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CHARACTERIZATION OF THE 3α-HYDROXYSTEROID DEHYDROGENASE OF DOG PROSTATE

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SUMMARY

The effects of 31 different steroids on the formation of 3α-androstanediol from [1,2-^{3}H]-dihydrotestosterone were assessed in both microsomal and cytosolic fractions of dog prostate. Several features of the substrate specificity of the reaction were deduced from these studies. First, 4-ene-3-keto steroids and 5β-reduced steroids were weak or inactive inhibitors of the reaction. Second, oxo substitution at C_6 or C_{11} abolished the inhibitory ability of 5α-reduced steroids. Third, 2α-halogenated androstanes were good inhibitors, and, in particular, the 2α-bromo steroids (2α-bromo-androstanedione and -dihydrotestosterone) were at least five fold more inhibitory than the parent androstane. Fourth, the 3-keto function of the steroid nucleus is critical whereas a 17-oxo-group (hydroxyl or keto) is not essential. Fifth, the relative inhibition of the microsomal and cytosolic enzyme correlated closely, a finding compatible with enzymic identity of the two intracellular fractions.

INTRODUCTION

The administration to the castrate dog of pharmacological amounts of 3α-androstanediol,* alone or in combination with oestradiol-17β, results in profound growth of the prostate, comparable to that found in the naturally occurring canine hypertrophic prostate [1]. In contrast, the administration of testosterone or 5α-dihydrotestosterone in similar amounts is less effective in causing prostatic growth [1, 2]. It has also been noted that the activity of microsomal and cytoplasmic enzymes that convert 5α-dihydrotestosterone to 3α-androstanediol are on average 10-fold higher in the hypertrophic than in the immature dog prostate [3]. These findings suggest the possibility that the formation of 3α-androstanediol may play a role in the regulation of prostatic growth in the dog.

Although considerable information is available as to the substrate specificity and kinetic characteristics of the cytosolic 3α-hydroxysteroid dehydrogenase of rat prostate [4], the analogous enzyme(s) of dog prostate have not been investigated in such detail [3]. The current studies were conducted in an attempt to gain insight into the process by which 3α-androstanediol is formed in dog prostate. The effects of thirty-one steroids on the activity of the 3α-hydroxysteroid dehydrogenase have been assessed in cytosol and microsomes, and the results indicate that several modifications of the A and B rings of potential substrates profoundly affect enzyme activity.

* The trivial names used are: 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid: NAD (P)$^+$ oxidoreductase EC 1.1.1.50; 5α-dihydrotestosterone hemisuccinate, 3-oxo-5α-androstane-17β-yl hemisuccinate; 3α-androstanediol, 5α-androstane-3α,17β-diol; 3β-androstanediol, 5α-androstane-3β,17β-diol; cortexolone, 17,21-dihydroxy-4-pregnene-3,20-dione.

MATERIALS AND METHODS

Prostates (12.1 ± 3.3 S.E.M. g in weight) were removed from mongrel dogs immediately after death and stored in ice cold saline. The adherent connective tissue was removed, and the prostates were processed immediately. The prostates were homogenized and separated into 104,000 *g* supernatant (cytosol) and 104,000 *g* sediment (microsomes) as before [3].

Two assay procedures were used: In experiments utilizing a fixed inhibitor and variable substrate concentration, the assay contained 1 μM inhibitor, 0.05–2.5 μM [1,2-^{3}H]-5α-dihydrotestosterone, 0.5 mM NADH, cytosol or microsomes, 0.5 mM EDTA, and 0.1 M Tris-chloride and 0.1 M Na citrate, pH 6.5, in a total vol. of 0.2 ml. In experiments utilizing a fixed substrate and variable inhibitor, the assay contained 0.05 μM [1,2-^{3}H]-5α-dihydrotestosterone, 0.1–2.5 μM inhibitor, 0.5 mM NADH, cytosol or microsomes, 0.5 mM EDTA, and 0.1 M Tris-chloride and 0.1 M Na citrate pH 6.5 in a total vol. of 0.2 ml. The quantity of microsomal or cytosolic protein used in each inhibitor study was that amount required to catalyze reduction of 50% of the dihydrotestosterone substrate (at 0.05 μM concentration); these quantities, determined by a preliminary experiment at 5 different dilutions of each fresh enzyme preparation were 20–400 μg cytosolic protein and 2–20 μg microsomal protein respectively. Substrate and inhibitor steroids were dissolved in chloroform–methanol (2:1, v/v) at a concentration of 10 mM, and appropriate dilutions were prepared in the same solvent. The indicated amounts of steroids in 0.1 ml chloroform–methanol (2:1, v/v) were added to the assay tubes and reconstituted in the final reaction mixture after evaporation of the organic solvent. The samples were incubated for 30 min at 37° in a shaking water bath.

