

THE INHIBITIVE EFFECTS OF STEROID ANALOGUES IN THE  
BINDING OF TRITIATED 5 $\alpha$ -DIHYDROTESTOSTERONE TO  
RECEPTOR PROTEINS FROM RAT PROSTATE TISSUE

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Received: 11/25/74

ABSTRACT

The relationship between molecular structure and the binding potential of steroids to receptor proteins was investigated. Twenty-four selected steroids were studied in minced incubations of rat prostate tissue. Measurements of the inhibitory effects of the steroids on the binding of tritiated 5 $\alpha$ -dihydrotestosterone to the receptor proteins were obtained in the 100,000 x g dialysed supernatant and the purified nuclear component of the prostate cells. The steroids that achieved the highest degree of inhibition were those compounds that exhibited a generally planar geometric shape and were known to possess potent androgenic activity. Several of the compounds were shown to possess a higher degree of inhibition than that of testosterone or 5 $\alpha$ -dihydrotestosterone.

The data is further supportive of the two step theory that necessitates the complexing of the free steroids to the receptor proteins in the cytosol before transport to the nuclei. Evidence is also suggestive of the presence of 17-esterase activity. The inhibitory effect of the steroids apparently involves the binding to the intracellular receptors and is not related to the uptake of 5 $\alpha$ -dihydrotestosterone into the cell.

INTRODUCTION

The cytoplasm from prostate tissue contains binding proteins which possess a strong affinity for 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT) (1-7). Evidence has accumulated which suggests that these receptor proteins function to specifically transport 5 $\alpha$ -DHT from the cytoplasm into the nucleus (1,2,5,7). Until recently few attempts have been made to assess the structural requirements of steroids for interaction with the receptor proteins (5,8,9). Even less information is available,

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relating steroid structure to androgen or anti-androgen activity on a subcellular level. Liao, *et al.*, in a recent comprehensive study has contributed significantly toward a more definitive understanding of these relationships (9).

This paper reports the results from studies dealing with the ability of testosterone analogues to prevent the binding of  $5\alpha$ -DHT- $^3\text{H}$  to the protein receptors in prostate minces. The inhibitory nature of 24 steroids was studied in both the 100,000 x g supernatant and nuclei of the prostate cell. Selection of the steroids was based upon their structural relation to  $5\alpha$ -DHT as well as their known androgenic or anti-androgenic activities.

#### MATERIALS AND METHODS

##### Materials

Sprague-Dawley rats weighing between 300-350 grams were used. Tritiated dihydrotestosterone-1, 2- $^3\text{H}$ , with a specific activity of 44 curies/mM, was purchased from the New England Nuclear Corp. The authors are grateful to Dr. P. D. Klimstra of Searle Laboratories for furnishing samples of 5-7, 16 and 21 (see Table 1) to Dr. J. C. Babcock of the Upjohn Co. for steroids 3 and 4, and to Dr. R. Neri of Schering Corp. for steroid 23. The other steroids were obtained from commercial sources or prepared previously by one of us (REC). A purity check of the steroids was accomplished by t.l.c. systems of isopropyl ether:acetone (4:1) and benzene:ethyl acetate (2:1).

##### Methods

Rats castrated via a scrotal incision were sacrificed 24 hours later (10). The prostatic tissue from 15 rats was removed, trimmed of extraneous tissue and pooled. The pooled tissue was minced with scissors and 0.5 gm aliquots were incubated in 3.0 ml of Eagles Basal Medium that contained glutamine and Earle's salts. The incubation medium included  $5\alpha$ -DHT- $^3\text{H}$  at a concentration of 16.7 nanomolar, unless stated otherwise. The steroid under investigation was added in 0.10 ml of absolute ethanol. Control incubations also contained 0.10 ml of absolute ethanol. Incubations were performed at  $30^\circ\text{C}$  with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  as the gas phase. After 30 minutes the incubations were transferred to test tubes and chilled on ice. The tissue was separated from the incubation medium by brief centrifugation and washed 3 times with 10.0 ml of Eagles Basal Medium. The washed tissue was homogenized in 3 volumes (1.5 ml) of 0.88 M sucrose + 1.5 mM  $\text{CaCl}_2$  in a glass-teflon homogenizer. The crude nuclear fractions were sedimented by centrifu-

gation at 800 x g for 10 min. The 800 x g supernatants were centrifuged at 100,000 x g for 60 min. to obtain the 100,000 x g supernatant fractions (11).

