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# CHARACTERIZATION OF A [$^3$H]METHYLTRIENOLONE (R1881) BINDING PROTEIN IN RAT LIVER CYTOSOL

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**Summary**—The binding of radiolabelled methyltrienolone 17$\beta$-hydroxy-17$\alpha$-methyl-estra-4,9,11-trien-3-one (R1881) to adult male rat liver cytosol has been characterized in the presence of Na-molybdate to stabilize steroid-hormone receptors, and triamcinolone acetonide to block progestin receptors. Using sucrose density gradient analysis, male liver cytosol contains a [$^3$H] R1881 macromolecular complex which sediments in the 8–9S region. 8S binding of R1881 to male rat serum, female liver cytosol or cytosol from a tfm rat cannot be demonstrated. Further metabolism of [$^3$H] R1881 following 20h incubation with male rat liver cytosol was excluded: In the 8S region 97% of [$^3$H] R1881 was recovered by thin layer chromatography. Characteristics of this [$^3$H] R1881-8S binding protein include high affinity ($K_d = 2.3 \pm 41$ nM) and low binding capacity ($18.8 \pm 3.3$ fmol/mg cytosol protein), precipitability in 0–33% ammonium sulfate, and translocation to isolated nuclei following *in vivo* R1881 treatment. Whereas, the cytosol R1881-receptor is competed for by dihydrotestosterone, testosterone, and estradiol, [$^3$H] estradiol binding in the 8S region is not competitive with androgens but does compete with diethylstilbestrol. The nuclear androgen binding site has a $K_d = 2.8$ nM for [$^3$H] R1881, and is androgen specific (testosterone > 5$\alpha$-dihydrotestosterone > estradiol > progesterone > cyproterone acetate > diethylstilbestrol > dexamethasone > triamcinolone). Since a number of liver proteins including the drug and steroid metabolizing enzymes are, in part, influenced by the sex-hormone milieu, the presence of a specific androgen receptor in male rat liver may provide valuable insight into the regulation of these proteins.

## INTRODUCTION

Sexual dimorphism is expressed by a number of enzyme pathways in rat liver. Notable examples include the drug and steroid metabolizing enzymes and xanthine oxidase [1–3]. The hepatic synthesis of additional specific proteins such as serum testosterone–estrogen binding globulin, thyroxine binding globulin, and a recently described 4S-cytosolic estrogen–androgen binder is also influenced by the sex-hormone milieu of the animal [4–6]. While the precise mechanism of steroid-hormone action is not fully understood, the concept that these agents mediate their effect through binding to intracellular protein receptors is generally accepted [7]. If receptor proteins are required for hormone action, then the sensitivity of a target tissue to endocrine influence should be related to the presence of specific cytoplasmic receptor proteins.

A number of investigators have documented the presence of at least two species of estrogen receptors in rodent liver. The first type exhibits specific estrogen receptor properties including high affinity binding ($K_d = 10^{-10}$ M), low binding capacity (12 fmol/mg cytosol protein), and saturability and specificity for both steroidal and nonsteroidal estrogens [8]. This estrogen receptor sediments in sucrose gradients at 8–9S and is a minor species in male liver. A second species of estrogen binding protein which sediments in the 3–4S region on sucrose gradients appears to be unique to male liver although it can be induced in ovariectomized females treated with dihydrotestosterone [8–10]. The properties of this 3–4S estrogen binder which distinguish it from the 8–9S receptor include moderate binding affinity ($K_d = 10^{-7}$ M), high binding capacity (196 fmol/mg cytosol protein), and specificity for both dihydrotestosterone and steroidal estrogens. The function of this 3–4S male estrogen–androgen binding protein is unknown.

In contrast to the presence of estrogen receptors in rodent liver, specific, high affinity binding and low capacity androgen receptor proteins, and retention of androgen in liver cell nuclei have not been demonstrated. Differences in hepatic ethylmorphine *N*-demethylase activity in male rodents with the androgen insensitivity syndrome, a sex linked autosomal recessive trait, suggest the absence of specific androgen receptors in mutants and their presence in normal males [11]. This tfm model is characterized by an external female phenotype, inguinal testis and lack of androgen receptors in kidney, preputial and submaxillary glands [12].

Reproducible techniques for demonstrating specific androgen receptor proteins have been hindered by problems relating to the metabolism of the

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ligand employed and receptor inactivation during preparative procedures. Through the use of a non-metabolizable synthetic androgen, methyltrienolone (R1881), and the addition of Na-molybdate to stabilize receptor proteins [13], we have characterized a high affinity, low capacity androgen receptor in rodent liver which sediments at 8–9S in sucrose gradients that is absent in the tfm rat. Nuclear translocation of this ligand–receptor complex can also be demonstrated.

