J. steroid Biochem. Vol. 23, No. 4, pp. 405–413, 1985
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0022-4731/85 $3.00 + 0.00
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A NEW EXCHANGE PROCEDURE FOR THE QUANTITATION OF PROSTATIC ANDROGEN RECEPTOR COMPLEXES FORMED *IN VIVO*

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(*Received* 10 *December* 1984)

Summary—A procedure is described for the measurement of rat prostatic androgen receptor saturated *in vivo* with non-radioactive androgen. While NaSCN alone induces irreversible dissociation (denaturation) of androgen from the receptor, the combination of this chaotropic salt (0.15 M) with sucrose (15%) and sodium molybdate (10 mM) allows the exchange of R DHT with [^{3}H]DHT at 0°C with only minimal receptor denaturation. The validity of the present exchange assay is based on the following: a similar quantity of androgen receptor was detected when binding was measured directly after *in vivo* treatment with radioactive androgen or indirectly by [^{3}H]DHT exchange after treatment with non-radioactive androgen. Steroid specificity, sedimentation analysis and equilibrium association constants indicated that this exchange assay labels the androgen receptor without interference from other prostatic steroid binding proteins. With this method it is now possible to quantitate not only prostatic androgen receptors bound to androgens *in vitro* but also hormone–receptor complexes formed in intact animals under the influence of endogenous androgen.

INTRODUCTION

The lack of adequate methods for the quantitation of androgen receptors (AR) prevents the study of some of the processes involved in the mechanism of androgen action. The ease of measurement of free cytoplasmic receptor sites is of little consequence, since in the non-castrated animal the high levels of endogenous androgen keep most of the available receptor sites saturated and presumably localized in the nucleus. Deeper knowledge of hormone action requires the ability to exchange quantitatively these filled receptor sites in the soluble and particulate fractions of tissue homogenates. Despite reports of a number of such procedures [1–10], none have proven to be satisfactory, since they are not quantitative. Indeed, so uncertain are these methods, that the generally accepted concept of hormone-dependent depletion of cytosolic receptor coupled to nuclear accumulation has been questioned [7]. More importantly, correlation of receptor concentration with hormonal sensitivity of tumors, found so useful in therapeutic management of breast cancer, has yet to be established for prostatic cancer. The principal difficulties in the development of an exchange assay for the androgen receptor are the instability of the receptor at elevated temperatures [8, 9, 11] and the slow dissociation of unlabeled ligand at 0–4°C [8, 12–15].

We have described the use of mersalyl acid, an –SH directed organic mercurial reagent, to promote the rapid dissociation of steroid from the androgen receptor complexes formed *in vitro* [16]. Attempts to apply this exchange assay to the measurement of androgen receptor complexes formed *in vivo*, however, were only partially successful, due to the inability of mersalyl to induce complete dissociation of *in vivo* bound DHT [15]. We now describe an exchange assay which allows quantitation of prostatic androgen receptor complexes formed *in vivo* or *in vitro*.

EXPERIMENTAL

Isotopes and chemicals

The following compounds were obtained from New England Nuclear Corp., (Boston, MA): [1,2,6,7-^{3}H]-Testosterone ([^{3}H]T, 93.9 Ci/mmol); [1,2,4,5,6,7-^{3}H]dihydrotestosterone ([^{3}H]DHT, 51.6–123 Ci/mmol); 17β-hydroxy-17α-methyl [^{3}H]estra-4,9,11-trien-3-one, ([^{3}H]R1881, 87 Ci/mmol); bovine serum [^{14}C]albumin ([^{14}C]BSA), human [^{14}C]γ-globulin, R1881. Non-radioactive steroids were obtained from Steraloids (Wilton, NH). All other reagents used were of analytical grade and purchased from commercial sources.

This work was supported by Research Grant CA28856 from the National Cancer Institute, Bethesda, MD. The following abbreviations have been used: R: receptor; AR: androgen receptor complex; T: testosterone; DHT: dihydrotestosterone; HAP: hydroxylapatite; DCC: dextran coated charcoal. This is publication No. 121 from the Hubert H. Humphrey Cancer Research Center at Boston University.

Animals

Male Sprague–Dawley rats (250–350 g b.wt) were purchased from Charles River Breeding Laboratories (Wilmington, MA) or from ESS Laboratories Inc. (Lynnfield, MA). Animals were either kept intact or castrated via the scrotal route 18–24 h before further experimentation.