The loosely bound or free  $5\alpha$ -DHT- $^3$ H was removed from the 100,000 x g supernatants by dialysis against 2 liters of 0.01 M Tris HCl pH 7.0 that contained 0.05 mM EDTA, 5.0 mM  $MgCl_2$  and 0.5 mM mercaptoethanol. The dialysis was performed at 4°C for 24 hr. with a change of dialysis buffer after 12 hrs. The amount of  $5\alpha$ -DHT- $^3$ H in the dialysed supernatants was determined in a liquid scintillation spectrometer with aquasol as the scintillation fluid.

The crude nuclear fractions were purified by sedimentation through a sucrose gradient (11). The gradient was prepared by adding, successively, 5.0 ml of 2.2 M sucrose + 0.5 mM  $CaCl_2$ , 5.0 ml of 1.8 M sucrose + 0.5 mM  $CaCl_2$  and 20 ml of 0.88 M sucrose + 1.5 mM  $CaCl_2$ . The crude nuclei were suspended in 1.0 ml of 0.88 M sucrose + 1.5 mM  $CaCl_2$  and layered on top of the gradients. The gradients were then centrifuged for 90 min. at 33,000 x g in a Beckman I2-65 centrifuge with the SW 27.1 rotor. The purified nuclear pellets were suspended in 3.0 ml of 0.01 M Tris-HCl pH 7.0 that contained 0.05 mM EDTA, 5.0 mM  $MgCl_2$ , 0.5 mM mercaptoethanol and 0.6 M NaCl. These suspensions were sonicated at 65 W for 3 min. with an Ultrasonic Model W 185 sonifier. Samples of these suspensions were taken for the determination of radioactivity in a liquid scintillation spectrometer with aquasol as the scintillation fluid.

Supernatant protein was determined by the Lowry method (12) with bovine serum albumin as the standard. Nuclear DNA was determined with the Burton method (13). DNA type IV (Sigma) was used as the standard.

## RESULTS AND DISCUSSION

### System Development:

The screening system employed in these investigations is a combination of several procedures previously published by other authors. We performed several preliminary experiments in order to define and validate the system.

The molar concentration of  $5\alpha$ -DHT- $^3$ H used routinely in the system and the length of incubation time were determined by experiments reflected in Figures I-IV. In Figure I, the relationship between the concentrations of  $5\alpha$ -DHT- $^3$ H and the amount of binding to receptor proteins indicated that a concentration of approximately 16.7 nanomolar would be satisfactory for the routine screening of competitive binding

