Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 335–343. Pergamon Press. Printed in Great Britain

ANDROGEN BINDING IN RAT UTERUS CYTOSOL. STUDY OF THE SPECIFICITY

W. HEYNS, G. VERHOEVEN and P. DE MOOR

Laboratorium voor Experimentele Geneeskunde, Katholieke Universiteit Leuven, Rega Instituut, Minderbroedersstraat 10, B-3000 Leuven, Belgium

(*Received* 28 *August* 1975)

SUMMARY

During *in vivo* infusion of [^{3}H]-testosterone, the rat uterus accumulates the radioactivity in the form of testosterone (T) and the prostate in the form of 5α-dihydrotestosterone (DHT), as shown by a DHT/T ratio of 18.1 for the radioactivity extracted from the prostate and 0.014 for the uterus. *In vitro* the binding of T in uterus cytosol appears to be stronger than the binding of DHT, as opposite to the prostate. Competition studies, involving a large number of steroids do not reveal major differences in specificity of the T-binding component in the uterus and the T- or DHT-binding component in the prostate which have also a similar concentration (66.5 vs 43.3 fmol/mg protein) and affinity for T (K_D 1.1 vs 1.2 nM). Evidence is presented that the apparently weaker binding of DHT in uterus cytosol is not due to the androgen-binding component itself, but to indirect factors, such as non-specific binding and metabolic conversion of the steroid. Indeed, uterus cytosol contains a 3α-hydroxysteroid dehydrogenase with high activity even at low temperature. The high affinity androgen binding components of uterus and prostate cytosol have thus almost the same specificity with intrinsically preferential binding of DHT, and do not explain the difference in androgen retention observed *in vivo*.

INTRODUCTION

The specific high-affinity steroid-binding proteins or "receptors", found in the cytosol of hormone-responsive mammalian cells are considered to play an important role in the mechanism of action of steroid hormones [1–3]. In androgen-dependent tissues, such as the rat prostate, the androgen receptor is characterized by a particularly strong binding of 5α-dihydrotestosterone, and this is found as well in studies *in vivo* as *in vitro* [4–6]. In other organs, however, preferential uptake and binding of testosterone has been reported, for instance in the immature rat uterus [7–9], the mouse kidney [10, 11] and the musculus levator ani [12]. These organs are not androgen-dependent but are androgen-responsive with regard to some parameters. These observations led to the opinion that the first group of organs contain a 5α-dihydrotestosterone receptor, whereas the receptor of the second group is a testosterone-binding protein [3]. To check this hypothesis we performed a study of androgen accumulation and binding in the uterus of the adult rat and compared our results with similar data for the rat prostate. Our study suggests that the preferential retention and apparent preferential binding of testosterone or 5α-dihydrotestosterone is not due to a difference in the specificity of binding to androgen receptors in uterus or prostate, but to other factors, such as a difference in enzymatic conversion of the steroid.

MATERIALS AND METHODS

Animals. Unless stated otherwise, adult Wistar rats (weight 200–300 g) were used in this study. Male rats were orchidectomized under ether anesthesia about 17 h before death. Before removal of uterus or prostate the animals were anesthetized by ether and then bled to death by section of the carotid artery.

Chemicals. [1, 2, 6, 7-^{3}H]-Testosterone (87 Ci/mmol) and [1, 2-^{3}H]-5α-dihydrotestosterone (48 Ci/mmol) were obtained from New England Nuclear (Langen, Germany). Unlabelled steroids were supplied by Merck (Darmstadt, Germany) and by Ikapharm (Ramat–Gan, Israel). Cyproterone and cyproterone acetate were a gift from Schering A.G.; N-(3,5-dimethyl-4-isoxazolylmethyl)-phtalimide (DIMP) and 6α-bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one (BOMT) were provided by Hoffman–La Roche. Medroxyprogesterone acetate and chlormadinone acetate were obtained from Upjohn and Ayerst Laboratories respectively. T.l.c. plates were supplied by Merck (Darmstadt, Germany) as were most analytical grade reagents and solvents, Dimilume by Packard Instrument, Sephadex G50 and Dextran T70 by Pharmacia (Upssala, Sweden). Protamine sulfate (from salmon milt) was supplied by BDH (Poole, England) and Norit A by Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.). Unless stated otherwise, the buffer used in this study consisted of 50 mM Tris HCl buffer (pH 7.4 at 4°C).

Study of in vivo *retention during constant infusion of* [^{3}*H*]*-testosterone.* Unspayed adult female rats or adult male rats, 17 h after gonadectomy, were infused with [^{3}H]-testosterone by catheterization of the jugular vein performed under ether anesthesia. Fifteen μCi of [^{3}H]-testosterone were given as priming dose, followed by 6 h of infusion at a rate of 3 μCi (0.6 ml)/h.

The labelled steroid was dissolved in 5% ethanol in saline. At the end of the infusion the rats were anesthetised with ether and bled to death by section of the opposite carotid artery. The uterus or prostate was removed immediately, cleaned, weighed and homogenized in 2 ml of buffer using the Ultraturrax, as described for the preparation of cytosol. The homogenate was extracted twice with 4 ml of ethylacetate-cyclohexane, various unlabelled steroids were added and after evaporation of the solvent at 40°C under a nitrogen stream the extracts were spotted on silicagel t.l.c. plates. The plates were run twice in the system dichloromethane–ether (85:15, v/v) and the marker steroids were localized by U.V. absorption or by primulin spray [13] and fluorescence in U.V. light. Thereafter the appropriate zones were scraped off in counting vials. The radioactivity in these vials was measured by liquid scintillation counting after addition of 10 ml of Dimilume. A similar procedure was followed for rat plasma. Aliquots of the total homogenate, the total extract and the aqueous phase were counted as well.

Preparation of cytosol. Three uteri were homogenized in 6 ml of buffer by means of an Ultraturrax (model TP 18/2N; Ika Werk, Germany) using three times 15 seconds of homogenization at full speed with intermittent cooling in ice for 1 min. This homogenate was submitted to ultracentrifugation for 45 min at 45.000 rev./min (98.700*g*) using and M.S.E. Superspeed 65 centrifuge (England) equipped with an angle rotor (8 × 25 ml aluminium). The supernatant of this centrifugation is the cytosol. The whole procedure was performed at 0 to 4 C.