## EXPERIMENTAL

### *Preparation of cell fractions*

Following cervical dislocation, liver tissue was obtained from young adult (300–400 g) Sprague–Dawley rats (supplied by King Animal Laboratories, Inc., Oregon, WI), and the tfm rat (OKR strain supplied by the University of Oklahoma, Animal Resource Facility, Oklahoma City, OK) [14]. Fresh tissue was homogenized in receptor buffer (RB) containing 10 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol, glycerol 10% (v/v), and 25 mM Na-molybdate at 0°C in a 3:1 vol to weight ratio with a Polytron PT10-35 (Brinkmann Instruments, Inc., Westbury NY). The tissue was subjected to 4 bursts, 15 s at an intensity setting of 5 within cooling in-between. The homogenate was centrifuged at 100,000 *g* for 1 h at 2°C in a Beckman L2-65 ultracentrifuge (Beckman Instruments, Inc., Lincolnwood, IL) and the cytosol supernatant was carefully removed for binding studies.

### *Cytosol androgen receptor assay*

Binding studies to characterize the liver cytosol R1881-receptor were performed by sucrose density gradient centrifugation according to the method of Hawkins *et al.* with minor modifications [13]. Aliquots of cytosol (350 $\mu$l) were incubated in RB for 5 h at 0°C with 11.6 nM [$^3$H] R1881 (87 Ci/mmol, New England Nuclear Corp., Boston, MA) and 5 $\mu$m unlabelled triamcinolone acetonide (Sigma, St Louis, MO) in the presence or absence of 1 $\mu$M R1881. The addition of triamcinolone acetonide prevents R1881 binding to both progestin and glucocorticoid receptors [15, 16]. Various potential competitors were studied in an identical fashion except that unlabelled R1881 was replaced by 100-fold excess of the competitor. Also, triamcinolone acetonide was excluded from competition studies. Incubates were analyzed on sucrose gradients (see below). Stock steroid solutions were prepared in absolute alcohol and stored at −20°C. Prior to use, the solvent was evaporated under nitrogen. Incubations were terminated by removing free and loosely bound steroids with Dextran-coated charcoal (DCC) prepared from 0.05% (w/v) Dextran T70 (Pharmacia Fine Chemicals, Piscataway, NJ), and 0.5% (w/v) activated charcoal (Sigma Corp., St Louis, MO) in 350 $\mu$l RB. The DCC was pelleted at 1,000 *g* (International Centrifuge) for 2 min at 0°C and the supernatant was discarded. Incubates were transferred to the DCC pellets, vortexed and held at 0°C for 1 min and centrifuged at 1,000 *g* for 2 min at 0°C.

Continuous 5 to 20% sucrose gradients in polyallomer tubes (Beckman Instruments, Inc., Lincolnwood, IL) were prepared from 5 and 20% (w/v) sucrose solutions in RB using a gradient mixer (Buchler Instruments, Fort Lee, NJ). Two-hundred and fifty $\mu$l aliquots of DCC extracted cytosol were carefully layered onto the gradients. To separate 5 to 20% sucrose gradients, 250 $\mu$l each of human IGG [7.1S] (Cappell Laboratories, Cochranville, PA) in a final concentration of 10 mg/ml, catalase (11.3S) and bovine serum albumin (4.6S), both in a final concentration of 5 mg/ml (Sigma, St Louis, MO) were layered; all gradients were centrifuged at 160,000 *g* for 18 h at 2°C in a Beckman L2-65 ultracentrifuge with a SW 50.1 rotor (Beckman Instruments, Inc., Lincolnwood, IL). Following centrifugation, the bottom of the tubes was punctured and 2-drop fractions were collected by gravity displacement and counted in 10 ml 3A70b counting flor in a scintillation counter (Tricor Isocap 300, Nuclear Chicago, DesPlaines, IL). The counting efficiency for tritium was 45% using an external standard supplied by the manufacturer. The marker proteins were determined spectrophotometrically by the absorbence at 280 nM following dilution in 1 ml RB. Cytosol protein concentrations were determined by the method of Bradford[17].

### *Nuclear exchange assay*

Documentation of nuclear binding, the kinetics of R1881-receptor translocation into liver cell nuclei, and nuclear competition data were studied using an *in vitro* exchange assay [18, 19]. Adult Sprague–Dawley, males castrated 18 h earlier, and intact females were injected subcutaneously with 100 $\mu$g unlabelled R1881 in 0.1 ml propylene glycol. For kinetic studies animals were sacrificed at 0, 1, 5 and 24 h following R1881 treatment. Assessment of equilibrium conditions, Scatchard plot, and competition data were obtained from animals sacrificed at 1 h following R1881 treatment.