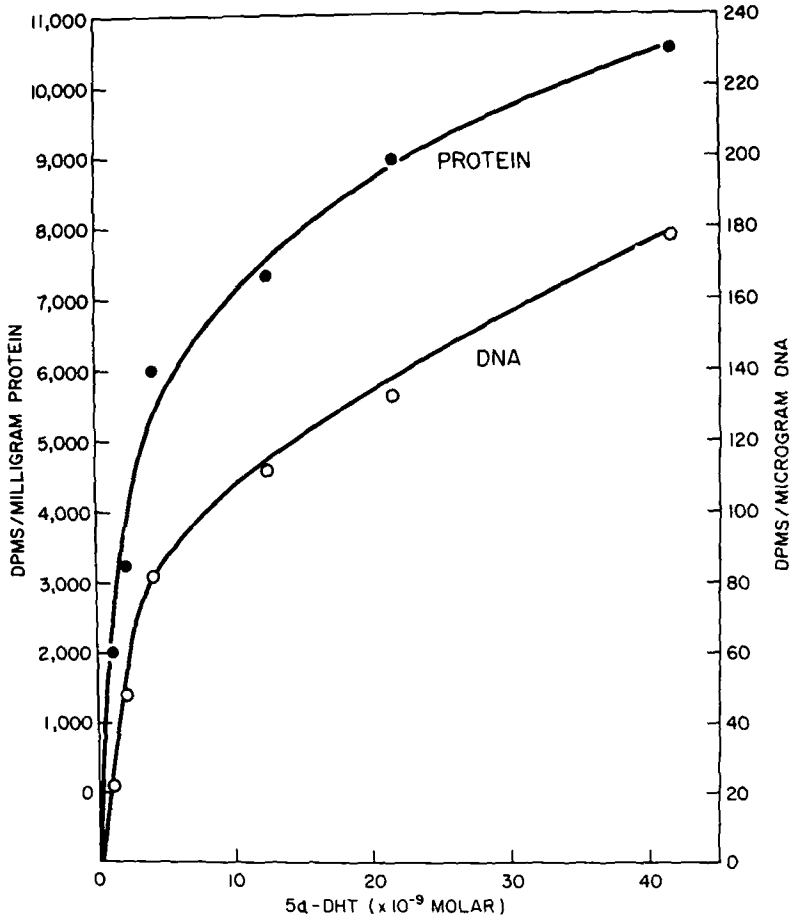


Fig. I. Binding of  $5\alpha$ -DHT- $^3$ H at varying nanomolar concentrations.

0.5 gm. of minced prostate tissue was incubated at  $30^{\circ}\text{C}$  for 30 minutes in 3.0 ml. Eagles Basal Medium with Earle's salts and L-glutamine. ●—● Binding in the 100,000 x g supernatant; ○—○ Binding in the purified nuclei.

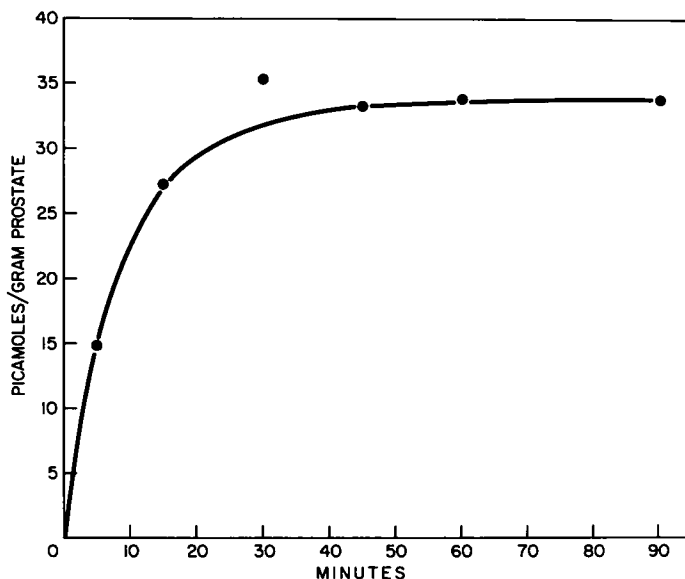


Fig. II. Uptake of  $5\alpha$ -DHT- $^3$ H by minced prostatic tissue.

0.5 gm. of tissue was incubated at  $30^{\circ}\text{C}$  in 3.0 ml. Eagles Basal Medium with Earle's salts and L-glutamine. Concentration of  $5\alpha$ -DHT- $^3$ H was  $1.67 \times 10^{-8}$  M. Aliquots of the whole homogenate were counted and equated to tissue weight by the total volume of the homogenate.

