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### Research Article

Previous studies have suggested that dihydrotestosterone accumulation in the prostate may be involved in the pathogenesis of prostatic hyperplasia in man and dog. However, the fact that the administration of 10 mg dihydrotestosterone/d to castrated, mongrel dogs (0.5 mg/kg body wt) causes little growth in the prostate, whereas identical doses of 3 $\alpha$ - androstanediol regularly induce prostatic hyperplasia (> 14 g weight) has raised the possibility that the dihydrotestosterone accumulation may be the result rather than the cause of the pathology. To investigate the mechanism of this phenomenon, we measured the levels of dihydrotestosterone and 3 $\alpha$ -androstanediol in prostates from 75 dogs. In both naturally occurring and 3 $\alpha$ -androstanediol-induced prostatic hyperplasia, the levels of dihydrotestosterone were high (>5 ng/g), whereas in immature glands and glands from dihydrotestosterone-treated animals, levels were similar (2.1 and 2.6 ng/g, respectively). 3 $\alpha$ -Androstanediol levels were no different in animals treated with dihydrotestosterone or 3 $\alpha$ -androstanediol.

Therefore, because exogenous 3 $\alpha$ -androstanediol is a better precursor of prostatic dihydrotestosterone than exogenous dihydrotestosterone itself, the effects of treatment with larger doses (2.5 mg/kg per d) of dihydrotestosterone and 3 $\alpha$ -androstanediol for 12 wk were examined. In these amounts, dihydrotestosterone was as effective as 3 $\alpha$ -androstanediol in inducing the development of prostatic hyperplasia and in elevating prostatic dihydrotestosterone concentration.

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# Concentration of Dihydrotestosterone and 3 $\alpha$ -Androstanediol in Naturally Occurring and Androgen-Induced Prostatic Hyperplasia in the Dog

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**ABSTRACT** Previous studies have suggested that dihydrotestosterone accumulation in the prostate may be involved in the pathogenesis of prostatic hyperplasia in man and dog. However, the fact that the administration of 10 mg dihydrotestosterone/d to castrated, mongrel dogs (0.5 mg/kg body wt) causes little growth in the prostate, whereas identical doses of 3 $\alpha$ -androstanediol regularly induce prostatic hyperplasia (>14 g weight) has raised the possibility that the dihydrotestosterone accumulation may be the result rather than the cause of the pathology. To investigate the mechanism of this phenomenon, we measured the levels of dihydrotestosterone and 3 $\alpha$ -androstanediol in prostates from 75 dogs. In both naturally occurring and 3 $\alpha$ -androstanediol-induced prostatic hyperplasia, the levels of dihydrotestosterone were high (>5 ng/g), whereas in immature glands and glands from dihydrotestosterone-treated animals, levels were similar (2.1 and 2.6 ng/g, respectively). 3 $\alpha$ -Androstanediol levels were no different in animals treated with dihydrotestosterone or 3 $\alpha$ -androstanediol.

Therefore, because exogenous 3 $\alpha$ -androstanediol is a better precursor of prostatic dihydrotestosterone than exogenous dihydrotestosterone itself, the effects of treatment with larger doses (2.5 mg/kg per d) of dihydrotestosterone and 3 $\alpha$ -androstanediol for 12 wk were examined. In these amounts, dihydrotestosterone was as effective as 3 $\alpha$ -androstanediol in inducing the development of prostatic hyperplasia and in elevating prostatic dihydrotestosterone concentration.

Because dihydrotestosterone accumulates in spontaneous prostatic hyperplasia, because the administration of sufficient amounts of dihydrotestosterone to the

castrated dog can induce the development of prostatic hyperplasia, and because 3 $\alpha$ -androstanediol induces the development of hyperplasia via conversion to dihydrotestosterone, we conclude that accumulation of dihydrotestosterone is the cause of canine prostatic hyperplasia.

## INTRODUCTION

Benign prostatic hyperplasia that results in obstruction to the urethra and/or rectum occurs in males of two species, man and dog (1-3). Although there are histological differences, the natural history, course, and incidence of prostatic hyperplasia in the two species are similar (4, 5). And, in both, it is clear that the process develops only in the presence of an intact testis (6, 7).

In the prostate (as well as in other androgen target tissues) testosterone is irreversibly 5 $\alpha$ -reduced to dihydrotestosterone<sup>1</sup> which can undergo a reversible conversion to both 3 $\alpha$ - and 3 $\beta$ -androstanediols. A growing body of evidence has been assembled over the past several years to suggest that the accumulation of dihydrotestosterone within the prostate may be involved in the pathogenesis of prostatic hyperplasia in man and dog. In brief, these data can be summarized as follows: First, dihydrotestosterone is the intracellular hormone that mediates androgen-induced growth in many tissues (8, 9). Second, the concentration of dihydrotestosterone is elevated in the hyperplastic prostates of man (10, 11) and dog (12, 13). Third, the plasma levels of dihydrotestosterone are elevated in men with hyperplastic prostates (14, 15). Fourth, both canine and