Reactions were stopped by extraction with 1 ml chloroform–methanol (2:1, v/v), and the mixture was vortexed 10 times and centrifuged at 1000 rev./min for 5 min. The organic solvent phase was separated, mixed with 10 μg each of dihydrotestosterone and 3α-androstanediol, and spotted for t.l.c. on plastic sheets precoated with silica gel-G-HY. Following chromatography in the solvent system dichloromethane–ethyl acetate–methanol (85:15:3, by vol.), the steroids were visualized by spraying with anisaldehyde reagent (100 ml glacial acetic acid, 2 ml concentrated H_2SO_4, 1 ml *p*-anisaldehyde) and heating at 100° for 5 min. The zones corresponding to the reference steroids were marked, cut with scissors, added to 10 ml of 0.4% 2,4-diphenyloxazole in toluene–methanol (10:1, v/v), and assayed for radioactivity. 3α-Androstanediol formation was estimated from the fraction of total radioactivity recovered in the area corresponding to 3α- and 3β-androstanediol. In some experiments, t.l.c. was also performed on plastic sheets precoated with aluminum oxide as before in order to separate 3α- and 3β-androstanediols [3]. Calculated K_i values were similar by the two methods.

[1,2-^{3}H]-dihydrotestosterone (45 Ci/mmol) was obtained from New England Nuclear and used at a concentration of 0.05 μM in the assays. Nonradioactive dihydrotestosterone was added to achieve higher concentrations as indicated. Plastic sheets precoated with silica gel G-HY were obtained from the Machery–Nagel Co., and plastic sheets precoated with aluminum oxide F254 (type E) were obtained from E. Merck, Darmstadt. Several of the steroids used (# 10, 11, 13, 15, 16, Table 1) were the gift of Dr. John Babcock of the Upjohn Company, Kalamazoo, MI; the remainder were obtained from Steraloids, Inc. NADH was from P-L Biochemicals.

RESULTS

Previous studies in homogenates of dog prostate have demonstrated that NADH is a more effective cofactor than NADPH for the formation of 3α-androstanediol and that 3α-androstanediol formation is on an average about 20 times that of 3β-androstanediol [3]. Therefore, in the present studies, NADH only was used as coenzyme, and a silica gel t.l.c. system that does not separate 3α- from 3β-androstanediol was routinely utilized. The validity of this assay procedure was substantiated by an experiment in which it was demonstrated that one in-