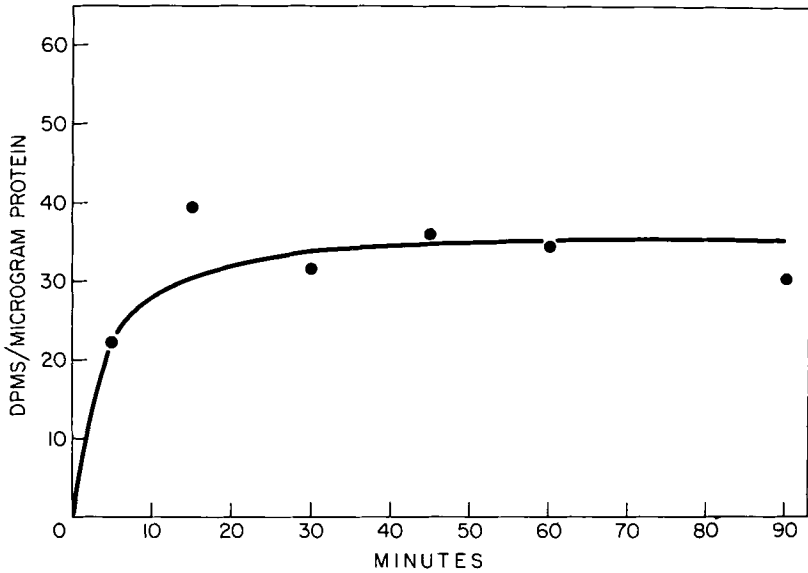


Fig. III. Saturation of the binding sites of the receptor proteins in the 100,000 x g supernatant.  
Incubation data same as for Figure II.

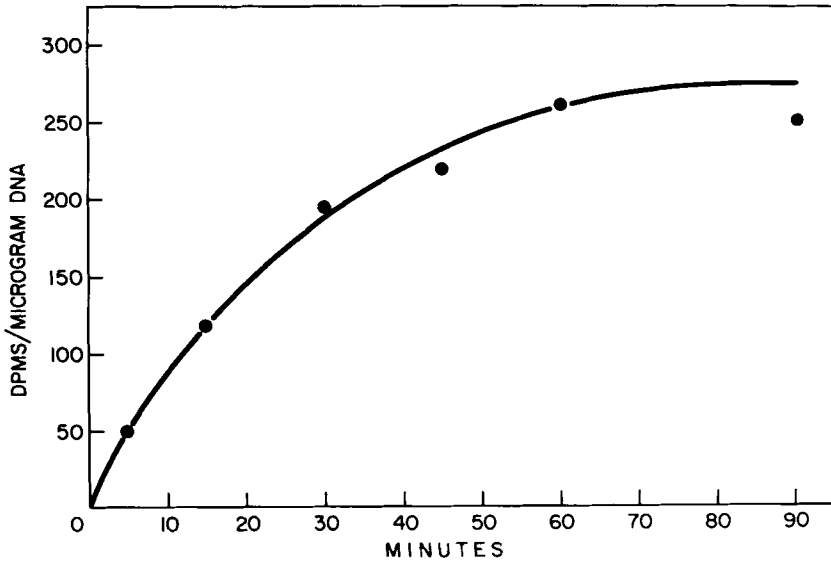


Fig. IV. Nuclear retention of tritiated 5 $\alpha$ -DHT.  
Incubation data same as for Figure II.

of other steroids. This concentration precluded any possible masking of inhibitory responses of the test steroids by using excessive amounts of tritiated  $5\alpha$ -DHT and yet was sufficient to approach the saturation levels of the protein receptors. Figures II-IV demonstrate the amount of radioactivity taken up during varying incubation periods. From this data a 30 minute incubation period was routinely instituted. The time lag for the nuclei, when compared to the supernatant, to reach saturation was expected and is in agreement with the two step theory, which necessitates first the complexing of androgens to receptor proteins in the cytosol before transport into the nuclei (5,7).

When 0.5 gm of minced tissue was incubated under the above prescribed conditions a 41.6 percent uptake of total radioactivity was obtained. Analysis of the 100,000 x g supernatants from these experiments by gel-filtration on a G-200 Sephadex column revealed an association of radioactivity with the protein peak and also the presence of free or unbound radioactivity. However, exhaustive dialysis of the supernatant prior to gel-filtration excluded the unbound radioactivity and retained the radiolabeled protein profile. Dialysis caused a 56.3 percent loss of radioactivity in the supernatant. The elution peaks in both cases are similar to those reported by Fang, et al. for complex II (2).