Injection of steroids

Animals were injected s.c. with 0.2 ml of ethanol containing the desired dose of radioinert or radioactive steroids (0.5–1 Ci/mmol). One hour after injection, the animals were killed by cervical dislocation, their ventral prostate excised, stripped of fat and mesenteric tissue and either used immediately or stored in liquid nitrogen for 1–2 weeks.

Buffers and solutions

Buffer TEGM: 50 mM Tris, 1.5 mM EDTA, 10% v/v glycerol, 10 mM Na_2MoO_4, pH 7.4 at 4°C. Buffer TEDGM: buffer TEGM with 1 mM dithiothreitol NaN_3 (0.02%) was added to all buffers to prevent microbial growth. Dithiothreitol was added immediately prior to use to a final concentration of 1 and 10 mM respectively. Phenylmethylsulfonylfluoride (PMSF) and leupeptin were included in the homogenization buffer at concentrations of 0.5 and 1 mM, respectively.

Preparation of cytosolic and nuclear fraction

Unless otherwise stated all manipulations were carried out at 0–4°C. Frozen tissue samples were pulverized using a Thermovac tissue pulverizer precooled in liquid nitrogen. Fresh tissue or tissue powder was homogenized in 3–4 vol of buffer using a Duall glass–glass homogenizer with a motor driven pestle. The homogenate was either used directly or further centrifuged at 100,000 *g* for 30 min to yield the high speed supernatant (cytosol) and the crude nuclear pellet.

Binding of [^{3}H]DHT to the cytosolic androgen receptor

Aliquots (0.1–0.2 ml) of cytosol were mixed with an equal volume of buffer containing [^{3}H]DHT with (non-specific binding) or without (total binding) a 100-fold excess of unlabeled DHT. All samples were kept at 0°C for the times indicated; bound [^{3}H]DHT was measured by the HAP technique as described previously [15]. Specific binding was calculated by subtracting non-specific from total binding.

Dissociation of [^{3}H]DHT from cytoplasmic androgen receptor complexes formed in vitro

Cytosolic R[^{3}H]DHT complexes were mixed with an equal volume of buffer containing 30% sucrose with or without 0.3 M NaSCN. Each sample was further divided and incubated at 0°C either with (irreversible dissociation of [^{3}H]DHT) or without (stability control) 2×10^{-6} M R1881. At the desired time points duplicate 0.1–0.2 ml aliquots were removed and receptor bound radioactivity measured by HAP-assay.

Dissociation of [^{3}H]DHT from androgen receptor complexes formed in vivo

R[^{3}H]DHT was formed *in vivo* by injecting 75 μg of [^{3}H]T (sp. act. 0.5 Ci/mol) into castrated animals [15]. To determine non-specific binding, animals were injected with 7.5 mg of unlabeled testosterone 5 min prior to administration of the radioactive steroid. One hour after injection the animals were killed and prostatic tissue was removed and homogenized. The homogenate was further diluted with an equal volume of buffer containing 30% sucrose with or without 0.3 M NaSCN. Aliquots of the homogenate were incubated with (dissociation) or without (stability control) 2×10^{-6} M R1881; stability samples were first reconstituted with [^{3}H]DHT (4×10^{-8} M) of the same specific activity (0.5 Ci/mmol). At the desired time aliquots (0.1–0.2 ml) were removed and added to an HAP slurry (0.5 ml) for measurement of R[^{3}H]DHT complexes.

Analysis of R[^{3}H]DHT on sucrose density gradients

Linear 5–20% sucrose gradients in buffer TEDGM containing various concentrations of NaSCN were prepared in polyallomer tubes and kept 1–2 h at 0°C before use. Cytosol samples (0.1 ml) were layered on the gradients; [^{14}C]BSA (4.6 S) and human [^{14}C]γ-globulin (7S) were added to each gradient as internal sedimentation markers. Centrifugation was performed in a Beckman SW60 rotor at 55,000 rpm for 18–20 h at 2°C. Fractions (0.1 ml) were collected in scintillation vials containing 0.2 ml of water, mixed with 4 ml Liquiscint and the radioactivity counted. Water was added to increase the counting efficiency of the ^{14}C-labeled proteins in the scintillation fluid [17].

Miscellaneous

Protein concentration was measured by the method of Lowry *et al.*[18]. DNA determination was made by the modified diphenylamine assay of Burton[19]. To reduce interference by contaminating glycoproteins, the 800 or 100,000 *g* pellets were washed 3 times with ice-cold buffer and once with ice-cold 0.5N perchloric acid prior to DNA solubilization.