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<sup>1</sup>Abbreviations and nomenclature used in this paper: androstanediol, 5 $\alpha$ -androstane-3,17-dione; 3 $\alpha$ -androstanediol, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol; 3 $\beta$ -androstanediol, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol; dihydrotestosterone, 17 $\beta$ -hydroxy-5 $\alpha$ -androstane-3-one; PBS, phosphate-buffered saline.

human prostatic hyperplasia are accompanied by an increase in the activity of 3 $\alpha$ -hydroxysteroid dehydrogenase (16, 17), and (in the human prostate at least) this increased activity is paralleled by a decreased tissue concentration of 3 $\alpha$ -androstenediol (11). Considered together, these data are compatible with the hypothesis that the accumulation of dihydrotestosterone leads to the hyperplasia of the tissue in the male. Furthermore, the fact that 17 $\beta$ -estradiol enhances androgen-mediated growth (18, 19) and the amount of the cytoplasmic receptor for dihydrotestosterone in the dog prostate (20) suggests that the pathologic consequences of dihydrotestosterone accumulation may be more severe under circumstances of estrogen excess. It is also known that estrogen formation increases in aging men (21).

Attractive as this thesis may be, it has not yet been possible to prove that dihydrotestosterone accumulation is causally linked to the etiology. Indeed, in experiments lasting as long as 2 yr, it was not possible to induce prostatic hyperplasia in the castrated dog by the administration of either dihydrotestosterone or testosterone (22). In contrast, the administration of 3 $\alpha$ -androstenediol (alone or in combination with 17 $\beta$ -estradiol) causes the development of prostatic hyperplasia within 3 mo in the castrated dog (18, 19). This phenomenon suggested the possibility that some androgen metabolite other than dihydrotestosterone might be the mediator of the enlargement.

To provide further insight into this process, we have measured the tissue content of testosterone, dihydrotestosterone, and 3 $\alpha$ -androstenediol in the prostates of 36 control dogs and 39 castrated dogs treated with a variety of hormonal regimens for 3 mo. The results suggest that under all circumstances (even in prostatic hyperplasia induced by 3 $\alpha$ -androstenediol) the size of the prostate gland correlates with the content of dihydrotestosterone plus testosterone in the tissue. Furthermore, when given in sufficient quantities, dihydrotestosterone itself induces the development of prostatic hyperplasia in the castrated dog. We interpret these

findings as support for the thesis that accumulation of dihydrotestosterone may be causally linked to the development of prostatic hyperplasia.

## METHODS

*Animals.* 75 mongrel dogs weighing 14–28 kg (average weight: 21.2 kg) were treated by one of three protocols. In the first study (Table I), 30 control male dogs of varying ages were killed, and the prostates were removed, cleaned, weighed, and frozen in a Revco freezer (Revco, Inc., West Columbia, S. C.) at  $-80^{\circ}\text{C}$ . The average time between death and assays was 2 wk. In the second study (Table II), frozen prostates from a previous study (19) were used. The protocol for hormone-replacement studies in castrated dogs has been described (19). In brief, under anesthesia, dogs were castrated, and the size of the prostate was estimated from three-dimensional measurements (19). Noncastrates and castrates were treated with an injection of either 1 ml of triolein three times a week or 1 ml of triolein that contained testosterone, dihydrotestosterone, 3 $\alpha$ -androstenediol, dihydrotestosterone plus 17 $\beta$ -estradiol, or 3 $\alpha$ -androstenediol plus 17 $\beta$ -estradiol. The total weekly doses were 75 mg for the three androgens and 750  $\mu\text{g}$  for 17 $\beta$ -estradiol. After 12 wk the animals were killed, and the prostates were processed as above. These prostates were frozen for an average of 18 mo before extraction. In the third study (Table III), prostates were measured, and the dogs were castrated and treated three times a week with 2.5 ml of triolein that contained pharmacological (fivefold) amounts of 3 $\alpha$ -androstenediol or dihydrotestosterone. The total weekly dose in each instance was 375 mg. After 12 wk the dogs were killed, and the prostates were frozen for 1 wk before extraction.

In three instances dogs were castrated and adrenalectomized and maintained only on dexamethasone (4 mg/wk for 3–8 wk before death).