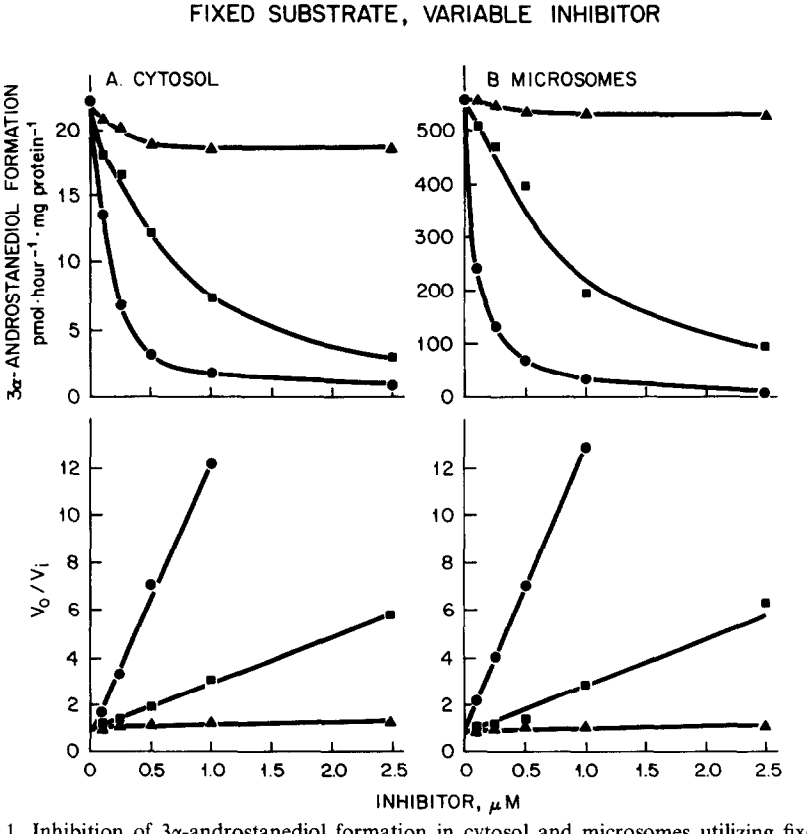

Fig. 1. Inhibition of 3α-androstanediol formation in cytosol and microsomes utilizing fixed substrate and variable inhibitor concentrations. Each assay tube contained 0.05 μM [1,2-^{3}H]-5α-dihydrotestosterone, 0.1–2.5 μM inhibitor, 0.5 mM NADH, cytosol (0.5 mg protein) or microsomes (0.02 mg protein), 0.05 mM EDTA, and 0.1 M Tris-chloride and 0.1 M Na citrate, pH 6.5 in a total vol. of 0.2 ml. In the upper panels are plotted the rates of formation of 3α-androstanediol, and in the lower panels are shown least squares plots of the ratio of the rates in the absence (Vo) or presence (Vi) of inhibitor steroid as a function of inhibitor concentration. ▲——▲, 5α-androstane-3,11,17-trione; ■——■, 2α-cyano-5α-dihydrotestosterone; ●——●, 2α-bromo-5α-dihydrotestosterone.

hibitor of 3α-androstanediol formation (2α-bromo-17β-hydroxy-5α-androstan-3-one) had similar inhibitory effects on the formation of 3α- and 3β-androstanediols by microsomes and cytosol (results not shown).

Two protocols were initially compared to investigate the ability of various steroids to inhibit 3α-androstanediol formation. In the first, the effects of varying the concentration of inhibitor steroid from 0.1 to 2.5 μM on the formation of 3α-androstanediol from 0.05 μM [1,2-^{3}H]-5α-dihydrotestosterone were examined, and the apparent K_i values were estimated from the ratio of the rates of formation in the presence and absence of inhibitor (Fig. 1). In the second the effects of 1 μM inhibitor on the formation of 3α-androstanediol from [1,2-^{3}H]-5α-dihydrotestosterone that varied in concentration from 0.05 to 2.5 μM were examined, and the apparent K_i values were determined from double reciprocal plots (Fig. 2). In these experiments, the apparent K_i values were similar for the two methods (0.50 and 0.52 μM for microsomes and 0.42 and 0.31 μM for cytosol for 2α-cyano-dihydrotestosterone and 0.08 and 0.08 μM for microsomes and 0.05 and 0.09 μM for cytosol for 2α-bromo-dihydrotestosterone). Thus, in the ensuing studies a single method utilizing fixed substrate and variable inhibitor was used.

