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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1. An [³H]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 60 min at 20°C in the presence of [³H]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 0.4 M-KCl extract of testicular nuclei remained constant during incubation at 20°C. For uterine nuclei incubated with [³H]oestradiol at 37°C a shift of specifically bound [³H]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 (0.5–1 nM) 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 [³H]-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 500 ng of oestradiol-17 β or 500 ng of [^3H]oestradiol-17 β in 0.2 ml of 0.15 M-NaCl containing 2.5% (v/v) ethanol. The rats were decapitated 1 h after the injection. In experiments designed to examine the effect of human choriongonadotropin, a daily dose of 50 i.u. of the hormone (WHO, 1975), dissolved in 0.1 ml of 0.15 M-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- ^3H]Oestradiol-17 β (sp. radioactivity 85 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was examined for purity by t.l.c. Diethylstilboestrol and oestradiol-17 β 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 10 nM-oestradiol in 2.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.2% glucose (Umbreit *et al.*, 1964). Incubations were carried out in an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5) for 60 min at 32°C for testicular tissue and at 37°C for uterine tissue. In control studies tissues were incubated with either 10 nM-[^3H]oestradiol or 10 nM-[^3H]oestradiol plus 10 μM -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 10 vol. of 10 mM-Tris/HCl buffer, pH 7.4, containing 1.5 mM-EDTA and 0.02% NaN_3 (TEN buffer) with six strokes of a Potter-Elvehjem homogenizer at 1100 rev./min. The homogenate was rehomogenized in an all-glass Potter-Elvehjem homogenizer and centrifuged at 500g for 10 min at 0°C. The 500g pellet was washed once with 3 ml of TEN buffer, twice with 3 ml of TEN buffer containing 0.2% Triton X-100 and another time with 3 ml of TEN buffer. Uterine tissue after incubation was suspended in 3 ml of TEN buffer and homogenized by hand with two strokes in an all-glass Kontes homogenizer. The homogenate was centrifuged at 800g for 10 min at 0°C and the pellet was washed three times with 3 ml 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 0.4 M-KCl.

Hormone-binding assay

(a) *Determination of nuclear binding, after incubation or injection with [^3H]oestradiol.* Isolated nuclei were extracted with 0.4 M-KCl in TEN buffer, pH 8.5, during 60 min at 0°C. The extract was centrifuged at 0°C for 10 min 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 1 ml of 1 M-NaOH, and 100 μl was counted for radioactivity after addition of 50 μl of 3 M- 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 120 min at different temperatures with either 10 nM-[^3H]oestradiol or 10 nM-[^3H]oestradiol plus 10 μM -diethylstilboestrol. After the incubation, nuclei were extracted as described under (a). To remove the excess of unbound steroid, the nuclear suspension in 0.4 M-KCl was treated for 10 min with dextran-coated charcoal (final concentrations: 0.5% charcoal and 0.05% dextran T 300) at 0°C and was subsequently centrifuged for 30 min at 105000g_{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 3 ml of ethanol. The ethanol extract was removed for assay of the amount of radioactivity.

Sucrose-density-gradient centrifugation

A 200–300 μ l portion of the KCl-extractable nuclear fraction was layered on top of a linear 5–20% (w/v) sucrose gradient prepared in TEN buffer, pH 8.5, containing 0.4 M-KCl. The gradients were centrifuged at 260000 g_{av} for 18 h at 0°C in a Beckman SW 65 rotor. On separate gradients bovine serum albumin (200 μ g) was run as a sedimentation marker (4.6S). After centrifugation, each gradient was fractionated into 27 fractions of 0.2 ml, 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 10 nM-[3 H]oestradiol and with 10 nM-[3 H]oestradiol plus 10 μ M-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 0.1 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] and 4.8 g 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 0.4 M-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 60 min at 37°C with 20 nM-oestradiol. Isolated nuclei were incubated for 60 min at 37°C with either 10 nM-[3 H]oestradiol or 10 nM-[3 H]oestradiol plus 10 μ M-diethylstilboestrol. Testicular tissue was incubated for 60 min at 32°C with 10 nM-oestradiol. Isolated nuclei were incubated for 60 min at 20°C or 32°C with either 10 nM-[3 H]oestradiol or 10 nM-[3 H]oestradiol plus 10 μ M-diethylstilboestrol. Incubated uterine and testicular nuclei were washed twice with TEN buffer and extracted with 3 ml 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 $\pm$ 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 $\pm$ 323 (5)	263 $\pm$ 59 (5)	748 $\pm$ 267 (5)
	Control	1089 $\pm$ 200 (3)	68 $\pm$ 6 (3)	1021 $\pm$ 195 (3)
Testis	Exchange at 20°C	2011 $\pm$ 98 (5)	1932 $\pm$ 64 (5)	79 $\pm$ 36 (5)
	Exchange at 32°C	1891 $\pm$ 50 (4)	1763 $\pm$ 80 (4)	126 $\pm$ 82 (4)
	Control	92 $\pm$ 11 (3)	30 $\pm$ 5 (3)	62 $\pm$ 6 (3)