*Homogenization of prostates.* Samples weighing 2.5 g were minced with scissors, transferred to 25  $\times$  200-mm test tubes, and mixed with 3 vol of water. A mixture of authentic [1,2,6,7- $^3\text{H}$ ]testosterone, [1,2,4,5,6,7,16,17- $^3\text{H}$ ]dihydrotestosterone, [1,2,4,5,6,7,16,17- $^3\text{H}$ ]3 $\alpha$ -androstenediol, and, in the initial experiments, [1,2,4,5,6,7,16,17- $^3\text{H}$ ]3 $\beta$ -androstenediol (5,000–6,000 dpm of each steroid) in 200  $\mu\text{l}$  of methanol was added to serve as recovery standards. The tubes were immersed in ice, and the mixtures were homogenized with a Polytron PT30 tissue homogenizer (Polytron Corp., Elkhart, Ind.) set at 90% of maximal intensity for three 20-s periods with cooling for 30-s periods after each homogenization. The final 25% wt/vol homogenate was decanted into 125-ml glass bottles. After 5 vol of dichloromethane was added, the bot-

TABLE I  
Androgen Content of Prostate in Intact Control Dogs

Group	Mean prostate weight	Androgen content		
		Dihydrotestosterone	Testosterone	3 $\alpha$ -Androstenediol
	<i>g</i> *	<i>ng/g</i>		
I. Immature	(8)	2.4 $\pm$ 0.6	0.29 $\pm$ 0.15	0.31 $\pm$ 0.22
II. Mature	(10)	10.8 $\pm$ 1.8	1.37 $\pm$ 0.38	0.76 $\pm$ 0.28
III. Hypertrophic	(12)	25.7 $\pm$ 2.7	3.48 $\pm$ 1.20	1.28 $\pm$ 0.48

Normal dogs were killed, and the prostates were removed, weighed, and frozen. Number of dogs shown in parentheses.

\* All values shown are mean $\pm$ SEM.

TABLE II  
Androgen Content of Prostate of Dogs Treated with Conventional Amounts of Androgens for 12 wk

Group		Mean prostate weight		Androgen content		
		Initial	Final	Dihydro- testosterone	Testosterone	3 $\alpha$ -Androstanediol
<i>g*</i>						
I. Intact controls	(6)	10.8 $\pm$ 2.3	11.4 $\pm$ 3.1	3.65 $\pm$ 0.92	0.78 $\pm$ 0.36	1.79 $\pm$ 0.16
II. Castrate controls	(5)	11.3 $\pm$ 2.8	3.8 $\pm$ 0.8	0.25 $\pm$ 0.03	0.05 $\pm$ 0.02	1.43 $\pm$ 0.25
III. Castrate plus testosterone	(4)	11.0 $\pm$ 4.1	6.0 $\pm$ 2.5	2.12 $\pm$ 0.70	0.37 $\pm$ 0.14	1.63 $\pm$ 0.22
IV. Castrate plus dihydrotestosterone	(5)	12.5 $\pm$ 2.8	7.4 $\pm$ 1.4	2.64 $\pm$ 0.75	0.16 $\pm$ 0.10	1.87 $\pm$ 0.35
V. Castrate plus 3 $\alpha$ -androstanediol	(7)	10.7 $\pm$ 2.2	19.7 $\pm$ 3.4	8.82 $\pm$ 1.31	0.16 $\pm$ 0.06	2.14 $\pm$ 0.43
VI. Castrate plus dihydrotestosterone plus 17 $\beta$ -estradiol	(6)	5.3 $\pm$ 0.9	6.5 $\pm$ 1.4	2.70 $\pm$ 0.89	0.05 $\pm$ 0.01	2.10 $\pm$ 0.90
VII. Castrate plus 3 $\alpha$ -androstanediol plus 17 $\beta$ -estradiol	(5)	8.4 $\pm$ 2.1	43.5 $\pm$ 9.6	4.40 $\pm$ 0.52	0.05 $\pm$ 0.01	1.8 $\pm$ 0.24

The prostates used in this experiment were from dogs that have been described previously (19). In brief, the size of the prostates was estimated at abdominal laparotomy. Intact and castrated controls were given 1 ml of triolein intramuscularly three times a week. The remaining castrated dogs were treated three times a week with 1 ml of triolein that contained testosterone, dihydrotestosterone, 3 $\alpha$ -androstanediol, dihydrotestosterone plus 17 $\beta$ -estradiol, or 3 $\alpha$ -androstanediol plus 17 $\beta$ -estradiol. The weekly doses were 75 mg for the three androgens and 750  $\mu$ g for 17 $\beta$ -estradiol. After 3 mo, the prostates were removed and frozen before processing. Number of dogs shown in parentheses.

\* All values shown are mean $\pm$ SEM.

tles were covered with Saran Wrap (Dow Chemical, Midland, Mich.), tightly capped, and shaken in a wrist-action shaker at 0–4°C for 30 min. The emulsion was centrifuged at 2,000 rpm (1,250 g) for 10 min. The lower phase (dichloromethane) was aspirated with a 50-ml glass syringe fitted with a stainless steel cannula and evaporated at 37°C under a stream of N<sub>2</sub>. The residues were dissolved in 1.25 ml of iso-octane (saturated with ethylene glycol) and stored at 0–4°C. Plasma (2.5 ml) was extracted with 9 vol of ether by shaking as above, and the mixture was stored overnight at –20°C. The ether phase was then decanted from the frozen aqueous phase and evaporated to dryness in a 37°C water bath under a stream of N<sub>2</sub>. The residues were dissolved in iso-octane saturated with ethylene glycol as above.

