High Affinity Protein-Binding and Enzyme-Inducing Activity of Methyltrienolone in *Pseudomonas testosteroni*

Åke Pousette[a*] and Kjell Carlström[b]

[a]Department of Clinical Chemistry, [b]Department of Obstetrics and Gynecology, Karolinska Institutet, Huddinge University Hospital, S-141 86 Huddinge, Sweden

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The synthetic androgen methyltrienolone (R 1881) was shown to increase steroid Δ^1 dehydrogenase activity when added to cultures of *Pseudomonas testosteroni* at concentrations of 10^{-10}-10^{-8}M. Incubation with a soluble extract of *P. testosteroni* showed that (^{3}H)-R 1881 was bound to a macromolecule with high affinity (K_d 0.6 × 10^{-9}M) and low capacity (number of binding sites 120 × 10^{-15} mol/mg of protein). The (^{3}H)-R 1881-macromolecule complex was partially destroyed following treatment with protease, was precipitated by addition of ammonium sulfate at 20 % of saturation, sedimented at 6.3 S both in 0.01 and 0.4 M KCl solutions, and had an isoelectric point of pH 6.3. The complex was partially bound to DNA-cellulose. Analysis by sucrose gradient centrifugation indicated that neither (^{3}H)-testosterone and (^{3}H)-estradiol-17β nor (^{3}H)-corticosterone were bound with high affinity to the (^{3}H)-R 1881-binding macromolecule. It is suggested that the partially characterized R 1881-binding macromolecule, which at least in certain respects resembles androgen receptors described in mammalian cells, is involved in the inductive effect of R 1881 on the Δ^1 dehydrogenase activity in *P. testosteroni*.

In mammalian species including man, the action of androgens, as well as of other steroids, is considered to be mediated by specific receptor proteins present in the target tissues. While some of these steroid-receptor mechanisms (estrogen, glucocorticoid) have been rather easily studied in mammalian tissues, investigations on androgen-receptor interaction are frequently hampered by the low concentration and instability of the androgen receptor *in vitro*[1]. A suitable, stable, experimental, model system is therefore highly desirable.

The microorganism *Pseudomonas testosteroni* is well known for its steroid metabolizing capacity and for its ability to utilize steroids as the sole carbon source. Steroid-metabolizing enzymes can be induced by steroids, notably androgens[2], but the mechanism of this action in *P. testosteroni* is not known. Although most workers have failed to detect any steroid receptors in microorganisms (for a review see Ref. 1), Watanabe and coworkers reported on the presence of an androgen receptor in *P. testosteroni*[3-5]. However, since this androgen-binding protein was present only in steroid – induced cultures, it is probably not involved in the initial androgen action.

With the aim of finding a suitable experimental model for the study of androgen – receptor interaction, we have investigated the effects of the synthetic androgen R 1881 (methyltrienolone) upon the steroid metabolism of *P. testosteroni*. The present paper describes the induction of Δ^1 dehydrogenase activity by R 1881 and the partial characterization of a high affinity, receptor-like binding protein for R 1881 in uninduced *P. testosteroni*.

Materials and methods

Steroids: (1,2,6,7-^{3}H)-Testosterone (specific activity 84 Ci/mmol), (1,2,6,7-^{3}H)-4-androstene-3,17-dione (specific radioactivity 89 Ci/mmol), (2.4.6.7−^{3}H)-estradiol-17β (specific radioactivity 90.8 Ci/mmol) and (1,2,6,7−^{3}H)-corticosterone

*To whom correspondence should be addressed.

(specific radioactivity 80.5 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. 6,7-(^{3}H)-Methyltrienolone (R 1881, 17β-hydroxy,17α-methylestra-4,9,11-trien-3-one, specific radioactivity 52.8 Ci/mmol), unlabelled R 1881 as well as (^{3}H)-labelled and unlabelled 17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione (R 5020) were purchased from New England Nuclear Chemicals GmbH, Dreieichenheim, W. Germany. The chemical purity of the labelled compounds (99 %) was checked by thin layer chromatography. Unlabelled testosterone, 4-androstene-3,17-dione and 5α-dihydrotestosterone were kindly supplied by Dr. J. Babcock, Upjohn Co., Kalamazoo, MI, USA. 1,4-Androstadiene-3,17-dione was purchased from Steraloids Inc., Wilton, NH, USA.