Separation of bound and unbound steroid by gel filtration. Columns of Sephadex G 50 were prepared in buffer. The internal diameter was 1.1 cm. and the bed height 31.5 cm., corresponding to a bed vol. of 30 ml. After application of the sample, consisting of 0.5 ml of cytosol incubated with labelled steroid, the column was eluted with the same buffer. Four fractions were collected; a first fraction of 10 ml. preceding the protein, a protein fraction of 4 ml, a second fraction of 4 ml and finally a fraction of 25 ml containing the unbound steroids. The radioactivity in these fractions was measured by liquid scintillation counting. The whole procedure was performed at 4 °C using jacketed columns and a thermostatic circulator.

Separation of bound and unbound steroid by competitive adsorption to Dextran-coated charcoal. To aliquots (0.5 ml) of the cytosol incubated with labelled steroid, 1 ml of a magnetically stirred Dextran-coated charcoal suspension was added. After 15 min of incubation at 0°C the tubes were centrifuged for 15 min at 8000 rev./min (7700*g*) in a Sorvall RC2B cooled centrifuge. Thereafter, the radioactivity in the supernatant (bound fraction) was estimated. The unbound fraction was obtained by subtracting the bound radioactivity from the total radioactivity in the cytosol. Unless stated otherwise, the composition of the Dextran-coated charcoal suspension was 1 g charcoal (Norit A) and 0.1 g Dextran T 70 for 100 ml of water.

Precipitation of bound radioactivity with protamine sulfate. To aliquots (0.5 ml) of cytosol incubated with labelled steroid 1 ml of a solution of protamine sulfate (0.2 mg/ml) in buffer was added. After 15 min of incubation at 0°C, the samples were centrifuged for 5 min at 8000 rev./min in the cooled centrifuge and the supernatant discarded. Thereafter, the pellets were rinsed with 5 ml of buffer at 0°C and centrifuged again under the same conditions. The radioactivity in the pellets was measured after incubation for 30 min at 50°C with 0.5 ml of Soluene and addition of 10 ml of Dimilume.

Other techniques. Radioactivity due to [^{3}H]-labelled steroids was measured by liquid scintillation counting in a Packard Liquid Scintillation Counter Model 2425 after addition of 10 ml of Dimilume to the samples. Quenching was corrected for by external standardisation, using a quench curve prepared in the same solvents. Protein was measured by the method of Hartree[14] using BSA as standard.

RESULTS

In vivo *accumulation of radioactivity during constant infusion of [^{3}H]-testosterone.* As shown in Table 1, the composition of radioactivity extracted from uterus or prostate after constant infusion of [^{3}H]-testosterone is different for both organs. Indeed, in the prostate this radioactivity consists for the largest part

Table 1. Composition of unconjugated radioactivity in rat prostate, uterus or plasma after 6 h i.v. infusion of [^{3}H]-testosterone

Origin	% of extracted radioactivity (mean ± S.D.) as polar metabolites	as testosterone	as 5α-dihydrotestosterone
prostate (n = 6)	3.3 ± 0.3	4.4 ± 1.4	79.8 ± 4.8
uterus (n = 6)	37.9 ± 7.0	48.2 ± 6.7	0.7 ± 0.2
plasma (male) (n = 4)	46.1 ± 6.5	28.5 ± 6.0	1.3 ± 0.7
plasma (female) (n = 5)	61.6 ± 3.2	23.8 ± 2.7	0.9 ± 0.1

of 5α-dihydrotestosterone and contains only a small fraction of testosterone. In the uterus, on the other hand, the major radioactive component is testosterone and almost no [^{3}H]-5α-dihydrotestosterone is detected. The unidentified polar metabolites are more important than in the prostate. The ratio of [^{3}H]-5α-dihydrotestosterone to [^{3}H]-testosterone is 18.1 for the prostate and 0.014 for the uterus. This shows clearly that during infusion of [^{3}H]-testosterone there is a preferential retention of radioactivity under the form of [^{3}H]-5α-dihydrotestosterone by the prostate and under the form of [^{3}H]-testosterone by the uterus. In addition, the accumulation of radioactivity is much lower for the uterus than for the prostate. Indeed, the tissue/plasma ratio of radioactivity present under the form of testosterone and 5α-dihydrotestosterone added together was 4.0 ± 1.8 (mean ± S.D.; $n = 5$) for the uterus and 25.9 ± 11.2 ($n = 4$) for the prostate.

In vitro *demonstration of high-affinity testosterone binding in the uterus of the adult rat.* The accumulation of testosterone by the uterus of the immature rat has been explained by the presence of a saturable testosterone-binding protein [7, 8]. Consequently, the finding of testosterone retention by the uterus of the adult rat suggested that a similar binding protein is present during adulthood. This was demonstrated by incubation of uterus cytosol at 0°C with a constant amount of [^{3}H]-testosterone and various amounts of unlabelled testosterone. Thereafter, the binding of testosterone was measured by various techniques, which allow high affinity (or slowly dissociating) binding to be distinguished from low affinity binding, such as gel filtration at 4°C, charcoal adsorption or selective precipitation with protamine sulfate (Fig. 1) and expressed according to Scatchard[15]. These experiments clearly show that cytosol from the uterus of adult rats contains a limited amount of a testosterone-binding component with a high affinity for this steroid.

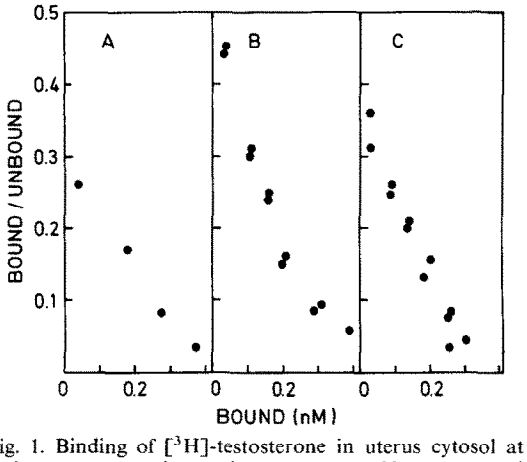

Fig. 1. Binding of [^{3}H]-testosterone in uterus cytosol at various concentrations of testosterone. Uterus cytosol (protein concentrations: 5 to 7.2 mg/ml) was incubated at 0°C with 0.2 nM of [^{3}H]-testosterone and various amounts of non-labelled testosterone. Thereafter, binding was measured (A) by gel filtration, (B) by precipitation with protamine sulfate or (C) by competitive adsorption to Dextran-coated charcoal (1 vol; 4 g charcoal and 0.2 g Dextran per 100 ml). The results are expressed according to Scatchard[16] without correction for non-specific binding.