RESULTS

Effect of NaSCN on the stability of the androgen receptor at 0°C

NaSCN (0.4M) has been used to enhance the dissociation of estrogen from cytosolic and nuclear receptors at 0–4°C [20, 21]. In preliminary dissociation experiments (Fig. 1A) a concentration dependent irreversible loss of [^{3}H]DHT binding was observed. In this respect the androgen receptor behaves like glucocorticoid [22], Vitamin D_3 [23] and

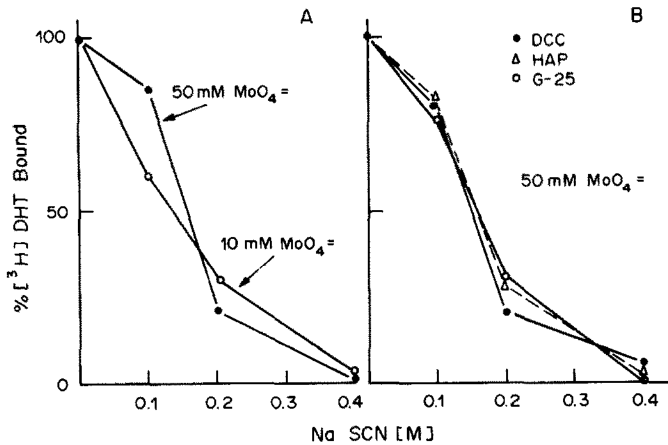

Fig. 1. Effect of NaSCN on the stability of cytosolic androgen receptors. Panel A: Prostatic cytosol (10–12 mg protein/ml) of castrated rats prepared in buffers containing 10 or 50 mM molybdate was incubated at 0°C for 2 h with [^{3}H]DHT (2×10^{-8} M) in the absence (total binding) or presence (non-specific binding) of 2×10^{-6} M unlabeled DHT. The samples were further incubated at 0°C for 20 h in the absence or presence of various concentrations of NaSCN. [^{3}H]DHT bound in each sample was assayed with HAP. All data represent specific binding. Panel B: Cytosol incubation and treatment with NaSCN were as in panel A. Measurements of bound radioactivity were made with HAP (△---△), DCC (●---●) and Sephadex G-25 (○---○).

progesterone [24] receptors. Na_2MoO_4, a reagent that stabilizes steroid receptors [8, 16, 25–27], offered no protection against NaSCN denaturation of AR.

Since HAP adsorption was used to determine the bound radioactivity, it was necessary to see if NaSCN interfered with receptor binding to HAP. Cytosol labeled with [^{3}H]DHT for 2 h and then incubated with NaSCN was assayed for specific binding by HAP, DCC, or Sephadex G-25 centrifugal gel filtration [15]. As shown in Fig. 1b, regardless of the method used, loss of receptor binding capacity was observed. A small fraction of R[^{3}H]DHT complexes was stable at intermediate concentrations of NaSCN. It is possible that the chaotropic salt altered DHT interaction with the binding site of the receptors by unfolding the latter; a similar effect on other proteins has been reported [28].

The effects of NaSCN on receptor sedimentation are shown in Fig. 2. In the absence of NaSCN (Fig. 2a) R[^{3}H]DHT sedimented as a 10S macromolecule in low salt gradients and at 7S in 0.4M KCl. A gradual decrease in the sedimentation constant was

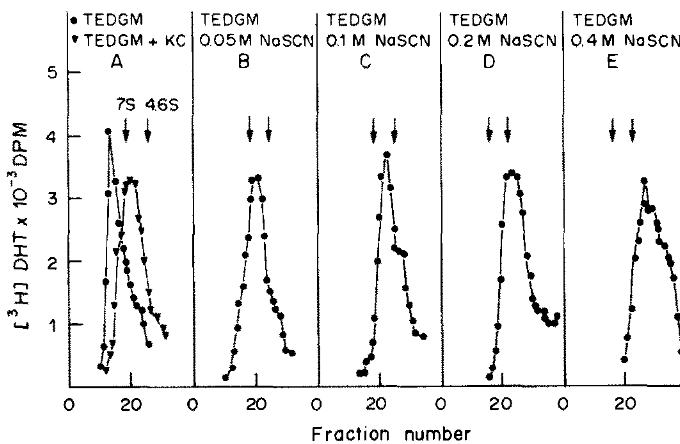

Fig. 2. Effect of NaSCN on androgen receptor sedimentation. Prostatic cytosol (17 mg protein/ml) prepared in buffer TEDG containing 50 mM molybdate was incubated at 0°C for 2 h with [^{3}H]DHT (2×10^{-8} M). To determine non-specific binding, parallel samples were incubated with [^{3}H]DHT in the presence of 2×10^{-6} M unlabeled DHT. Aliquots of each incubation were added to a DCC pellet prepared from identical volumes of DCC suspension, mixed, and kept on ice for 10 min. After centrifugation, the supernatant was removed and aliquots (0.1 ml) were layered on 5–20% sucrose density gradients prepared in the same buffer with (▼---▼) (panel A) or without (panel A–E) (●---●) 0.4M KCl. Gradients B–E contained the indicated concentrations of NaSCN. Only specific binding is shown in the figure. Arrows indicate positions of ^{14}C-labeled protein markers.