#### Effect of steroid analogues on the binding of $5\alpha$ -DHT- $^3\text{H}$

The ability of different steroids to inhibit the binding of  $5\alpha$ -DHT- $^3\text{H}$  to both supernatant and nuclei of prostate minces is shown in Table 1. The steroids were placed in two groups depending on whether they were derivatives of 19-nor- $5\alpha$ -DHT (Group I) or  $5\alpha$ -DHT (Group II). In Group I, all compounds except 7 displayed a strong inhibitory effect.

The low activity of the  $5\beta$  analog (7) which contains the A/B cis stereochemistry is consistent with the lack of in vivo androgenic activity of such compounds. These results confirm the previous noted importance of an essentially planar ring system for optimal hormonal activity (5,9).

The availability of a larger number of  $5\alpha$ -DHT analogs (Group II) afforded a better opportunity to explore the effects of structural modification on binding activity within this group. The binding affinities for these steroids were also consistent with the reported in vivo androgenicity of the compounds except for the variable results shown by the 17-acetate derivatives.

Previous in vivo studies dealing with testosterone esters have been controversial. It has been postulated that they become biologically active only after hydrolysis to the free alcohol, testosterone (15,16,17). Under the conditions of the present experiments an esterase present in the minces during incubation could give rise to the free alcohol and would explain the high activity of 9, 11 and 13 which was indistinguishable from the free alcohol 8, 10 and 12, respectively.

If a specific esterase for testosterone esters is present in the incubation media, this could account for the lower activity shown for the  $2\alpha$ -bromo (19) and  $2\alpha$ -fluoro (21) esters. Such modified testosterone esters would be less suitable as substrates for a specific testosterone esterase and would remain intact for a longer period of time. Such speculation is supported by the recent discovery of a specific testosterone esterase in rat brain (15).

Moderate alterations in the A ring are acceptable in terms of maintaining inhibitory activity. A methyl group at the  $1-\alpha$  position (16), seemingly does not alter the inhibitory activity. The substitu-



TEST STEROIDS (Table I)

GROUP I	19-Nor-5 $\alpha$ -Dihydrotestosterone Derivatives	% INHIBITION	
		SUPERNATANT	NUCLEI
1.	19-Nor-5 $\alpha$ -DHT .....	84.0 .....	89.1
2.	17-Acetate .....	89.8 .....	90.3
3.	7 $\alpha$ -Methyl-4-Dehydro .....	86.1 .....	90.9
4.	7 $\alpha$ , 17 $\alpha$ -Dimethyl-4-Dehydro .....	86.5 .....	90.9
5.	2 OXA-4, 9-Dehydro .....	90.5 .....	94.4
6.	17 $\alpha$ -Methyl-2OXA-4, 9-Dehydro .....	88.1 .....	93.5
7.	19-Nor-5 $\beta$ -DHT-17-Acetate .....	34.1 .....	39.8
GROUP II			
	5 $\alpha$ -Dihydrotestosterone Analogues		
8.	5 $\alpha$ -DHT .....	88.6 .....	88.1
9.	17-Acetate .....	79.7 .....	80.1
10.	4-Dehydro (Testosterone) .....	91.2 .....	92.4
11.	4-Dehydro-17-Acetate .....	86.2 .....	89.0
12.	1-Dehydro .....	90.9 .....	93.6
13.	1-Dehydro-17-Acetate .....	90.7 .....	89.6
14.	1-Dehydro-3, 17-Acetate .....	37.4 .....	26.5
15.	17 $\alpha$ -Methyl-1-Dehydro .....	86.1 .....	94.1
16.	1 $\alpha$ -Methyl .....	92.2 .....	93.4
17.	1 $\alpha$ , 2 $\alpha$ -Epoxy .....	28.8 .....	44.7
18.	2 $\alpha$ -Bromo .....	91.7 .....	91.1
19.	2 $\alpha$ -Bromo-17-Acetate .....	40.2 .....	33.3
20.	2 $\alpha$ -Fluoro .....	71.0 .....	76.4
21.	2 $\alpha$ -Fluoro-17-Acetate .....	41.4 .....	37.0
22.	17-Dehydro (Androstanedione) .....	57.9 .....	47.5
GROUP III			
23.	Cyproterone .....	77.1 .....	80.2
24.	17 $\beta$ -Estradiol .....	57.8 .....	24.7

Table 1. The competitive effects of various non-radiolabeled steroids on the binding of 5 $\alpha$ -DHT-<sup>3</sup>H in both the supernatant and nuclei of prostatic minces.