Pseudomonas testosteroni ATCC 11996 was obtained from the American Type Culture Collection, Rockville, MD, USA. The bacteria were grown in a medium containing 1g $NH_4H_2PO_4$, 1g $(NH_4)_2HPO_4$, 2g KH_2PO_4, 10 g Difco yeast extract, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 mg NaCl, 5 mg $ZnSO_4$, 5 mg $MnSO_4 \cdot 3H_2O$, 0.5 mg $CuSO_4 \cdot 5H_2O$ and 0.1 ml H_2SO_4 per l, adjusted to pH 6.65[3]. The cultures were grown on a shaking table at 28 °C. For binding studies, the bacteria were harvested 48 h after inoculation. For induction, R 1881 dissolved in ethanol was added 48 h after inoculation, giving a final ethanol concentration of 0.5 %. The induced cultures were harvested 4 days after inoculation. The bacteria were harvested by centrifugation for 10 min at 8000 × *g*, and the wet weight determined. This and the following procedures were carried out at 0–4 °C. The cells were washed twice in buffer 1 (0.15 M NaCl, 50 mM Tris-HCl, pH 9.0) and resuspended in 2 ml of buffer 2 (50 mM Tris, 5 mM EDTA and 1 mM dithiotreitol, pH 9.0) per g of bacteria. The bacteria were ruptured by sonication (2 × 20 s) using an MSE 500 W sonicator at full output. The cell debris was removed by centrifugation at 8000 × *g* for 10 min. Streptomycin sulfate was added to the cell-free extract to a final concentration of 1 % (w/v) and after continuous stirring for 2 h, the precipitated DNA was removed by centrifugation (8000 × *g* for 10 min). The supernatant was dialyzed against 100 volumes of buffer 2 for 18 h.

Cell extracts intended for binding studies were generally stored at −20 °C until the experiments were performed, but studies of metabolism were usually carried out on the day of extract preparation. The protein concentration of the bacterial extract was about 0.2 mg/ml.

Assay of metabolites. The incubation mixture contained 1 ml of bacterial extract, 10^6 dpm (^{3}H)-4-androstene-3,17-dione diluted with unlabelled steroid (final concentration 10^{-6}-10^{-4}M), 10^{-3}M NADH or 10^{-3}M phenazine methosulfate (Sigma Chemical Co., St. Louis, MO, USA). Incubation was performed at 30 °C for 20 min and terminated by the addition of 5 ml chloroform: methanol, 2:1 (v/v). The phases were allowed to separate; the chloroform phase was dried under nitrogen and analyzed by thin layer chromatography and radio-gas chromatography. The extract was applied to precoated silica gel plates (250 μm, Merck A. G., Darmstadt, W. Germany) that were developed in the solvent system chloroform: ethyl acetate, 4:1 (v/v). The radioactive metabolites were localized and quantitated with a thin layer scanner (Berthold model II, Wildbad, W. Germany). For identification of metabolites, the radioactive zones were scraped off and the steroids eluted from the silica gel with methanol. After trimethylsilylation of the steroids, they were analyzed by radio-gas chromatography[6]. A steroid was considered as identified if it had the same thin layer chromatographical mobility and the same retention time on SE-30 and OV-17 as the reference steroid. Metabolism of (^{3}H)-androstenedione was linear with respect to time and enzyme concentration.

Assay for binding. Bacterial extract (0.2 ml) was added to 1 ml glass tubes containing increasing amounts of (^{3}H)-R 1881 or (^{3}H)-R 5020 in the presence or absence of a 100-fold excess of the respective unlabelled steroid. Incubations were performed for 16 h at 0–4 °C. For measurement of total radioactivity, 50 μl were taken from the incubation mixture and 50 μl of a suspension of dextran-coated charcoal (0.5 % w/v washed charcoal, 0.05 % w/v dextran T-500) were then added to each tube. After 20 min at 4 °C, the charcoal was sedimented at 6700 × *g* for 20 min and 50 μl of the supernatant was taken off for measurement of radioactivity. Radioactivity measurements were performed using Instagel (Packard Instrument Co., Inc., Warrenville, Downers Grove, IL, USA) as scintillation liquid. Radio-

activity was measured as dpm using the external standard technique. Specific binding was calculated as the difference between bound radioactivity in the absence (total binding) and presence (unspecific binding) of unlabelled steroid. Calculation of K_d and binding sites was performed according to Scatchard[7].