Studies of the effects of 31 different steroids on 3α-hydroxysteroid dehydrogenase activity are summarized in Table 1. The table is a composite of studies utilizing four different fresh homogenates of dog prostate. In each preparation the apparent K_M values for dihydrotestosterone were similar (0.62 ± 0.18 μM S.E.M. for microsomes and 0.65 ± 0.05 μM S.E.M. for cytosol), and as a consequence it was considered valid to group the data together. The data are of interest in several regards. First, steroids with a 4-5 double bond (testosterone, progesterone, deoxycorticosterone, corticosterone, and cortexolone) and 5β-reduced steroids (5β-androstane-3,17-dione, 5β-dihydrotesterone and 5β-pregnane-3,20-dione), were weak inhibitors of the reaction, implying a critical function for 5α-reduction of the 4-5 double bond in order for a steroid to be an effective inhibitor. Second, oxo-substitutions on the 11 and 6 positions prevented the inhibition completely (5α-androstane-3,6,17-trione, 5α-androstane-3,11,17-trione, and 11β,21-dihydroxy-5α-pregnane-3,20-dione). Third, 2α-bromo- and 2α-fluoro- compounds were more effective inhibitors than the parent steroids (2α-bromo-dihydrotestosterone, 2α-bromo-5α-androstane-3,17-dione, and 2α-fluoro-5α-androstane-3,17-dione), whereas 2α-iodo-5α-androstane-3,17-dione was approximately as effective as 5α-androstane-3,17-

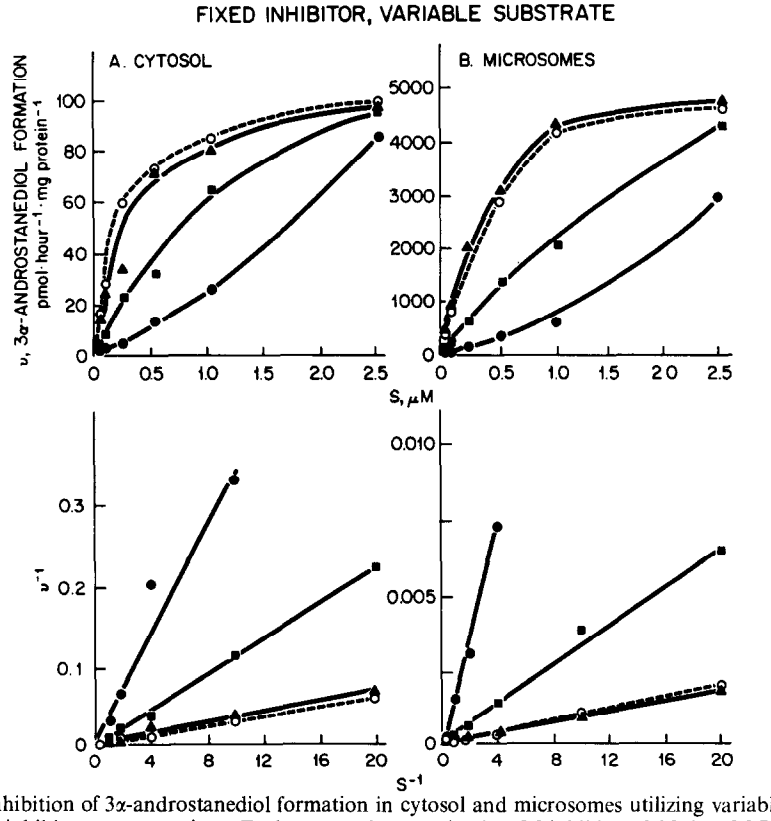

Fig. 2. Inhibition of 3α-androstanediol formation in cytosol and microsomes utilizing variable substrate and fixed inhibitor concentrations. Each assay tube contained 1 μM inhibitor, 0.05–2.5 μM [1,2-^{3}H]-5α-dihydrotestosterone, 0.5 mM NADH, cytosol (0.5 mg protein) or microsomes (0.02 mg protein), 0.5 mM EDTA, and 0.1 M Tris-chloride and 0.1 M Na citrate, pH 6.5 in a total vol. of 0.2 ml. In the upper panels are plotted the rates of formation of 3α-androstanediol, and in the lower panels are shown double reciprocal plots. ▲——▲, 5α-androstane-3,11,17-trione; ■——■, 2α-cyano-5α-dihydrotestosterone; ●——●, 2α-bromo-5α-dihydrotestosterone; ○——○, no additions.