observed with increasing concentrations of NaSCN. This could be due to dissociation of the 10S receptor into subunits and/or to NaSCN induced protein unfolding. Intermediate concentrations of NaSCN in the sucrose gradient did not cause significant dissociation of [^{3}H]DHT from R[^{3}H]DHT complexes; 0.4M NaSCN, however, induced dissociation of the androgen–receptor complexes as shown by the presence of [^{3}H]DHT in the upper fractions of the gradient.

The principal difference between the experiments shown in Fig. 1 and those shown in Fig. 2 is that in the latter the receptor was exposed to NaSCN in the presence of sucrose, a sugar known to stabilize proteins against unfolding [29]. To verify this stabilization by sucrose, [^{3}H]DHT receptor complexes were incubated with various concentrations of NaSCN with or without sucrose, and specific binding was measured. In the presence of sucrose (15%) and 0.1–0.2M NaSCN no significant loss of specifically bound [^{3}H]DHT was observed. At higher concentrations of NaSCN, however, progressive loss of specific [^{3}H]DHT binding was observed even with sucrose (data not shown). Thus, 0.15M NaSCN was used in subsequent experiments.

Effect of NaSCN and sucrose on the dissociation of [^{3}H]DHT from in vitro *and* in vivo *labeled androgen receptor*

Cytosolic [^{3}H]DHT complexes formed *in vitro* were incubated with sucrose (15%) in the presence or absence of 0.15M NaSCN. Irreversible dissociation of [^{3}H]DHT at 0°C was initiated by addition of a small volume (1:100) of ethanol containing 2×10^{-4} M R1881; stability incubations received ethanol only. At the times indicated in Fig. 3A aliquots were removed and bound radioactivity measured. In the absence of NaSCN the dissociation of [^{3}H]DHT from androgen receptor complexes was very slow ($t_{1/2} = 62$ h) [8, 12, 15]. In the presence of NaSCN, however, [^{3}H]DHT dissociated much faster, with a half time of 18–20 h. Under these conditions receptor inactivation was minimal as shown by the small loss of specifically bound radioactivity in the stability incubation. Equilibrium binding studies in the presence of NaSCN (data not shown) showed that the affinity of AR for DHT decreased 7–8-fold; the K_d increased from 0.4 to 3 nM. This is in agreement with the increased rate of [^{3}H]DHT dissociation observed in Fig. 3.