*Celite chromatography.* Celite was heated overnight in a muffle furnace at 600°C and stored at room temperature in a

dessicator. A mixture of celite:ethylene glycol (1.0:0.5) was prepared and packed into 5-ml disposable serological pipets. The beds of celite, supported by 3-mm glass beads, contained a volume of 1.5 ml ( $\approx$ 0.9 g of celite:ethylene glycol). With the aid of N<sub>2</sub> under pressure, columns were washed twice with 3.5 ml of 50% benzene in iso-octane and twice with 3.5 ml iso-octane. The 50% benzene wash removed significant amounts of nonspecific immunoreactive substances and resulted in reduction of the radioimmunoassay “blank.” After the second wash with iso-octane the columns were used immediately or stored in iso-octane.

Samples in 1.0 ml of iso-octane (saturated with ethylene glycol) were applied to the columns. After the samples had entered the column bed, 1.0-ml aliquots of iso-octane were used to rinse the walls of the pipets. Sequential elutions were performed first with 3 ml iso-octane and then with increasing

TABLE III  
Androgen Content in the Prostate of Dogs Treated with Supraphysiological Doses of Dihydrotestosterone and 3 $\alpha$ -Androstanediol for 12 wk

Group		Mean prostate weight		Androgen content		
		Initial	Final	Dihydrotestosterone	Testosterone	3 $\alpha$ -Androstanediol
<i>g*</i>						
<i>ng/g</i>						
I. Castrate plus fivefold dihydrotestosterone	(4)	9.2 $\pm$ 2.1	22.4 $\pm$ 1.8	19.4 $\pm$ 4.6	0.11 $\pm$ 0.01	4.98 $\pm$ 1.37
II. Castrate plus fivefold 3 $\alpha$ -androstanediol	(3)	9.0 $\pm$ 1.6	21.5 $\pm$ 3.2	16.8 $\pm$ 2.3	0.10 $\pm$ 0.01	8.25 $\pm$ 0.96

The size of the prostate was estimated at abdominal laparotomy, and the dogs were castrated. The dogs were then treated three times a week with 2.5 ml of triolein that contained dihydrotestosterone or 3 $\alpha$ -androstanediol. The weekly dosages were 375 mg for each of the androgens. After 3 mo, the prostates were removed and frozen before processing. Number of dogs shown in parentheses.

\* All values shown are mean $\pm$ SEM.

concentrations of benzene in iso-octane: 3.5 ml 5% benzene, 11.0 ml 20% benzene, 5.5 ml 35% benzene, and 5.5 ml 50% benzene. Elution was performed under  $N_2$  pressure providing flow rates of  $\approx 0.3$  ml/min. Fractions (64 drops,  $\approx 1.0$  ml) were collected, and 0.1-ml aliquots were analyzed for  $^3H$  after the addition of 4.5 ml of toluene that contained 0.5% diphenyloxazole and 2% methanol. From the  $^3H$ -radioactivity elution profile, the zones corresponding to each authentic steroid were identified and pooled by combining the peak fraction plus one fraction on either side. Each combined extract (volume:  $\approx 2.7$  ml) was evaporated to dryness, and the steroids were dissolved in 1.0 ml methanol/g equivalent of tissue. Aliquots were taken for determination of hormone content by radioimmunoassay. For testosterone and dihydrotestosterone assay, duplicate aliquots of 25 and 100  $\mu$ l were used, and for  $3\alpha$ - and  $3\beta$ -androstanediol assays aliquots of 100 and 250  $\mu$ l were used. In addition, aliquots (200  $\mu$ l) were taken for the estimation of recoveries.

**Radioimmunoassay.** Samples of standards (5–500 pg) or unknowns (in methanol) were added to  $12 \times 75$ -mm assay tubes, evaporated under vacuum, and reconstituted in 0.2 ml of phosphate-buffered saline (PBS)-gelatin (0.01  $Na_2HPO_4$ , pH 7.8, 0.9% NaCl, 0.1% gelatin, and 0.01%  $NaN_3$ ) that contained [1,2,4,5,6,7,16,17- $^3H$ ]dihydrotestosterone (7,300 dpm, 5 pg). After mixing, the tubes were allowed to stand at room temperature for  $\approx 10$  min before the addition of 0.1 ml of PBS-gelatin representing a 1:10,000 dilution of an antiserum prepared against 3-carboxymethoxime-testosterone hemocyanin (23). The final dilution of antiserum in the assay mixture was 1:30,000. The assay mixtures (0.3 ml) were incubated at 60°C for 10 min followed by 30°C for 30 min. The tubes were then transferred to an ice bath. After 10 min at 0°C, 0.5 ml of 1% charcoal in PBS-gelatin was rapidly added, and the tubes were vortexed. After 10 additional min at 0°C, centrifugation was performed in the Sorvall HS-4 rotor (Ivan Sorvall, Inc., Norwalk, Conn.) at 3,250 rpm for 5 min. The supernates were decanted into scintillation vials, 10 ml of toluene that contained 0.5% diphenyloxazole and 2% methanol was added, and the two phases were mixed by shaking vigorously for 60 s. The counting efficiency for  $^3H$  in the two-phase counting mixture averaged 54%. Steroids were estimated by logit transformation of the appropriate radioimmunoassay data after correction for nonspecific binding (which averaged  $<1\%$  of the total assay radioactivity).