Density gradient centrifugation. Aliquots (0.2 ml) of cell extracts were incubated with 8×10^{-9}M (^{3}H)-R 1881, (^{3}H)-testosterone, (^{3}H)-R 5020, (^{3}H)-estradiol-17β or (^{3}H)-corticosterone. After treatment with dextran-coated charcoal, the incubation mixtures were passed through Sephadex G-25 columns equilibrated in buffer 2, 0.01 or 0.4 M with respect to KCl. The void volumes (0.3 ml) were layered on top of linear 5 ml 5–20 % (w/v) sucrose gradient in buffer 2, 0.01 or 0.4 M with respect to KCl. The tubes were centrifuged in an SW 50.1 Beckman rotor. At the end of the centrifugation, the bottoms of the tubes were punctured and 3–4 drop fractions were collected and measured for radioactivity. The following markers were used: alcohol dehydrogenase (7.4 S) and catalase (11.3 S).

Sephadex G-200 chromatography. Generally, incubation mixtures were applied on Sephadex G-200 columns equilibrated in TGE buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM EDTA and 10 % w/v glycerol). The following calibration standards were used: dextran blue (void volume), alcohol dehydrogenase (Stokes radius (SR) 46 Å), bovine serum albumin (SR 35 Å), hemoglobin (SR 24 Å) and cytochrome c (SR 17 Å).

Isoelectric focusing was carried out essentially as earlier described[8].

Ion exchange chromatography. One ml of cell extract was incubated with 2×10^{-9}M (^{3}H)-R 1881 for 18 h at 0 °C. After treatment with dextran-coated charcoal, the supernatant was passed trough a Sephadex G-25 column equilibrated in TE buffer, pH 7.4. The void volume was applied on a DEAE-cellulose column (10 × 30 mm) that was eluted with 10 ml portions of TE buffer, 0.05, 0.1, 0.2, 0.4, and 0.6 M, respectively, with regard to KCl. An aliquot was taken from each fraction for measurement of radioactivity.

Other procedures. Ammonium sulfate precipitation, DNA-cellulose chromatography and protease treatment were performed using the protein-bound fraction obtained after filtration of the dextran-charcoal-treated (^{3}H) R 1881-labelled bacterial extract through a Sephadex G-25 column equilibrated in TE buffer (50 mM Tris, 5 mM EDTA, pH 9.0). Ammonium sulfate was added to yield final concentrations of 20 %, 40 % and 60 % of saturation. Precipitates were removed by centrifugation and redissolved in buffer before aliquots were taken for measurement of radioactivity. DNA-cellulose chromatography was carried out as earlier described[2]. An aliquot of the sample was also chromatographed on a cellulose column to determine unspecific binding to cellulose. The samples were put on DNA-cellulose columns (10 × 10 mm) applied in TE buffer and the columns were washed with TE buffer until no more radioactivity could be eluted. The columns were then eluted with TE buffer, 0.4 M with respect to KCl. Aliquots were taken from each fraction for measurement of radioactivity.

In order to investigate the nature of the androgen-binding macromolecule, the (^{3}H)-R 1881-macromolecule complex was treated with protease (1 mg/ml, type IV, Sigma) for 4 h at 20 °C. The remaining complex was measured after filtration through a Sephadex G-25 column. Control samples not treated with protease were run in parallel in order to measure the spontaneous degradation and dissociation of the complex.

Results

During the investigation of the metabolism of androstene-3,17-dione in extracts from uninduced and R 1881-induced bacteria, only one metabolite was detected by thin layer chromatography (Fig. 1). This metabolite was further characterized by radio-gas chromatography, where the retention time relative to testosterone was 0.75 (1 % SE-30) and 2.10 (1 % OV-17). These characteristics were identical to those of the reference compound 1,4-androstadiene-3,17-dione, a known metabolite of 4-androstene-3,17-dione in *P. testosteroni*[9].