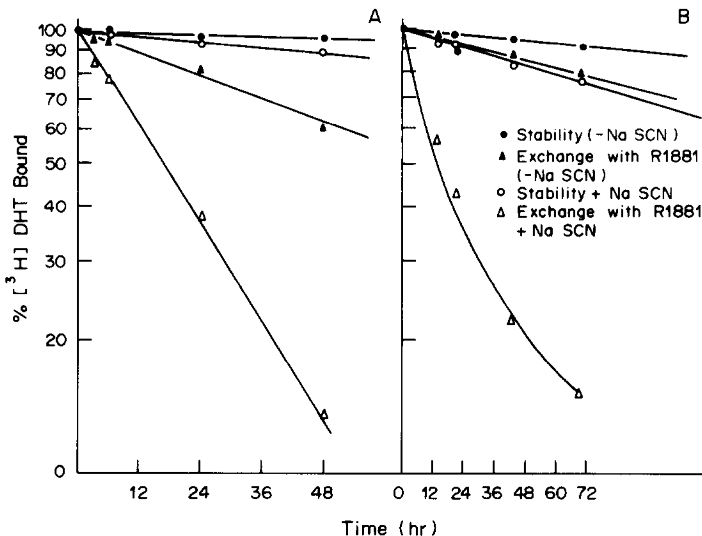

Fig. 3. Effect of NaSCN on the dissociation of [^{3}H]DHT from androgen receptor complexes formed *in vivo* and *in vitro*. Panel A: Prostatic cytosol (20.8 mg protein per ml) of castrated rats was incubated at 0°C for 2 h with [^{3}H]DHT (3×10^{-8} M) in the absence or presence of 3×10^{-6} M unlabeled DHT. Aliquots of these incubations were mixed with equal volumes of buffer containing 30% sucrose without (stability incubation ●---●) or with (dissociation ▲---▲) 2×10^{-6} M unlabeled R1881. Furthermore, cytosol labeled as above was mixed with an equal volume of buffer containing 30% sucrose and 0.3 M NaSCN in the absence (stability incubation ○---○) or presence (dissociation △---△) of 2×10^{-6} M unlabeled R1881. At the times indicated duplicate samples (0.1 ml) were removed and bound [^{3}H]DHT was measured with HAP. All data points represent specific binding. Panel B: [^{3}H]testosterone (75 μg 0.5 Ci/mmol) was injected into castrated rats, with or without injection of 7.5 mg of unlabeled testosterone. One hour later animals were sacrificed, and prostates were excised and homogenized. Total homogenate (cytosol and nuclei) was used for this experiment. To assess receptor stability, aliquots of the homogenate were first reconstituted with [^{3}H]DHT (4×10^{-8} M; sp. act. 0.5 Ci/mmol) and subsequently incubated with sucrose buffer in the presence (○---○) or absence (●---●) of NaSCN as described for Panel A. Similarly, aliquots of the homogenate were mixed with buffer containing sucrose, and unlabeled R1881 in the presence (△---△) or absence (▲---▲) of NaSCN (dissociation). At the indicated times aliquots were assayed for bound radioactivity with HAP. Data were corrected for non-specific binding (< 5% of total binding).

Since DHT dissociates at different rates from hormone receptor complexes formed *in vitro* and *in vivo* [15] it was essential to demonstrate that NaSCN also accelerated the rate of steroid dissociation from those complexes formed *in vivo*. Castrated rats were injected with [^{3}H]T or [^{3}H]T + T, sacrificed 1 h later, and then the prostates were excised and homogenized. To initiate irreversible ligand dissociation R1881 (2×10^{-6} M) was added. At the times indicated in Fig. 3B [^{3}H]DHT remaining bound to the receptor was measured. To assess receptor stability in the presence or absence of NaSCN aliquots of the homogenate were mixed with an equal volume of buffer containing 30% sucrose with or without 0.3M NaSCN. Since NaSCN decreases the receptor affinity for DHT 7–8-fold, [^{3}H]DHT (4×10^{-8} M) with the same specific activity as the injected [^{3}H]T (0.5 Ci/mmol) was included in the stability incubation to compensate for the decrease in steroid concentration caused by homogenization; this prevents a decrease in receptor bound [^{3}H]DHT caused by ligand dissociation rather than through receptor denaturation. In the absence of NaSCN R[^{3}H]DHT complexes formed *in vivo* were stable at 0°C, with only a 15% loss of binding after 72 h (solid circles); [^{3}H]DHT dissociated very slowly, with a $t_{1/2}$ of 160 h (solid triangles). In the presence of NaSCN only 25% of the R[^{3}H]DHT complexes were denatured after 72 h of incubation (open circles), while dissociation was greatly accelerated ($t_{1/2}$ = 20–30 h) and was almost complete (85%) after 72 h (open triangles). These data demonstrate that NaSCN promotes dissociation of [^{3}H]DHT from androgen receptor complexes formed both *in vitro* and *in vivo*.

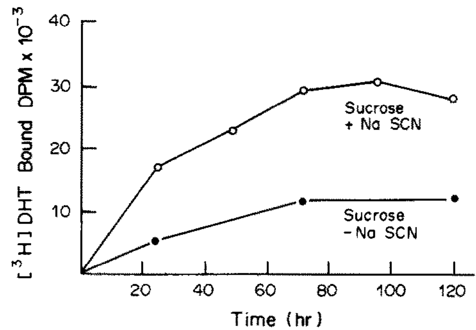

Fig. 4. Effects of NaSCN on the [^{3}H]DHT exchange of DHT receptor complexes formed *in vivo*. Prostatic tissue of intact rats was removed and homogenized. Samples (0.2 ml) of the homogenate (155 μg DNA/ml) were incubated with [^{3}H]DHT (4×10^{-8} M) and sucrose (15%), with or without NaSCN (0.15 M). Parallel samples were incubated as above but in the presence of 4×10^{-6} M unlabeled DHT for determination of non-specific binding. At the indicated times [^{3}H]DHT binding was measured with HAP. Only specific binding is represented.