The relative cross-reactivities of the antibody with the four steroids assayed are shown in Fig. 1A. The displacement curves for dihydrotestosterone and testosterone were similar.  $3\alpha$ -Androstanediol had a cross-reactivity  $\approx 20\%$  as effective as testosterone or dihydrotestosterone.  $3\beta$ -Androstanediol was bound less tightly than  $3\alpha$ -androstanediol by the antiserum and had a relative cross-reactivity of  $\approx 10\%$  that of testosterone or dihydrotestosterone. Logit transformations of the binding curves are summarized in Fig. 1B. The between-assay reproducibility of the method was independent of the steroid being assayed. Average values for picograms of steroid corresponding to logit  $B/B_0 = 0$  were  $21.7 \pm 0.82$  pg for dihydrotestosterone (36 standard curves),  $23.3 \pm 0.95$  pg for testosterone (28 standard curves),  $118 \pm 14$  pg for  $3\alpha$ -androstanediol (14 standard curves), and  $204 \pm 16$  pg for  $3\beta$ -androstanediol (4 standard curves).

The specificity of the antibody to a variety of other steroids had been previously established, and several C18, C19, and C21 steroids, including estradiol, dehydroepiandrosterone, and progesterone have cross-reactivities  $<0.05\%$  (23). Steroids with a cross-reactivity  $>1\%$  are 19-nortestosterone (39%) and androsterone (18%) (24).

Recoveries were estimated routinely from known amounts of authentic  $^3H$ -steroids added before organic solvent extrac-

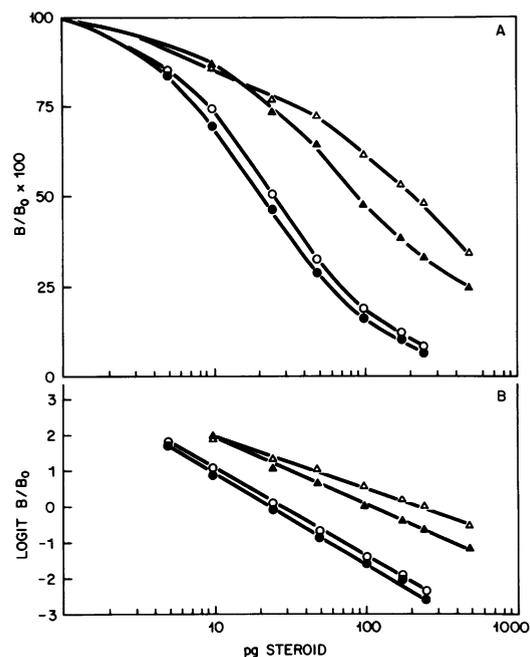


FIGURE 1 Radioimmunoassay standard curves for dihydrotestosterone (●), testosterone (○),  $3\alpha$ -androstanediol (▲), and  $3\beta$ -androstanediol (△). A. Relative binding as a function of increasing concentrations of each steroid. Radioimmunoassays were performed as described in the text. Binding (B) was corrected for nonspecific binding ( $<1\%$ ) and plotted as a percentage of the radioactive [ $^3H$ ]dihydrotestosterone bound in the absence of added steroid ( $B_0$ ). Values for  $B_0$  varied between 39 and 49% of the total  $^3H$ -radioactivity. B. Logit transformation of radioimmunoassay standard curves ( $\ln [B/B_0]/(1-[B/B_0])$ ). The data of panel A have been transformed to generate linear functions for each steroid standard curve, and the best fit was determined by linear regression. Correlation coefficients ranged from 0.992 to 0.999 for each set of standards. Apparent equilibrium constants (picograms of steroid at logit  $B/B_0 = 0$ ) correspond to 22, 26, 101, and 208 pg, respectively, for dihydrotestosterone, testosterone,  $3\alpha$ -androstanediol, and  $3\beta$ -androstanediol.

