appears that such an interaction, if present, could represent a low affinity interaction without physiological meaning.

It has been shown by Tata and Baker (208,211) and Chesterton et al. (209) that sonication and sucrose gradient centrifugation of rat liver nuclei could achieve separation of euchromatin and heterochromatin fractions. This has been supported by results of transcription on DNA stretches in vitro, the localization of nascent RNA molecules and the precipitation of heterochromatin in the presence of 5 mM $MgCl_2$. Due to the presence of Mg^{2+} ions in the fractionation buffer this simple method could not be applied to the isolated uterine subnuclear fractions. In the present study it could be demonstrated that rapidly labelled RNA, after in vitro incubation of uterus, was predominantly located in the upper layers of the fractionation gradient (Table II), suggesting that euchromatin is present in these fractions. It cannot be excluded, however, that as a result of the isolation and fractionation of nuclei RNA chains become detached from their respective templates and interact with other DNA stretches. Nevertheless a random redistribution of labelled RNA can be excluded on basis of the results presented in Table II.

From the results presented in this chapter it is evident that the localization of oestradiol-receptor complexes in subnuclear fractions of uterus requires still further investigations. In this respect the influence of the different fractionation techniques on the subnuclear localization of steroid receptor molecules creates a challenging problem. Another difficulty is caused by the different ionic conditions which are required for the isolation of the 'physiological' form of those components which are supposed to be obligatory for the ultimate effect of steroid hormones, i.e. steroid hormone receptor associated with chromatin, RNA-polymerases and chromatin. Furthermore the question remains whether the isolated chromatin fractions are really representative for regions which are active and inactive in

transcription in vivo. In two studies (229,230) it has been reported that chromatin could be separated in euchromatin and heterochromatin, which differed in protein composition as well in their activity in transcription with RNA-polymerase in vitro. However DNA-DNA reassociation experiments performed with radioactive cDNA, obtained after transcription of RNA molecules with reverse transcriptase, did not provide any evidence for an unequal distribution of the RNA genes over both chromatin fractions. However the usefulness of endogenous genomes as probes for the structure of chromatin has been demonstrated in these studies. Perhaps the use of such experiments will assist in future attempts to relate in vitro definitions of fractionated chromatin to activities in vivo.

In conclusion the results in this chapter indicate that:

1. Oestradiol-receptor complexes with different affinities for the chromatin were present in subnuclear fractions isolated from uterine tissue.
2. Oestradiol-receptor complexes were located in both the light and heavy chromatin fractions, which might be representative for euchromatin and heterochromatin respectively.
3. Oestradiol-receptor complexes loosely bound to the chromatin were located in the light chromatin fractions, whereas tightly bound oestradiol-receptor complexes were located predominantly in the heavy chromatin fractions.

General discussion and conclusions

The work presented in the previous chapters concerning the regulation of the concentration and the subcellular localization of steroid receptors in target cells was started because almost nothing is known about the mechanisms regulating the amount and localization of steroid receptors. These receptors, which are supposed to be obligatory for steroid hormone effects, are located in the cytoplasmic fraction of a target cell, but are translocated into the nucleus after having formed a steroid-receptor complex with their specific steroid. Therefore changes in the number of cytoplasmic receptor sites might have a direct effect on the number of receptor sites, which can be translocated and this could ultimately result in changes of physiological effects i.e. the response of a tissue to the hormone.

6.1 Regulation of cytoplasmic and nuclear receptor sites for oestradiol in testicular tissue

It was attempted to investigate possible control mechanisms which might be important in controlling the number of oestradiol receptor sites in cytoplasmic and nuclear fractions of Leydig cells from rat testis.

Effect of oestradiol

From the results in appendix paper I it appears that oestradiol administration results in a maximal depletion of the oestradiol receptor from the cytoplasmic fraction isolated from both mature and immature rat testis within 1 h. For mature rats it has also been demonstrated that this depletion is accompanied by a rapid increase and a subsequent decrease of plasma oestradiol levels, suggesting that oestradiol from the rat plasma penetrates into the Leydig cell and induces a shift in the subcellular distribution of receptor molecules towards the nucleus. Replenishment of cytoplasmic receptor sites was shown to occur between 3 and 5 h after the administration of oestradiol.

For uterine and hypothalamic tissue it has been demonstrated that the replenishment of receptor sites involves both synthesis of new receptor molecules and recycling of receptors from the nucleus to the cytoplasm (98,143,168). These two processes resulted in a restoration of the cytoplasmic receptor levels to values similar to control levels between 11 and 15 h after the hormone administration.

The results obtained in the present studies for testicular tissue appeared different from those described for uterine and hypothalamic tissue. From the data obtained 3 and 5 h after the administration of oestradiol it is evident that the replenishment of cytoplasmic receptor sites in testis occurs at a faster rate. Similar differences in the rate of restoration of cytoplasmic receptor levels for oestradiol have been observed between pituitary tissue of male and female rats (231). It has also been observed that the replenishment of receptors in the pituitary of neonatally androgenized female rats follows the same pattern as was observed for the pituitary of either normal or androgenized male rats (231). Considering these results, it is tempting to speculate about a sex related difference between the replenishment of receptor molecules in male and female rats which might be caused by an interaction of androgens with the process. This might result either in differences in the de novo synthesis of receptor sites or in differences in the rate of transfer of nuclear receptor sites into the cytoplasm. The finding that the nuclear retention of oestradiol receptor sites in testis is relatively short when compared to that of the uterus (appendix paper III) is in favour of the latter possibility.

Effect of hypophysectomy

In order to study the influence of oestradiol on the amount of receptor sites for oestradiol, the number of cytoplasmic receptor sites in interstitial tissue from mature rat testis and the number of total available receptor sites

in immature rat testis were measured at different times after hypophysectomy. In appendix paper I it was shown that no changes in the oestradiol receptor concentration (expressed as fmol/mg interstitial cytosol protein) could be observed in adult rats between 1 and 10 days after hypophysectomy. During this period the weight of the testis does not change considerably. In studies of de Jong (42) it was shown that hypophysectomy for periods of 6-12 days drastically decreased the testicular oestradiol concentrations.

The observation that oestradiol receptor levels remain unaffected, while oestradiol concentrations decrease leads to the conclusion that oestradiol itself is not necessary for the maintainance of a constant number of cytoplasmic receptor sites per Leydig cell. It can, however, not be excluded that small but significant amounts of oestradiol produced extratesticularly play a role in maintaining constant receptor levels.

From studies performed with immature rats 5 days after hypophysectomy (Table 2, appendix paper II) it became clear that the total number of oestradiol receptor sites per testis was influenced by a factor which is under the control of the pituitary-hypothalamic system. This factor might be oestradiol, LH or some other steroid or trophic hormone. Because no data are available concerning the number of Leydig cells in testes of hypophysectomized immature rats it remains uncertain whether the observed changes reflect also a change in the number of receptor sites per Leydig cell.

Effect of choriogonadotropin

The decrease in the total available number of receptor sites per immature rat testis after hypophysectomy might be the result of a decrease in the number of Leydig cells. This view is supported by the observation that LH administration to hypophysectomized rats could prevent or even reverse the atrophy of the interstitial tissue (232). This view also fits the data obtained after injection of immature rats

with HCG, presented in appendix paper II. Administration of HCG to immature rats has a stimulatory effect on the number of Leydig cells per testis (233). It appears from this observation and the data presented in appendix paper II that HCG can stimulate the number of mitosis in Leydig cells which results in an increase of the number of Leydig cells also containing oestradiol receptors. Possibly as a result of the increase in production of oestradiol after HCG administration, as was shown for mature rats (42), the fraction of the receptor population which could be recovered from the nuclear fraction was increased. This suggests that the distribution of oestradiol receptor molecules over the cytoplasmic and the nuclear compartment of the Leydig cell is under the control of testicular oestradiol concentrations. The number of available receptor sites may also be influenced by the hormonal environment. Further studies are needed to elucidate whether LH and oestradiol are the only two hormones involved in the regulation of receptor sites for oestradiol and whether this regulation has any physiological significance in the development of the rat testis.

Ontogeny

In order to investigate the ontogeny of the oestradiol receptor in the testis, testicular tissue from immature rats was used. As a result of the presence of a second oestradiol binding protein (called α-foetoprotein) it was only possible to perform accurate quantitative measurements of cytoplasmic receptors in testicular tissue of rats older than 14 days. A rather constant amount of receptor sites (9 fmol/mg cytosol protein) appeared to be present in the cytoplasm of rats between 14 and 35 days of age. In mature rat testis an amount of approximately 18 fmol/mg cytosol protein has been measured. The increase in the receptor amount which appears from these data is in agreement with the results of Pahnke et al. (234). They observed a steep increase in the number of Leydig cells per testis between 30 and 60 days of age concomitant with a

parallel increase in testis weight.

A nuclear form of the oestradiol receptor could be demonstrated in testicular tissue of 4 days old rats, because contaminations originating from the plasma or the cytoplasm were effectively removed during the isolation of nuclei. Because no accurate data are available about the change of the number of Leydig cells during early development of the testis it is difficult to interpret these data per Leydig cell. Moreover, quantitative data would only be reliable if a method had been used, which estimates the number of receptor sites indepently from the amounts of endogenous oestradiol present in the testis from rats of different ages. Such a method has been developed for testicular tissue of immature rats and is described in appendix paper II.

Data on the amount of α-foetoprotein in the plasma of immature male rats are presented in appendix paper I. The change in the number of binding sites in plasma with age was compared with the amount measured in female rat plasma (235). It appears from the data in Table 1 of appendix paper I that the amount of α-foetoprotein in plasma of male rats is about 100 times lower, but the disappearance of the plasma protein occurs with a half life of about 5.5 days, which is in good agreement with the half life of 4 days observed in plasma of female rats.

Demonstration of occupied nuclear receptor sites

With the exchange method described in appendix paper II it could be shown that in nuclei isolated from intact immature rats (25 days of age), a considerable fraction of the total receptor population was present in the nuclear fraction isolated from Leydig cells. This observation suggests that sufficient oestradiol is present in the interstitial tissue compartment in order to keep the oestradiol-receptor complexes in the nuclear fraction. Nothing is known however about the origin of the oestradiol. It could have been produced from intratesticular as well as from peripheral

sources. Also nothing is known about its physiological role. If one assumes that under the influence of endogenous oestradiol also a significant fraction of the non-KCl extractable fraction of receptors is located inside the nucleus it is tempting to relate some Leydig cell function with it, because this fraction of receptors was shown to correlate with an oestradiol effect in the uterus (140).

6.2 Characterization and retention of nuclear receptor complexes

In appendix paper III studies concerning the nuclear translocation and nuclear binding of oestradiol receptor sites in testis and uterus are presented. This comparative study was performed in order to relate these processes in a cell with a poorly defined response, like the immature Leydig cell (236), with those in a cell, with a well-known response, the uterine cell.

Characterization of nuclear receptor sites in testis and uterus

From the results presented in appendix paper II it became apparent that certain differences concerning the retention of nuclear oestradiol-receptor complexes did exist between testicular and uterine tissue. In both tissues oestradiol is retained by two different nuclear receptor sites which could be distinguished according to their differences in extraction from nuclei with 0.4M KCl.

In order to obtain further information about the characteristics of the non-KCl extractable oestradiol receptor in testicular tissue, it was tried to solubilize the oestradiol-receptor complexes with either DNAse, trypsin or deoxycholate. All three different treatments did not result in a considerable release of oestradiol-receptor complexes. Only treatment with trypsin gave some information about the interaction of receptor complexes with the chromatin. From the altered sedimentation value of the complex after trypsin treatment, 4S instead of 5S, it was concluded that the enzyme

cuts off one part of the receptor protein containing the chromatin interaction site, while leaving the oestradiol binding site intact. In this respect the testicular oestradiol receptor does not differ from the oestradiol receptors found in liver and uterus (142,237).

The results presented in appendix paper II show that incubation of testicular nuclei, loaded with oestradiol-receptor complexes, for 60 min at 20°C in the presence of ^{3}H-oestradiol was sufficient for a complete exchange of the radioactive steroid with the non-radioactive steroid. It was also demonstrated that only 40% of the total receptor population (the fraction recovered in the KCl extract of the nuclei) could be measured by this method. In this respect two differences between testicular and uterine nuclei were observed. The oestradiol receptor in uterine nuclei can be readily extracted with 0.4M KCl (60 to 80% has been recovered in the extractable fraction) and in the exchange procedure routinely used for uterine nuclei by others (143, 164), a shift of receptor sites from the extractable fraction into the so-called nuclear residual fraction was observed. Whether this change is merely artificial or has any physiological significance is not clear.

Effect of energy and the release of nuclear bound receptor sites

It is generally accepted that nuclear oestradiol-receptor complexes, after being bound by the chromatin, can return into the cytoplasm via a recycling process (143,168). This suggests that after the interaction with the chromatin, enzymes must be involved in the release of nuclear receptor complexes. From the data in Figure 3 and Figure 4 in appendix paper III it can be concluded that different mechanisms might be involved in the retention of hormone-receptor complexes in nuclei of testicular and uterine tissue which could be related to differences in enzyme activities, necessary for the detachment of receptor sites.

From the studies performed with KCN it seems very likely that a continuous supply of energy is also important for the release of receptor complexes from the nuclei. In the non-KCl extractable fraction of both uterus and testis, addition of KCN drastically increased the number of oestradiol binding sites. This might be due to inhibitory action of KCN, mediated via ATP, on those enzymes which probably are necessary for the release of receptor sites associated tightly with the chromatin. In the literature other data are available which are in favour of this explanation concerning the release of chromatin bound steroid-receptor complexes. In studies performed on erythrocyte chromatin it has been demonstrated that the presence of Mg^{2+}-ions resulted in a complete contraction of the chromatin (238). Equal molar amounts of Mg^{2+} and ATP were found to inhibit this contraction. Therefore a decrease in the cellular (nuclear) amount of ATP as a result of the addition of KCN might result in a relative increase of the free Mg^{2+} concentration. The subsequent change in conformation of the chromatin might make the steroid receptor acceptor sites less susceptible for enzymes involved in the release of receptor molecules.

Retention of nuclear receptor sites in testis and uterus

The exact nature of the interaction of oestradiol-receptor complexes with the chromatin and the mechanism of release with KCl is still unclear. Therefore one can only speculate about the factors which determine the observed differences in the retention of nuclear receptor sites, shown in appendix paper III. One possible explanation might be found in differences in the affinity of the oestradiol-receptor complexes for acceptor sites on the chromatin. This could be due to variations in the nonhistone chromatin protein fraction among various tissues, because this group of proteins is thought to contain the so-called acceptor protein (121,124).

For uterine and prostatic tissue it has been suggested

that the retention of steroids by nuclear residual receptor sites (probably receptor sites with a very high affinity for the chromatin) are obligatory for hormone effects (140). This type of nuclear retention was shown to be absent in testicular tissue during longer incubation periods and may be due to the presence of acceptor sites with a relatively low affinity for the steroid-receptor complexes. This makes it very unlikely that an oestradiol effect in the testicular Leydig cell, if present, is mediated by an interaction of oestradiol-receptor complexes with acceptor sites in the non-KCl extractable nuclear fraction.

6.3 Subnuclear distribution of oestradiol-receptor complexes in uterus

From the results obtained in appendix paper III it was concluded that due to the lack of prolonged retention of oestradiol-receptor complexes in the non-KCl extractable fraction of testicular nuclei, the uptake and the subsequent binding of receptor complexes in testis and uterus might follow different pathways. One way to obtain further information about the mechanism of action of steroid hormones via their receptors in any cell type is to unravel the molecular mechanisms inside the nucleus, which are involved in the expression of known steroid effects. Therefore one has to characterize and localize the locus on the chromatin which interacts with receptor molecules. A possible correlation of the binding of receptor complexes with this locus and a specific hormone effect inside the nucleus, i.e. RNA-polymerase activity increase or synthesis of specific mRNA species, might provide the evidence necessary for the elucidation of the mechanism of action of steroid hormones. Preliminary results concerning the interaction of oestradiol-receptor complexes with subnuclear fractions isolated from uterine tissue are presented in chapter 5.