The concentrations of 5 $\alpha$ -DHT-<sup>3</sup>H and the test steroids that were added to the incubation media, were 1.67 x 10<sup>-8</sup> M and 1.67 x 10<sup>-6</sup> M respectively. The inhibitory values listed in this table were produced by comparing the test steroids results with control values in individual experiments on a basis of dpm/mgm protein in the dialysed 100,000 x g supernatant and dpm/microgram DNA in the nuclei. Values listed represent duplicate samples with less than a 5.0 percent variance between samples.

The IUPAC nomenclature for the trivial names of the steroids listed table 1 are as follows: (1.) 17 $\beta$ -Hydroxy-5 $\alpha$ -estran-3-one (2.) 17 $\beta$ -Hydroxy-5 $\alpha$ -estran-3-one Acetate (3.) 17 $\beta$ -Hydroxy-7 $\alpha$ -methylene-4-en-3-one

(4.) 17 $\beta$ -Hydroxy-7 $\alpha$ ,17 $\alpha$ -dimethylestra-4-en-one (5.) 17 $\beta$ -Hydroxy-2-oxa-estra-4,9-diene-3-one (6.) 17 $\beta$ -Hydroxy-17 $\alpha$ -methyl-2-oxa-estra-4,9-diene-3-one (7.) 17 $\beta$ -Hydroxy-5 $\beta$ -estran-3-one Acetate (8.) 17 $\beta$ -Hydroxy-5 $\alpha$ -androstan-3-one (9.) 17 $\beta$ -Hydroxy-5 $\alpha$ -androstan-3-one Acetate (10.) 17 $\beta$ -Hydroxy-4-androsten-3-one (11.) 17 $\beta$ -Hydroxy-4-androsten-3-one Acetate (12.) 17 $\beta$ -Hydroxy-5 $\alpha$ -androst-1-en-3-one (13.) 17 $\beta$ -Hydroxy-5 $\alpha$ -androst-1-en-3-one Acetate (14.) 5 $\alpha$ -Androst-1-en-3 $\beta$ ,17 $\beta$ -diol Diacetate (15.) 17 $\beta$ -Hydroxy-17 $\alpha$ -methyl-5 $\alpha$ -androst-1-en-3-one (16.) 17 $\beta$ -Hydroxy-1 $\alpha$ -methyl-5 $\alpha$ -androstan-3-one (17.) 1 $\alpha$ ,2 $\alpha$ -Epoxy-17 $\beta$ -hydroxy-5 $\alpha$ -androstan-3-one (18.) 2 $\alpha$ -Bromo-17 $\beta$ -hydroxy-5 $\alpha$ -androstan-3-one (19.) 2 $\alpha$ -Bromo-17 $\beta$ -hydroxy-5 $\alpha$ -androstan-3-one Acetate (20.) 2 $\alpha$ -Fluoro-17 $\beta$ -hydroxy-5 $\alpha$ -androstan-3-one (21.) 2 $\alpha$ -Fluoro-17 $\beta$ -hydroxy-5 $\alpha$ -androstan-3-one Acetate (22.) 5 $\alpha$ -Androstan-3,17-dione (23.) 6-Chloro-1 $\beta$ ,2 $\beta$ -dihydro-17-hydroxy-3'H-cyclopropo [1,2]pregna-1,4,6-triene-3,20-dione Acetate (24.) 1,3,5(10)-Estratriene-3,17 $\beta$ -diol.

tution of a halogen at the 2 $\alpha$ -position, 18 and 20, also does not affect inhibition. The 1-dehydro derivatives, 12, 13 and 15, are equally as active as testosterone. This activity may reflect intrinsic activity of the compounds or it may represent the ability of the prostate cells to enzymatically reduce the 1-dehydro position as well as the 4-dehydro position. Regardless of the exact inhibitory activity, all steroids investigated gave approximately the same extent of inhibition of 5 $\alpha$ -DHT binding to supernatant and nuclei.