Liver cell nuclei and nuclear androgen receptors were isolated from a separate pool of liver (4 g) using hexylene glycol and pyridoxal 5′-phosphate according to the method of Isomaa *et al.*[19]. Briefly, liver tissue was homogenized in 15 vol buffer composed of 1 M hexylene glycol, 0.1 mM $MgCl_2$, 2 mM dithiotreitol, 5 mM EDTA, 1 mM Pipes, pH 7.5 and centrifuged at 1500 *g* for 10 min at 0°C. Nuclei were washed in 10 vol of hexylene glycol buffer and resuspended in 10 vol 2 M sucrose in buffer containing 50 mM Tris–HCl, pH 7.5, 2.5 mM KCl, 2 mM $MgCl_2$ and 1 mM dithiothreitol and sedimented at 80,000 *g* for 1 h in an SW 27.1 swinging bucket rotor.

Nuclear androgen receptors were next extracted for 30 min at 0°C in barbitol buffer (2 ml/g equivalent liver tissue) containing 20 mM barbitol, pH 8.0, 1.5 mM EDTA, 150 mM KCl, 5 mM dithiothreitol, 20% (v/v) glycerol and 5 mM pyridoxal 5′-phosphate. The extraction efficiency for this method is 87% [19]. Following extraction, nuclei were sedimented at 80,000 *g* for 30 min and the pellet was used for DNA determinations [20]. Supernatants were assayed in triplicate for androgen receptors in barbitol buffer for 18 h at 0°C containing 15 nM [$^3$H] R1881 in the presence and absence of 1 $\mu$M unlabelled R1881. Potential competitors were studied in a similar fashion except that unlabelled R1881 was replaced by 1–1000-fold excess of competitor. Bound and free ligand were separated by hydroxylapatite as previously described [19]. Ethanol extracts of hydroxylapatite pellets were counted for radioactivity. Results of both cytosolic and extracted nuclear receptors in the exchange assay are expressed per mg DNA.

Following DCC treatment duplicate aliquots (350 $\mu$l) of liver cytosol were incubated in RB with 11.6 nM [$^3$H]R1881 and 5 $\mu$M unlabelled triamcinolone acetonide in the presence and absence of 1 $\mu$M R1881 as described above except that incubations were carried out for 18 h. Bound radioactivity in cytosol was determined by gradient analysis as described above.

*Thin layer chromatography (TLC)*

Both the purity and extent of metabolism of R1881 were determined by TLC. The purity of R1881 was assessed by applying 6 pmol [$^3$H]R1881 and 10 $\mu$g each of the unlabelled steroids R1881, dehydroepiandrosterone, 5 $\alpha$-dihydrotestosterone, 5 $\beta$-androstan 3-17-dione, $\Delta^4$-androsten-3-17-dione and testosterone (Sigma, St Lousi, MO) to 20 × 20 cm precoated silica-gel sheets with fluorescent indicator (Eastman Chromatogram Sheets, Eastman Kodak Company, Rochester, NY). The sheets were developed with two ascents of the solvent system chloroform–methanol (97.5:2.5, v/v), air dried and viewed under a fluorescent light (Chromato-Vue, Ultra-Violet Products, Inc., San Gabriel, CA) for relative mobility. The entire lane to which $^3$H was applied was cut out and counted in a scintillation counter. [$^3$H]R1881 was found to be 90% pure. For analysis of steroid metabolites of [$^3$H]R1881 in cytosol preparations, incubates were layered onto 5–20% sucrose gradients and centrifuged at 160,000 *g* for 18 h at 20°C. The 8S region of the gradient was extracted with methylene–chloride saturated with water, evaporated to dryness, redissolved in 0.05 ml absolute ethanol and applied to silica-gel sheets and developed as for the studies of steroid purity.

## RESULTS

Sucrose density gradient analysis of [$^3$H]R1881 cytosol preparations from mature male, female, and tfm rat liver shows a single peak of radioactivity in the 8–9S region in the control male only, and absence of 8S [$^3$H]-labelling in both female and tfm liver cytosol (Fig. 1). Also, no specific R1881 binding is seen in the 4S region in cytosol from control male (Fig. 1A). One-hundred-fold excess of unlabelled