Addition of R 1881 (10^{-10}–10^{-8} M) to cultures of *P. testosteroni* induced Δ^1 dehydrogenase activity (Fig. 2), but no effect of R 1881 addition

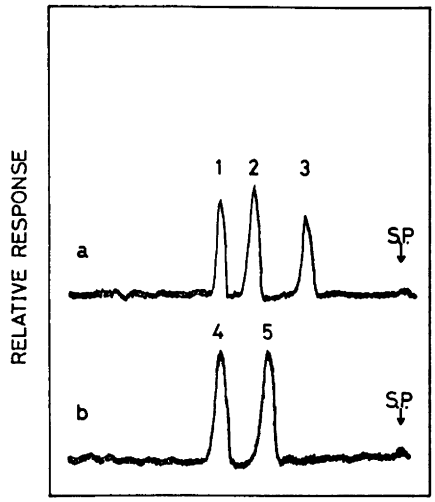

Fig. 1. Radioactivity scanning of thin layer plates with a) the reference compounds (1: (^{3}H)-4-androstene-3,17-dione; 2: (^{3}H)-5α-dihydrotestosterone and 3: (^{3}H)-testosterone) and b) metabolites formed during incubations of 10^{-6}M (^{3}H)-4-androstene-3,17-dione with cell-free extracts from induced (10^{-9}M R 1881) cultures of *P. testosteroni*. The steroids in the incubation extract were identified as 4: 4-androstene-3,17-dione and 5: (^{3}H)-1,4-androstadiene-3,17-dione. S.P.: Starting Point.

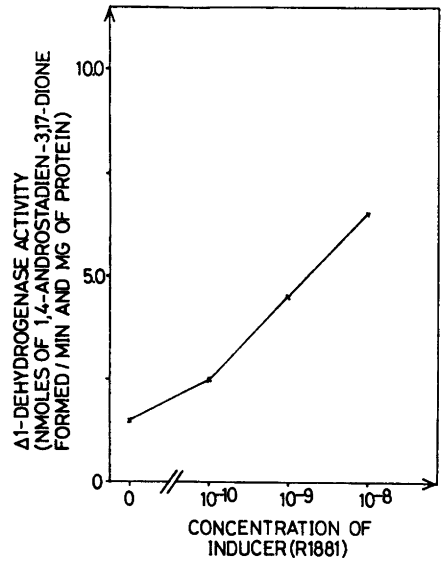

Fig. 2. Δ^1 dehydrogenase activity in cell-free extracts from *P. testosteroni* grown in the presence of different concentrations of R 1881. The enzyme activity was calculated from the amount of 1,4-androstadiene-3,17-dione formed.

could be detected on the yield of bacteria (calculated as their total wet weight). Storage of the bacterial extract at −20 °C totally destroyed the Δ^1 dehydrogenase activity.

In view of the enzyme-inducing activity of R 1881 in *P. testosteroni*, it was of interest to investigate whether the bacteria contained any receptor-like protein-binding for R 1881. Extracts from uninduced bacteria were found to bind (^{3}H)-R 1881 with high affinity and low capacity. No metabolism of R 1881 could be detected in such extracts. Analysis according to Scatchard[7] revealed a K_d of 0.6×10^{-9} M and a number of binding sites of 120–150 $\times$ 10^{-15} mol/mg of protein (Fig. 3). No specific binding could be detected when (^{3}H)-R 5020 was used as ligand.

Density gradient centrifugation. When extracts from uninduced cultures of *P. testosteroni* were incubated with 8×10^{-9} M (^{3}H)-R 1881, a complex was formed which sedimented at 6.3 S when analyzed on sucrose gradients in TE buffer, 0.01

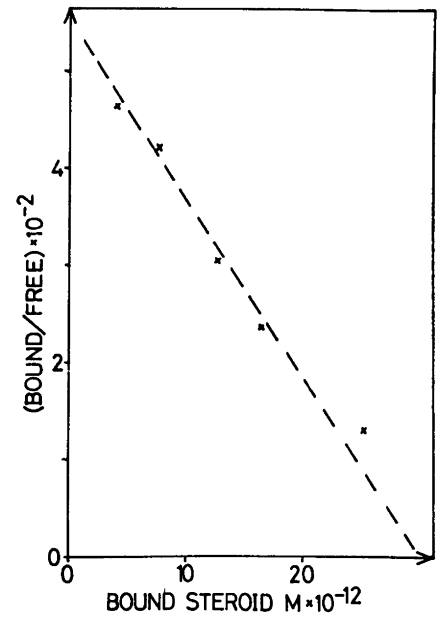

Fig. 3. Scatchard plot of specific binding of (^{3}H)-R 1881 in cell-free extract from uninduced cultures of *P. testosteroni*.