The inhibitory activity of several of the most active compounds was investigated in more detail. These included the two oxa-derivatives, 19-nor-5 $\alpha$ -DHT-17-acetate, 1-dehydro-5 $\alpha$ -DHT, 1-dehydro-5 $\alpha$ -DHT-17-acetate, 17- $\alpha$ -methyl-1-dehydro-5 $\alpha$ -DHT, and 1- $\alpha$ -methyl-5 $\alpha$ -DHT. The amount of inhibition of binding of 5 $\alpha$ -DHT-3H to supernatant and nuclei by prostate minces was determined with different concentrations of the steroid analogues. The results are compared in Figure V with the inhibition of binding obtained with unlabeled 5 $\alpha$ -DHT and testosterone. Again, the amount of inhibition produced in the supernatant and nuclei are similar at all concentrations studied. The oxa-estratrienes (5 and 6) were the most active inhibitors. Compound 6 gave 50% inhibition at

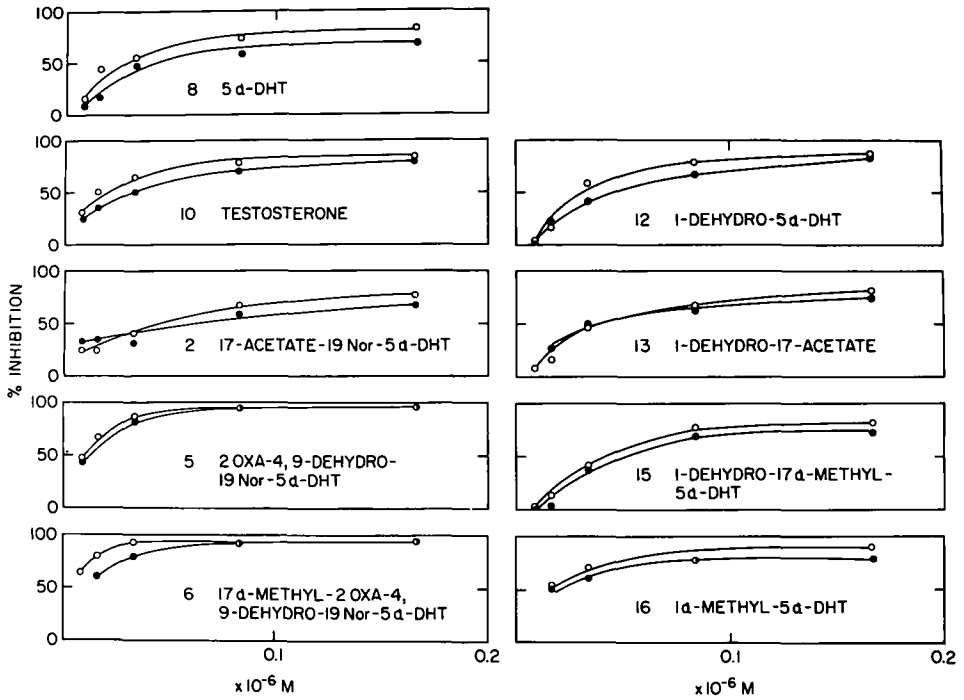


Fig. V. Inhibition curves of some of the more active test steroids.

The test steroids concentrations varied between  $8.33 \times 10^{-9}$  M and  $1.67 \times 10^{-6}$  M. The concentration of  $5\alpha$ -DHT- $^3$ H and incubation data are the same as for Figure II, with the exception that incubation period was 30 minutes. ○—○ 100,000 x g supernatant; ●—● purified nuclei.

about 0.007 M. Compound 5 appeared to be slightly less active with a 50% inhibition at 0.010 M. These activities can be compared to 5 $\alpha$ -DHT which gave 50% inhibition at 0.028 M. The results are consistent with the results of Liao *et al.* (9) who reported the oxa-estratrienes to be more potent androgens than 5 $\alpha$ -DHT.