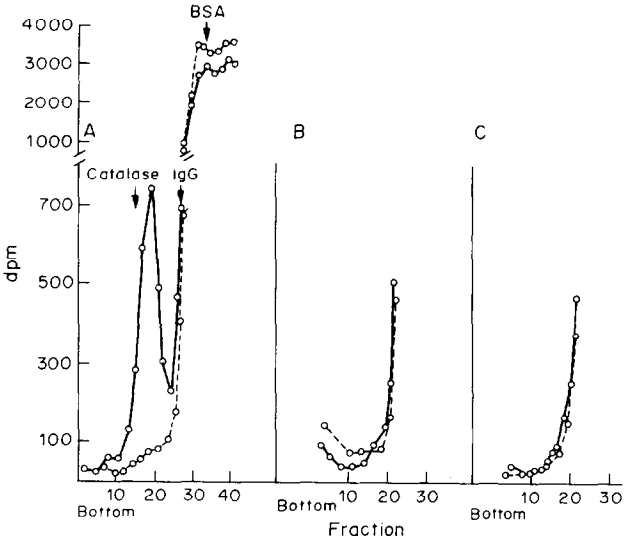


Fig. 1. Density gradient centrifugation of liver cytosol in (A) control male, (B) tfm male, and (C) control female after incubation with [$^3$H]R1881. Aliquots of cytosol prepared as described in the Experimental section were incubated for 5 h at 0°C with 11.6 nM [$^3$H]R1881 with (– – –) and without (——) the addition of 100-fold excess cold R1881 and extracted with DCC. 250 $\mu$l were applied to 5–20% sucrose gradients and centrifuged at 160,000 *g* for 18 h. Two drop fractions were collected and assayed for radioactivity. The positions of human IgG (7.1s), catalase (11.3s), and BSA (4.6s) markers are indicated. Note the absence of 8S-R1881 binding in both the tfm and female rat liver cytosol.

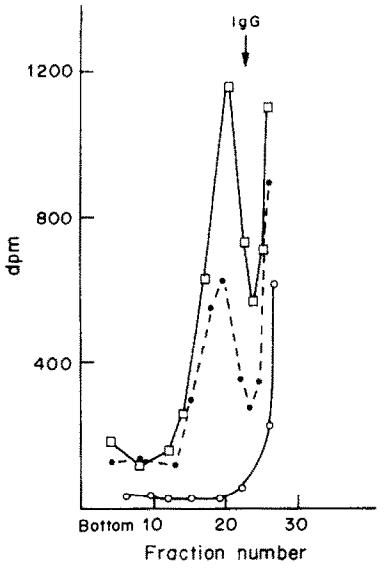


Fig. 2. Effect of Na-molybdate on the binding of [$^3$H]R1881 by rat liver cytosol. Cytosol (protein = 3 mg/ml) prepared under standard conditions described in the text (molybdate was absent in control RB) was incubated at 0°C for 5 h with 11.6 nM [$^3$H]R1881 with (——) or without (---) 25 mM Na-molybdate. Following brief DCC treatment, the ligand–receptor complex was layered onto 5–20% sucrose gradients and centrifuged for 18 h at 160,000 *g*. Fractions were counted in a scintillation counter. The absence of binding of [$^3$H]R1881 in the 8S region by plasma under identical incubation conditions in the presence of 25 mM Na-molybdate is also shown (◎——◎).

Table 1. Effect of various potential competitors on the retention of [$^3$H]R1881 or [$^3$H]estradiol by 8S binding protein of male liver cytosol*

| Competitor | Per cent depression of R1881 | Per cent depression of estradiol |
|---|---|---|
| R1881 | 100 | 15 |
| Estradiol | 73 | 100 |
| Diethylstilbestrol | 0 | 100 |
| Testosterone | 100 | 0 |
| Dihydrotestosterone | 87 | 0 |

*Cytosol was incubated for 5 h at 0°C with 11.6 nM [$^3$H]R1881 or 16 nM [$^3$H]estradiol in the presence or absence of 100-fold excess potential competitor. Incubates were subjected to DCC treatment and analyzed on sucrose gradients.

R1881 abolishes the radioactive peak demonstrating the specificity of this binding component in the 8S region of sucrose gradients. Similar to the results of others, we have found that the addition of Na-molybdate (25 mM) enhances specific [$^3$H]R1881 binding in the 8S region of sucrose gradients (Fig. 2).

In order to determine if the 8S binding was due to the presence of contaminating plasma proteins, a similar experiment to that described in Fig. 1 was performed on plasma samples from a control male. A sedimentation profile shown in Fig. 2 failed to demonstrate plasma R1881 binding in the 8–9S region in contrast to liver cytosol. The specificity of the assay for binding in the 8S region was further confirmed by noting that [$^3$H]R1881 incubated overnight with liver cytosol and extracted with methylene–chloride was essentially unmetabolized when determined by TLC; 97% of the $^3$H-labelled steroid migrated with carrier R1881.