It became apparent that the subnuclear fractionation method by Tata and Baker (208) as such could not be applied

to uterine tissue in order to obtain information about the subnuclear distribution of oestradiol receptor sites. It was also found that a classical method for the analysis of nuclear receptor sites i.e. sucrose density gradient centrifugation in high salt, was not suitable for the demonstration of receptor sites in the different subnuclear fractions. To circumvent these difficulties we made appropriate changes in the fractionation buffer (citrate was replaced by chloride) and decided to use hydroxylapatite column chromatography in 0.4M KCl as a method for separation of nuclear receptor sites. This method has the advantage that it is suitable for the separation of KCl extractable and non-KCl extractable receptor sites as was previously demonstrated by Gschwendt for the oestradiol receptor in rat liver nuclei (183).

Application of the described method to nuclei obtained after in vitro incubation of uterus with oestradiol gave indications for the presence of steroid receptor molecules in both the euchromatin and heterochromatin fraction. The relatively small amount (in the order of 10%) of receptor sites which could be demonstrated in the nucleoplasm is probably released from nuclei during mild sonication and might represent a fraction of steroid-hormone complexes present in the nucleoplasm in vivo.

From the data presented in chapter 5 it is evident that the method used for the subcellular fractionation of uterine nuclei in order to correlate uterine oestradiol effects within the nucleus with the presence of oestradiol-receptor complexes, still requires improvement. It may be worthwhile to compare the different techniques which are available for the fractionation of intact nuclei in order to obtain an unambigous characterization of the isolated fractions. Furthermore the use of more sensitive methods for the detection of steroid-hormone complexes, like the antibody assay technique developed by Castaneda and Liao (239) or a technique which uses fluorescence labelled receptors, might improve our knowledge about the role of receptors in the mechanism of action of steroid hormones.

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Summary

Effects of steroid hormones in target cells are thought to be mediated via the interaction of steroids with specific receptor molecules. The complex formed between the receptor and the steroid is translocated into the nucleus and initiates a cascade of events, which ultimately results in the response ascribed to a specific hormone.

In chapter 2 the successive steps between the initial interaction of the steroid with its receptor and the final tissue response are discussed.

In chapter 3 a summary of methods used for the determination of specific steroid binding sites is given.

In order to obtain information about a possible function of the oestradiol receptor in the testicular Leydig cell we have compared several characteristics obtained for the testicular and uterine oestradiol receptor. In chapter 4 and appendix papers I and II the experiments on the regulation of the number of cytoplasmic and nuclear receptors are introduced and discussed.

It was found that after administration of oestradiol to intact mature and immature rats a decrease in the testicular concentration of cytoplasmic receptor sites was observed within 1 h. The binding capacity was replenished starting about 3 h after oestradiol administration and after 5 h the receptor levels were completely restored (appendix paper I).

In mature animals which were hypophysectomized for periods up to 10 days a constant level of cytoplasmic receptor sites was measured in total testicular tissue and dissected interstitial tissue (appendix paper I). From these results it is concluded that neither gonadotrophins nor steroid hormones are necessary for maintaining constant oestradiol receptor levels in the cytoplasm.

The testicular receptor for oestradiol was demonstrated in its nuclear form in rats from 4 days of age onwards. No accurate figures could be obtained for the number of receptor sites during the development of the rat (appendix paper I).

By means of an exchange method, in which receptor bound oestradiol is substituted for ^{3}H-oestradiol, it was demonstrated that testicular tissue of intact immature rats (25 days old) contained a considerable amount of nuclear receptors, occupied with endogenous oestradiol (20%) (appendix paper II). This amount was considerably increased in rats which had been treated with human choriogonadotropin for five successive days. Concomitant with this shift in favour of the nuclear fraction it was observed that the total amount of receptor sites per testis, was increased threefold. In contrast, after hypophysectomy the total amount of receptor sites per testis was only 50% of the amount measured in intact rats. In these animals no nuclear receptor sites occupied with endogenous oestradiol could be observed (appendix paper II). These observations suggest that gonadotrophins and oestradiol may be important in the in vivo regulation of the amount and the subcellular distribution of receptor sites in immature rat testicular tissue.

In chapter 4 and appendix paper III the results obtained in a comparative study with the uterus are described. It was observed that inside rat testicular nuclei oestradiol is specifically bound by two different classes of receptor sites, which differ in extractability with KCl. The amount of 'extractable' nuclear binding sites could be increased by mild trypsin treatment. The receptor sites, after being released with trypsin, showed a sedimentation value of 4S on sucrose density gradients, in contrast to the value of 5S which was observed for KCl extracted receptors.

The number of non-KCl extractable receptor sites in both testis and uterus was increased significantly after incubation of the tissues in the presence of KCN. This observation suggests that energy might be involved in either the binding or the subsequent release of nuclear oestradiol-receptor complexes.

In vitro incubation of testicular and uterine tissue was used as a technique for studying the rate of translocation and retention of nuclear oestradiol-receptor complexes in

both tissues (appendix paper III). The number of KCl extractable receptor sites in testis remained constant after 30 min of incubation; the number of non-KCl extractable receptor sites decreased continuously after incubation periods longer than 30 min. In contrast the number of both types of binding sites in the uterine nuclei after an initial increase, decreased to 50% of the maximum value between 30 and 60 min of incubation. On basis of these results it seems very likely that oestradiol-receptor complexes in testis and uterus interact with different acceptor sites on the chromatin.

In chapter 5 the experiments on the subnuclear distribution of oestradiol-receptor complexes in uterine tissue are presented. It was observed that oestradiol-receptor complexes were localized in a transcriptionally active chromatin fraction (euchromatin) and a chromatin which is repressed in transcription (heterochromatin). Also in the nucleoplasmic fraction oestradiol-receptor complexes could be demonstrated.

In chapter 6 of this thesis, the results obtained in the appendix papers and in chapter 5 are discussed in more detail and are compared with results presented in the literature.

Samenvatting

De interaktie van steroidhormonen met specifieke receptor eiwitten speelt waarschijnlijk een rol in het tot stand komen van steroidhormoon effekten. Het steroid-receptor-komplex dat gevormd wordt in het cytoplasma van een cel, verhuist naar de kern en heeft een reeks van gebeurtenissen tot gevolg, die resulteren in de veranderingen, die toegeschreven worden aan een bepaald hormoon.

In hoofdstuk 2 is een overzicht gegeven van de gebeurtenissen die plaats vinden tussen de interaktie van het steroid met de receptor en de uiteindelijke veranderingen in de cel.

In hoofdstuk 3 worden de methoden beschreven, die voor de bepaling van steroidreceptoren gebruikt zijn.

De Leydig cel in de testis van de rat bevat een receptor eiwit, dat specifiek oestradiol bindt. Om voor dit eiwit een mogelijke funktie op te sporen zijn in dit proefschrift enkele eigenschappen van deze receptor vergeleken met die van de oestradiol receptor in uterus weefsel. In hoofdstuk 4 worden de experimenten beschreven die de regulatie van cytoplasmatische receptoren en kernreceptoren bestuderen. Na toediening van oestradiol aan intakte volwassen of jonge ratten neemt de hoeveelheid cytoplasmatische receptor in de testis binnen 1 uur af. Reeds na 3 uur neemt de hoeveelheid receptor weer toe en na 5 uur worden weer kontrole waarden gemeten (appendix publikatie I).

Na hypofysektomie van volwassen ratten blijft de hoeveelheid receptor gedurende 10 dagen konstant zowel in totaal testisweefsel als in interstitieel weefsel (appendix publikatie I). Op grond van deze resultaten is het onwaarschijnlijk dat gonadotrofines en steroidhormonen nodig zijn voor de handhaving van receptor gehaltes in het cytoplasma.

De oestradiol receptor kon al aangetoond worden in kernfrakties geïsoleerd uit de testis van 4 dagen oude ratten. Het was niet mogelijk kwantitatieve gegevens te verkrijgen voor de hoeveelheid receptor gedurende de ontwikkeling van

de rat (appendix publikatie I).

In appendix publikatie II wordt een methode beschreven, waarmee een hoeveelheid kernreceptor gemeten kan worden in aanwezigheid van grote hoeveelheden niet-radioaktief oestradiol. Met behulp van deze methode, waarbij niet-radioaktief steroid uitgewisseld wordt tegen radioaktief steroid, is aangetoond, dat in de testis van 25 dagen oude ratten 20% van de totale hoeveelheid receptor zich in de kernfraktie bevindt (appendix publikatie II). Na toediening van HCG nam zowel de totale hoeveelheid receptor als het percentage receptor dat zich in de kern bevindt aanzienlijk toe. Door hypofysektomie verminderde de totale hoeveelheid receptor molekulen per testis tot 50% van de kontrole waarde. Deze receptoren werden alleen in het cytoplasma en niet in de kern aangetroffen (appendix publikatie II). Op grond van deze resultaten is het waarschijnlijk dat gonadotrofines en oestradiol een rol spelen in de regulering van de hoeveelheid en de subcellulaire verdeling van de receptoren tussen cytoplasma en kern van de testis van jonge ratten.

In hoofdstuk 4 en appendix publikatie III zijn de eigenschappen van de oestradiol receptor in testis en uterus met elkaar vergeleken. In de kernfraktie van de testis bindt oestradiol zich aan twee verschillende receptor eiwitten. Deze eiwitten onderscheiden zich van elkaar op grond van hun extraheerbaarheid met KCl. Trypsine behandeling van kernen heeft een toename van de hoeveelheid KCl extraheerbare receptor tot gevolg. Op sucrose dichtheidsgradienten vertonen de oestradiol-receptor komplexen na trypsine behandeling een sedimentatiewaarde van 4S. Dit in tegenstelling met de 5S waarde, die gemeten wordt voor de trypsine behandeling.

Wanneer testis- en uterusweefsel met oestradiol geïnkubeerd werden in aanwezigheid van KCN resulteerde dit in een toename van de hoeveelheid kernreceptoren, die bestand zijn tegen KCl extraktie. Deze waarneming maakt het waarschijnlijk dat energie een rol speelt in de binding van receptoren in de kern of de daaropvolgende verwijdering van receptoren uit de kern.

De snelheid van verplaatsing naar en de verblijftijd van receptoren in de kernfraktie van testis en uterus zijn bestudeerd met behulp van weefselinkubatie (appendix publikatie III). De hoeveelheid KCl extraheerbare receptor nam toe tot een maximum na 30 min., waarna deze waarde konstant bleef. De hoeveelheid receptor die niet met KCl geëxtraheerd wordt bereikte eveneens na 30 min. een maximum, maar nam bij langere inkubatieduur sterk af. Tijdens inkubatie van de uterus werd voor beide typen kernreceptoren na 30 min. een maximumhoeveelheid receptor gemeten. In dit geval had een langere inkubatieduur een afname tot 50% van de maximale waarden tot gevolg. De verschillen in kinetiek van de kernbinding maken het aannemelijk dat oestradiol-receptor-komplexen in de kernfrakties van testis en uterus op verschillende wijze worden gebonden.

In hoofdstuk 5 wordt de verdeling van oestradiol-receptor komplexen in verschillende kernfrakties van de uterus beschreven. De komplexen werden zowel aangetoond in een chromatine fraktie die aktief is in transkriptie (euchromatine) als in een fraktie, welke inaktief is (heterochromatine). In het nucleoplasma werden eveneens oestradiol-receptor-komplexen aangetoond.

In hoofdstuk 6 van dit proefschrift zijn de verkregen resultaten besproken in het kader van in de literatuur verschenen waarnemingen.

Abbreviations

ATP	- adenosine 5'-triphosphate
CBG	- corticoid-binding globulin
DBG	- dihydrotestosterone-binding globulin
DNA	- deoxyribonucleic acid
DNAse	- deoxyribonuclease
dpm	- disintegrations per minute
EBG	- estrogen-binding globulin
ECHTAM	- epichlorohydrin-tris(hydroxymethyl)-aminomethane
E.coli	- Escherichia coli
E_{260}	- absorbance at 260 nm; 1 cm light-path
FSH	- follicle stimulating hormone
g	- relative centrifugal force
h	- hour
HCG	- human choriogonadotropin
Hn-RNA	- heterogeneous nuclear RNA
IP	- induced protein
K_a	- association constant
KRBG	- Krebs Ringer bicarbonate glucose
lac-operon	- lactose operon
LH	- luteinizing hormone
M^{-1}	- litres per mole
(m)M	- (milli)moles per litre
mA	- milliampère
mRNA	- messenger RNA
PBG	- progesterone-binding globulin
poly-A	- polyadenylic acid
RNA	- ribonucleic acid
RNAse	- ribonuclease
RNP-particles	- ribonucleoprotein particles
rRNA	- ribosomal RNA
S	- Svedberg unit
SBG	- sex steroid-binding globulin
sec	- seconds
sp.act.	- specific activity

$t_{1/2}$	- half life time
Tris	- Tris(hydroxymethyl)aminomethane
tRNA	- transfer RNA

Nawoord

Vele mensen en ratten hebben aan het tot stand komen van dit proefschrift hun bijdrage geleverd. Enkelen wil ik in het bijzonder bedanken.
Mijn promotor, Henk van der Molen, voor de kritische begeleiding van alle in dit proefschrift beschreven experimenten en voor de vele korrekties, die nodig waren voor publikaties en het proefschrift.
Eppo Mulder, die in zijn rol als advokaat van de duivel, vele beantwoorde en onbeantwoorde vragen kon oproepen.
Joan de Vries, die vrijwel alle experimenten tot een goed einde bracht, danzij haar grote dosis nauwkeurigheid, volharding en inzicht.
Wilma van Beurden, voor de stimulerende samenwerking en het inzicht in het verrichte onderzoek.
Marjan Peters en Bep Roodnat, voor de vele niet-biochemische intermezzo's voor, tijdens of na thee- en koffiepauzes.
Pim Clotscher, die altijd weer de ultracentrifuges aan de gang wist te houden.
De beide coreferenten, Professor M. Gruber en Professor W.C. Hülsmann, voor het kritisch doorlezen van het manuskript en de geleverde kommentaren.
Marja Decae, die in staat was zelfs twee proefschriften gelijktijdig in een definitieve vorm te typen.
Rien Blankenstein, voor de allerlaatste noodzakelijke korrekties en
Nico Woudstra, voor de artistieke bijdrage aan dit proefschrift.

Curriculum vitae

Op 21 maart 1948 ben ik te Joure geboren.In 1965 behaalde ik het getuigschrift HBS-B aan het Openbaar Lyceum te Heerenveen en in hetzelfde jaar begon ik de studie chemie aan de Rijksuniversiteit te Groningen.In 1972 heb ik het doctoraal examen afgelegd met als hoofdvak biochemie en als bijvak klinische chemie.Sinds januari 1973 ben ik werkzaam als wetenschappelijk medewerker bij de afdeling Biochemie II (Chemische Endocrinologie) van de Erasmus Universiteit te Rotterdam.

Appendix papers

J. Endocr. (1976), 70, 397–407 *Printed in Great Britain*

EFFECTS OF OESTRADIOL-17β, HYPOPHYSECTOMY AND AGE ON CYTOPLASMIC OESTRADIOL-17β RECEPTOR SITES IN RAT TESTIS INTERSTITIAL TISSUE

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(*Received* 20 *October* 1975)

SUMMARY

After administration of oestradiol-17β to intact mature and immature rats, a decrease in the testicular concentration of specific oestradiol-binding sites was observed within 1 h. The binding capacity was replenished starting about 3 h after oestradiol administration and after 5 h the oestrogen receptor level had returned to control values. Exposure of intact animals to oestradiol-17β for longer periods (up to 24 h) did not result in an increase of receptor levels in testicular cytosol.