Time course of [^{3}H]DHT exchange in the presence of NaSCN

To establish the optimal incubation times for the *in vitro* exchange of androgen receptor complexes formed *in vivo*, prostatic homogenates from intact rats were incubated with [^{3}H]DHT and 15% sucrose in the presence or absence of 0.15M NaSCN. At the times indicated in Fig. 4, samples were assayed with HAP for bound radioactivity. Exchange of endogenously bound DHT with [^{3}H]DHT was 3 times more efficient in the presence of NaSCN and was complete within 72–96 h.

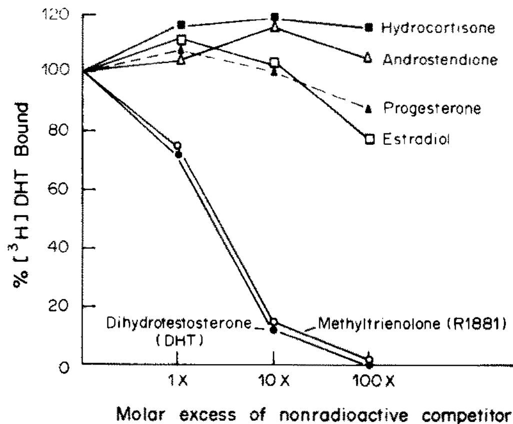

Fig. 5. Steroid specificity of the androgen binding sites in the presence of NaSCN. Prostatic tissue obtained from intact rats was homogenized and aliquots (0.2 ml) of the homogenate were mixed with an equal volume of buffer containing 6×10^{-8} M [^{3}H]DHT, 30% sucrose, 0.3 M NaSCN in the absence (100% control) or presence of various concentration of unlabeled steroids. To determine non-specific binding parallel incubation was made in the presence of 3×10^{-6}M unlabeled DHT. All samples were kept at 0°C for 72 h and assayed for bound [^{3}H]DHT with HAP. Specific binding was plotted as % of control vs molar ratio of unlabeled competitor to [^{3}H]DHT.

Steroid specificity of androgen receptor in the presence of NaSCN

To demonstrate that under the exchange assay conditions described here [^{3}H]DHT bound specifically to the androgen receptor, we incubated prostatic homogenates from intact rats with [^{3}H]DHT, NaSCN and sucrose at 0°C for 72 h with increasing concentrations of various unlabeled steroids. Non-specific binding was determined by incubation with [^{3}H]DHT and a 100-fold excess of DHT. At the end of the incubation [^{3}H]DHT binding was measured. The data in Fig. 5 show that only unlabeled DHT and R1881 effectively competed for [^{3}H]DHT binding. Estradiol and progesterone were very weak competitors, while cortisol and androstenedione were ineffective. These data suggest that [^{3}H]DHT exchanged with receptor androgen complexes formed *in vivo* without interference from other steroid binding proteins.

Analysis of the exchanged material on low salt sucrose density gradients (Fig. 6) further demonstrated that the [^{3}H]DHT was bound to a macromolecule with a sedimentation coefficient of 4.6S.

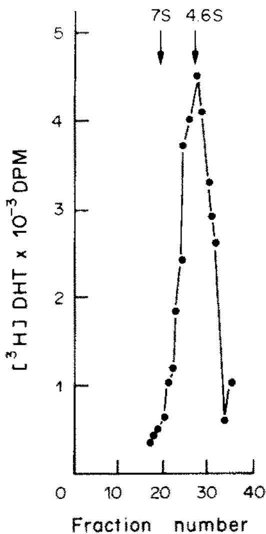

Fig. 6. Sucrose density gradient analysis of androgen receptor complexes formed *in vivo* and exchanged *in vitro* with [^{3}H]DHT. Prostatic tissue of intact animals was homogenized and incubated with [^{3}H]DHT (3×10^{-8} M) in presence of sucrose (15%) and NaSCN (0.15 M) for 72 h at 0°C. Non specific binding was determined by parallel incubation in presence of a 100-fold molar excess unlabeled DHT. The samples were centrifuged at 100,000 *g* for 30 min and the supernatant (cytosolic and nuclear extract) was centrifuged through Sephadex G-25. Aliquots of the filtrate were centrifuged on low salt sucrose density gradients. The radioactivity profile represents only specific binding.