The ligand-specificity of the cytosolic R1881 binding component present in the 8S region is different from that exhibited by the 8S-estrogen receptor. In contrast to the 8S-estrogen receptor, the male-specific 8S-R1881 binding component does not bind diethylstilbestrol (Table 1). Moreover, testosterone and dihydrotestosterone while having no effect on 8S-estradiol binding, compete for the 8S-R1881 binding sites. Contrariwise, R1881 is without effect on the binding of radiolabelled estradiol in the 8S region of sucrose gradients. Additional studies using 16 nM (2,4,6,7-$^3$H[N]) estradiol (115 Ci/mMol, New England Nuclear Corp., Boston, MA) incubated with cytosol from both the tfm and control male show binding in both the 4S and 8S region on sucrose gradients (Fig. 3). These results suggest the presence of an 8S androgen receptor in liver cytosol which is separate from the 8S estradiol receptor and absent in the tfm model. Furthermore, the 4S male-specific estradiol–androgen binding protein is present in the tfm model independent of the 8S androgen receptor.

The characterization of the androgen receptor including quantitation of receptor sites and its binding properties in male liver cytosol were performed by brief DCC treatment, and sucrose gradient centrifu-

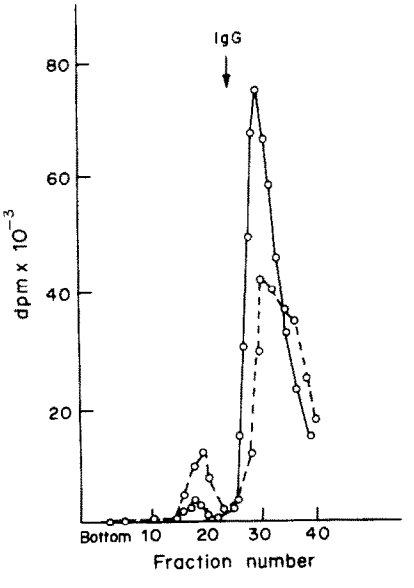


Fig. 3. Density gradient centrifugation of liver cytosol after incubation with [$^3$H]estradiol. Aliquots of cytosol from tfm (---) and control male (——) under standard conditions described for the androgen receptor assay excluding R1881 were incubated at 0°C for 5 h with 16 nM [$^3$H] estradiol and extracted with DCC. Two-hundred and fifty $\mu$l were applied to 5 to 20% sucrose gradients, centrifuged at 160,000 *g* for 18 h and fractions were counted in a scintillation counter. Note the presence of [$^3$H]estradiol binding in the 8S region for both tfm and control male liver cytosol.

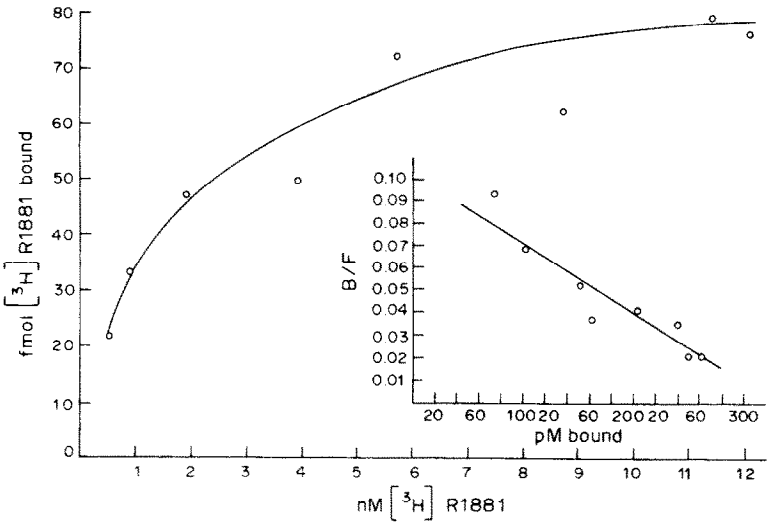


Fig. 4. Scatchard plot analysis of [$^3$H]R1881 binding in the 8S region of rat liver cytosol. Cytosol (protein = 3 mg/ml) prepared under standard conditions as described in the text was incubated at 0°C for 5 h with concentrations of [$^3$H]R1881 ranging from 500 pM to 11.6 nM. Free steroid was removed with DCC, and ligand–receptor complexes were layered onto sucrose gradients and centrifuged at 160,000 *g* for 18 h. Fractions were counted in a scintillation counter. Data are expressed in the form of a saturation curve and Scatchard plot analysis of radioactivity in the 8S region. The estimated number of binding sites and the $K_d$ are given in the text. Each data point represents the mean value of three separate determinations.