Mature animals which were hypophysectomized for periods of up to 10 days did not show a significant change in the number of specific oestradiol-binding sites in either total testicular tissue or dissected interstitial tissue. At 15 days or longer periods after hypophysectomy, an apparent increase in receptor concentrations in total testicular cytosol was observed due to a relative increase in the amount of interstitial tissue.

A specific oestradiol-binding protein is present in plasma of immature male rats aged less than 30 days. This plasma protein could also be demonstrated in the cytosol of testes of immature rats. In contrast to the cytosol receptor, which shows a moderate affinity for diethylstilboestrol (DES), the plasma protein did not bind DES. The sedimentation values of the plasma protein and the oestradiol receptor were 4 S and 8 S respectively. These differences in characteristics made it possible to demonstrate the presence of the oestradiol receptor in addition to the binding protein in testicular cytosol of rats from 14 days of age onwards. The nuclear receptor for oestradiol-17β could be demonstrated after incubation of testicular tissue of rats from 4 days of age onwards.

INTRODUCTION

In the course of a study on steroid hormone receptors in testicular tissue we have previously demonstrated the presence of a specific oestradiol receptor in the cytosol and nuclear fractions of rat testicular interstitial tissue (Brinkmann, Mulder, Lamers-Stahlhofen, Mechielsen & van der Molen, 1972; Mulder, Brinkmann, Lamers-Stahlhofen & van der Molen, 1973). This receptor is specific for oestradiol ($K_a = 10^{10}$ l/mol) and has a negligible affinity for androgens (van Beurden-Lamers, Brinkmann, Mulder & van der Molen, 1974). The possible physiological meaning of the specific binding of oestradiol by interstitial cells is not clear. Studies by de Jong, Hey & van der Molen (1973, 1974) have shown that endogenous oestradiol can be demonstrated in testicular (10^{-10} mol/l) and interstitial ($0{\cdot}5$–1×10^{-9} mol/l) tissue. Actions of exogenous oestradiol on testicular DNA, RNA and protein synthesis have been reported for Balb/c mice (Samuels, Uchikawa & Huseby, 1967). An effect of oestradiol on testosterone concentrations in the circulation of male rats without a concomitant change

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in luteinizing hormone (LH) levels, which might imply a direct effect of oestradiol on steroidogenesis in testicular tissue, has been observed. (Danutra, Harper, Boyns, Cole, Brownsey & Griffiths, 1973; Danutra, Harper & Griffiths, 1973; Chowdhury, Tcholakian & Steinberger, 1974; Tcholakian, Chowdhury & Steinberger, 1974).

It is now widely accepted that steroid hormones exert their effects through an interaction with subcellular receptors. After binding of the hormone to cytoplasmic receptors the receptor–hormone complex is translocated into the nuclear fraction and becomes bound to the chromatin. This interaction between the receptor–hormone complex and the chromatin might further regulate gene expression. Thus the hormonal control of target cells could depend both on variations in hormone concentrations and on changes in the amount of receptor proteins.

The purpose of the present study was to investigate the testicular concentration of receptor sites for oestradiol-17β under various conditions. This included the influence of oestradiol-17β administration on the concentration of cytoplasmic receptor sites, the effect of gonadotrophins in the regulation of receptor concentrations and the ontogeny of the testicular oestradiol receptor in immature rats.

MATERIALS AND METHODS

Materials

[2, 4, 6, 7-^{3}H]Oestradiol-17β (sp.act. 105 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. The radiochemical purity was verified by thin-layer chromatography. Diethylstilboestrol and oestradiol-17β were obtained from Steraloids Inc, Pawling, N.Y., U.S.A.

Preparation of subcellular fractions and incubation procedures

Mature (3 months old) and immature rats of the R-Amsterdam strain were used. The animals were injected with 500 ng (mature animals) or 100 ng (immature animals) oestradiol-17β dissolved in 0·15 M-saline containing 2 % ethanol where indicated. For studies *in vitro* decapsulated testicular tissue of immature rats was incubated in 2·0 ml Krebs–Ringer–bicarbonate buffer (pH 7·4), containing 0·2 % glucose and 2×10^{-8} M-[^{3}H]oestradiol-17β. Incubations were carried out at 32° C for 60 min in an atmosphere of 95 % O_2:5 % CO_2. Interstitial tissue was obtained by wet dissection of decapsulated whole testicular tissue at 0 °C (Christensen & Mason, 1965). The isolated tissue was homogenized in 10 mM-Tris–HCl buffer (pH 7·4), (1 ml/g tissue) containing 1·5 mM-EDTA and 0·02 % NaN_3, with three strokes of a Potter–Elvehjem homogenizer at 1100 rev./min. The homogenate was centrifuged at 105000 *g* for 60 min at 0 °C. The 105000 *g* supernatant (cytosol) was incubated with steroids for 16–24 h at 0 °C to reach saturation. For the preparation of nuclei, total testicular tissue was homogenized in ice-cold Tris–HCl buffer (pH 7·4), containing 1·5 mM-EDTA and 0·02 % NaN_3 with six strokes of a Potter–Elvehjem homogenizer at 1100 rev./min. The homogenate was centrifuged at 500 *g* for 10 min at 0 °C. The 500 *g* pellet was washed once with homogenization buffer containing 0·2 % Triton X-100 and twice with homogenization buffer. A nuclear extract was prepared by extraction of the nuclear fraction with 0·4 M-KCl in 10 MM Tris–HCl (pH 8·5), containing 1·5 mM-EDTA and 0·02 % NaN_3, for 60 min at 0 °C, followed by centrifugation at 105000 *g* for 30 min at 0 °C.

Measurement of steroid binding

When cytosol was incubated with oestradiol-17β the steroid was bound to specific and non-specific binding sites. In preliminary experiments it was shown that 2×10^{-8} M-oestradiol saturates all specific binding sites. The cytosol was incubated with either [^{3}H]oestradiol-17β

to determine total binding or [^{3}H]oestradiol-17β plus a 200-fold excess of unlabelled oestradiol-17β to determine non-specific binding (Williams & Gorski, 1973). The amount of specifically bound steroid was calculated by subtracting the value for [^{3}H]oestradiol-17β bound in the presence of unlabelled oestradiol-17β (non-specifically bound oestradiol-17β) from the value for total [^{3}H]oestradiol-17β binding.

The bound and unbound steroid fractions after incubation of cytosol with steroids were separated by one of the following techniques.

Sephadex chromatography

Sephadex chromatography was performed as described by Williams & Gorski (1973). A 50 μl portion of incubated cytosol was layered on a column ($8 \times 0{\cdot}5$ cm) of Sephadex G-25 Superfine grade. The column was eluted with homogenization buffer and the excluded volume (bound radioactivity) was collected in a vial and the radioactivity was measured.

Gradient centrifugation

After incubation of the cytosol with steroid, 200 μl were layered on 5 ml of a 5–20 % sucrose density gradient prepared in homogenization buffer. After centrifugation in a Beckman L2–65B centrifuge at 0 °C for 16 h at 150000 g_{av} in a SW65 rotor, the bottom of the tube was pierced and 30 fractions were collected. Radioactivity was measured in each fraction. Nuclear extracts were layered on 5 ml of a 5–20 % sucrose density gradient prepared in extraction buffer and run for 18 h at 260000 g_{av}. Bovine serum albumin (BSA) and alcohol dehydrogenase (ADH) were used as markers with sedimentation values of 4·3 S and 7·6 S respectively.

Agar-gel electrophoresis

Agar-gel electrophoresis was performed essentially as described by Wagner (1972). A 50 μl portion of incubated cytosol was applied on an agar plate ($100 \times 85 \times 5$ mm thick) kept at 0 °C (agar Noble; Difco, Detroit, Michigan, U.S.A.). After electrophoresis for 90 min at 130 mA per plate (200–250 V) at 0 °C, the plate was cut into ten strips, each containing one sample, and each strip was divided into 20 fractions of 4 mm. To count the radioactivity, steroid from the individual agar fractions was dissolved by shaking for 12 h at room temperature in 10 ml Triton/scintillation fluid mixture.

Pretreatment with charcoal

Excess unbound steroid was in some experiments removed by adding 0·5 mg dextran-coated charcoal to 200 μl incubated cytosol. After mixing, the suspensions were incubated for 15 min at 0 °C and charcoal was removed by centrifugation for 10 min at 1200 g.

Protein determination

The protein content of the isolated cytosols was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. Generally the cytosol of mature rat testis contained 20–25 mg/ml and immature rat testicular cytosol contained 5–15 mg/ml.

Estimation of oestradiol-17β

Oestradiol-17β was measured by a radioimmunoassay technique as described by de Jong *et al.* (1974).

Measurement of radioactivity

Radioactivity was measured in a Packard model 3375 liquid scintillation spectrometer. The scintillation fluid consisted of a mixture of Triton X-100 (Rohm and Haas, Philadelphia, U.S.A.) and toluene (1:2, v/v) containing 0·1 g POPOP (1,4-bis-(5-phenyloxazol-2-yl)

benzene)/l and 4·8 g PPO (2,5-diphenyloxazole)/l (Packard Instrument S.A. Benelux, Brussels, Belgium).

RESULTS

Effects of exogenous oestradiol-17β on cytoplasmic receptor levels

Receptor concentrations were estimated by Sephadex chromatography and sucrose gradient centrifugation. With both these techniques specific steroid binding is estimated by subtracting the radioactivity bound in the presence of the competing non-radioactive oestradiol from the radioactivity retained in the absence of the competing steroid. It has previously been shown that these two methods give similar results (van Beurden-Lamers *et al.* 1974) which are also in good agreement with the results estimated using a Scatchard-type plot obtained by charcoal assay (Brinkmann *et al.* 1972).

Mature rats received a single injection of 500 ng oestradiol-17β. One and 3 h after injection, the values obtained were significantly lower than the control levels, but levels 5 and 24 h after injection of oestradiol-17β were similar to those of the control (Fig. 1*a*).

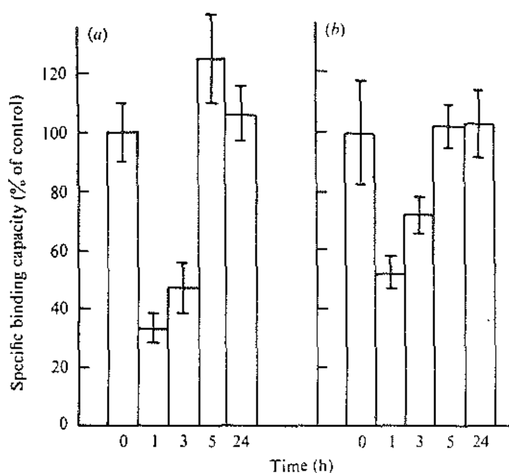

Fig. 1. Effect of oestradiol-17β on cytoplasmic receptor concentration. Rats were exposed to a single dose of oestradiol-17β for various periods. Testicular cytosol was incubated for 24 h at 0 °C with 2×10^{-9} M-[³H]oestradiol-17β or with 2×10^{-9} M-[³H]oestradiol-17β in the presence of 4×10^{-7} M-oestradiol-17β. After incubation, binding was determined by Sephadex G-25 chromatography and sucrose density gradient centrifugation. Specific binding is expressed as a percentage of control values ±S.D. for rats not injected with oestradiol-17β (n = 4). (*a*) Injection of mature rats with 500 ng oestradiol-17β. (*b*) Injection of immature rats with 100 ng oestradiol-17β.

Immature rats (23 days) were injected with 100 ng oestradiol-17β. Within 1 h after oestradiol administration, receptor levels decreased significantly; levels similar to those of the control were observed again at 5 and 24 h after hormone injection (Fig. 1*b*).

Binding of [³H]oestradiol to cytoplasmic receptor molecules was measured after incubation at 0 °C. At this temperature only free receptor sites are estimated. Receptor sites which were already occupied by unlabelled oestradiol remain unaffected due to the slow dissociation of the receptor–hormone complex at 0 °C. From other data (van Beurden-Lamers *et al.*

1974; de Jong *et al.* 1974) it has been calculated that in the order of 10 % of the available receptor sites in testicular cytosol of untreated rats are maximally occupied by endogenous oestradiol. To measure the total amount of cytoplasmic receptor sites at various times after hormone injection mature and immature animals were injected with 500 and 100 ng [^{3}H] oestradiol respectively. The specific activity of the injected steroid (105 Ci/mmol) is not influenced by the endogenous amount of oestradiol present in rat testicular tissue, because this amount is negligible compared with the amount of injected steroid. Testicular cytosols were isolated and incubated with [^{3}H]oestradiol of the same specific activity as that used for injection. During this incubation process only free receptor sites are labelled, any receptor sites already labelled *in vivo* and still present in the isolated cytosol would remain occupied. The amount of specifically bound hormone in the cytosol estimated after incubation *in vitro* was similar after injection of unlabelled oestradiol or after injection of [^{3}H]oestradiol. This may reflect the fact that the receptor sites which are present in the cytoplasm at any time after oestradiol injection are all available for the binding of [^{3}H]oestradiol in the subsequent incubation procedure. In previous studies the presence of specific nuclear receptors for oestradiol was demonstrated in interstitial tissue (Mulder *et al.* 1973). Therefore it seems very likely that the observed decrease in cytosol receptor concentrations is a result of the translocation of receptor–hormone complexes into the nuclei.

At various times after injection of 500 ng oestradiol-17β into mature rats, oestradiol-17β concentrations in plasma were measured (Fig. 2). One hour after injecting the oestradiol-17β the concentration was raised while 3, 5 and 24 h after administration plasma concentrations of oestradiol-17β did not differ significantly from the control values measured before administration.

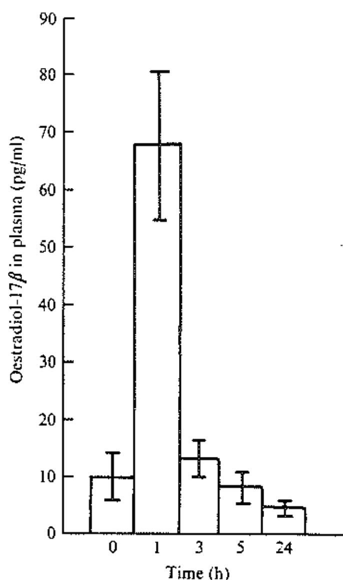

Fig. 2. Concentration of oestradiol-17β in rat plasma at various times after s.c. injection of 500 ng oestradiol-17β. After collection of plasma the concentration of oestradiol-17β was estimated. Results are means ± S.D. (n = 3).

Effect of hypophysectomy on cytoplasmic receptor levels

At various times after hypophysectomy receptor concentrations in total testicular tissue and interstitial tissue were determined. Up to 15 days after hypophysectomy no change could be observed in oestradiol receptor concentration in total testicular tissue (Fig. 3). Similar results were obtained for cytosol receptor concentrations in dissected interstitial tissue isolated from rats 1–10 days after hypophysectomy. For periods longer than 15 days after hypophysectomy an increase in receptor concentrations in total testicular tissue was observed (Fig. 3.).