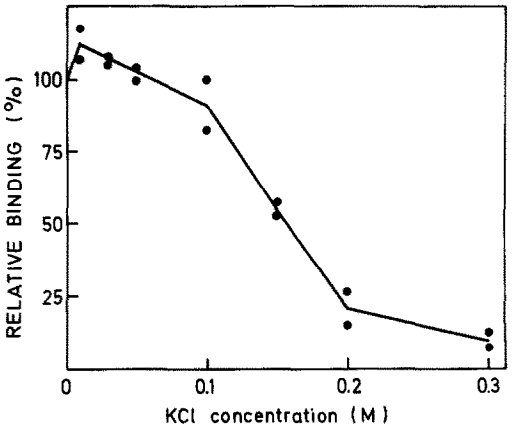

Fig. 2. Effect of KCl on testosterone binding. After homogenization of 4 uteri in 6 ml of Tris-buffer, aliquots of the homogenate were diluted with the same buffer, containing various amounts of KCl and cytosol was prepared as usual. The cytosol preparation were incubated for 3 h with 0.2 nM [^{3}H]-testosterone and the binding of testosterone was measured by gel filtration. The whole procedure was performed at 0 to 4°C. The results are expressed as percentage of the binding observed without addition of KCl and plotted as a function of the concentration of KCl.

Quantitative evaluation of testosterone binding in uterus cytosol. In order to obtain an idea of the concentration of the testosterone binding component in uterus cytosol, the experiment shown in Fig. 1c was repeated several times on different preparations of cytosol. The results of these experiments were analysed by means of Scatchard-plots [15] and corrected for non-specific binding [16]. This yielded an estimate of the concentration of the binding site, which was expressed as a function of the protein content of the sample and gave an estimate of the apparent equilibrium constant of dissociation. As shown in Table 2, the concentration of the binding site for testosterone is about 66 fmol per mg protein, and the apparent dissociation constant about 1.1 nM. These values are

Table 2. Concentration and apparent equilibrium constant of dissociation (K_d) for testosterone of the androgen binding component of uterus and prostate cytosol (mean ± S.D.)

Tissue	Concentration (fmol/mg protein)	K_d (nM)
uterus (n = 7)	66.5 ± 19.4	1.10 ± 0.31
prostate (n = 3)	43.3 ± 5.1	1.22 ± 0.83

Cytosol was prepared from 3 uteri or from 2 prostates in 7.5 ml of buffer and incubated for 3 h at 0°C with various amounts of testosterone. Thereafter, the bound fraction was estimated by means of the Dextran-coated charcoal technique.

Table 3. Effect of various components on the binding of [^{3}H]-testosterone in rat uterus cytosol

Added component	Concentration (mM) 0.5	1.0	1.5	3.0	5.0	10.0
$MgCl_2$		100.9		85.3		75.0
$CaCl_2$	100.4		77.5		64.8	
EDTA	116.3		122.6		115.0	
thioglycerol	99.4		98.7		104.3	
dithiothreitol	101.3		94.3		85.7	

For each experiment 3 uteri were homogenized in 4.5 ml of buffer and aliquots of the homogenate were diluted with an equal volume of buffer containing the added component at twice the indicated concentration. After ultracentrifugation the samples were incubated for 3 h at 0°C with [^{3}H]-testosterone (0.2 nM) and binding was measured by gel filtration. The results are expressed as a percentage of the binding measured without addition.

close to the corresponding values, obtained by this technique in the prostate of the adult rat, 17 h after castration.

Factors affecting the binding of [^{3}H]-testosterone in rat uterus cytosol. The addition of various ions to cytosol had a variable effect on the degree of binding of testosterone in rat uterus cytosol (Table 3). The most marked effect was observed with KCl which very strongly diminished binding when added at a concentration of 0.15 M before incubation of the cytosol with [^{3}H]-testosterone (Fig. 3). This effect of KCl was non-reversible and proportional to the duration of preincubation with KCl: it is thus probably due to a loss of binding ability of the binding protein. A similar effect was observed with NaCl. Low concentration (10–30 mM of KCl), on the other hand, enhanced the binding slightly, as compared to Tris–HCl buffer. Addition of KCl (0.25 M) after incubation of cytosol with [^{3}H]-testosterone produced a more gradual decrease in binding, showing a protective effect by the bound steroid. Addition of $CaCl_2$ or $MgCl_2$, even at relatively low concentrations, produced some decrease in binding, whereas EDTA had a protective effect. Finally, almost no effect was seen with thioglycerol, dithiothreitol and sodium azide (0.1 mg/ml).

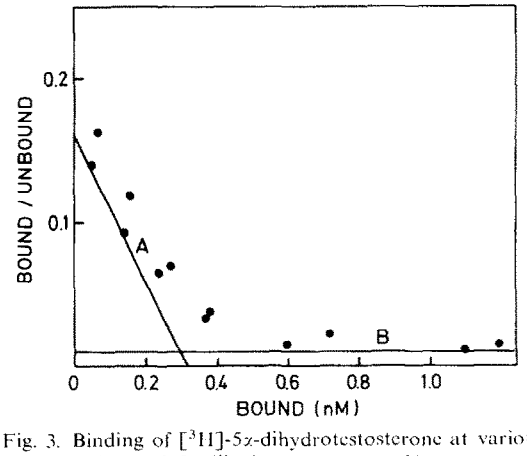

Fig. 3. Binding of [^{3}H]-5α-dihydrotestosterone at various concentrations of 5α-dihydrotestosterone. Uterus cytosol (protein concentration 6.3 mg/ml) was incubated for 3 h at 0°C with 0.4 nM of [^{3}H]-5α-dihydrotestosterone and various amounts of unlabelled 5α-dihydrotestosterone. Thereafter, binding was measured by means of gel filtration. The results are plotted according to Scatchard[15]. The continuous lines represent high affinity binding (A: K_d: 2 nM) and non-specific binding (B), derived by the method of Rosenthal[16]