tion. Approximately 90% of each steroid was recovered in the dichloromethane extracts. After celite chromatography, overall recoveries for 24 samples of prostate averaged  $69.5 \pm 4.2$ ,  $69.4 \pm 2.1$ , and  $71.5 \pm 4.4\%$ , respectively, for tritiated dihydrotestosterone, testosterone, and  $3\alpha$ -androstanediol. When 5 ng of each steroid was added to the extracts, the final recoveries averaged 74.7, 83.5, and 74.6%, respectively for dihydrotestosterone, testosterone, and  $3\alpha$ -androstanediol. Neither the mass nor the  $^3H$ -radioactivity of the recovery standards significantly affected the results of the radioimmunoassay. Samples (25 and 100  $\mu$ l) used for the assay of testosterone or dihydrotestosterone contained on average  $<0.1$  pg of the respective  $^3H$ -recovery standard, well below the sensitivity (2.5 pg) of the assay. In these same samples, the  $^3H$  content, as a result of the presence of recovery standards, averaged 25 and 100 cpm, corresponding to 0.7 and 2.7% of the total radioactivity of the radioimmunoassay. For the assay of  $3\alpha$ - or  $3\beta$ -androstanediol, the larger portions (100 and 250  $\mu$ l) used contained  $<0.3$  pg of steroid and on average 100 and 250 cpm of  $^3H$ . Because antibody binding of both androstanediols was

approximately one-third that of dihydrotestosterone or testosterone, the error introduced by the presence of recovery standards in the radioimmunoassays for each of the steroids was minimal.

**Materials.** All glassware was either disposable or was rinsed with methanol before use. [1,2,6,7-<sup>3</sup>H]Testosterone (85 Ci/mmol) and [1,2,4,5,6,7,16,17-<sup>3</sup>H]dihydrotestosterone (190 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). [1,2,4,5,6,7,16,17-<sup>3</sup>H]3 $\alpha$ -Androstenediol was synthesized enzymatically from dihydrotestosterone with 3 $\alpha$ -hydroxysteroid oxidoreductase from rat ventral prostate cytosol as previously described (25). [1,2,4,5,6,7,16,17-<sup>3</sup>H]3 $\beta$ -Androstenediol was synthesized enzymatically by incubating 200  $\mu$ Ci ( $\approx$ 1 nmol) of [1,2,4,5,6,7,16,17-<sup>3</sup>H]dihydrotestosterone in 0.033 M Na phosphate, pH 6.0, which contained 0.5 mM NADH and 0.1  $\mu$ g of a 3 $\beta$ -hydroxysteroid dehydrogenase preparation from *Pseudomonas testosteronii* (Catalog No. H-9004, Sigma Chemical Co., St. Louis, Mo.) at 25°C for 15 min. All <sup>3</sup>H-steroids were purified by celite chromatography before use. Nonradioactive steroids were from Steraloids, Inc. (Pawling, N. Y.). Solvents (distilled in glass) were from Burdick & Jackson Laboratories, Inc. (Muskegon, Mich.). Celite was from Fisher Scientific Co. (Pittsburgh, Pa.), and chromatography ethylene glycol was from Matheson, Coleman & Bell (East Rutherford, N. J.). Gelatin was type I (swine skin) from Sigma Chemical Co. Charcoal obtained from Mallinckrodt, Inc. (St. Louis, Mo.) was carefully prepared for use by suspending at a concentration of 1% in H<sub>2</sub>O and aspirating the fine particles three times with a J-shaped needle. The charcoal was then sedimented, washed in PBS-gelatin, suspended in PBS-gelatin, and stored at 0–4°C.

## RESULTS

The initial experiments were designed to establish the validity of the chromatographic and radioimmunoassay procedures and to determine whether any androgens other than dihydrotestosterone, testosterone, 3 $\alpha$ -androstenediol, and 3 $\beta$ -androstenediol were present in extracts of plasma or prostate. The antibody chosen for this study is one that reacts with the three principal active androgens (testosterone, dihydrotestosterone, and 3 $\alpha$ -androstenediol), and we assumed that if another 17 $\beta$ -hydroxy androgen were present in large concentrations it would probably cross-react with the antibody in sufficient degree to allow detection. Therefore, the steroid extracts of plasma and prostate from intact controls were applied to celite columns and eluted with increasing concentrations of benzene in iso-octane (Fig. 2). Each fraction was then assayed by radioimmunoassay. In plasma the two major peaks of immunoreactivity corresponded with the elution peaks of authentic [<sup>3</sup>H]dihydrotestosterone and [<sup>3</sup>H]testosterone. No other significant peaks of immunoreactivity were detected. In prostate, four peaks of cross-reacting material were found, corresponding to the elution of dihydrotestosterone, testosterone, 3 $\alpha$ -androstenediol, and 3 $\beta$ -androstenediol. In addition, cross-reacting material was eluted in the initial fractions (fractions 1–10); this material corresponds to the elution profile of several less-polar steroids (including androstenedione, progesterone, and androstenedione) but was not