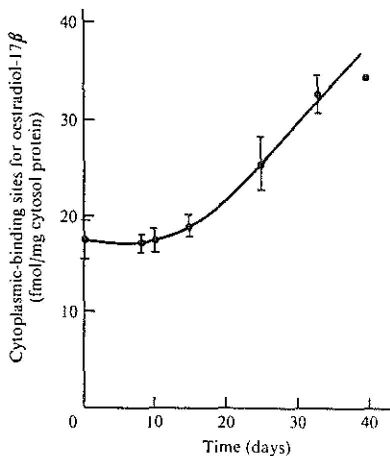

Fig. 3. Effect of duration of hypophysectomy on oestradiol-binding capacity in rat testicular cytosol. Testicular cytosol was incubated for 16 h at 0 °C with either 2×10^{-9} M-[^{3}H]oestradiol-17β or 2×10^{-9} M-[^{3}H]oestradiol-17β in the presence of 4×10^{-7} M-oestradiol-17β. After incubation binding was determined by Sephadex G-25 chromatography. Each point represents the mean (± S.D.) of four different animals. Value at day 38 is the mean of two estimations.

Oestradiol binding sites in plasma and testicular cytosol of immature rats

Plasma of immature rats 4–35 days old was incubated with either [^{3}H]oestradiol-17β or [^{3}H]oestradiol-17β in the presence of a 200-fold excess of non-radioactive oestradiol-17β. Specific binding was determined using agar-gel electrophoresis (Table 1) or sucrose density gradient centrifugation (Fig. 4*a*). High levels of a specific oestradiol-binding protein with a sedimentation value of 4 S were demonstrated immediately after birth while decreasing levels were measured during the onset of pubescence. No specific binding could be demonstrated in plasma of 30-day-old rats.

When testicular cytoplasm of 20-day-old rats was incubated with [^{3}H]oestradiol-17β and analysed for binding using sucrose density gradients at low ionic strength two binding proteins with different sedimentation values of 8 and 4S respectively could be demonstrated (Fig. 4*b*). Binding of [^{3}H]oestradiol-17β to the 8 S component was blocked by DES and by oestradiol-17β whereas DES did not affect the 4S binding (Fig. 4*b*). Thus it is possible to distinguish between the cytoplasmic oestradiol receptor and the plasma binding protein using the differences in sedimentation valu e and in binding affinity for DES. After incubation

Table 1. *Effect of age on the presence of oestradiol-17β-binding sites in testicular cytosol and plasma of immature rats*

(Testicular cytosol and 1 :50 diluted plasma were incubated for 24 h at 0 °C either with 2×10^{-9} M-[^{3}H]oestradiol-17β or with 2×10^{-9} M-[^{3}H]oestradiol-17β in the presence of 4×10^{-7} M-diethylstilboestrol. After incubation, cytosol samples were applied to sucrose density gradients and run for 16 h at 150000 g_{av} in a SW 65 rotor. Specific binding in plasma samples was determined using agar-gel electrophoresis. In column II binding in plasma is given. In column III the range of the measured values in the cytosol is given, n.d. means that 8 S binding macromolecules were not detectable. For comparison the total amount of oestradiol-17β-binding protein in cytosol is given in column IV. The results represent the mean of 2 to 3 experiments.)

Age (days) of rats (I)	Plasma binding (fmol/μl undiluted plasma) (II)	8S-binding cytosol (fmol/mg protein) (III)	Total amount of binding protein in cytosol (fmol/mg protein) (IV)
4	187	n.d.	525
7	125	n.d.	510
10	133	n.d.	110
14	59	10–13	200
20	29	6–9	70
26	44	7–11	125
30	0·6	8–10	15
35	0·6	6–9	20

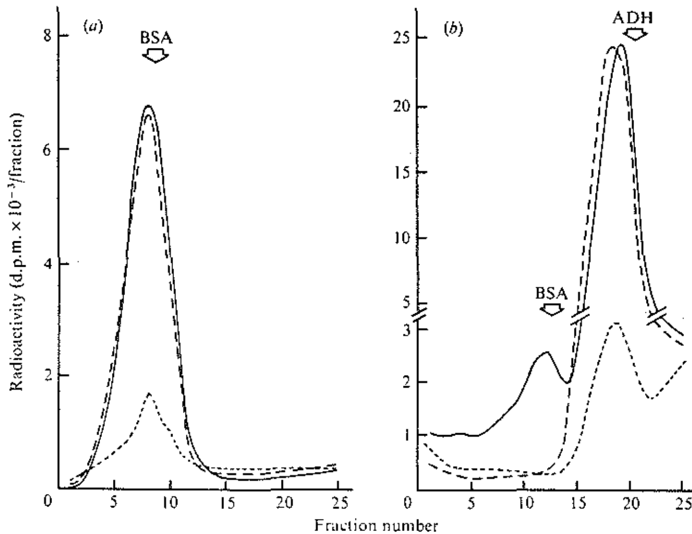

Fig. 4. Oestradiol-binding proteins in plasma and testicular cytosol of 20-day-old rats. Testicular cytosol and 1 :50 diluted plasma were incubated with 2×10^{-9} M-[^{3}H]oestradiol-17β (solid line), with 2×10^{-9} M-[^{3}H]oestradiol-17β plus 4×10^{-7} M-diethylstilboestrol (broken line), or with [^{3}H]-oestradiol-17β plus 4×10^{-7} M-oestradiol-17β (dotted line). After incubation at 0 °C for 24 h and removal of unbound steroids with charcoal, samples were applied to sucrose density gradients. Plasma samples (Fig. 4*a*) were run for 18 h at 260000 g_{av}; cytosol samples (Fig. 4*b*) for 16 h at 150000 g_{av} in a SW65 rotor. Fractions were collected from the bottom of the tube. The arrows indicate the positions of bovine serum albumin (BSA) (4·3 S) and alcohol dehydrogenase (ADH) (7·6 S).

of testicular cytosol from immature rats with [^{3}H]oestradiol-17β in the presence of a 200-fold excess of DES the 8 S receptor for oestradiol-17β could be demonstrated for rats from the age of 14 days onwards (Table 1). The last column of Table 1 represents the specific binding of [^{3}H]oestradiol-17β to both the cytoplasmic receptor and the 4 S binding protein in the testicular cytosol of immature rats as determined by agar-gel electrophoresis.

To investigate the presence of testicular oestradiol receptors in rats younger than 14 days, total testicular tissue was incubated with [^{3}H]oestradiol-17β or [^{3}H]oestradiol-17β in the presence of a 200-fold excess DES. In the nuclear extract prepared from testicular tissue of rats from 4 days of age onwards a specific binding peak for [^{3}H]oestradiol-17β was found (Fig. 5).

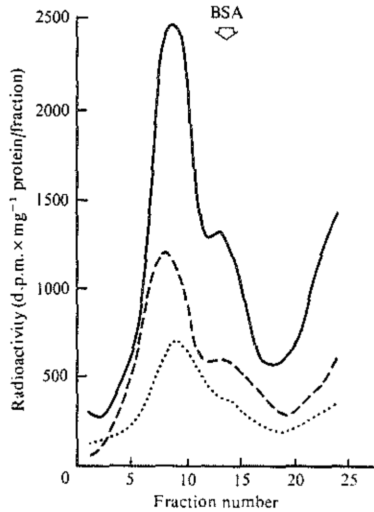

Fig. 5. Nuclear oestradiol-binding proteins from testicular tissue of immature rats. Total testis tissue was incubated for 60 min with either 2×10^{-8} M-[^{3}H]oestradiol-17β or with 2×10^{-8} M[^{3}H]-oestradiol-17β plus 4×10^{-6} M-diethylstilboestrol. Nuclei and nuclear extract were prepared as described in Materials and Methods. Nuclear extracts (200 μl) were applied to sucrose density gradients containing 0·4 M-KCl and run for 18 h at 260000 g_{av} in a SW65 rotor. Values are corrected for non-specific binding. The protein concentrations of the nuclear extracts were 2·7, 2·0 and 3·2 mg/ml for 4 (solid line), 7 (broken line) and 10 (dotted line) day-old-rats respectively. The arrow indicates the position of bovine serum albumin (BSA) (4·3 S).

DISCUSSION

There is no general concept about the regulation of steroid hormone receptor concentrations. For rat prostate it has been shown that after castration the amount of cytoplasmic receptor for dihydrotestosterone decreased to undetectable levels (Jung & Baulieu, 1971; Mainwaring & Mangan, 1973; Sullivan & Strott, 1973; Bruchovsky & Craven, 1975), but after longer periods of castration almost complete restoration of receptor levels was observed (Sullivan & Strott, 1973). In guinea-pig uterus, administration of oestradiol-17β results in an enhancement of cytoplasmic receptor levels for progesterone while administration of progesterone itself causes a decrease of receptor levels (Milgrom, Thi, Atger & Baulieu, 1973; Freifeld, Feil & Bardin, 1974). Treatment of immature rats with oestradiol-17β results in an increase

in the level of uterine receptors for oestradiol-17β (Sarff & Gorski, 1971). The present data indicate that administration of 100 and 500 ng unlabelled oestradiol-17β to immature and mature rats respectively caused a rapid decrease of oestradiol-17β receptor sites available in testicular cytoplasm (Fig. 1). The time-course of the disappearance and reappearance of cytoplasmic receptor sites after oestradiol-17β administration closely parallels the entry of oestradiol into and its removal from the blood circulation. Injection of [^{3}H]oestradiol-17β and the subsequent incubation of cytosol with [^{3}H]oestradiol-17β at 0°C resulted in a similar decrease of receptor levels as measured after injection of unlabelled oestradiol-17β. Therefore the observed decrease in the amount of specifically bound radioactivity must be the result of the disappearance of receptor molecules from the cytoplasm. Previously the existence of a nuclear form of oestradiol-17β receptor in interstitial tissue has been demonstrated (Mulder *et al.* 1973). For uterine tissue Williams & Gorski (1972) observed an equilibrium between the concentration of receptor-bound steroid in the cytosol and receptor-bound steroid in the nuclei. Therefore the observed changes in testicular receptor concentration after hormone injection may also reflect the translocation process of hormone molecules into the nuclei. A comparison of the processes of nuclear translocation and cytoplasmic restoration of receptor molecules between testicular tissue and other oestrogen-sensitive tissues shows several differences. In uterine tissue of immature rats the lowest cytoplasmic receptor levels have been measured 3–6 h after administration of 100 ng oestradiol-17β (Sarff & Gorski, 1971; Cidlowski & Muldoon, 1974). Control levels were reached only after 12 h (Sarff & Gorski, 1971). Administration of 1 μg oestradiol-17β to adult female rats resulted in a maximal depletion of cytoplasmic receptor molecules in pituitary, hypothalamus and uterus within 1 h. The reappearance of receptor molecules in uterine tissue and pituitary occurred at 15 h after injection reaching levels slightly below those of the control. In the hypothalamus, however, a plateau (60 % of the control value) was reached 5 h after injection. In addition an enhancement of cytoplasmic receptor concentrations, as observed in uterine tissue 24 h after oestradiol administration, was not observed in testicular tissue. Thus testicular tissue belongs to the group of tissues which in response to oestradiol administration shows a depletion and subsequent reappearance of cytosol receptor molecules. The magnitude and the rate of both processes seems to vary among various tissues.

Six to 12 days after hypophysectomy in rats, plasma levels of gonadotrophins and testicular levels of oestradiol-17β and testosterone are decreased (Gay & Sheth, 1972; de Jong, 1974). In the present experiments cytoplasmic levels of oestradiol-17β receptor were not affected after hypophysectomy. The initial decrease in oestradiol concentration in testicular tissue after hypophysectomy might have resulted in a proportional increase in unoccupied and therefore measurable receptor sites. A constant amount of measurable receptor sites might therefore accompany a decrease in the total receptor concentration (unoccupied and occupied sites) during the first days after hypophysectomy. It has been calculated, however, that only in the order of 10 % of the total receptor sites in testicular cytosol of intact rats can be maximally occupied by endogenous oestradiol-17β (van Beurden-Lamers *et al.* 1974; de Jong *et al.* 1974). Therefore it appears very unlikely that gonadotrophins, oestradiol-17β and testosterone are important for the maintenance of the concentration of oestradiol-17β receptors in testicular cytoplasm. The increase in receptor sites which is found at 15 days or longer periods after hypophysectomy is an apparent increase, reflecting the relative increase in the amount of interstitial tissue.

Testicular cytosol of immature rats older than 14 days contained two binding proteins for oestradiol-17β with sedimentation values of 4S and 8S respectively (Fig. 4). This is similar to the observations for oestradiol-17β binding by uterine cytosols of immature rats (Michel, Jung & Baulieu, 1974; Sömjen, Kaye & Lindner, 1974). The binding capacity of plasma of immature male rats for oestradiol-17β showed a decrease of more than two orders of

magnitude between birth and puberty, which paralleled the decrease in 4 S oestradiol-17β-binding protein in testicular cytoplasm. It is therefore very likely that the oestradiol-17β-binding plasma protein is responsible for (part of) the 4 S oestradiol-17β binding found in the testicular cytoplasm of immature rats. This suggestion is supported by the similar behaviour of both the plasma component and the 4 S testicular cytosol component towards diethylstilboestrol.

The very large concentration of 4 S oestradiol-17β binding protein in testicular tissue made the quantitative measurement of receptor concentrations less accurate. Therefore small amounts of oestradiol-17β receptor molecules, even if present in the testicular cytosol of rats younger than 14 days, might have escaped detection. However, the nuclear form of the testicular oestradiol-17β receptor could be demonstrated in rats from 4 days of age onwards. Similar observations have been made for other receptors both for gonadal and adrenal steroids. The hepatic receptor for glucocorticoids could be demonstrated in liver cytosol of foetuses and immature rats (Feldman, 1974). The epididymal androgen receptor has been detected in 20-day-old rats (Calandra, Podesta, Rivarola & Blaquier, 1974) and the presence of oestradiol-17β-binding proteins with a sedimentation value of 8 S has been demonstrated in uteri of 5-day-old rats (Michel *et al.* 1974).

The physiological meaning of the testicular oestradiol-17β receptor is not yet clear. It has been reported that administration of oestradiol benzoate (50 μg) to adult male rats results in a rapid decrease in testicular testosterone levels within 2 h without changes in LH levels in the circulation (Tcholakian *et al.* 1974). The present results showed that injection of oestradiol-17β into mature and immature rats caused a rapid decrease in testicular receptor levels in the cytoplasm. Therefore a possible direct inhibiting effect of oestradiol-17β on testosterone synthesis could be mediated by the binding of oestradiol-17β to the cytoplasmic receptor and the subsequent binding of the receptor–hormone complex to the chromatin. Whether the endogenous concentration of oestradiol-17β in mature rats is high enough to exert the same regulatory effect on testosterone synthesis is unknown. The presence of very large amounts of oestradiol-17β-binding protein in immature rat plasma which decreased during the onset of pubescence might be sufficiently high to trap all the endogenous oestrogen. This would indicate that the suggested regulatory role of oestrogens on steroid biosynthesis in testicular tissue can only be important if the oestradiol-17β binding in testicular cytosol exceeds the binding in plasma.

This work was supported (in part) by the Foundation for Medical Research FUNGO, which is subsidized by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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Biochem. J. (1977) **162**, 331–339
Printed in Great Britain

Comparative Study of Nuclear Binding Sites for Oestradiol in Rat Testicular and Uterine Tissue

DETERMINATION OF LOW AMOUNTS OF SPECIFIC BINDING SITES BY AN [3H]OESTRADIOL-EXCHANGE METHOD

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(*Received* 4 *August* 1976)

1. An [^{3}H]oestradiol-exchange method was developed for the determination of oestradiol–receptor complexes in the nuclear fraction of immature rat testicular tissue. This method permits the determination of nuclear oestradiol-receptor sites in the presence of a relatively large amount of non-specific oestradiol binding present in testicular nuclei. After incubation of nuclei for 60min at 20°C in the presence of [^{3}H]oestradiol with or without a 1000-fold excess of non-radioactive diethylstilboestrol, specific binding can be determined quantitatively in the KCl-extractable fraction, which contains 40% of the total receptor population. 2. The amount of receptor-bound steroid present in the 0.4M-KCl extract of testicular nuclei remained constant during incubation at 20°C. For uterine nuclei incubated with [^{3}H]oestradiol at 37°C a shift of specifically bound [^{3}H]oestradiol occurred from the KCl-soluble fraction to the KCl-insoluble fraction. 3. In intact rat testis, about 20% of the total receptor concentration was present in its nuclear form. Hypophysectomy 5 days before measurement resulted in a twofold decrease in the amount of receptor, which was present mainly in the cytosol. After injection of choriogonadotropin to intact animals, the total receptor concentration increased threefold. 4. This nuclear exchange method might be useful for determination of occupied specific receptor sites in tissues with relatively low contents of specific receptors.