Table 4. Competition of various compounds with the binding of [^{3}H]-testosterone (T) or [^{3}H]-5α-dihydrotestosterone (DHT) in rat uterus and prostate cytosol

cytosol	Uterus		Prostate	
labelled ligand	T	T	T	DHT
method	A	B	B	C
Competitor	*Apparent K_i* (nM)			
testosterone	1.1		1.2	3.3
19-nortestosterone	1.1			1.0
17β-hydroxy-17α-methyl-1,4-androsten-3-one	3.3	5.4		2.0
epitestosterone	33			33
5α-dihydrotestosterone	4.3			2.0
5α-androstane, 3α, 17β-diol	39	25		35
5α-androstane, 3β, 17β-diol	7.2			9.9
5β-dihydrotestosterone	34			
4-androstene-3,20-dione	> 50			62
3β-hydroxy-5-androsten-3-one	> 50			
5-androstene-3β, 17β-diol	42			
estradiol	6.1	6.8	10.1	12
corticosterone		> 100	409	
progesterone	27	31	30	11
20β-hydroxy-4-pregnen-3-one	30			
17α-hydroxyprogesterone		140	162	250
17α-acetoxyprogesterone		5.4	11.0	7.2
cyproterone		22	65	31
cyproterone acetate	2.6	2.9	5.2	4.1
chlormadinone acetate	3.5	1.6	5.6	4.5
medroxyprogesterone acetate		2.2	5.3	1.4
B.O.M.T.	> 50	52	116	31
D.I.M.P.	21	26	61	44

Uterus or prostate cytosol was incubated for 3 h at 0°C with 0.2 nM of labelled steroid and 6 different concentrations of competitor. Thereafter, the bound steroid was measured by gel filtration (A), competitive adsorption (B), or ammonium sulfate precipitation (C, Ref. 11). The apparent K_i corresponds to the concentration which gives 50% inhibition of the binding in the absence of competitor, and is estimated on a logit/log plot.

Competition of various steroids with the binding of testosterone in uterus cytosol. The specificity of the testosterone-binding component in the rat uterus cytosol was evaluated by means of competition experiments as described in the legend of Table 4. The results of this study, which includes a large group of steroids and the non-steroidal antiandrogen DIMP are given in Table 4. As expected testosterone is one of the most tightly bound components, but some steroids produce a similar degree of competition. The most competitive steroids are found in the group of components which have a structural relationship to testosterone or to 5α-dihydrotestosterone and contain

a 17β-hydroxy and 3-oxo group. Usually, these steroids have androgenic or anabolic activity. When the 3-oxo group is reduced, as in 5α-androstane-3α, 17β-diol and 5α-androstane-3β, 17β-diol the degree of competition diminishes markedly. In contrast to 5α-dihydrotestosterone, 5β-dihydrotestosterone is a poor competitor. This is not unexpected in view of its different steric configuration. Estradiol, on the other hand, is surprisingly competitive. Most C^{21}-steroids have little or no competitive effect. An exception is formed by some derivatives of 17α-hydroxyprogesterone. This steroid itself is not very competitive, but acetylation of the 17α-hydroxy group markedly enhances competition. The same effect is seen when comparing cyproterone and cyproterone acetate and probably explains the marked competitive effect of chlormadinone acetate and medroxyprogesterone acetate. Finally, the steroidal antiandrogen BOMT and the non-steroidal antiandrogen DIMP are only relatively weak competitors.

In order to compare the specificity of androgen binding in uterus and prostate, some data are included in Table 4 on the competition of various compounds with the binding of [^{3}H]-testosterone and [^{3}H]-5α-dihydrotestosterone in prostate cytosol. The results on competition with [^{3}H]-testosterone were obtained in this study by the charcoal technique: the data on competition with [^{3}H]-5α-dihydrotestosterone come from a previous study [11], wherein ammonium sulphate was used for the precipitation of the specifically bound steroid. In spite of numerous factors, which may affect the results, there is a marked similarity in the competition pattern for androgen binding in uterus and prostate cytosol.

5α-dihydrotestosterone binding in uterus cytosol. In contrast to androgen-binding proteins found in some other organs (e.g. the prostate) which preferentially bind 5α-dihydrotestosterone, the androgen-binding protein described in the uterus of the immature rat is believed to be specific for testosterone [7–9]. In view of the selective uptake of testosterone, observed *in vivo* this was expected to be also the case for the uterus of the adult rat. When, however, the experiment of Fig. 1 was repeated with [^{3}H]-5α-dihydrotestosterone and unlabelled 5α-dihydrotestosterone, high affinity 5α-dihydrotestosterone binding was detected in uterus cytosol of adult rats (Fig. 3). This binding of 5α-dihydrotestosterone was weaker (apparent K_D for 5α-dihydrotestosterone 2 nM) than the binding of testosterone and variable from experiment to experiment. Whereas 5α-dihydrotestosterone showed marked competition with the binding of [^{3}H]-testosterone (see Table 3), testosterone was a strong competitor with the binding of [^{3}H]-5α-dihydrotestosterone, the K_i being appr. 2.1 nM. These data are in agreement with the hypothesis that the "high affinity" androgen-binding protein(s) of uterus cytosol bind both testosterone and 5α-dihydrotestosterone, but have a higher apparent affinity for testosterone. In the next section we will make a more detailed comparison of the binding of these two androgens in rat uterus cytosol.

Comparison of 5α-dihydrotestosterone and testosterone binding. Some indirect arguments favour the hypothesis that the binding of 5α-dihydrotestosterone in uterus cytosol is only apparently weaker than the binding of testosterone. A first argument is the observation that 5α-dihydrotestosterone binding prevails during experiments, such as ultracentrifugation, wherein the steroid–protein complex is subjected for a prolonged time to conditions favouring dissociation. This is confirmed by measurements of the *dissociation rate* under chase conditions (Fig. 4). Indeed, at 0°C very little dissociation of bound [^{3}H]-5α-dihydrotestosterone was observed even after 120 min of incubation, whereas 80% of the bound [^{3}H]-testosterone remained in the bound fraction at that time. At 25°C the dissociation rate constant was 0.0288 min^{-1} for [^{3}H]-testosterone and only 0.0024 min^{-1} for [^{3}H]-5α-dihydrotesterone. Incidentally, the dissociation rate constant for [^{3}H]-testosterone bound in prostate cytosol was 0.023 min^{-1} at 25°C, a value close to the one found for testosterone binding in uterus cytosol.