Table 1. Effects of 31 steroids on 3α-androstanediol formation by cytosol and microsomes of dog prostate. The protocol described in Fig. 1 utilizing fixed substrate and variable inhibitor concentration was used in these experiments. Each value represents either a single determination or a mean of 2–3 assays

	Microsomes		Cytosol	
	Ki, μM	$\frac{Km^{DHT}}{Ki^{steroid}}$	Ki, μM	$\frac{Km^{DHT}}{Ki^{steroid}}$
1. 5α-androstane	*	—	*	—
2. 5α-androstan-3-one	2.6	0.2	2.1	0.3
3. 5α-androstan-17β-ol	*	—	*	—
4. 5α-androstane-3,16-dione	2.1	0.3	1.2	0.5
5. 5α-androstane-3,17-dione	1.3	0.5	1.4	0.5
6. 5α-androstane-3,6,17-trione	*	—	5.5	0.1
7. 5α-androstane-3,11,17-trione	*	—	*	—
8. 17α-methyl-5α-dihydrotestosterone	2.3	0.3	2.5	0.3
9. 5α-dihydrotestosterone hemisuccinate	3.7	0.2	3.4	0.2
10. 2α-cyano-17β-methoxy-5α-androstan-3-one	0.81	0.8	0.7	0.9
11. 2α-hydroxymethylene-17β-methoxy-5α-androstan-3-one	6.1	0.1	2.0	0.3
12. 2α-bromo-5α-androstane-3,17-dione	0.12	5.2	0.13	5.0
13. 2α-bromo-5α-dihydrotestosterone	0.08	7.8	0.09	7.6
14. 2α-iodo-5α-androstane-3,17-dione	0.90	0.7	1.0	0.6
15. 2α-fluoro-5α-androstane-3,17-dione	0.28	2.2	0.52	1.3
16. 2α-cyano-5α-dihydrotestosterone	0.87	0.7	0.93	0.9
17. 3β-chloro-5α-androstan-17-one	*	—	*	—
18. 5α-pregnane-3,20-dione	0.60	1.0	0.83	0.8
19. 21-hydroxy-5α-pregnane-3,20-dione	0.87	0.7	0.78	0.8
20. 17,21-dihydroxy-5α-pregnane-3,20-dione	3.9	0.2	2.5	0.3
21. 11β,21-dihydroxy-5α-pregnane-3,20-dione	*	—	*	—
22. 5β-androstane	*	—	*	—
23. 5β-androstane-3,17-dione	*	—	*	—
24. 5β-dihydrotestosterone	2.7	0.2	*	—
25. 5β-pregnane-3,20-dione	5.6	0.1	*	—
26. Testosterone	*	—	*	—
27. Progesterone	*	—	*	—
28. Deoxycorticosterone	*	—	*	—
29. Cortexolone	*	—	*	—
30. Corticosterone	*	—	*	—
31. 2α-cyano-17β-methoxy-4-androsten-3-one	6.1	0.1	*	—

* Inhibitory effect too low to measure.

dione. Fourth, a 2α-carbonitrile substituted steroid (2α-cyano-dihydrotestosterone) was also approximately as effective as the parent steroid, whereas 2α-hydroxymethylene substitution blocked the ability to inhibit. Fifth, whereas a 3-oxo-group was absolutely essential (5α-androstane-17β-ol was totally ineffective), substitution in the 17 position was not an absolute requirement (5α-androstan-3-one was about 1/5 as effective as dihydrotestosterone). Sixth, some substitutions on the 17 carbon have little effect (5α-pregnane-3,20-dione and 21-hydroxy-5α-pregnane-3,20-dione are about as effective as dihydrotestosterone) whereas in two instances 17α-substitutions appeared to reduce the capacity of the steroid to inhibit (17,21-dihydroxy-5α-pregnane-3,20-dione was less effective than 21-hydroxy-5α-pregnane-3,20-dione and 17α-methyl-5α-dihydrotestosterone and 5α-dihydrotestosterone hemisuccinate were less effective than 5α-dihydrotestosterone itself).

Finally, it was of considerable interest that each of the various steroids examined exhibited a similar ability to inhibit the activity of the NADH-linked 3α-hydroxysteroid dehydrogenase in cytosol and microsomes [Fig. 3]. That is, the more potent inhibitors of the cytosolic enzyme were the more potent inhibitions of the microsomal enzyme.