Investigations of a biochemical explanation for the mechanism of action of steroids have revealed the presence of specific receptors for steroid hormones in target tissues. For uterine tissue it is well documented that oestradiol–receptor complexes migrate into the nucleus, bind to receptor sites on the chromatin and initiate a sequence of events which result in the hormone effect (Clark *et al.*, 1973; O'Malley & Means, 1975). The interstitial tissue of the testis from rats of 4 days of age onwards contains a specific receptor for oestradiol (Brinkmann *et al.*, 1972; Mulder *et al.*, 1973; de Boer *et al.*, 1976). Oestradiol is endogenously produced in the testis and it appears that oestradiol concentrations in rat testis interstitial tissue (0.5–1nM) are higher than those in seminiferous tubules (de Jong *et al.*, 1974), but it is still uncertain whether oestradiol has a physiological function in testicular tissue. The well-known decrease in testicular testosterone production after administration of oestradiol could be fully explained by a negative feedback of oestrogens on lutropin (luteinizing hormone) secretion (W. M. O. van Beurden-Lamers, unpublished work), although it has been suggested that oestradiol might have a local intratesticular effect on testosterone production.

Possible direct effects of oestradiol mediated by the oestradiol receptor on Leydig-cell functions have not been studied in detail. On the assumption that an effect of steroid hormones is preceded by binding of the steroid–receptor complex in the nucleus, it was the purpose of the present study to investigate a possible translocation of the oestradiol receptor into the nucleus of testicular tissue of intact animals. In addition, the effects of oestradiol and administration of choriogonadotropin on the concentration and localization of the receptor were studied.

For a quantitative determination of nuclear receptor sites in intact animals it is necessary to use a method that can distinguish between the total available amount of receptor sites and the number of receptor sites that is occupied by endogenous oestradiol. Anderson *et al.* (1972) have developed an [^{3}H]-oestradiol-exchange method for determination of the number of nuclear oestradiol-receptor sites in the

presence of endogenous oestradiol. Because a considerable amount of non-specific oestradiol-binding sites was observed in testicular tissue, it was necessary to modify this nuclear exchange method. We have therefore compared this modified hormone-exchange assay for determination of nuclear oestradiol receptors in testicular tissue with the assay described by Anderson *et al.* (1972) for uterine tissue.

Materials and Methods

Preparations of animals and materials

Immature male and female rats (21–35-days-old) of the R-Amsterdam strain were used in this study. In some experiments animals were hypophysectomized 5 days before the experiments. Male rats were injected subcutaneously with a solution of either 500ng of oestradiol-17β or 500ng of [^{3}H]oestradiol-17β in 0.2ml of 0.15M-NaCl containing 2.5% (v/v) ethanol. The rats were decapitated 1h after the injection. In experiments designed to examine the effect of human choriogonadotropin, a daily dose of 50i.u. of the hormone (WHO, 1975), dissolved in 0.1ml of 0.15M-NaCl, was subcutaneously injected for 5 successive days. Testicular tissue was removed after decapitation of the animals and placed on a Petri dish on ice before incubation or isolation of nuclei.

[2,4,6,7-^{3}H]Oestradiol-17β (sp. radioactivity 85Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was examined for purity by t.l.c. Diethylstilboestrol and oestradiol-17β were obtained from Steraloids Inc., Pawling, NY, U.S.A.

Incubation of whole tissues in vitro

One decapsulated testis or one uterus, stripped of adhering fat and mesentery, from immature rats was incubated with 10nM-oestradiol in 2.0ml of Krebs–Ringer bicarbonate buffer, pH7.4, containing 0.2% glucose (Umbreit *et al.*, 1964). Incubations were carried out in an atmosphere of O_2+CO_2 (95:5) for 60min at 32°C for testicular tissue and at 37°C for uterine tissue. In control studies tissues were incubated with either 10nM-[^{3}H]oestradiol or 10nM-[^{3}H]oestradiol plus 10μM-diethylstilboestrol.

Uterine and testicular tissue of immature rats contains, in addition to the receptor, a plasma binding protein (α-fetoprotein), which has a rather high affinity for oestrogens (Raynaud *et al.*, 1971; Aussel *et al.*, 1974; de Boer *et al.*, 1976). However, oestradiol bound to the plasma protein cannot be displaced by an excess of diethylstilboestrol. Therefore excess of diethylstilboestrol rather than oestradiol was used in incubations where the non-specific binding was estimated. The specific oestradiol binding to receptor molecules was then defined as the difference between the total binding and the non-specific binding and is expressed as fmol/mg of protein or as fmol/two testes or fmol/uterus. Previously it has been shown that the affinity of diethylstilboestrol for the testicular oestradiol receptor is one-quarter of the affinity of oestradiol (van Beurden-Lamers *et al.*, 1974). The amount of non-specific binding, obtained after incubation of mature rat testis and uterus *in vitro*, tissues that do not contain α-fetoprotein or other specific oestradiol-binding plasma proteins, did not differ if a 100-fold excess of oestradiol or a 1000-fold excess of diethylstilboestrol was used.

Preparations of nuclei

After isolation and incubation the testicular tissue was homogenized in 10vol. of 10mM-Tris/HCl buffer, pH7.4, containing 1.5mM-EDTA and 0.02% NaN_3 (TEN buffer) with six strokes of a Potter–Elvehjem homogenizer at 1100rev./min. The homogenate was rehomogenized in an all-glass Potter–Elvehjem homogenizer and centrifuged at 500g for 10min at 0°C. The 500g pellet was washed once with 3ml of TEN buffer, twice with 3ml of TEN buffer containing 0.2% Triton X-100 and another time with 3ml of TEN buffer. Uterine tissue after incubation was suspended in 3ml of TEN buffer and homogenized by hand with two strokes in an all-glass Kontes homogenizer. The homogenate was centrifuged at 800g for 10min at 0°C and the pellet was washed three times with 3ml of TEN buffer. The resulting nuclear pellets were used either for the hormone-exchange assay of the nuclear oestradiol-binding sites or for immediate extraction with 0.4M-KCl.

Hormone-binding assay

(*a*) *Determination of nuclear binding, after incubation or injection with [^{3}H]oestradiol.* Isolated nuclei were extracted with 0.4M-KCl in TEN buffer, pH8.5, during 60min at 0°C. The extract was centrifuged at 0°C for 10min at 1500g, and a KCl-extractable and a residual nuclear fraction were obtained as the supernatant and pellet fractions. The bound radioactivity in the KCl-extractable fraction was measured by sucrose-density-gradient centrifugation. The residual nuclear fraction was dissolved in 1ml of 1M-NaOH, and 100μl was counted for radioactivity after addition of 50μl of 3M-$HClO_4$.

(*b*) *Hormone-exchange assay followed by KCl extraction of nuclei.* Isolated nuclei were incubated in TEN buffer containing 25% (v/v) glycerol for periods up to 120min at different temperatures with either 10nM-[^{3}H]oestradiol or 10nM-[^{3}H]oestradiol plus 10μM-diethylstilboestrol. After the incubation, nuclei were extracted as described under (*a*). To remove the excess of unbound steroid, the nuclear suspension in 0.4M-KCl was treated for 10min with dextran-coated charcoal (final concentrations: 0.5% charcoal and 0.05% dextran T 300) at 0°C and was subsequently centrifuged for 30min at 105000$g_{av.}$ in a SW 65 rotor to remove the charcoal. Specific binding in the super-

natant fraction was measured by using sucrose-density-gradient centrifugation. In this assay system the radioactivity in the residual nuclear fraction could not be measured, owing to the sedimentation of charcoal in this fraction.

(*c*) *Hormone-exchange assay technique* (*Anderson et al.*, 1972). Isolated nuclei were incubated as described under (*b*). After the incubation, nuclei were washed twice with TEN buffer and were immediately extracted with 3ml of ethanol. The ethanol extract was removed for assay of the amount of radioactivity.

Sucrose-density-gradient centrifugation

A 200–300μl portion of the KCl-extractable nuclear fraction was layered on top of a linear 5–20% (w/v) sucrose gradient prepared in TEN buffer, pH8.5, containing 0.4M-KCl. The gradients were centrifuged at 260000$g_{av.}$ for 18h at 0°C in a Beckman SW 65 rotor. On separate gradients bovine serum albumin (200μg) was run as a sedimentation marker (4.6S). After centrifugation, each gradient was fractionated into 27 fractions of 0.2ml, and each fraction was assayed for radioactivity. The amount of specifically bound [^{3}H]oestradiol was determined as the difference between the amount of radioactivity sedimenting in the 5S region of the incubations with 10nM-[^{3}H]oestradiol and with 10nM-[^{3}H]oestradiol plus 10μM-diethylstilboestrol respectively as described for whole tissue (see also Fig. 1).

General procedures

Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard. Radioactivity was measured in a Packard model 3375 liquid-scintillation spectrometer. The scintillation fluid consisted of a mixture of Triton X-100 (Rohm and Haas, Philadelphia, PA, U.S.A.) and toluene (1:2, v/v) containing 0.1g of POPOP [1,4-bis-(5-phenyloxazol-2-ylbenzene] and 4.8g of PPO (2,5-diphenyloxazole)/litre (Packard Instrument S.A., Benelux, Brussels, Belgium).

Results

Exchange assay of nuclear binding sites for oestradiol in testicular and uterine tissue by using ethanol extraction

Initially we attempted to measure nuclear binding of oestradiol in testicular and uterine tissue using the method of Anderson *et al.* (1972) as described in the Materials and Methods section. Table 1 shows that for uterine tissue 74% of the total amount of steroid in the nuclear sample was due to specifically bound steroid. This amount is in close agreement with the amount of specifically bound steroid observed in experiments where the specifically bound steroid was measured after incubation of uterine tissue with radioactive steroid and immediate extraction of isolated nuclei.

In testicular tissue after exchange at 20°C, as well as at 32°C, only a small percentage of the steroid appeared to be specifically bound. Consequently unreliable data with a large S.D. were obtained for the amount of specific binding sites in testicular tissue. In control nuclei obtained from testicular tissue, directly labelled *in vitro* with radioactive oestradiol without nuclear exchange, the non-specifically bound oestradiol represents only 30% of the total amount of bound oestradiol. In this case an accurate estimate of specific binding by ethanol extraction is possible.

Determination of specific oestradiol binding in KCl extracts of nuclei after nuclear exchange

The relative amount of non-specifically adsorbed steroid on testicular nuclei could be lowered by extraction of nuclei with 0.4M-KCl and removal of excess of free steroid by charcoal adsorption as described in the Materials and Methods section. Specific binding in the KCl extract was measured by sucrose-gradient

Table 1. *Exchange assay of nuclear binding sites for oestradiol in testicular and uterine tissue*

Uterine tissue was incubated for 60min at 37°C with 20nM-oestradiol. Isolated nuclei were incubated for 60min at 37°C with either 10nM-[^{3}H]oestradiol or 10nM-[^{3}H]oestradiol plus 10μM-diethylstilboestrol. Testicular tissue was incubated for 60min at 32°C with 10nM-oestradiol. Isolated nuclei were incubated for 60min at 20°C or 32°C with either 10nM-[^{3}H]oestradiol or 10nM-[^{3}H]oestradiol plus 10μM-diethylstilboestrol. Incubated uterine and testicular nuclei were washed twice with TEN buffer and extracted with 3ml of ethanol. Uterine and testicular nuclei, obtained after incubation of tissues with either [^{3}H]oestradiol or [^{3}H]oestradiol plus diethylstilboestrol, were extracted immediately after isolation and were used as a control. Results are given in fmol of [^{3}H]oestradiol bound per uterus or per two testes ± S.D. for the numbers of experiments shown in parentheses.

	Assay conditions	Total binding	Non-specific binding	Specific binding
Uterus	Exchange at 37°C	1011 ± 323 (5)	263 ± 59 (5)	748 ± 267 (5)
	Control	1089 ± 200 (3)	68 ± 6 (3)	1021 ± 195 (3)
Testis	Exchange at 20°C	2011 ± 98 (5)	1932 ± 64 (5)	79 ± 36 (5)
	Exchange at 32°C	1891 ± 50 (4)	1763 ± 80 (4)	126 ± 82 (4)
	Control	92 ± 11 (3)	30 ± 5 (3)	62 ± 6 (3)

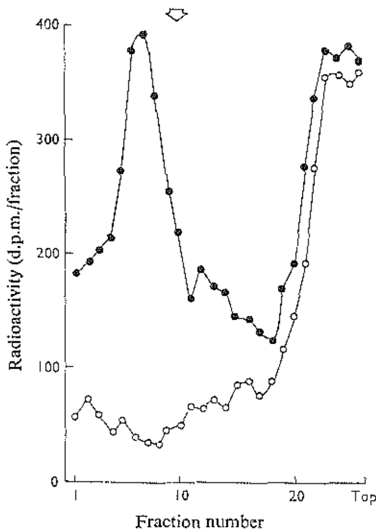

Fig. 1. *Binding of [³H]oestradiol in the KCl-extractable fraction of testicular nuclei*

Testicular tissue was incubated for 60min at 32°C with either 10nM-[³H]oestradiol (●) or 10nM-[³H]-oestradiol plus 10μM-diethylstilboestrol (○). Nuclei were isolated and extracted as described in the Materials and Methods section. A 200μl portion of the nuclear extracts was centrifuged on sucrose gradients for 18h at $260000g_{av.}$. Specific binding of oestradiol in the KCl extract was estimated by calculating the difference in radioactive steroid sedimenting in the 5S region of the gradient in the presence and in the absence of the excess of competing steroid. The arrow indicates the position of bovine serum albumin (4.6S) run in a separate gradient.

centrifugation (Fig. 1). The amount of specifically bound steroid recovered in the KCl-extractable fraction represents 39±9% (S.D.) ($n = 6$) from the total amount of specific nuclear binding as determined by ethanol extraction (Table 1, line 5). To determine the amount of binding sites in the KCl extract, an almost complete exchange should be obtained at a temperature where no appreciable decomposition of binding sites occurs. The effect of different incubation temperatures on the stability of nuclear oestradiol binding is shown in Fig. 2. In the absence of excess of [³H]oestradiol in the incubation medium, specific binding in both the KCl-extractable and residual nuclear fraction decreases considerably during 1h of incubation (Fig. 2*a*). In the presence of oestradiol, dissociation of binding sites in the KCl-extractable fraction at 20°C was less than 5% (Fig. 2*b*). Dissociation of the binding sites in the residual nuclear fraction in the presence of added ³H-labelled steroid could not be measured, owing to the large amount of free and non-specifically bound oestradiol present in this fraction. For uterine tissue both the KCl-extractable and the non-KCl-extractable nuclear receptors (pellet) could be measured, because in contrast with testicular nuclei, it was possible to remove excess of unbound steroid by washing the uterine nuclei. In the presence of added [³H]oestradiol, a loss of binding sites in the KCl-extractable fraction was accompanied by an increase in the amount of non-KCl-extractable binding sites (Fig. 3*b*). As a consequence the total binding of oestradiol remained nearly constant. In the absence of [³H]oestradiol the amount of both KCl-extractable and non-KCl-extractable binding sites in uterine nuclei were considerably decreased (Fig. 3*a*).