A more direct proof that the binding of 5α-dihydrotestosterone in uterus cytosol is intrinsically stronger than the binding of testosterone is found in the effect of charcoal pretreatment of the cytosol. Indeed, this procedure results in a moderate rise of the binding of testosterone, and in a marked increase of the binding of 5α-dihydrotestosterone (Fig. 5). Consequently, the binding of 5α-dihydrotestosterone becomes stronger than the binding of testosterone, at about the same number of binding sites. In fact, the flattened

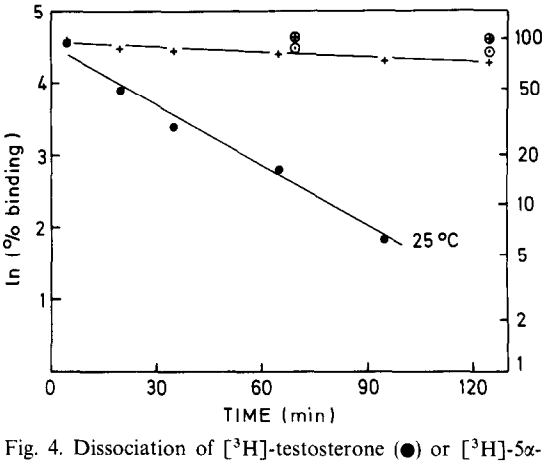

Fig. 4. Dissociation of [^{3}H]-testosterone (●) or [^{3}H]-5α-dihydrotestosterone (+) bound in uterus cytosol at 25°C or at 0°C (circled symbols). For each curve uterus cytosol (protein concentration approx. 5 mg/ml) was prepared from 2 uteri in 4 ml of buffer and incubated for 3 h at 0°C with 0.2 nM of [^{3}H]-testosterone or of [^{3}H]-5α-dihydrotestosterone. Thereafter, unlabelled steroid was added in 0.3 vol. of buffer, resulting in a steroid concentration of 3.5 μM, the tubes were incubated for various amounts of time at 25°C (or at 0°C) and the bound radioactivity measured by gel filtration at 4°C. The results are expressed as percentage binding with respect to time 0 on a ln scale.

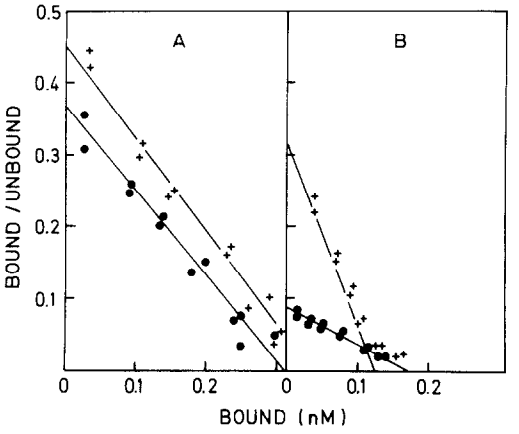

Fig. 5. Effect of charcoal pretreatment on the binding of [^{3}H]-testosterone (A) and of [^{3}H]-5α-dihydrotestosterone (B). Cytosol (protein concentration 8.1 mg/ml for A and 6.6 mg/ml for B) was prepared from 6 uteri in 10 ml of buffer. To one half of the cytosol 0.4 vol. of water (o) or 0.4 vol. of Dextran-coated charcoal (+); (4 g charcoal and 0.2 g Dextran per 100 ml) were added, the samples were incubated for 30 min at 0°C and centrifuged for 10 min at 8000 c.p.m. Thereafter, the binding of either steroid in the supernatant was measured and expressed as in Fig. 1C. For testosterone the apparent K_d was 0.9 nM for the diluted (●) and 0.8 nM for the charcoal-treated (+) cytosol. For 5α-dihydrotestosterone these values were respectively 2.1 nM and 0.38 nM. The experiments of Fig. 5a and Fig. 5b were performed on two different days.

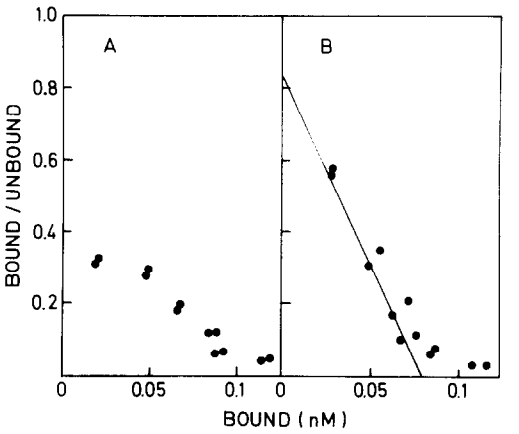

Fig. 6. Effect of prolonged incubation with [^{3}H]-5α-dihydrotestosterone on its binding. Uterus cytosol was prepared and charcoal-treated as in Fig. 5. The binding of [^{3}H]-5α-dihydrotestosterone was measured by means of charcoal adsorption after 3 h (A) or 5 hrs (B) of incubation at 0°C with the labelled and unlabelled steroid. The K_D for 5α-dihydrotestosterone in Fig. 6b was 0.096 nM.

shape of the Scatchard plot obtained for 5α-dihydrotestosterone after charcoal treatment suggest that the binding of this steroid was still underestimated. That this is the case, is shown by the effect of prolongation of the incubation time (Fig. 6), which results in a further increase of 5α-dihydrotestosterone binding.