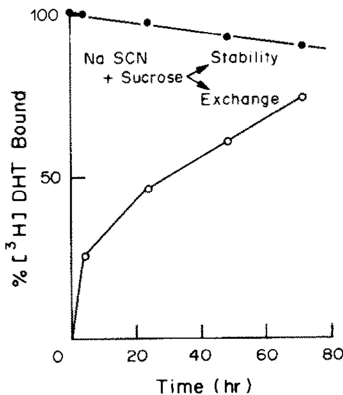

Fig. 7. NaSCN induced [^{3}H]DHT exchange of cytosolic DHT receptor complexes formed *in vitro*. Prostatic cytosol (15.8 mg protein/ml) of castrated rats was incubated for 20 h at 0°C with 2×10^{-8} M non-radioactive DHT (○---○) or [^{3}H]DHT (●---●) in the presence or absence of a 100-fold excess unlabeled DHT. Free steroid was removed by Sephadex G-25 centrifugal gel filtration. The eluates were then reincubated at 0°C with buffer containing [^{3}H]DHT (3×10^{-8} M) sucrose (15%) and NaSCN (0.15M). Parallel samples were incubated with 4×10^{-6} M unlabeled DHT to determine non-specific binding. At the indicated times samples were removed and assayed for bound [^{3}H]DHT with HAP. Specifically bound [^{3}H]DHT in the samples preincubated with [^{3}H]DHT represent stability in presence of NaSCN. Binding in the samples preincubated with unlabeled DHT represent exchange.

As already shown in Fig. 2, exposure to 0.1–0.2M NaSCN yields an entity which sediments at 4–5S. This entity represents cytosolic and nuclear androgen receptors since NaSCN extracts 87% of nuclear bound [^{3}H]DHT (data not shown).

Exchange of in vitro *bound unlabeled DHT with [^{3}H]DHT*

To test the quantitativeness of the NaSCN sucrose exchange assay, prostatic cytosol from castrated rats was incubated with [^{3}H]DHT with or without DHT at 0°C for 20 h. Furthermore, aliquots of the same cytosol were incubated with DHT at 0°C for 20 h. After removing free steroid by centrifugal gel filtration [15] all samples were reconstituted with [^{3}H]DHT (3×10^{-8} M), NaSCN (0.15M) and sucrose (15%) in the presence or absence of a 100-fold excess of DHT. As shown in Fig. 7 exchange of DHT with [^{3}H]DHT (open circles) reached a maximum within 70–72 h. The concentration of [^{3}H]DHT receptor complexes formed by exchange was approx. 80% of that obtained by direct receptor labeling with tritiated ligand followed by a 72 h incubation with [^{3}H]DHT (stability incubation, solid circles).

Validation of the exchange assay with androgen receptors bound to unlabeled androgen in vivo

Twenty-four hours after castration rat prostatic androgen receptor decreases from 10 to about 2 pmol per mg DNA [30]. Treatment with testosterone replenishes the binding sites [15, 31] by a process believed to require protein synthesis. To validate the NaSCN–sucrose exchange assay it was necessary to compare the prostatic AR concentrations measured 1 h after injection of [^{3}H]T into castrated rats with those measured by *in vitro* exchange of androgen receptor complexes of prostates from animals which had been treated with an identical dose of unlabeled testosterone. Injection of 10, 30 and 75 μg of [^{3}H]T to castrated rats gave receptor concentrations of 6, 10 and 12 pmol/mg DNA respectively (Table 1). Thus, as previously shown [15, 31], it appears that the replenishment of prostatic androgen receptor after castration is hormone and dose dependent.

In a parallel experiment castrated rats were injected with 10, 30 or 75 μg of unlabeled testosterone and the concentration of prostatic androgen receptor was measured by [^{3}H]DHT exchange. As shown in Table 1, the androgen receptor values found after exchange were similar to those obtained by direct labeling, demonstrating the quantitativeness of this procedure.

DISCUSSION

Exchange assays currently used for measurement of androgen receptors occupied *in vivo* by androgen are not quantitative for the following reasons:

(a) At 0°C DHT dissociates slowly from the receptor with a half time exceeding 65 h [8, 13, 15]; thus,

Table 1. Validation of androgen receptor measurements by exchange assay in the presence of NaSCN

Steroid administered [^{3}H]T* Dose (μg/rat)	Unlabeled T† AR pmol/mg DNA (Direct labeling)	(Exchange)
10	6	7.28
30	10	9.69
75	12	13.7

*Castrated rats (2 animals/group) were injected with 10, 30 or 75 μg of [^{3}H]T (sp. act. 0.5 Ci/mmol). To determine non-specific binding groups of castrated rats were injected with 1,3 and 7.5 mg of unlabeled T 5 min before injection of [^{3}H]T. One hour after injection, animals were sacrificed and ventral prostates were excised and homogenized. Aliquots of the homogenates were used to determined bound radioactivity. All data were corrected for non-specific binding and normalized per mg DNA.