The time-course of the [³H]oestradiol exchange by KCl-extractable nuclear receptor sites in testicular tissue was studied after labelling of the tissue *in vivo* by injection of oestradiol. Fig. 4 shows that the hormone exchange in the KCl-extractable fraction is completed within 15min. The amount of specific binding measured after the exchange procedure [21.9fmol±2.7 (S.D.), $n = 13$] did not differ significantly from control values [22.4fmol±2.5 (S.D.), $n = 3$], which were obtained after injection of [³H]oestradiol *in vivo*. Therefore the hormone-exchange procedure *in vitro* during 60min of incubation of nuclei with [³H]oestradiol at 20°C gives reliable results for oestradiol-receptor concentrations.

Determination of occupied nuclear oestradiol receptors in testicular tissue of intact immature rats and the effects of hypophysectomy on the amount of nuclear receptor sites

Fig. 5(*a*) shows that the KCl-extractable fraction of testicular nuclei isolated from intact immature rats and subjected to the hormone-exchange procedure contains oestradiol-binding sites in the 5S region of the gradient. In contrast, in testicular tissue of 5-day hypophysectomized rats little or no specifically bound oestradiol could be demonstrated in the 5S region of sucrose gradients after application of the exchange technique (Fig. 5*b*). Table 2 shows the number of nuclear receptor sites per two testes of intact and hypophysectomized animals and the total available amounts of nuclear receptor sites measured after injection of rats with 500ng of non-radioactive oestradiol. As a result of hypophysectomy the total available number of receptor sites is decreased by a factor of two.

Effect of choriogonadotropin treatment on the amount of specific nuclear receptor sites

Administration of 50i.u. of choriogonadotropin/

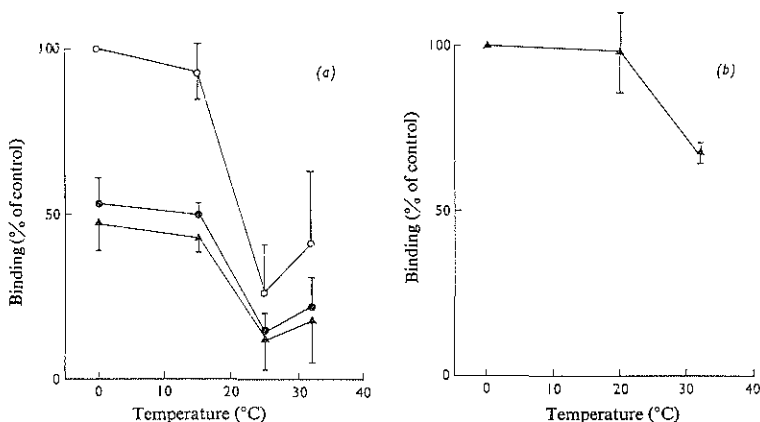

Fig. 2. *Effect of temperature on the stability of testicular nuclear oestradiol receptors*
Testicular tissue was incubated for 60min at 32°C with either 10nM-[^{3}H]oestradiol or 10nM-[^{3}H]oestradiol plus 10μM-diethylstilboestrol. Nuclei were isolated and nuclear suspensions were kept for 60min at different temperatures either without [^{3}H]oestradiol (*a*) or with 10nM-[^{3}H]oestradiol (*b*) in the incubation medium. Thereafter nuclei were washed twice with TEN buffer and extracted with 0.4M-KCl as described in the Materials and Methods section. In (*a*) specific binding in the KCl-extractable (▲) and residual nuclear fraction (●) was calculated as a percentage of the total nuclear binding (○) obtained after incubation of nuclei at 0°C [61.3 ± 4.9(4) fmol/two testes]. In (*b*) specific binding in the KCl-extractable fraction is given as the percentage of the specific binding obtained after incubation of nuclei at 0°C [23.0 ± 3.4(3) fmol/two testes]. Each value is the mean ± S.D. of two to four determinations.

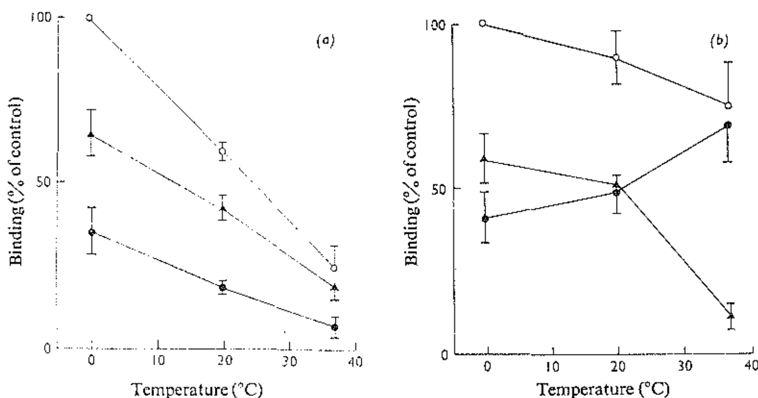

Fig. 3. *Effect of temperature on the stability of uterine nuclear oestradiol receptors*
Uterine tissue was incubated for 60min at 37°C with either 20nM-[^{3}H]oestradiol or 20nM-[^{3}H]oestradiol plus 4μM-diethylstilboestrol. Nuclei were isolated and nuclear suspensions were kept for 60min at different temperatures either without [^{3}H]oestradiol (*a*) or with 10nM-[^{3}H]oestradiol (*b*) in the incubation medium. Thereafter nuclei were washed twice with TEN buffer and extracted with 0.4M-KCl as described in the Materials and Methods section. Specific binding in the KCl-extractable (▲) and residual nuclear fraction (●) was calculated as the percentage of the total nuclear binding (○) obtained after incubation of nuclei at 0°C [985 ± 231 (5) fmol/uterus]. Each value is the mean ± S.D. of two to five determinations.

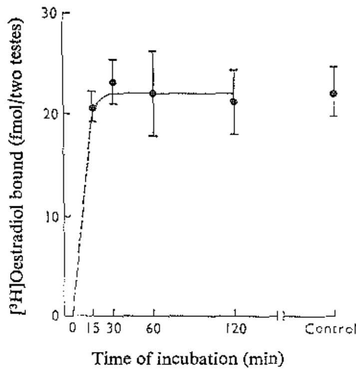

Fig. 4. *Time-course of oestradiol exchange with the nuclear receptor at 20°C after injection of rats with oestradiol in vivo*

Immature rats were subcutaneously injected with 500ng of oestradiol, and 1h later testicular nuclei were prepared and incubated for different time-periods at 20°C with either 10nM-[³H]oestradiol or 10nM-[³H]oestradiol plus 10μM-diethylstilboestrol. After incubation the nuclei were extracted with 0.4M-KCl and specific binding in the KCl-extractable fraction was estimated by sucrose-density-gradient centrifugation as described in the Materials and Methods section. The number of specific oestradiol-binding sites in the KCl-extractable nuclear fraction obtained after injection of animals with 500ng of [³H]oestradiol *in vivo* was used as a control. Results are expressed in fmol per two testes and represent the mean±S.D. obtained from three to four experiments.

day for 5 successive days resulted, 20h after the last injection, in an increased number of KCl-extractable nuclear binding sites as measured by the hormone-

Table 2. *Determination of occupied KCl-extractable nuclear binding sites for oestradiol in testicular tissue of intact rats, hypophysectomized rats and choriogonadotropin-treated rats*

Nuclei were isolated from testicular tissue from intact rats, from rats that had been hypophysectomized 5 days before or from rats that had been treated with 50i.u. of choriogonadotropin for 5 successive days. For determination of the total amount of nuclear binding sites all groups of animals were injected with 500ng of non-radioactive oestradiol 60min before the animals were killed. Nuclei were incubated for 60min at 20°C with either 10nM-[³H]oestradiol or 10nM-[³H]oestradiol plus 10μM-diethylstilboestrol. Incubated nuclei were washed with TEN buffer and extracted with 0.4M-KCl. The specific oestradiol binding in the KCl-extractable fraction was measured by sucrose-gradient centrifugation as described in the Materials and Methods section. Results are expressed in fmol per two testes and represent the mean±S.D. for the numbers of experiments shown in parentheses.

	Occupied nuclear binding sites (fmol/two testes)
Intact	4.7± 1.5 (6)
Intact+oestradiol	21.8± 2.7 (8)
Hypophysectomized	0.5± 0.6 (4)
Hypophysectomized+oestradiol	12.4± 0.8 (3)
Choriogonadotropin	47.5± 1.7 (3)
Choriogonadotropin+oestradiol	65.0±10.4 (4)

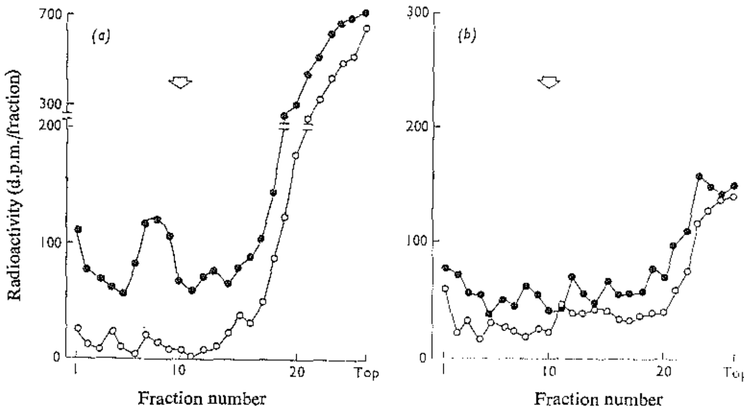

Fig. 5. *Presence of occupied nuclear receptors for oestradiol in testicular tissue of intact and hypophysectomized rats*

Testicular nuclei were isolated from intact rats (*a*) or from rats that had been hypophysectomized 5 days before the experiments (*b*). Nuclei were incubated for 60min at 20°C with either 10nM-[³H]oestradiol (●) or 10nM-[³H]oestradiol plus 10μM-diethylstilboestrol (○). Incubated nuclei were extracted with 0.4M-KCl and the specific binding in the KCl-extractable fraction was estimated by sucrose-density-gradient centrifugation as described in the Materials and Methods section. The arrows indicate the position of bovine serum albumin (4.6S) run in a separate gradient.

exchange assay (Table 2). To measure the total number of receptor sites, testicular nuclei, isolated from choriogonadotropin-treated animals injected with 500ng of oestradiol 60min before death, were subjected to the exchange procedure. In choriogonadotropin-treated rats the testicular receptor concentration was three times that in intact animals (see Table 2).

Discussion

The accumulation of specific receptor–steroid complexes in target-cell nuclei appears to precede the observed effects of steroids on synthesis of new RNA and protein (van der Berg *et al.*, 1974; Lazier, 1975; Tsai *et al.*, 1975; Jänne *et al.*, 1976; Hardin *et al.*, 1976). This has stimulated the interest in quantitative evaluations of nuclear steroid receptors as a possible indicator of physiological actions of steroids, even if in some cases the actual physiological importance of a steroid is not yet known. Such a situation exists for oestradiol in the rat testis. Oestradiol is produced and present in the testis, and there is now ample proof of the occurrence of specific cytoplasmic and nuclear receptors in rat testis interstitial tissue (Brinkmann *et al.*, 1972; Mulder *et al.*, 1973; de Boer *et al.*, 1976). However, there is no certainty about the possible action of oestradiol or the significance of the oestradiol receptors in testicular tissue. Therefore in the present study we attempted to investigate the biochemical behaviour of oestradiol in nuclei of testis interstitial tissue as a possible indicator of a physiological effect.

For our studies we required a reliable method for the quantitative determination of nuclear receptor–oestradiol complexes. In order to obtain information about the amount of receptor that was already occupied by endogenous unlabelled steroid, we were interested in a method that could distinguish between the total amount (occupied and unoccupied) of oestradiol receptor and the amount occupied. Methods that have been used for labelling receptors *in vitro* with negligible amounts of radioactive ligand (followed by separation of unbound and receptor-bound radioactive ligand) only give an indication of the number of unoccupied receptor sites.

Anderson *et al.* (1972) were the first to introduce the so-called nuclear exchange method, which uses the exchange of ^{3}H-labelled steroid with the endogenous receptor-bound steroid in the nuclear fraction for quantitative determination of the number of occupied nuclear receptor sites. In this method ^{3}H-labelled steroid, accumulated in the nucleus after the exchange procedure, is extracted with ethanol (total radioactivity T). In control experiments exchange is performed in the presence of ^{3}H-labelled steroid and an excess of non-radioactive steroid. It is assumed that in this case the amount of ^{3}H-labelled steroid extracted with ethanol represents the non-specifically bound steroid (N). Specific binding of [^{3}H]oestradiol to nuclear receptor molecules is defined as the difference between total and non-specific binding (T−N). The practicability and reliability of this approach for measuring nuclear steroid-hormone receptors in several tissues has been reported (Anderson *et al.*, 1973; Hsueh *et al.*, 1974; Sanborn *et al.*, 1975; Teng & Teng, 1976). An indication of the total amount of receptor present should be obtained if, before the exchange labelling, tissues and animals are treated with excess of unlabelled steroid, so that all cytoplasmic receptor molecules are transferred to the nuclear fraction (Anderson *et al.*, 1972).

In initial experiments with testicular tissue, when we tried to use the conditions described by Anderson *et al.* (1972) for uterine tissue, it became evident that in testis nuclei, almost all [^{3}H]oestradiol (96%) was retained by non-specific binding sites (Table 1). Therefore measurements of occupied nuclear receptor sites in testicular tissue became inaccurate. For uterine tissue, which was used for comparison, 26% of the total amount of radioactive oestradiol in nuclei was retained by non-specific binding sites (Table 1). The amount of non-specifically bound steroid in the nuclear extract could be decreased if, after the exchange procedure, both the amount of non-specific binding sites and the amount of free steroid were decreased. This could be achieved if, after incubation with steroids, nuclei were extracted with 0.4M-KCl and subsequently treated with charcoal to remove excess of free steroid. Others have observed that the number of cytoplasmic oestradiol-binding sites measured by a charcoal-adsorption method in the presence of 0.4M-KCl was underestimated as a result of a partial dissociation of the hormone from the oestradiol–receptor complex (Peck & Clark, 1974). In our studies we measured the number of receptor sites in nuclei obtained after injection of animals with [^{3}H]oestradiol. Under these conditions the measured number of receptor sites did not differ significantly from the number of sites obtained after injection of animals with non-radioactive oestradiol and by the exchange procedure (Fig. 4). Therefore it seems unlikely that, in the exchange procedure using charcoal adsorption, a considerable underestimation of the number of oestradiol-binding sites occurs. The modified method did result in much lower numbers of non-specific binding sites (10–20% of the total binding). This procedure has, however, the disadvantage that only in the KCl-extractable nuclear fraction can specific binding be measured. Because free steroid cannot be removed from the residual nuclear fraction, the difference between the total binding and the non-specific binding is too small to obtain reliable steroid-receptor measurements.

Of the total amount of receptor present, about 40% could be extracted from testicular nuclei with

0.4M-KCl; 60% was recovered in the residual nuclear fraction. Comparable results have been obtained after extraction of nuclei from kidney and hen oviduct (Best-Belpomme *et al.*, 1975; Jänne *et al.*, 1976), but the extraction efficiencies for chick liver nuclei and immature rat uterine nuclei were slightly lower (Lebeau *et al.*, 1974; Mester & Baulieu, 1975). Even if only part of the total nuclear receptor population can be measured quantitatively in this way, information indicating the number of occupied receptor sites present in testicular nuclei of immature rats under different physiological conditions might be obtained. The KCl solution appears to extract a nuclear non-histone fraction (Kostraba *et al.*, 1975), and the KCl-extractable radioactivity may reflect the steroid–receptor complexes associated with non-histone proteins.