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

Testicular tissue was incubated for 60 min at 32°C with either 10 nM - ^3H -oestradiol ($\bullet$) or 10 nM - ^3H -oestradiol plus $10\text{ }\mu\text{M}$ -diethylstilboestrol ($\circ$). Nuclei were isolated and extracted as described in the Materials and Methods section. A $200\text{ }\mu\text{l}$ portion of the nuclear extracts was centrifuged on sucrose gradients for 18 h at $260\,000g_{\text{av}}$. 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 $39 \pm 9\%$ (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 1 h of incubation (Fig. 2a). In the presence of oestradiol, dissociation of binding sites in the KCl-extractable

fraction at 20°C was less than 5% (Fig. 2b). 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. 3b). 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. 3a).

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 15 min. The amount of specific binding measured after the exchange procedure [$21.9\text{ fmol} \pm 2.7$ (s.d.), $n = 13$] did not differ significantly from control values [$22.4\text{ fmol} \pm 2.5$ (s.d.), $n = 3$], which were obtained after injection of [^3H]oestradiol *in vivo*. Therefore the hormone-exchange procedure *in vitro* during 60 min 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. 5b). 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 500 ng 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 50 i.u. of choriogonadotropin/

Fig. 2. Effect of temperature on the stability of testicular nuclear oestradiol receptors

Testicular tissue was incubated for 60 min at 32°C with either 10 nM-[³H]oestradiol or 10 nM-[³H]oestradiol plus 10 μ M-diethylstilboestrol. Nuclei were isolated and nuclear suspensions were kept for 60 min at different temperatures either without [³H]oestradiol (a) or with 10 nM-[³H]oestradiol (b) in the incubation medium. Thereafter nuclei were washed twice with TEN buffer and extracted with 0.4 M-KCl as described in the Materials and Methods section. In (a) specific binding in the KCl-extractable (Δ) and residual nuclear fraction ($\bullet$) was calculated as a percentage of the total nuclear binding ($\circ$) 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 $\pm$ 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 60 min at 37°C with either 20 nM-[³H]oestradiol or 20 nM-[³H]oestradiol plus 4 μ M-diethylstilboestrol. Nuclei were isolated and nuclear suspensions were kept for 60 min at different temperatures either without [³H]oestradiol (a) or with 10 nM-[³H]oestradiol (b) in the incubation medium. Thereafter nuclei were washed twice with TEN buffer and extracted with 0.4 M-KCl as described in the Materials and Methods section. Specific binding in the KCl-extractable (Δ) and residual nuclear fraction ($\bullet$) was calculated as the percentage of the total nuclear binding ($\circ$) obtained after incubation of nuclei at 0°C [985 ± 231 (5) fmol/uterus]. Each value is the mean $\pm$ 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 500 ng of oestradiol, and 1 h later testicular nuclei were prepared and incubated for different time-periods at 20°C with either 10 nM-[³H]oestradiol or 10 nM-[³H]oestradiol plus 10 μM-diethylstilboestrol. After incubation the nuclei were extracted with 0.4 M-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 500 ng 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, 20 h 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 50 i.u. of choriogonadotropin for 5 successive days. For determination of the total amount of nuclear binding sites all groups of animals were injected with 500 ng of non-radioactive oestradiol 60 min before the animals were killed. Nuclei were incubated for 60 min at 20°C with either 10 nM-[³H]oestradiol or 10 nM-[³H]oestradiol plus 10 μM-diethylstilboestrol. Incubated nuclei were washed with TEN buffer and extracted with 0.4 M-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 60 min at 20°C with either 10 nM-[³H]oestradiol (●) or 10 nM-[³H]oestradiol plus 10 μM-diethylstilboestrol (○). Incubated nuclei were extracted with 0.4 M-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 500 ng of oestradiol 60 min 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 ^3H -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 ^3H -labelled steroid, accumulated in the nucleus after the exchange procedure, is extracted with ethanol (total radioactivity T).¹ In control experiments exchange is performed in the presence of ^3H -labelled steroid and an excess of non-radioactive steroid. It is assumed that in this case the amount of ^3H -labelled

steroid extracted with ethanol represents the non-specifically bound steroid (N). Specific binding of [^3H]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 [^3H]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 [^3H]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 [³H]oestradiol at this temperature was completed within 5 min (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 hypo-

physectomy. 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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