In Figure VI the kinetics of uptake and binding in the presence of 2-oxa-4, 9-dehydro-19-nor-5 $\alpha$ -DHT (5) in timed incubations show that even though there was considerable uptake of 5 $\alpha$ -DHT-<sup>3</sup>H into the prostate cells in the presence of the test compound there was virtually no binding of 5 $\alpha$ -DHT-<sup>3</sup>H in either the supernatant or nuclei. In this experiment the amount of 5 $\alpha$ -DHT-<sup>3</sup>H in the homogenate after incubation was reduced by approximately 40.0 per-cent. In order to more clearly establish whether 2-oxa-4, 9-dehydro-19-nor-5 $\alpha$ -DHT had any effect upon the prostate cells an additional experiment was performed. Prostate minces were incubated for 30 minutes in the presence of 5 $\alpha$ -DHT-<sup>3</sup>H and 2-oxa-4, 9-dehydro-19-nor-5 $\alpha$ -DHT. Inulin-<sup>3</sup>H was included in control incubations to measure the volume of extracellular space, thus enabling the calculation of the amount of 5 $\alpha$ -DHT-<sup>3</sup>H present intracellularly. The amount of intracellular 5 $\alpha$ -DHT-<sup>3</sup>H was calculated to be 25.1 pico moles in the control and 24.7 pico moles in the presence of 2-oxa-4, 9-dehydro-19-nor-5 $\alpha$ -DHT. These pico mole concentrations represent approximately 92.0 per-cent of the total prostatic uptake of radioactivity. This strongly suggests that the effect of the steroid analogue is not on the uptake process but is specifically associated with the binding to the receptor proteins.

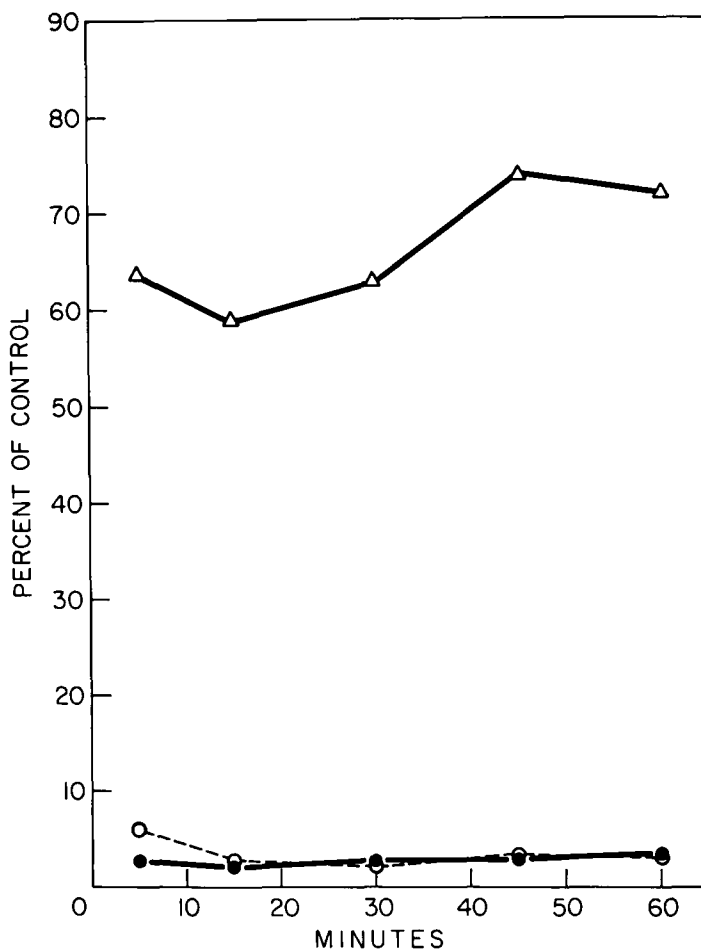


Fig. VI. Timed incubations of derivative 5, 2-oxa-4, 9-dehydro-19-nor-5 $\alpha$ -DHT, compared to timed control incubations.

▲—▲ whole homogenate; o----o 100,000 x g dialysed supernatant; ●—● purified nuclei.

## ACKNOWLEDGMENTS

This investigation was funded by the Veterans Administration, project number 7169:02.

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