Two factors may explain, at least in part, why the binding of 5α-dihydrotestosterone is underestimated in uterus cytosol. A first factor is the higher non-specific binding observed with [^{3}H]-5α-dihydrotestosterone. Indeed, where equilibrium dialysis was performed at 0°C on cytosol (protein concentration 7.3 mg/ml) with [^{3}H]-5α-dihydrotestosterone or [^{3}H]-testosterone and unlabelled steroid at a total concentration of 3.5×10^{-9}M the ratio of bound to unbound radioactivity was 5.7 for 5α-dihydrotestosterone and 1.2 for testosterone. The extensive metabolism of 5α-dihydrotestosterone in uterus cytosol during incubation at 0°C forms the second factor. After 3 h of incubation of [^{3}H]-5α-dihydrotestosterone in uterus cytosol at 0°C the radioactivity in the total cytosol and in the unbound fraction consists for the largest part of 5α-androstane-3α, 17β-diol, a component which does not bind strongly to the cytosolic binding protein, whereas the specifically bound fraction contains mainly untransformed 5α-dihydrotestosterone (Table 5). The non-specifically bound fraction also consists mainly of 5α-androstane-3α, 17β-diol (Table 6). Under the same conditions testosterone is metabolized only to a limited extent. Because of this conversion of 5α-dihydrotestosterone during the incubation of cytosol our calculations, which involve the use of total radioactivity, overestimate the unbound concentration of this steroid and hence underestimate the association constant. The increase of binding observed after charcoal pretreatment may be due to a similar mechanism. Indeed, this procedure results in a marked reduction of this metabolism, since 66.8% of the [^{3}H]-5α-dihydrotestosterone remained unchanged after 3 h of incubation in charcoal-pretreated cytosol at 0°C.

3α-hydroxysteroid dehydrogenase activity in uterus cytosol. As already shown in Table 5 [^{3}H]-5α-dihydrotestosterone is metabolized extensively in rat uterus cytosol. Under these conditions 5α-androstane-3α-

Table 5. Metabolism and binding of [^{3}H]-testosterone and [^{3}H]-5α-dihydrotestosterone after 3 h of incubation in uterus cytosol at 0°C

Incubated Steroid	% Unchanged steroid in total cytosol	% Unchanged steroid in bound fraction	% Binding in terms of total radioactivity	% Binding in terms of unchanged compound
[^{3}H]-testosterone	85.7	92.2	15.9	16.9
[^{3}H]-5α-dihydrotestosterone	10.0	89.0	5.8	51.3

For each steroid cytosol was prepared from 2 uteri in 4 ml buffer and incubated for 3 h with 1.15 nM of [^{3}H]-testosterone or 1.92 nM of [^{3}H]-5α-dihydrotestosterone. The binding was measured by means of gel filtration and the composition of the radioactivity by use of extraction, t.l.c. and liquid scintillation counting.

Table 6. Non-specific binding and metabolism of 5α-dihydrotestosterone in uterus cytosol as measured by equilibrium dialysis (mean ± S.D.)

bound/unbound	total radioactivity	:	6.4 ± 0.8
	as 5α-dihydrotestosterone	:	4.5 ± 0.7
	as 5α-androstane-3α, 17β-diol	:	7.5 ± 0.8
5α-androstane-3α, 17β-diol	inside	:	22.4 ± 1.6
5α-dihydrotestosterone	outside	:	14.5 ± 0.7

Uterus cytosol (5.7 mg protein per ml) was incubated for 3 h at 0°C with 3μM of unlabelled 5α-dihydrotestosterone and 2 nM of [^{3}H]-5α-dihydrotestosterone. Thereafter, 0.5 ml aliquots were placed into dialysis bags and after 48 h of dialysis at 4°C against 2 ml of buffer the total radioactivity inside and outside and its composition was measured.

17β-diol is the only quantitatively important metabolite, as demonstrated by thin-layer chromatography and by paper chromatography in the Bush B_3 system [17]. In Fig. 7 the reaction is followed as a function of time: the conversion of 5α-dihydrotestosterone proceeds gradually and after 3 h the largest part of this steroid is converted into 5α-androstane-3α, 17β-diol. The speed of this reaction is increased markedly by the addition of the coenzyme NADPH. Indeed, in the presence of NADPH a similar degree of conversion is obtained after 15 min of incubation at 0°C and this in 10 times more diluted cytosol (Fig. 8). Incidentally, the addition of $NADP^+$, which is not expected to promote the conversion in this direction resulted also in a marked increase in the formation of 5α-androstane-3α, 17β-diol, probably because the coenzyme is rapidly reduced by other reactions which occur in the cytosol. The effect of substrate concentration on the speed of the reaction is shown in Fig. 9, which allows to estimate the K_m and V_{max}. At 0°C the K_m was 6.5×10^{-8}M and the V_{max} 10.9 nmol/g protein/min. The corresponding values at 37°C were 1.9×10^{-7}M and 222 nmol/g protein/min. The substrate specificity of the enzyme was not studied but marked competition was observed with 5α-pregnane-3, 20-dione: in the presence of 6×10^{-8}M of this steroid the conversion of 5α-dihydrotestosterone, measured at 0°C in diluted cytosol (protein concentration: 0.026 mg/ml) with added NADPH was 22.1% of the conversion observed without 5α-pregane-3, 20-dione. This observation suggests that this enzyme may play a role in the conversion of progesterone metabolites.

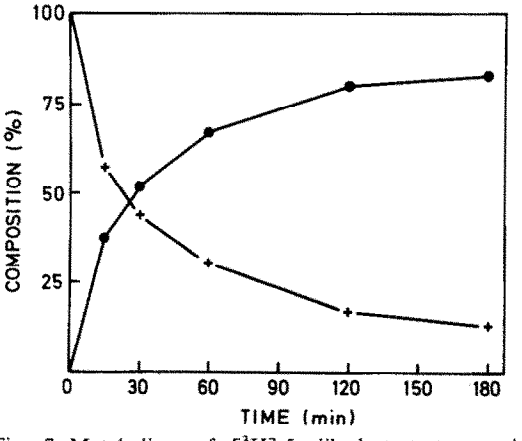

Fig. 7. Metabolism of [^{3}H]-5α-dihydrotestosterone in uterus cytosol at 0°C as a function of time. Cytosol from 3 uteri in 6 ml of buffer was incubated at 0°C with 1.9 nM of [^{3}H]-5α-dihydrotestosterone. At the indicated times 1 ml of cytosol was extracted with 5 ml cyclohexane–ethylacetate (1.1 v/v) and the composition of the radioactivity in the extracts was measured by t.l.c. and liquid scintillation counting. 5α-dihydrotestosterone: +; 5α-androstane-3α-17β-diol: ●.