gation as described in the Experimental section. A typical saturation curve for the binding of [$^3$H]R1881 is shown in Fig. 4. Specific binding is saturable at a low steroid concentration (5 nM) indicating a limited number of binding sites. Binding kinetics of [$^3$H]R1881 in liver cytosol were determined by the method of Scatchard[21] and are shown in the insert of Fig. 4. Analysis of three separate studies yielded a dissociation constant of 2.3 nM $\pm$ 0.41 (SE), and the number of apparent binding sites is 18.8 $\pm$ 3.3 (SE) fmol/mg cytosol protein. These binding characteristics are commensurate with the results of Gaubert *et al.* in studies of cytosolic androgen receptors in rat prostate using [$^3$H]R1881 and 20 mM Na-molybdate [22]. Further characterization of androgen receptor specificity was sought in studies using ammonium sulfate precipitation. Whereas, 25–28% specific binding was found in the 8S region of sucrose gradients in cytosol recovered in the 0–33% ammonium sulfate fraction, no specific binding in the 8S region was noted in cytosol recovered in the 33–60% fraction.

The specificity of a particular ligand–receptor complex should be enhanced by a relationship between hormone receptor binding and receptor initiation of response, the latter dependent upon nuclear steroid retention. Since previous studies have suggested that nuclear androgen receptor concentration in non-target organ cells of intact animals is very low, we chose to optimize androgen content in liver nuclei by treatment with R1881 (100 $\mu$g sq) for various time intervals prior to sacrifice [23]. An *in vitro* exchange assay as described in the Experimental section was used to demonstrate translocation of the R1881 receptor complex from cytosol to the nucleus. An 11-fold decline in the cytosol receptor level is seen at 1 h following a single *in vivo* injection (100 $\mu$g) of unlabelled R1881 (Fig. 5). Concomitantly with the decline in cytosolic receptors, nuclear receptors increased 8-fold within 1 h and were maintained for an additional 4 h falling toward pretreatment level at 24 h.

Since nuclear steroid receptors are associated with endogenous ligand that must be exchanged with $^3$H-labelled steroid, it was important to determine the optimum time to reach equilibrium for [$^3$H]R1881 binding. The time course of [$^3$H]R1881 binding at 4°C is shown in Fig. 6. Specific nuclear binding at 4°C

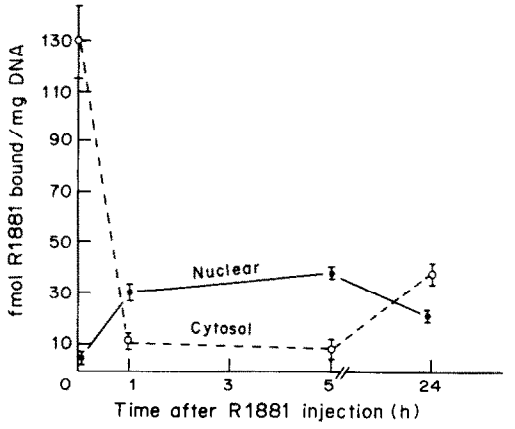


Fig. 5. Distribution of cytosol and nuclear [$^3$H]R1881-receptor in male liver after a single dose of unlabelled R1881 (100 $\mu$g sq). Following androgen injection, rats were sacrificed at the times indicated. [$^3$H]R1881-receptor in both cytosol and purified nuclei were determined by an exchange assay as described in the method section and expressed per mg DNA. Mean of triplicate values are shown.

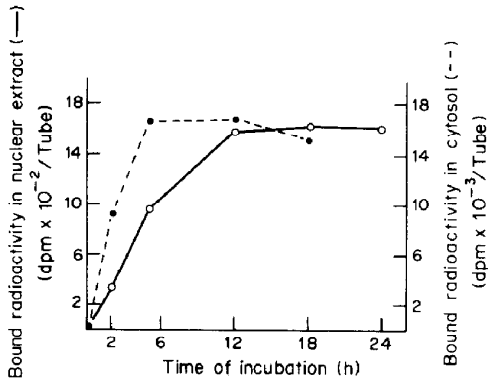


Fig. 6. Effect of *in vitro* incubation time on [$^3$H]R1881 binding in liver cytosol and nuclear androgen receptors. Nuclear receptors were extracted with barbital buffer containing 5 mM pyridoxal 5′-phosphate. The extract was incubated in triplicate with 15 nM [$^3$H]R1881 in the presence and absence of 100-fold excess unlabelled R1881 at 4°C. Cytosol was incubated in triplicate under standard conditions described in the text.