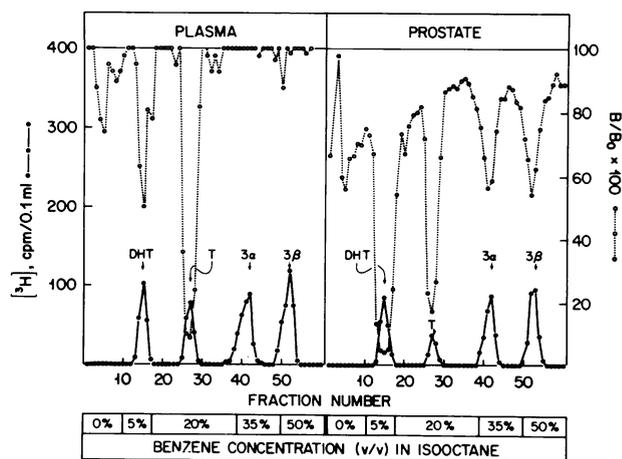


FIGURE 2 Celite chromatography of extracts of plasma and prostate from control dogs. Mixtures of [<sup>3</sup>H]testosterone (T), [<sup>3</sup>H]dihydrotestosterone (DHT), [<sup>3</sup>H]3 $\alpha$ -androstenediol (3 $\alpha$ ), and [<sup>3</sup>H]3 $\beta$ -androstenediol (3 $\beta$ ) were added to plasma (2.5 ml) or prostate (2.5 g). Steroid extraction was performed as described in the text, and the products were dissolved in iso-octane. Aliquots equivalent to 2.0 ml of plasma or 2.0 g of prostate were subjected to celite chromatography on 1.5-ml columns of celite:ethylene glycol (1.0:0.5), and elution was performed with increasing concentration of benzene in iso-octane. Flow rates of  $\approx$ 0.3 ml/min were effected by N<sub>2</sub> pressure. Fractions (32 drops,  $\approx$ 0.5 ml) were collected, and 0.1-ml aliquots were analyzed for <sup>3</sup>H-radioactivity. Other samples (0.1 ml) were evaporated to dryness and analyzed by radioimmunoassay as described in the text. v/v, vol/vol.

investigated further. After the addition of the maximal benzene concentration shown in Fig. 2, 22% ethyl acetate in iso-octane (which elutes 17 $\beta$ -estradiol) did not elute material that cross-reacts with the antibody (results not shown). We concluded from this study that the method does measure the major steroids that cross-react with this particular antibody, recognizing that any androgens that do not react with the antibody would not be detected.

In the prostates from castrated animals each of the C19 steroids was decreased to a fraction of the control level except for 3 $\alpha$ -androstenediol which was not significantly changed (Table II). To determine whether the 3 $\alpha$ -androstenediol in the castrate was derived from adrenal rather than testicular androgen, the content was measured in the prostates of three adrenalectomized and castrated dogs that were maintained on dexamethasone for 3–8 wk; the content of 3 $\alpha$ -androstenediol (1.48 ng/g tissue) was not different than in control animals (1.79 ng/g tissue). To be certain that the moiety eluting in the area of 3 $\alpha$ -androstenediol was in fact 3 $\alpha$ -androstenediol, the eluates containing radioactive 3 $\alpha$ -androstenediol from two prostates were combined and incubated with 3 $\alpha$ -hydroxysteroid dehydrogenase and NADP (25), and the products were rechromatographed on celite columns. Under these

conditions, 59% of the added [ $^3\text{H}$ ]3 $\alpha$ -androstenediol used for tracer and 61% of the immunoreactive material were converted to dihydrotestosterone (results not shown). We concluded that the material that elutes in the 3 $\alpha$ -androstenediol area is 3 $\alpha$ -androstenediol. In view of the fact that the 3-mo castrated prostate consists almost entirely of stromal cells, it is possible that the 3 $\alpha$ -androstenediol of dog prostate is localized in stromal cells and turns over very slowly.

Studies of the content of the three presumed active androgens—testosterone, dihydrotestosterone, and 3 $\alpha$ -androstenediol—in the prostates from 30 control dogs are summarized in Table I. The prostates have been divided into three groups: immature (<5 g), mature (5–14 g), and hyperplastic (>14 g), according to the criteria of Berg (26, 27). In all groups, dihydrotestosterone was the major androgen recovered, accounting for approximately two-thirds of the total. Mean dihydrotestosterone concentration increased from 2.42  $\pm$  0.56 to 4.26  $\pm$  0.81 and 5.18  $\pm$  0.68 ng/g in the mature and hyperplastic groups, respectively. There was also an increase in the mean concentration of testosterone and 3 $\alpha$ -androstenediol in the larger glands. These findings are in substantial agreement with previous measurements of androgen content in the dog prostate (12, 13).

Studies of the androgen content of the prostate in 38 dogs treated with varying regimens are summarized in Table II. In these animals the initial sizes of the prostates were fairly uniform, the mean weight varying from 5.3 to 12.5 g. In the intact controls, mean prostate size did not change significantly during the 12 wk of the study. Prostate size decreased in the animals subjected to castration alone (group II). Neither testosterone (group III) nor dihydrotestosterone (group IV) prevented prostate atrophy. In contrast, dihydrotestosterone plus estradiol-17 $\beta$  (group VI) prevented retrogression of prostate size, and treatment with 3 $\alpha$ -androstenediol, with or without 17 $\beta$ -estradiol (groups V and VII), caused enlargement characteristic of prostatic hyperplasia in 7 of 12 animals.