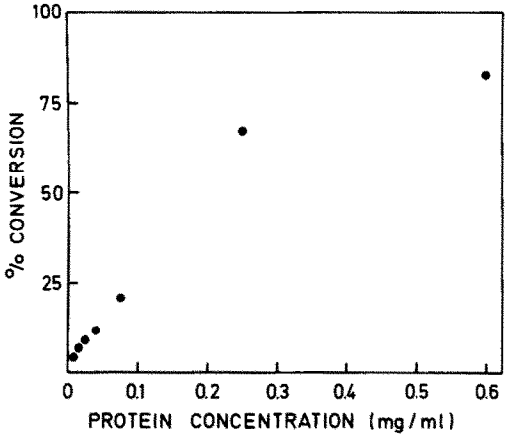

Fig. 8. Influence of the dilution of cytosol on the conversion of 5α-dihydrotestosterone into 5α-androstane-3α-17β-diol at 0°C in the presence of NADPH. Cytosol (protein concentration 6.0 mg/ml) was diluted 10 to 400-fold with buffer and incubated for 15 min at 0°C with 0.1 μC/ml of [^{3}H]-5α-dihydrotestosterone and 1 mg/ml of NADPH. Thereafter the conversion into 5α-androstane-3α, 17β-diol was measured as in Fig. 7.

DISCUSSION

High affinity androgen binding has been described in uterus cytosol of immature rats by Giannopoulos[7, 8]. This finding was confirmed by Rochefort and Lignon[9], who demonstrated that this androgen "receptor" was different from the estradiol receptor of uterus cytosol, although high concentrations of androgens could transfer the latter to the nucleus. In calf uterus also, the simultaneous presence of androgen and estradiol receptors has been shown [18].

In the present study we report on high affinity androgen binding in uterus cytosol of adult rats. Since such activity could not be detected in rat blood plasma, it is improbable that this binding is due to plasma contamination. As in immature rats [7–9], the

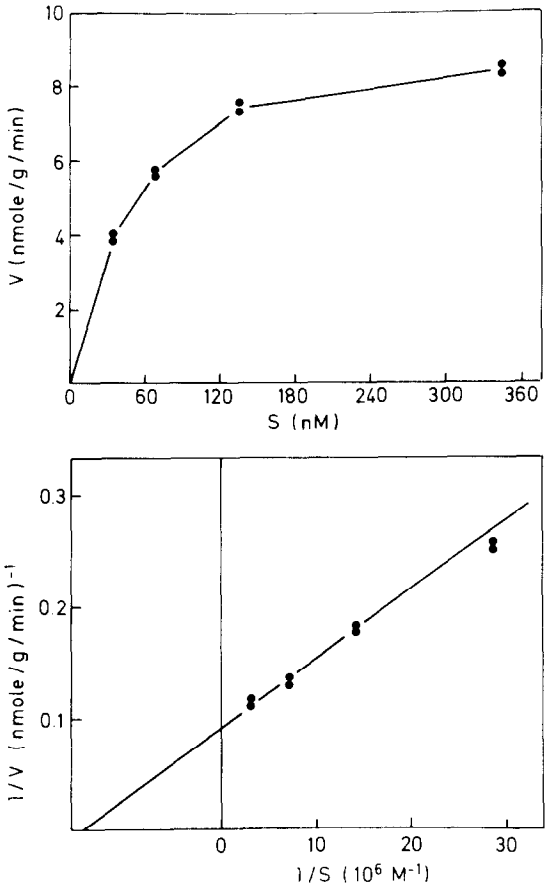

Fig. 9. Influence of substrate concentration on the conversion of 5α-dihydrotestosterone by uterus cytosol. Diluted cytosol (protein concentration 0.035 mg/ml) was incubated for 15 min at 0°C with 2 nM of [^{3}H]-5α-dihydrotestosterone and various concentrations of unlabelled steroid. Thereafter, the formation of 5α-androstane-3α, 17β-diol was measured by extraction, t.l.c. and liquid scintillation counting and expressed per g protein and per min (A). B shows a Michaelis-Menten plot of the same data.

binding of testosterone in uterus cytosol appeared to be stronger than the binding of 5α-dihydrotestosterone. This finding, which concurs well with the preferential accumulation of radioactivity in the form of testosterone during *in vivo* infusion of this [^{3}H]-steroid, seems to distinguish the androgen receptor in uterus cytosol from the one described in the prostate. Indeed, in the prostate, which accumulates [^{3}H]-5α-dihydrotestosterone during infusion of [^{3}H]-testosterone (Table 1), there is preferential binding of 5α-dihydrotestosterone [4–6]. On the other hand, the concentration of the high affinity binding site in both organs was very similar, a remarkable finding in view of the marked difference in androgen responsiveness. It was reasonable to expect that this difference in specificity of the androgen binding in uterus and prostate should be reflected also in competition studies. These experiments (Table 4), however, revealed a high degree of similarity, rather than striking differences, an observation, which could be extended to androgen binding in other organs [19]. In general, the competition pattern corresponds well with the biological profile of most steroids. In this respect the remarkable binding of 17α-acetoxyprogesterone and various derivatives, such as cyproterone acetate and some synthetic progestational steroids should be mentioned. Indeed, this finding agrees well with the observation of androgenic, synandrogenic and antiandrogenic properties of these components on mouse kidney and submaxillary gland [20, 21].

The remarkable similarity of the competition data led us to the hypothesis that the specificity of the androgen binding in uterus and prostate was essentially identical and that the preferential binding of testosterone by uterus cytosol was only apparent. Some other observations also point in this direction. Indeed, 5α-dihydrotestosterone, once bound to the androgen receptor seemed to be bound more tightly than testosterone, since 5α-dihydrotestosterone binding prevailed in experiments of long duration and since the dissociation rate constant for this steroid, bound to the androgen receptor was about ten times smaller than for testosterone. This seemed to indicate that the binding of 5α-dihydrotestosterone was intrinsically stronger than the binding of testosterone but that the association of 5α-dihydrotestosterone was impeded in uterus cytosol. In fact, as in mouse kidney [19] we found two factors which might produce this apparent decrease of 5α-dihydrotestosterone binding. Firstly, the presence of non-specific binding, which results in an overestimation of the unbound steroid, since a large part of the non-specifically bound steroid is included in the "unbound" fraction and, secondly the particularly strong metabolic conversion of 5α-dihydrotestosterone into 5α-androstane-3α, 17β-diol observed in uterus cytosol even during incubation at low temperature. The effect of charcoal pretreatment provides a more direct proof of stronger 5α-dihydrotestosterone binding in uterus cytosol. Indeed, after this procedure, which diminishes the conversion of 5α-dihydrotestosterone, probably by absorption of coenzyme and of related reducing substances, the binding of 5α-dihydrotestosterone clearly exceeds the binding of testosterone. It is possible that the conversion of 5α-dihydrotestosterone into 5α-androstane-3α, 17β-diol also leads to an underestimation of 5α-dihydrotestosterone binding in uterus cytosol of immature rats, since we found about the same 3α-hydroxysteroid dehydrogenase activity in adult and immature animals (results not shown).