For testicular nuclei it was necessary to use an exchange temperature of 20°C. It was shown that the exchange of [^{3}H]oestradiol at this temperature was completed within 5min (Fig. 4). At higher temperatures a considerable loss of KCl-extractable receptor sites was observed during incubations of intact nuclei (Fig. 2). Also for other steroid-hormone receptors, degradation has been observed during the hormone-exchange assay (Hsueh *et al.*, 1974; Mester & Baulieu, 1975). A good correlation was observed, however, between the depletion of cytoplasmic receptor sites and the increase in nuclear receptor sites measured by hormone exchange in Müllerian-duct cells after oestradiol administration (Teng & Teng, 1976). A possible difference in proteolytic enzyme activity in the isolated nuclei might offer an explanation for these contrasting findings.

In the present study, occupied oestradiol receptors could be demonstrated in the KCl-extractable fraction from nuclei of 25-day-old rats (4.7fmol/two testes). After administration of oestradiol the amount of receptor in the KCl extract increased to 21.8fmol/two testes (Table 2). As a consequence endogenous oestradiol in testicular tissue of intact immature rats could translocate 22% of the total receptor population into the nuclear fraction. For uterine tissue of intact immature rats, about 16% of the total oestradiol-receptor concentration was present in the nuclear fraction (Anderson *et al.*, 1972). In our studies, hypophysectomy of immature rats resulted, after 5 days, in a decrease in the number of KCl-extractable binding sites occupied with endogenous oestradiol (0.5fmol/two testes). The total amount of KCl-extractable binding sites in hypophysectomized animals measured after oestradiol administration also decreased (12.4fmol/two testes). Therefore the receptor population in hypophysectomized rats is decreased to about 50% of the value in intact rats. The small amount of receptor sites occupied by endogenous oestradiol does reflect the fall of the testicular oestradiol concentration after hypophysectomy. The decrease in testicular oestradiol-receptor content after hypophysectomy might be explained by a degeneration of Leydig cells or by a decrease in the number of these cells due to the disappearance of lutropin (Woods & Simpson, 1961; Gay & Sheth, 1972; Odell & Swerdloff, 1975). Similar observations have been made after hypophysectomy of mature female rats, which also results in a dramatic decrease in the number of available cytoplasmic receptor sites for oestradiol in the liver (Chamness *et al.*, 1975). The effect of choriogonadotropin was studied in order to investigate a possible role of gonadotropins on the number of oestradiol-receptor sites in testicular tissue. In intact immature rats, choriogonadotropin caused an increase in both the number of occupied receptor sites (47.5fmol/two testes) and the total amount of receptor sites (65.0fmol/two testes) in the KCl-extractable fraction. From these values it can be concluded that 73% of the total receptor population is present in the nuclear fraction and that choriogonadotropin administration results in a threefold increase in the receptor-site population per testis if compared with that in intact rats. The increase correlates very well with the observed increase in the number of Leydig cells in 21-day-old rats after choriogonadotropin treatment (15i.u./day for 5 successive days) owing to mitosis and cellular differentiation (Chemes *et al.*, 1976). It appears likely therefore that the number of oestradiol-receptor sites per Leydig cell does not change after choriogonadotropin treatment.

It is concluded that the number of total available and occupied oestradiol-receptor sites in the KCl-extractable fraction of testicular nuclei is under the potential control of the hormonal environment. Whether the observed changes in the number of receptor sites are correlated with similar changes in the activities of the RNA and protein-synthesizing processes still requires investigation.

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Journal of Steroid Biochemistry 1977 (accepted for publication)

Kinetics of in vitro binding of oestradiol in subcellular fractions of testicular and uterine tissue ; characterization of oestradiol binding in testicular nuclei

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(Received 7 December 1976)

Summary

1. Oestradiol in rat testicular and uterine tissue is specifically bound to nuclear receptor sites, which can be separated in KCl-extractable nuclear and nuclear residual (a nuclear fraction which resists KCl extraction) receptor sites.
2. The amount of 'extractable' nuclear binding sites for oestradiol in testis could be increased by mild trypsin treatment. Treatment of testicular nuclei with deoxycholate or DNAse resulted in a decrease of residual receptor sites and a concomitant increase of unbound oestradiol in the 'extractable' nuclear fraction.
3. The presence of KCN in vitro resulted in a relative increase in the number of oestradiol binding sites in the nuclear residual fraction in both uterine and testicular tissue; the number of binding sites in the KCl-extractable

fraction was not affected by KCN.

4. During in vitro incubations of testicular tissue the number of oestradiol binding sites in the KCl-extractable nuclear fraction reached a maximum and remained constant after 30 min of incubation; the number of binding sites in the nuclear residual fraction decreased after incubation periods longer than 30 min.
5. During in vitro incubations of uterine tissue the number of oestradiol binding sites in the KCl-extractable nuclear fraction and the nuclear residual fraction after an initial increase decreased to 50% of the maximal value between 30 and 60 min of incubation.
6. It is concluded, that the testicular oestradiol receptor shows certain characteristics comparable with those of the uterine receptor. However, regarding the differences in retention time of steroids in the nucleus, it seems very unlikely that the oestradiol effect in uterus and the oestradiol effect in testis, if present are mediated by identical receptor mechanisms.

Introduction

It is now well accepted that the cytoplasm of target cells for steroid hormones contains a specific binding protein called a receptor. The complete sequence of events in the response of a tissue to a steroid hormone is still unknown, but a postulated primary step is the interaction of the steroid with its receptor in the cytoplasm of the target tissue. The formed steroid-receptor complex migrates into the nucleus and binds to acceptor sites on the chromatin which ultimately results in a response of the tissue to the steroid via changes in RNA and protein synthesis [1,2,3,4].

The rat testicular Leydig cell contains a cytoplasm receptor for oestradiol which can be transported into the nucleus and which binds to the chromatin under the influence of endogenously produced oestradiol [5,6,7,8]. Whether the

binding of oestradiol-receptor complexes to nuclear acceptor sites results in a physiological effect in the Leydig cell is still unclear [9].

In order to gain further insight in a possible function of oestradiol receptors in the Leydig cell the processes of translocation and nuclear binding of receptor-oestradiol complexes in vitro in testicular tissue with similar processes in uterine tissue, a tissue which responds well to oestrogen administration, were compared [10,11].

In the present study we have also investigated the effects of trypsin, deoxycholate and DNAse treatment on the nature of nuclear oestradiol binding sites in testicular tissue. For uterine tissue it has been postulated that the number of nuclear residual oestradiol receptor sites (the fraction which resists KCl-extraction) determines the tissue response to oestradiol [12]. Therefore the distribution of KCl-extractable and nuclear residual binding sites was investigated after incubation of uterine and testicular tissue with oestradiol.

It has been reported that energy might be required for the action of glucocorticoids and progesterone [13,14,15, 16]. In this respect we have also studied the effect of energy deprivation on the distribution of oestradiol binding sites in KCl-extractable and nuclear residual fractions of uterine and testicular tissue.

Experimental

Materials. Unlabelled oestradiol and diethylstilboestrol (DES) were purchased from Steraloids Inc. Pawling, New York, U.S.A. ^{3}H-oestradiol (sp.act. 96 Ci/mmol) was purchased from Radiochemical Centre, Amersham, U.K. The purity of the steroids was determined by thin-layer chromatography. Trypsine (analytical grade) was obtained from Boehringer Mannheim, West Germany, sodium deoxycholate from Merck, Darmstadt, West Germany and DNAse from Sigma, St. Louis, U.S.A.

Source of tissues and treatments. Immature (25-30 day old) and mature (3 months old) rats of the R-Amsterdam strain were used in this study. Interstitial tissue of mature rat testis was obtained by wet dissection after incubation in vitro [17]. One decapsulated testis or one uterus stripped of adhering fat and mesentery, from immature rats, was incubated in 2.0 ml Krebs Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose. For mature rat testis 4.0 ml of incubation medium was used per testis. Incubations were carried out for different time periods in an atmosphere of 95% O_2 : 5% CO_2. In studies where the effect of KCN was investigated tissues were incubated in Krebs Ringer bicarbonate buffer, pH 7.4, containing 5×10^{-4}M KCN. Testicular tissue was incubated at 32°C; uterine tissue at 37°C.

Tissues were incubated either with 2×10^{-8}M ^{3}H-oestradiol (total binding) or with 2×10^{-8}M ^{3}H-oestradiol in the presence of 4×10^{-6}M DES (non-specific binding). Specific binding of ^{3}H-oestradiol is defined as the difference between the total binding and the non-specific binding.

Preparation of subcellular fractions. After incubation total testicular tissue and interstitial tissue were homogenized in 10 vol 10 mM Tris-HCl buffer, pH 7.4, containing 1.5 mM EDTA and 0.02% NaN_3 (TEN-buffer) with 6 strokes of Potter-Elvehjem homogenizer at 1100 rev./min. The homogenate was rehomogenized in an all-glass Potter-Elvehjem homogenizer and was subsequently centrifuged at 500 g for 10 min at 0°C. The 500 g pellet was washed once with TEN-buffer, twice with TEN-buffer containing 0.2% Triton X-100 and another time with TEN-buffer. Uterine tissue of immature rats was homogenized in an Ultraturrax homogenizer for 10 sec, rehomogenized in a Potter-Elvehjem homogenizer and centrifuged at 800 g for 10 min at 0°C. The 800 g pellet was further washed and centrifuged at 800 g as described for testicular tissue. The nuclear preparations thus obtained appeared pure by phase contrast microscopy and had a protein/DNA ratio in the range of 1.7-1.9. The isolated

pellets were used immediately after isolation for estimation of nuclear binding. Where indicated the 500 g and 800 g supernatants of the testicular and uterine tissue respectively were centrifuged at 105,000 g for 60 min to obtain the cytosol fraction.

Incubation of nuclei. In experiments concerning the nature of the oestradiol binding sites in testicular nuclei the following procedure was used. After incubation of testis tissue from mature rats in the presence of steroids for 60 min at 32°C interstitial tissue nuclei were isolated. Nuclei obtained from the interstitial tissue of one testis were suspended in 10 mM Tris buffer, pH 7.4 and incubated with DNAse (150 µg/ml; 30 min at 20°C), trypsin (150 µg/ml; 30 min at 10°C) or deoxycholate (1% DOC, 30 min at 0°C) in a volume of 1.0 ml. After incubation the nuclear suspensions were centrifuged for 30 min at 105,000 g and specific binding of ^{3}H-oestradiol was measured in the supernatant ('soluble') and pellet ('nuclear residual') fraction as described below. A KCl-extractable nuclear fraction and a nuclear residual fraction obtained after extraction of untreated nuclei with 0.4M KCl served as a control.

Estimation of specific ^{3}H-oestradiol binding. It was attempted to extract the ^{3}H-oestradiol in the nuclear pellet with 0.4M KCl in TEN-buffer, pH 8.5. The nuclear pellet was mixed with an equal volume of 0.8M KCl in TEN-buffer, pH 8.5. The final volume of the extraction buffer was adjusted to 800 µl with 0.4M KCl in TEN-buffer, pH 8.5. After incubation for 60 min at 0°C the extract was centrifuged for 30 min at 105,000 g and an aliquot of the supernatant fraction was analyzed on sucrose density gradients as described below (KCl-extractable nuclear binding). The residual nuclear pellet, from which no further ^{3}H-oestradiol could be extracted with KCl-TEN-buffer, was solubilized in 1 N NaOH and an aliquot was counted for radioactivity after addition of an equal volume of 3 N perchloric acid (residual nuclear bin-

ding). Specific binding in both fractions was estimated by substracting the non-specific binding from the total binding of ^{3}H-oestradiol and was expressed as fmol/mg protein, present in each fraction. In the 105,000 g supernatants specific cytosol binding was estimated by sucrose density gradient centrifugation as described below.

Sucrose gradient centrifugation. A 200 µl portion of the KCl-extractable nuclear fraction was layered on top of a linear 5-20% (w/v) sucrose density gradient prepared in TEN-buffer, pH 8.5, containing 0.4M KCl. Gradients were centrifuged at 260,000 g_{av} for 18 h at $0^{o}C$ in a Beckman SW65 rotor. Cytosol fractions (200-400 µl) were layered on top of linear 5-20% (w/v) sucrose gradients prepared in TEN-buffer and gradients were centrifuged at 150,000 g_{av} for 16 h at $0^{o}C$. On separate gradients 200 µg of bovine serum albumin (BSA) or alcohol dehydrogenase (ADH) were run as sedimentation markers (4.6S and 7.6S respectively). After centrifugation each gradient was fractionated in 27 fractions of 0.2 ml each and each fraction was assayed for radioactivity.

General procedures. Protein was determined by the procedure of Lowry et al. [18] with bovine serum albumin as a standard. Radioactivity was measured in a Packard model 3375 liquid scintillation spectrometer. The scintillation fluid consisted of a mixture of Triton X-100 (Rohm and Haas, Philadelphia, U.S.A.) and toluene (1:2 v/v) containing 0.1 g POPOP (1,4-bis-(5-phenyloxazol-2-yl) benzene)/l and 4.8 g PPO (2,5-diphenyloxazol)/l (Packard Instrument S.A., Benelux, Brussels, Belgium).

Results

Determination of two different types of specific oestradiol binding sites in testicular nuclei. Immature rat testicular tissue was incubated either with 10^{-8}M ^{3}H-oestradiol or with 10^{-8}M ^{3}H-oestradiol in the presence of increasing amounts non-radioactive DES (Fig. 1). Nuclei were isolated,

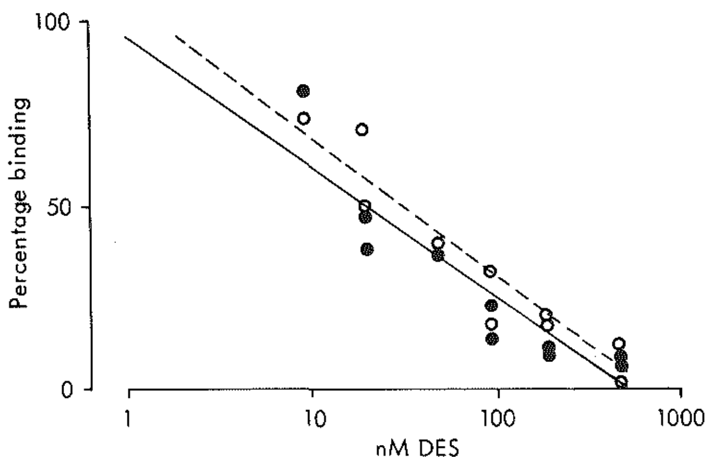

Figure 1 Determination of two different nuclear binding sites for oestradiol.
Testicular tissue from immature rats was incubated for 60 min at 32°C either with 10^{-8}M ^{3}H-oestradiol or with 10^{-8}M ^{3}H-oestradiol in the presence of increasing amounts of non-radioactive diethylstilboestrol. Nuclei were isolated and extracted with 0.4M KCl. Binding of oestradiol in the KCl-extractable fraction and the nuclear residual fraction was estimated as described in the methods section. The binding obtained in these two nuclear fractions in the absence of non-radioactive competitor is defined as the 100% value.
• - • nuclear extract; x --- x residual nuclear fraction.

extracted with 0.4M KCl and oestradiol binding sites present in the KCl-extractable fraction and the nuclear residual fraction (the fraction which resists extraction) was estimated.

In Fig. 1 it is shown that increasing amounts of non-radioactive DES decreased the binding of ^{3}H-oestradiol in both the KCl-extractable and the nuclear residual fraction. A two and three fold excess non-radioactive DES reduced the initial amount of binding by a factor two in the KCl-extractable fraction and nuclear residual fraction respectively.