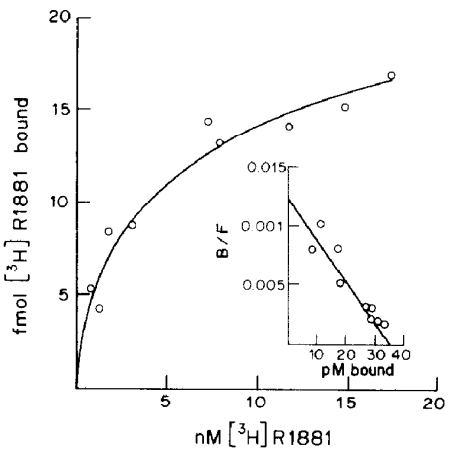


Fig. 7. Binding of [$^3$H]R1881 to androgen receptors extracted from liver cell nuclei isolated from R1881 (100 $\mu$g sq 1 h prior to sacrifice) treated adult male castrates. Nuclear extract was incubated for 18 h at 4°C with [$^3$H]R1881 at the indicated concentrations. Nonspecific binding was determined by assays containing 100-fold excess of R1881. Specific binding data according to the method of Scatchard are shown in the insert. Each data point represents the mean of triplicate determinations.

reached a maximum within 12 h and remained at this level through 24 h. Cytosolic binding reached a maximum at 5 h. Analysis of the specific binding data according to the method of Scatchard indicated the presence of a single class of high affinity binding sites with an apparent $K_d$ of 2.8 nM (Fig. 7). No cytosolic R1881 binding or nuclear translocation could be demonstrated in adult females (data not shown).

To ensure that only liver androgen receptors were measured with [$^3$H]R1881, steroid specificity for nuclear binding was assessed. The competition data are presented in Fig. 8. Of the different classes of steroids tested, all androgens were highly competitive for [$^3$H]R1881 binding to nuclear extract. Estradiol, cyproterone acetate, and progesterone displayed slight competition while diethylstilbestrol, dexamethasone, and triamcinolone were inactive.

## DISCUSSION

Data from a number of earlier studies suggest the presence of cytosolic androgen receptors in liver tissue. Two levels of observations suggested both their presence and possible significance. The first observation dealt with the modulating effect of androgen and estrogen on various microsomal enzyme systems and secretory proteins in rat liver mediated

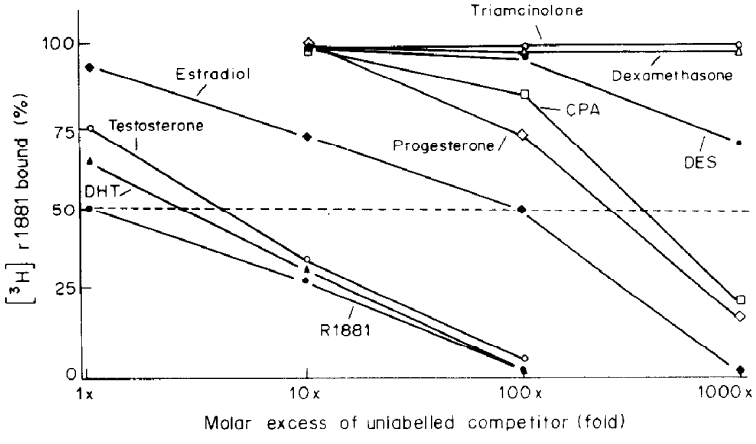


Fig. 8. Effect of various competitors on the retention of [$^3$H]R1881 by nuclear binding sites from male rat liver. R1881 binder was extracted with 5 mM pyridoxal 5′-phosphate from nuclei isolated from males castrated 18 h earlier and treated with 100 $\mu$g sq unlabelled R1881 for 1 h prior to sacrifice. Nuclear extracts were incubated with 15 nM [$^3$H]R1881 in the presence and absence of increasing concentrations of unlabelled competitor at 4°C for 18 h. Labelled nuclear binding sites were separated from free ligand by the hydroxylapatite method. Results represent the mean of triplicate determinations. Values are normalized to samples incubated with [$^3$H]R1881 alone, which is set at 100%. DHT, 5$\alpha$-dihydrotestosterone; CPA, cyproterone acetate; DES, diethylstilbestrol.

through putative sex hormone receptors [24–26]. The second important observation concerned the lack of sexually dimorphic differences in microsomal enzyme systems in male rodents with a normal chromosomal constitution but phenotypically expressing the testicular feminizing syndrome, a form of androgen insensitivity [11]. In addition, Roy *et al.* have shown the presence of androgen dependent synthesis of 2α-globulin by rat liver which seems to be dependent on the presence of a 4S cytosolic androgen binding protein [27]. Despite the strong arguments favoring the presence of specific androgen receptors in liver tissue, however, a high affinity binding macromolecular species and nuclear translocation of androgen–receptor complexes have not heretofore, been conclusively demonstrated [28].