During infusion of [^{3}H]-testosterone the prostate accumulates [^{3}H]-5α-dihydrotestosterone and the uterus [^{3}H]-testosterone (Table 1), and this at a markedly lower level than the prostate. Since the concentration (Table 2) and specificity of the androgen "receptors" (Table 4) were found to be similar in both organs, it is improbable that this difference in retention is due to these proteins. Furthermore, the composition of the circulating radioactivity (Table 1) does not provide an explanation for this difference. For this reason intracellular conversions of the steroid in

uterus and prostate probably play a major role in the specificity of androgen retention. The high 5α-reductase activity in the prostate [22] will then favour the accumulation in the form of 5α-dihydrotestosterone, with respect to other organs [23], whereas the high 3α-hydroxysteroid dehydrogenase activity in the uterus will preclude any 5α-dihydrotestosterone entering the cell or formed intracellularly from binding to the receptor. The intrinsically stronger binding of 5α-dihydrotestosterone as compared to testosterone may then lead to a higher level of androgen retention in the prostate.

The significance of androgen binding in the uterus is difficult to assess. Indeed, this organ is no typical target organ for androgens, although it is influenced by these hormones. Furthermore, testosterone and 5α-dihydrotestosterone seem to have different effects on the uterus [24]. This leads to the question whether the testosterone receptor complex has the same significance as the 5α-dihydrotestosterone receptor complex. In view of the lower stability of the testosterone–receptor complex it is conceivable that this complex will have a quantitatively weaker effect; it is possible also that the nature of the bound steroid affects the following steps of the mechanism of action of the hormone such as the activation of the hormone–receptor complex, the transfer to the nucleus and the interaction with the nuclear acceptor [25]. In addition, the heterogeneous composition of the uterus may reflect itself in the distribution of the androgen receptor and of the 3α-hydroxysteroid dehydrogenase and lead to a preferential uptake of testosterone in some cells and of 5α-dihydrotestosterone in other cells explaining the difference in response observed with both steroids. Finally, other metabolites (e.g. 5α-androstane-3α, 17β-diol) formed in the uterus or elsewhere may also be of some importance [26, 27].

REFERENCES

1. Gorski J., Toft D., Shyamala G., Smith D. and Notides A.: *Rec. Prog. Horm. Res.* **24** (1968) 45–80.
2. Baulieu E. E., Alberga A., Jung J., Lebeau M. C., Mercier-Bodard C., Milgrom E., Raynaud J. P., Raynaud-Jamet C., Rochefort H., Fruong H. and Robel P.: *Rec. Prog. Horm. Res.* **27** (1971) 351–412.
3. King R. J. B. and Mainwaring W. I. P.: *Steroid–Cell Interactions.* Butterworths, London (1974).
4. Fang S., Anderson K. M. and Liao S.: *J. biol. Chem.* **244** (1969) 6584–6595.
5. Mainwaring W. I. P.: *J. Endocr.* **45** (1969) 531–541.
6. Unhjem O., Tveter K. J. and Aakvaag A.: *Acta endocr., Copenh.* **62** (1969) 153–164.
7. Giannopoulos G.: *Biochem. biophys. Res. Commun.* **44** (1971) 943–951.
8. Giannopoulos G.: *J. biol. Chem.* **248** (1973) 1004–1010.
9. Rochefort H. and Lignon F.: *Eur. J. Biochem.* **48** (1974) 503–512.
10. Bullock L. P. and Bardin C. W.: *Endocrinology* **94** (1974) 746–756.
11. Verhoeven G., Heyns W. and De Moor P.: *Steroids* **26** (1975) 149–167.
12. Jung J. and Baulieu E. E.: *Nature New Biol.* **237** (1972) 24–26.
13. Wright R. S.: *J. Chromatog.* **59** (1971) 220–221.
14. Hartree E. F.: *Analyt. Biochem.* **48** (1972) 422–427.
15. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
16. Rosenthal H. E.: *Analyt. Biochem.* **20** (1967) 525–532.
17. Neher R.: *Steroid Chromatography.* Elsevier Publishing Company Amsterdam, London, New York (1964) p. 100.
18. Wagner R. K., Görlich L. and Jungblut P. W.: *Hoppe-Seyler's Z. physiol. Chem.* **353** (1972) 1654–1656.
19. De Moor P., Verhoeven G. and Heyns W.: *J. steroid Biochem.* **6** (1975) 437–442.
20. Mowszowicz J., Bieber J., Chung K. W., Bullock L. P. and Bardin C. W.: *Endocrinology* **95** (1974) 1589–1599.
21. Bullock L. P., Barthe P. L., Mowzowicz J., Orth D. N. and Bardin C. W.: *Endocrinology* **97** (1975) 189–195.
22. Wilson J. D. and Gloyna R. E.: *Rec. Prog. Horm. Res.* **26** (1970) 309–336.
23. Krieg M., Szalay R. and Voigt K. D.: *J. steroid Biochem.* **5** (1974) 453–459.
24. Gonzales-Diddi M., Komisaruk B. and Beyer C.: *Endocrinology* **91** (1972) 1129–1132.
25. Rousseau G. G.: *J. steroid Biochem.* **6** (1975) 75–89.
26. Huggins C., Jensen E. V. and Cleveland A. S.: *J. exp. Med.* **100** (1954) 225–240.
27. Robel P., Lasnitzki I. and Baulieu E. E.: *Biochimie* **53** (1971) 81–97.