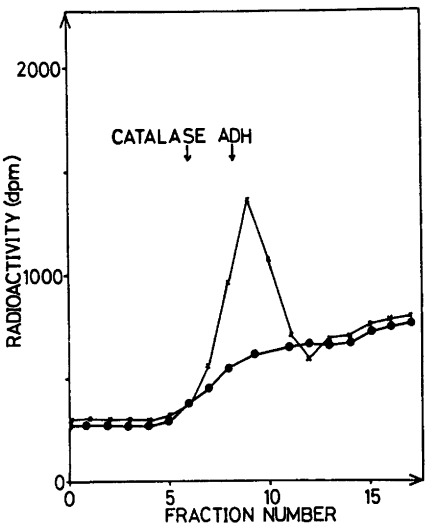

Fig. 4. Sucrose density gradient centrifugation of (^{3}H)-R 1881-macromolecule complex formed after incubation of cell-free extract from uninduced cultures of *P. testosteroni with* 2×10^{-9}M (^{3}H)-R 1881 in the absence (×---×) or presence (●-●-●) of a 100-fold excess of unlabelled R 1881.
Alcohol dehydrogenase (7.4 S) and catalase (11.3 S) were used as standards. The radioactive peak sedimented at 6.3 S.

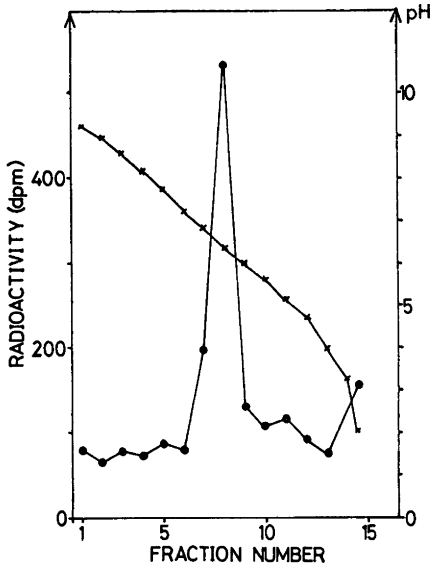

Fig. 5. Isoelectric focusing of the (^{3}H)-R 1881-macromolecule complex, formed by incubation of 2×10^{-9}M (^{3}H)-R 1881 with cell-free extracts from uninduced cultures of *P. testosteroni*, ●-●-● radioactivity, ×-×-×, pH.

or 0.4 M with respect to KCl (Fig. 4). The radioactivity was totally displaced when incubation was performed in the presence of 8×10^{-7} M unlabelled R 1881. It was not possible to detect any (^{3}H)-testosterone, (^{3}H)-R 5020, (^{3}H)-estradiol-17β or (^{3}H)-corticosterone-macromolecule complex in the region 3–10 S when sucrose density gradient centrifugation of incubation mixtures with extracts from *P. testosteroni* and (^{3}H)-labelled steroids was performed. These results are in agreement with earlier studies where no specific binding of (^{3}H)-testosterone, ^{3}(H)-estradiol-17β or (^{3}H)-corticosterone was found in cell extracts from uninduced cultures of *P. testosteroni*[10].

Isoelectric focusing indicated that the (^{3}H)-R 1881-macromolecule complex had an isoelectric point of pH 6.3 (Fig. 5). The radioactive peak was displaceable with a 100-fold excess of unlabelled R 1881.

Sephadex G-200 chromatography. When chromatographed on Sephadex G-200, the (^{3}H)-R 1881-macromolecule complex was eluted in the void volume of the column.

Ion exchange chromatography. The major part of the (^{3}H)-R 1881-macromolecule complex was eluted at 0.05 M KCl concentration. The complex was eluted at the same KCl concentration when the chromatography was performed at pH 8.5 instead of at pH 7.4 (data not shown).

DNA-cellulose and cellulose chromatography. Of the specifically bound (^{3}H)-R 1881-macromolecule complex, 13 % was retained on a DNA-cellulose column. The amount that was retained on the column was not increased when the (^{3}H)-R 1881-macromolecule complex was kept at 20 °C for 20 min prior to chromatography on DNA-cellulose (data not shown). No radioactivity was retained on the cellulose column (Table 1).