DISCUSSION

The 3-hydroxysteroid dehydrogenase system of dog prostate is exceedingly complicated in that both NADH and NADPH can serve as cofactors for 3-keto reduction, that both 3α- and 3β-hydroxysteroid dehydrogenase activities are present, and that the enzymes are located both in cytosol and in microsomes [3]. In the present study, no attempt was made either to purify these various activities or to characterize each of the various activities in unpurified homogenates. Instead, we have concentrated on the most active of the enzymes, namely the NADH-linked 3α-hydroxysteroid dehydrogenase of microsomes and cytosol [3]. Indeed, the latter two activities may actually be a single enzyme distributed in two subcellular compartments rather than separate enzymes.

With this view in mind, the abilities of 31 different steroids to inhibit competitively the NADH-linked 3α-hydroxysteroid dehydrogenase activities of dog prostate were examined. From these data a number

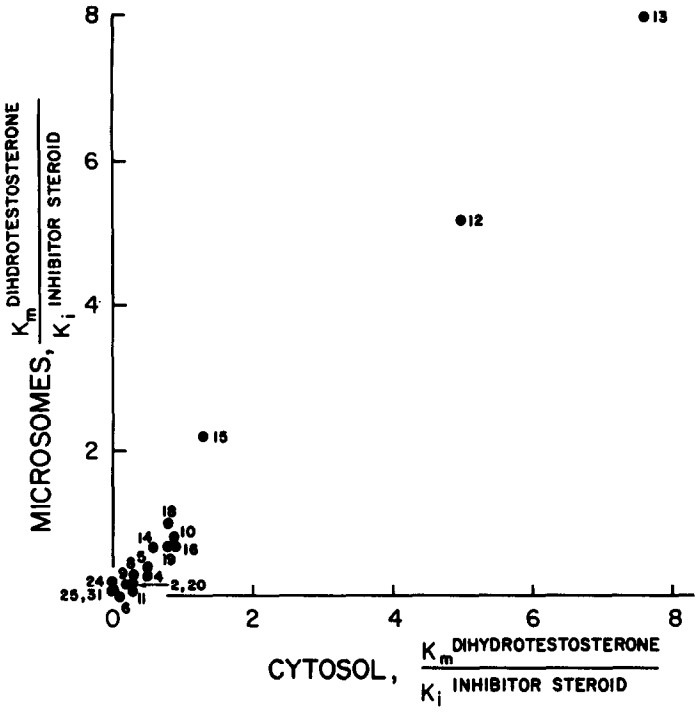

Fig. 3. Comparison of the ability of 19 steroids to inhibit the NADH-linked 3α-hydroxysteroid dehydrogenase activity of cytosol and microsomes relative to the ability of dihydrotestosterone to serve as substrate. The 19 steroids in Table 1 that inhibited the activity of either preparation have been plotted. The regression line was determined by the method of least squares ($y = 0.01 + 1.04\ X$, $r = 0.99$).

of generalizations can be made about the substrate specificity of the enzyme(s) involved. For example, the activity has a distinct property in common with the predominant 3α-hydroxysteroid dehydrogenase of rat prostate in that 11-oxo substitution blocks the ability of steroids to inhibit the activity [4]. However, several properties are different from those in the rat; namely 5β-dihydro derivatives are less effective inhibitors, and 4-ene-3-oxo steroids are totally ineffective as inhibitors in the dog whereas both are potent inhibitors in the rat [4]. Additional information has been derived in the present study that is not available for the rat. For instance, 6-oxo substitution blocks the ability to inhibit, whereas derivatives halogenated in the 2α position are potent inhibitors, particularly 2α-fluoro and 2α-bromo compounds. Likewise, the 2α-carbonitriles of two 5α-reduced steroids are approximately as effective as the parent compounds. Finally, it is of interest that a 3-oxo group is essential for this activity whereas 17 substitution is not required absolutely. These data should make it possible to design steroids than can block the activity of the enzyme at physiologically achievable intracellular concentrations.

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