†Three groups of castrated rats were injected with 10, 30 or 75 μg of unlabeled T. One hour after T administration all animals were sacrificed, and their ventral prostates excised and homogenized. The homogenates (280 μg DNA/ml for castrates injected with 10 μg T; 260 μg DNA/ml for castrates injected with 30 μg T, and 385 μg DNA/ml for castrates treated with 75 μg T) were further diluted with an equal volume of buffer containing 30% sucrose. Aliquots (0.2 ml, triplicate samples) of these homogenates were dispensed into a series of test tubes containing increasing concentrations of [^{3}H]DHT prepared in 0.4 ml of buffer containing 0.225 M NaSCN and 15% sucrose. Parallel incubations were made with [^{3}H]DHT in the presence of a 100-fold excess of unlabeled DHT to measure non-specific binding. After 72 h at 0–4°C protein bound [^{3}H]DHT was assayed by HAP adsorption technique. Only specific binding is shown.

exchange assays [5, 6, 8, 9, 32, 33] carried out at 0°C for as little as 20 h or as much as 96 h are incomplete.

(b) Acceleration of exchange by elevated temperature causes receptor inactivation (loss of binding capacity) [9, 11] even in the presence of molybdate [8], thus exchange assays [1–4, 7, 10] carried out at 15–30°C are non-quantitative.

Because of these limitations it has not been possible to demonstrate in the above studies that the concentration of AR measured by exchange assay in prostates of castrated rats injected with testosterone is equal to that measured by direct assay with [^{3}H]androgens in prostates of castrated rats injected with [^{3}H]T.

We have used NaSCN to accelerate exchange of unlabeled hormone from AR with [^{3}H]DHT. In the absence of protein stabilizing agents this salt inactivates AR (Fig. 1); however, our observation show that sucrose stabilizes AR against this inactivation (Fig. 2). NaSCN decreases the AR affinity for [^{3}H]DHT 7–8-fold, as determined by equilibrium binding, Scatchard analysis and dissociation kinetics (Fig. 3), thus allowing efficient ligand exchange.

To verify that under these conditions the androgen receptor maintained its steroid specificity, experiments presented in Fig. 5 were carried out. Only DHT and R1881 competed for [^{3}H]DHT binding; estradiol, progesterone and androstenedione did not do so significantly. These steroids are known to compete for DHT binding to the non-receptor steroid binding protein of rat prostate [34, 35]. Thus, the exchanged binding sites represent androgen receptor.

The decrease in AR affinity for DHT is, at least partially, caused by an increase in the rates of [^{3}H]DHT dissociation from its receptor (Fig. 3). The dissociative effects of NaSCN were observed both with androgen receptor complexes formed *in vitro* and *in vivo*. Furthermore, upon exchange in the presence of NaSCN, [^{3}H]DHT is specifically bound to a macromolecule which sediments at 4.6S on low salt sucrose density gradients (Fig. 6).

An important criterion to be met by any exchange assay is that the quantity of radiolabeled hormone receptor complexes generated through exchange must approach that measured in a parallel incubation in which the receptor had been labeled with radioactive ligand from the beginning. Failure to perform such control experiments prevents assessment of the quantitativeness of the procedure. The data in Fig. 7 demonstrate that with androgen receptor formed *in vitro* at least 80% of the available bound non-radioactive ligand is exchanged with [^{3}H]DHT within 72 h at 0°C (percentage calculations are corrected for stability). Analysis of the time course of the exchange reaction with androgen receptor complexes formed *in vivo* shows that (Fig. 4) exchange with [^{3}H]DHT is greatly accelerated by NaSCN and approaches completion within 72–96 h.

The quantitativeness of this exchange assay for androgen receptors occupied *in vivo* with unlabeled androgen was demonstrated by the experiments reported in Table 1. The AR values (pmol/mg DNA) measured with [^{3}H]DHT exchange in prostates of castrated rats injected with T were similar to AR values (pmol/mg DNA) measured by direct labeling of AR with [^{3}H] androgen in prostates of castrated rats injected with identical doses of [^{3}H]T.

The exchange assay described here overcomes difficulties of incomplete exchange at 0°C and receptor inactivation at elevated temperatures. Further-

more, it measures androgen receptor quantitatively regardless of whether the ligand was bound to the receptor *in vitro* or *in vivo*.

Note added in proof: Since submitting the manuscript a paper discussing the inadequacy of previous exchange assays has been published (Robel P., Eychenne B., Blondeau J.-P., Baulieue E.-E. and Hechter O.: Sex steroid receptors in normal and hyperplastic human prostate. *Prostate* **6** (1985) 255–269.

Acknowledgements—The skilful technical assistance of D. F. Williams is gratefully acknowledged.

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