In the order of 40% of the specifically bound steroid in testicular nuclei was generally recovered in the KCl extractable fraction; 60% resisted KCl extraction. The distribution of specific binding sites in the two nuclear fractions did not change if nuclei were extracted several times with 0.4M KCl. At 0.5M and higher salt concentrations the nuclear material became quite viscous and could not be sedimented by centrifugation.

Nature of the oestradiol binding sites in testicular nuclei. In order to investigate the nature of the oestradiol binding sites in testicular nuclei, testes from mature rats were incubated with oestradiol and the obtained nuclear suspension from interstitial tissue nuclei was incubated, either with trypsin, DNAse or deoxycholate.

The results in Fig. 2 show that treatment of nuclei with trypsin resulted in an increased amount of specifically bound ^{3}H-oestradiol in the soluble fraction. Further analysis of this soluble fraction on sucrose density gradients revealed that ^{3}H-oestradiol was specifically bound to a 4S sedimenting macromolecule. If nuclei were treated with DNAse or deoxycholate an oestradiol binding macromolecules with a sedimentation value of 5S was observed in sucrose density gradients. This value is similar to the value obtained after extraction of nuclei with 0.4M KCl. Treatment of nuclei with either DNAse or deoxycholate did not result in a further

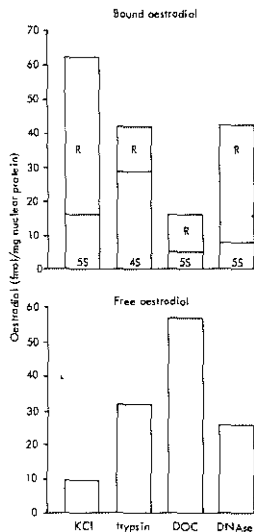

Figure 2 Effect of different treatments on the binding of oestradiol in testicular nuclei.
Testicular tissue from mature rats was incubated for 60 min at 32°C in the presence of steroids and after dissection of the interstitial tissue, nuclei were isolated. Aliquots of the nuclear suspension in 10 nM Tris-buffer, pH 7.4, were treated either with trypsin (150 µg/ml; 30 min at 10°C), with deoxycholate (1% DOC; 30 min at 0°C) or with DNAse (150 µg/ml; 30 min at 20°C). After incubation the suspensions were centrifuged at 105,000 g for 30 min and specific binding in the supernatant ('nuclear extractable') and pellet fraction ('residual nuclear fraction') were measured as described in Materials and Methods. Nuclei extracted with 0.4M KCl were used as a control. Radioactive steroid in the nuclei is subdivided in radioactivity bound by the nuclear extractable fraction (sedimentation value of 5S or 4S), free radioactive oestradiol in the extractable fraction and specifically bound residual nuclear radioactivity (R).

Uterus. Uterine tissue was incubated at $37^{o}C$ for different time periods after an initial incubation of 60 min at $0^{o}C$ in the presence of steroids. Nuclei were isolated and extracted with 0.4M KCl in order to obtain a KCl-extractable and nuclear residual fraction. Specifically bound oestradiol in both nuclear fractions increased during the time of incubation reaching a maximum after 30 min (Fig. 4). After continued incubation the specific binding of oestradiol in the KCl-extractable and nuclear residual fraction declined reaching a plateau after 60 min. During the first period of incubation the initial increase in nuclear oestradiol binding was accompanied by a decrease in the amount of specifically bound oestradiol in the cytosol fraction. After 60 min specific binding sites for oestradiol in the cytosol fraction became undetectable.

Effect of energy deprivation on the distribution of specifically bound ^{3}H-oestradiol in testicular and uterine nuclei. To determine the effect of energy deprivation on the distribution of oestradiol binding sites, testicular and uterine tissue were incubated in the presence of steroid and $5x10^{-4}M$ KCN. Tissues incubated in the presence of steroids but with the omission of KCN from the incubation medium served as control tissues. One hour after the addition of KCN the number of specific oestradiol binding sites in the nuclear residual fractions of both testicular (Fig. 5A) and uterine (Fig. 5B) tissue were enhanced significantly compared to controls. For testicular tissue a twofold increase was measured, for uterine tissue the increase was slightly lower (1.6 fold). The addition of KCN had no specific effect on the specific binding of oestradiol in the KCl-extractable nuclear fraction of both tissues.

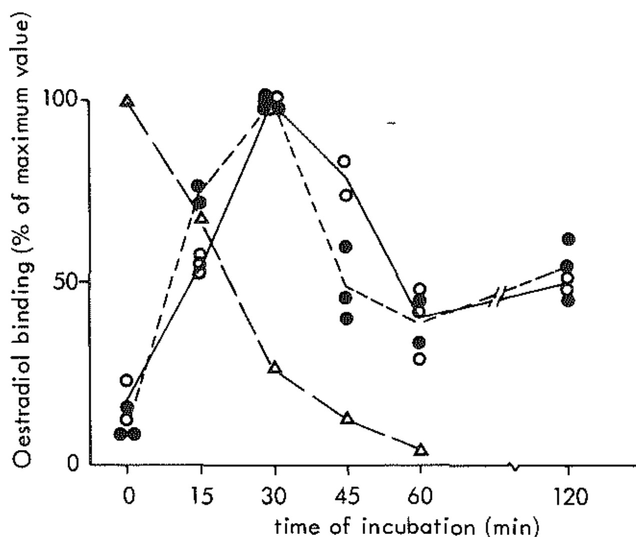

Figure 4 Time course of oestradiol binding in subcellular fractions after incubation of uterine tissue in vitro. Uteri from immature rats were preincubated at $0^{o}C$ for 60 min either with $2x10^{-8}M$ ^{3}H-oestradiol or with $2x10^{-8}M$ ^{3}H-oestradiol plus $4x10^{-6}M$ DES. After this incubation period the temperature was elevated to $37^{o}C$ and incubation was continued for different time periods. Uteri were rinsed with buffer, homogenized and nuclei were isolated. After extraction of nuclei with 0.4M KCl specific oestradiol binding was estimated in the KCl-extractable fraction and the nuclear residual fraction. Specific binding in the cytosol fraction, obtained as described in the method section, was also estimated. Each point reflects an individual estimation.
• - • nuclear extract; o --- o nuclear residual fraction;
Δ --- Δ cytosol fraction.

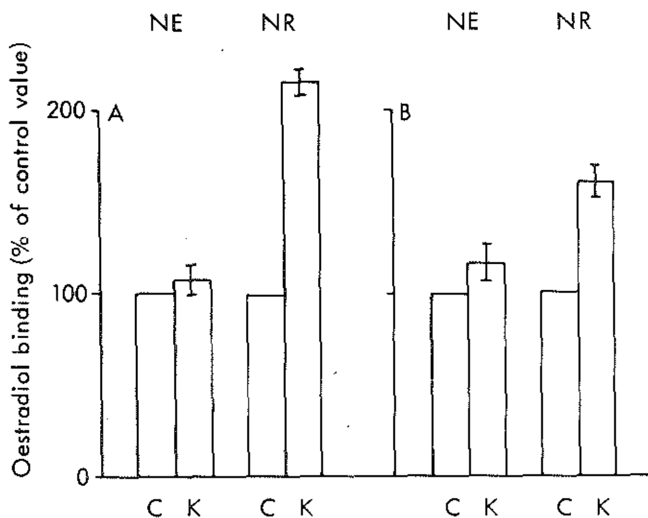

Figure 5 In vitro effect of KCN on the nuclear binding of oestradiol in testicular and uterine tissue.
Tissues were pre-incubated in KRBG-buffer at 32°C or 37°C for 60 min either with $2x10^{-8}$M ^{3}H-oestradiol or with $2x10^{-8}$M ^{3}H-oestradiol plus $4x10^{-6}$M DES. After this period the tissues were washed with KRB-buffer and further incubated in KRB-buffer for a period of 60 min at 32°C or 37°C in the presence of both steroids and $5x10^{-4}$M KCN. Nuclei were isolated and specific oestradiol binding in the KCl-extractable and nuclear residual fractions was estimated. The specific binding obtained after incubation of testis and uterus in KRBG for a period of 120 min in the presence of steroids is defined as the 100% value. Results are expressed as the mean of 3 experiments ± S.E.M.
C = control incubation. K = incubation in the presence of KCN.

Discussion

The testicular Leydig cell contains a receptor protein which is specific for oestradiol [7,19]. This oestradiol receptor has also been demonstrated in total testicular tissue from immature rats [20] and in dissected interstitial tissue of mature rats [5,20]. From the present results it appears that two different fractions of specifically bound oestradiol can be demonstrated in testicular nuclei: one fraction (40%) is extractable with 0.4M KCl, the other fraction resists KCl-extraction. The number of specific binding sites in the KCl-extractable fraction did not show any increase if nuclei were extracted repeatedly or if nuclei were extracted with concentrations of KCl above 0.4M. The present observations are compatible with those for steroid-hormone receptors in other tissues where nuclear binding could also be distinguished in a KCl-extractable and a nuclear residual form [12,13,21,22,24,25,26].

The role of different types of nuclear binding sites for steroid hormones is not yet clear. Evidence has been presented, however, that the residual nuclear binding proteins for oestradiol and testosterone might be important in the mechanism of action of steroid hormones [12,27,28]. The demonstration of two different nuclear binding sites for oestradiol in Leydig cells raises the possibility that actions of oestradiol in these cells can also be mediated via a similar receptor mechanism.

The in vitro incubations of testicular tissue with ^{3}H-oestradiol showed that both types of nuclear oestradiol binding were similarly affected by increasing amounts of non-radioactive DES (Fig. 1). The addition of a 2-3 fold excess of non-radioactive DES caused a 50% reduction of the ^{3}H-oestradiol binding in both nuclear fractions. This observation is in good agreement with those obtained with the cytoplasmic oestradiol receptor [19].

Different approaches were used to characterize the nature of the nuclear oestradiol binding sites in testicular tis-

sue. Mild trypsin treatment of nuclei, which contained oestradiol-receptor complexes, caused a release of binding sites for oestradiol with a sedimentation value of 4S. This value is different from the sedimentation value of 5S which is normally observed for KCl-extractable nuclear oestradiol receptors. Receptor complexes with a sedimentation value of 4S were also solubilized after treatment of liver and uterine nuclei with trypsin [22,23].

It has been reported previously that trypsin treatment of the oestradiol receptor from cytosols of calve uteri resulted in a loss of binding affinity of the receptor for DNA and it has been suggested [29] that trypsin treatment releases part of the receptor molecule, which contains the chromatin-binding unit, whereas the oestrogen binding unit remains unaffected and shows a sedimentation value of 4S on sucrose density gradients.

Extraction of isolated liver nuclei with NaCl solutions in a concentration range of 0.14M to 2M causes removal of DNA-histones and associated proteins; the residual protein fraction contains acidic proteins[30]. For other tissues it has been reported that nuclear steroid hormone receptors are associated with acidic nuclear proteins [27,31]. It has been reported by Wang [30], that deoxycholate (DOC) could solubilize 95% of the nuclear residual proteins from rat liver, including the acidic protein fraction. In the present experiments deoxycholate treatment of testicular nuclei, containing oestradiol-receptor complexes, caused the release of a considerable amount of total nuclear radioactive oestradiol, but only a minor fraction was bound to macromolecules with a sedimentation value of 5S. Addition of DOC to cytoplasmic oestradiol-receptor complexes did not result in a dissociation of the hormone from the receptor molecule. Therefore the observed release of unbound radioactivity from deoxycholate-treated nuclei might reflect a lowered affinity of the nuclear receptor molecule for the hormone subsequent to its binding by the chromatin.

DNAse was used in another attempt to characterize the na-

ture of the nuclear steroid binding. After treatment with DNAse of testicular nuclei, containing accumulated oestradiol-receptor complexes, a considerable increase of radioactive oestradiol in the soluble nuclear fraction was obtained, but again only a small part of the released oestradiol was bound to macromolecules.

From the observed effects of DNAse in the present experiments as well as in previous experiments by others [13, 32,33,34,35,36,37,38,39] it is not possible to derive unambiguous information about the nature of the nuclear acceptor sites which bind hormone-receptor complexes. From the available evidence [4,27,31,40,41,42] it appears most likely that the nuclear acceptor sites are composed of both DNA and nonhistone proteins.

The observed increase in the amount of nuclear binding sites in the KCl-extractable and nuclear residual fraction of testicular tissue during the first 30 min of incubation (Fig. 3) might reflect translocation of cytoplasmic receptor molecules.

Whereas the level of nuclear binding sites in the KCl-extractable fraction remained rather constant, the number of oestradiol binding sites in the nuclear residual fraction decreased, which might reflect either an inactivation of binding sites in this fraction or a redistribution of binding sites over nuclear and cytosol fractions. A possible return of nuclear binding sites into the cytoplasmic fraction could not be measured due to very high levels of nonspecific binding sites in this fraction.

Different results were obtained for uterine tissue (Fig. 4). After an initial increase, the specific binding to KCl-extractable and residual nuclear fractions declined to levels about 50% of the maximum value, which was observed at 30 min after the start of the incubation. This pattern is in close agreement with observations of Mester et al. for uterine tissue after in vivo injection of immature rats with oestradiol [24]. The measured amount of specific oestradiol binding sites in the nuclear residual fraction 60 min after

incubation (160 fmol/uterus) is comparable with the amount estimated by Clark et al. 1 and 6 h after in vivo injection of oestradiol [12]. These authors suggested that this rather constant amount of accumulated nuclear residual binding sites might be the important fraction for the response of the uterus to oestradiol [10,11]. In testicular tissue the amount of nuclear residual binding sites, after a maximum at t = 30 min, dropped gradually, without reaching a plateau.

From the results presented in Fig. 3 and Fig. 4 it can be concluded that the in vitro translocation of oestradiol-receptor complexes into the nuclear fractions of uterus and testis follow similar time courses. However, differences were observed for the retention times of the oestradiol-receptor complexes in the nuclear fractions of both tissues. The incubation temperatures used in the experiments were 37°C and 32°C for uterus and testis respectively. Therefore it cannot be excluded that the observed differences in the nuclear retention reflect a difference in the stability of the binding of hormone-receptor complexes to uterine and testicular chromatin under physiological conditions.

If hormone effects in cells are mediated via a receptor mechanism in the nuclear fraction, then the short retention time of tightly bound nuclear oestradiol receptor molecules (the binding sites in the residual nuclear fraction) could offer an explanation for the difficulties in the search for an oestrogen effect in the testis. In the literature some evidence is available that cellular ATP is involved in the mechanism of action of steroid hormones. It has been observed that the progesterone receptor in oviduct binds ATP [16] and that KCN could inhibit the glucocorticoid receptor release from fibroblast nuclei [13]. In our studies the presence of KCN increased the amounts of oestradiol binding in the residual nuclear fraction in testicular (2-fold increase) and uterine (1.6 fold) tissue, while the oestradiol binding in the KCl-extractable fraction remained unaffected. The increased residual nuclear binding in the presence of

KCN might indicate that a continuous production of ATP is necessary for the release of receptor molecules, possibly in an inactive form [43], into the cytosol. Further investigations are needed to elucidate the energy requirements in the mechanism of action of steroid hormones.

From the present results it can be concluded that the testicular Leydig cell shares the large group of cells that contain receptors for steroid hormones. Under the influence of added oestradiol the cytoplasmic receptor was translocated into the nuclei where it could be recovered from two different nuclear binding sites which differed in extractibility with KCl. The retention times of bound oestradiol in the nuclear fraction which resisted KCl extraction were different for uterine and testicular tissue. Therefore it seems very unlikely that the oestradiol effect in uterine tissue and in testicular tissue, if present, are mediated by identical nuclear receptor mechanisms.

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