Table 1. DNA-cellulose and cellulose chromatography of partly purified (^{3}H)–R 1881–macromolecule complex oblained from cell-free extracts from uninduced cultures of *P. testosteroni*. Radioactivity/dpm.

	DNA-cellulose	Cellulose
Specifically bound (^{3}H)–R 1881 applied on DNA-cellulose	3480	3600
Specifically bound (^{3}H)–R 1881 eluted with TE buffer	2970	3560
Specifically bound (^{3}H)–R 1881 eluted with TE buffer, 0.4M with respect to KCl	452	0

Table 2. Ammonium sulfate fractionation of purified (^{3}H)–R 1881–macromolecule complex from cell-free extracts from uninduced cultures of *P. testosteroni*.

Saturation of ammonium sulfate/%	Specific binding of (^{3}H)–R 1881 precipitated/% of total
0–20	80.6
20–40	14.8
40–60	3.2
60	1.4

Ammonium sulfate precipitation and protease treatment. Eighty % of the (^{3}H)-R 1881-macromolecule complex was precipitated by addition of ammonium sulfate to 20 % of saturation (Table 2); 70 % was destroyed by treatment with protease, indicating that the macromolecule was a protein.

Discussion

P. testosteroni is known to be responsive to steroids; androgens induce the synthesis of a number of steroid-metabolizing enzymes in this bacterium[1]. The mechanism of androgen action in bacteria is, however, obscure. Some authors have denied the existence of androgen receptor proteins in bacteria[2], whereas other workers have reported high affinity binding of androgens in bacterial cell extracts[3]. In a recent paper, we reported on the partial characterization of an (^{3}H)-androstenedione-receptor complex in induced cultures of *P. testosteroni*[10].

R 1881 (methyltrienolone) is not bound to sex hormone-binding globulin but binds to the androgen receptor in rat and human prostate with high affinity. In the present investigation, R 1881 was found to induce Δ^1 dehydrogenase activity in *P. testosteroni* when present in a concentration of 10^{-10}–10^{-8} M. The fact that an effect is seen at this low concentration indicates that a specific receptor participates in the action of R 1881 in *P. testosteroni*. This conclusion correlates well with the occurrence of a specific protein that binds (^{3}H) R 1881 with high affinity and low capacity. The Δ^1 dehydrogenase activity is essential for *P. testosteroni* since the catalyzed metabolic step initiates the breakdown of the steroid structure[11]. Previous studies have indicated that testosterone can also induce Δ^1 dehydrogenase activity[12]. However, in these experiments, testosterone was added in much higher concentrations.

The (^{3}H)-R 1881-macromolecule complex shows characteristics other than the earlier described (^{3}H)-4-androstene-3,17-dione-binding protein in androgen-induced cultures of *P. testosteroni* and appears to be a larger molecule than this protein[10]. The R 1881-macromolecule complex sediments at a higher S value (6.3 S compared to 3.5 S) and is excluded from a Sephadex G-200 column, whereas the 4-androstene-3,17-dione-protein complex has a Stokes radius of only 26 Å. The (^{3}H)-R 1881-macromolecule complex shows properties that, at least in certain respects, resemble those of androgen receptors in mammalian cells. The elution of the complex in the void volume of a Sephadex G-200 column seems to be a common characteristic of all described androgen receptors[2]. Furthermore, the (^{3}H) R 1881-macromolecule complex focused at pH 6.3 which is similar to the pI that has been described for activated 5α-dihydrotestosterone receptor complex in rat prostate[2]. Finally, the (^{3}H)-R 1881-macromolecule complex was at least partially bound to DNA-cellulose, a property typical for all steroid-receptor complexes. One can expect the described R 1881-binding macromolecule to show enzyme activity since it is quite probable that the same protein can act both as an enzyme and a transport protein.

The mechanism of androgen action in bacteria is still far from clear, but the fact that 10^{-10} M R 1881 induces Δ^1 dehydrogenase activity in *P. testosteroni* and that (^{3}H)-R 1881 is bound with high affinity and low capacity to a macromolecule in

the same bacterium indicate that androgen may act in a similar way as in eukaryotic cells. The present study indicates that *P. testosteroni* may provide an interesting model system for studies on mechanisms of androgen action.

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