The present data employing a recently characterized synthetic androgen, methyltrienolone, and a low ionic strength cytosol incubation buffer containing Na-molybdate demonstrate a high-affinity macromolecular complex that sediments in the 8–9S region on sucrose gradients. This androgen receptor appears to be male-specific and absent in the tfm rat. Using an *in vitro* exchange assay, we have shown nuclear translocation of the R1881 receptor to a single class of high affinity nuclear binding sites shortly after *in vivo* R1881 treatment; nuclear residence of R1881 binding sites is still apparent at 24 h. In addition, this R1881-receptor is not present in the 8S region of plasma from intact males when analyzed on sucrose gradients. While these properties of an intracellular androgen binding protein are not unequivocal proof of their participation in hormone action, they are, nonetheless, consistent with such a function.

Although we have not studied the affinities of additional androgens for this 8–9S androgen receptor in liver, the present nuclear competition studies suggest similarity to androgen receptors in both mouse kidney and adrenal cortex [19, 29]. Estradiol will modestly compete with androgen receptors in these tissues much as it does with the R1881 binder in liver tissue. By contrast, the 8S cytosolic estrogen receptor, which binds both steroidal and non-steroidal estrogens, does not bind R1881 nor androgens separating this 8S receptor protein from the androgen receptor in the present assay system. This notion is further supported by the presence of intracellular 8S-estradiol receptors in the tfm rat, a model which clearly lacks specific 8S-androgen receptors in all tissues studied [30].

The absence of cytosolic R1881 binding in the 4S region of sucrose gradients (Fig. 1), the lower affinity of the male-specific 4S estrogen–androgen binder in rodent liver ($K_d = 10^{-7}$ M), as well as the absence of *in vivo* nuclear translocation also separate this binding protein from the R1881 binder.

Furthermore, cyproterone acetate modestly competes with [$^3$H]R1881 nuclear binding sites whereas, according to Roy *et al.*, this anti-androgen does not bind to the 4S estrogen–androgen binding protein [28]. Similar competition has been observed in nuclei from mouse kidney [19]. The present cytosolic competition studies suggest, however, similarities between the 8S-R1881 receptor and the 4S cytosolic binder described by Roy *et al.* and Dickson *et al.*[10, 31]. Both bind dihydrotestosterone, testosterone and estradiol. Diethylstilbestrol does not compete with either the 4 or 8S cytosolic binder, or [$^3$H]R1881 binding sites translocated to the nucleus. While no direct evidence has linked the 4 and 8S male-specific androgen binding proteins in liver tissue, Griffin and Durant, using fibroblast cytosol, have suggested that the 8–9S moiety is an aggregate form of the physiological androgen receptor [32]. Whether R1881 binding represents a molybdate stabilized form of the 4S-androgen binder with the ability to translocate to nuclei must await studies done on purified preparations, as well as defining functional and developmenal relationships with liver proteins exhibiting sexual dimorphism.

Bonne and Raynaud first reported on the use of methyltrienolone to assay androgen binding sites [33]. The endogenous androgens including dihydrotestosterone and testosterone are limited in their use for measuring these receptor sites because of the formation of metabolic products with lower receptor site affinity. In addition, these endogenous androgens bind to contaminating serum proteins thus obscuring precise receptor measurements. R1881 has been studied in a number of tissues including rat and human prostate, a human breast cancer cell line, uterus, and other male accessory sex organs in the rat [13, 15, 29]. Whereas the synthetic ligand has a capacity to bind both androgen and progesterone receptors, the addition of 500-fold excess triamcinolone acetonide to the cytosol incubation buffer blocks R1881 binding to the progesterone binding sites allowing for selective characterization of androgen binding [15]. This method has been validated using a variety of techniques including sucrose gradients, ammonium sulfate and protamine sulfate precipitation, and nuclear translocation studies [19, 34]. Application of this method to liver tissue or the tfm model has not been previously reported.

Since the initial reports of Nielson *et al.* of glucocorticoid receptor stabilization, the addition of molybdate to other steroid receptor assay systems have included rat prostate androgen receptor, avian progesterone receptor, rabbit kidney aldosterone and rat intestinal vitamin D receptors [35–37]. Leach *et al.* have concluded that the molybdate ion interacts directly with the receptor to allow for physical stabilization of the protein [38]. They suggest that molybdate may interact with a phosphate moiety of the receptor protein stabilizing it to both inactivation, and blocking temperature dependent nuclear translocation.

In summary, our data indicate the presence of a specific 8–9S-androgen binding component in male rat liver cytosol that is retained in liver cell nuclei and

is absent in both the female and androgen insensitive rat. The posible relationship to the male-specific 4S-estrogen-androgen binding protein, and the development and function of this 8S androgen receptor remain a part of future investigations.

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