Dihydrotestosterone concentration in the prostates of castrates fell from 3.65 to 0.25 ng/g, but only minimal decrease was found in animals treated with testosterone or dihydrotestosterone. Dogs treated with 3 $\alpha$ -androstenediol (with or without estradiol) had elevated dihydrotestosterone levels (8.8 and 4.4 ng/g) that were similar to the values found in groups II and III of Table I. Testosterone concentrations were low in all castrated animals except those treated with exogenous testosterone. There were no consistent changes in the concentration of 3 $\alpha$ -androstenediol; for example, there was no difference between the 3 $\alpha$ -androstenediol levels of dogs treated with dihydrotestosterone (1.87  $\pm$  0.35 ng/g) and in those treated with 3 $\alpha$ -androstenediol (2.14

$\pm$  0.43 ng/g), despite the fact that the average weight of the latter was almost three times greater. The concentration of 3 $\alpha$ -androstenediol in animals treated with 3 $\alpha$ -androstenediol was no greater than in the controls, and the concentration of 3 $\alpha$ -androstenediol in the groups treated with androgens plus 17 $\beta$ -estradiol was similar, despite the fact that the weight of the prostates in the group treated with 3 $\alpha$ -androstenediol plus 17 $\beta$ -estradiol averaged sixfold greater than those in animals treated with dihydrotestosterone plus 17 $\beta$ -estradiol.

The relation between the mean concentration of dihydrotestosterone plus testosterone in the gland and the weight in the various groups described in Tables I and II is shown graphically in Fig. 3; for this analysis the sum of testosterone and dihydrotestosterone has been plotted because these hormones (but not 3 $\alpha$ -androstenediol) bind with approximately equal affinity to the androgen receptor of dog prostate (20). However, because the testosterone concentration is low in all castrated animals, the plot is similar to that for dihydrotestosterone alone, except in the intact controls. In all groups, mean concentration of the two steroids was below 3 ng/g in prostates with mean weights <10 g, whereas levels above 4 ng/g were found in the four groups with prostatic weight >10 g. The largest prostates (3 $\alpha$ -androstenediol plus 17 $\beta$ -estradiol) did not have as high a concentration of dihydrotestosterone plus testosterone as the glands from animals with naturally occurring prostatic hyperplasia or those treated with 3 $\alpha$ -androstenediol alone. In view of the fact that massively enlarged glands consist in large part of microcysts (12, 19), it is likely that if the data had been expressed per unit of cell mass the concentra-

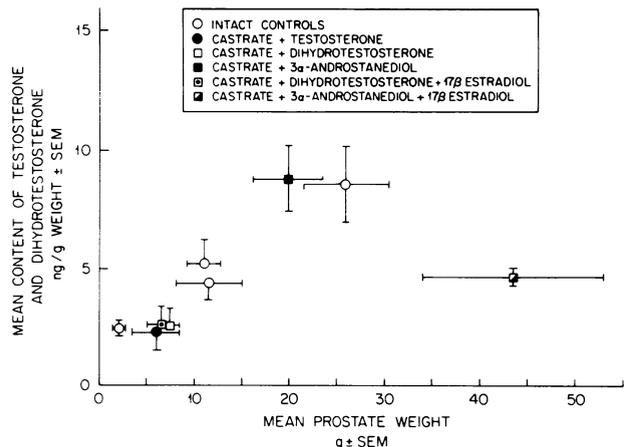


FIGURE 3 The relation between prostate weight and the mean concentration of testosterone plus dihydrotestosterone in the prostate. The intact control groups in Tables I and II are shown by the open circles, and the various treatment groups in Table II are designated by individual symbols.

tion of dihydrotestosterone plus testosterone in this group would have been higher. Unfortunately, DNA was not measured in these experiments.

Thus, pathologic growth of the prostate correlates better with the concentration of dihydrotestosterone plus testosterone than with that of  $3\alpha$ -androstanediol, even in animals in whom prostatic hyperplasia was induced by the administration of  $3\alpha$ -androstanediol. Because exogenously administered  $3\alpha$ -androstanediol is a better precursor of prostatic dihydrotestosterone than an equal dose of dihydrotestosterone, the apparent superiority of the former in inducing prostatic hyperplasia in the castrated dog must be a result of some pharmacological difference between the two steroids rather than of any unique role of  $3\alpha$ -androstanediol as an intracellular mediator of prostatic growth. Were this the case then the administration of a dose of dihydrotestosterone sufficient to cause elevation of prostatic dihydrotestosterone should be as effective as  $3\alpha$ -androstanediol in inducing prostatic growth. Therefore, castrated dogs were treated with extra-large amounts (375 mg/wk) of dihydrotestosterone or  $3\alpha$ -androstanediol for 12 wk (Fig. 4 and Table III). Under these circumstances dihydrotestosterone was as effective in promoting prostate growth as  $3\alpha$ -androstanediol ( $22.4 \pm 1.8$  vs.  $21.5 \pm 3.2$  g), and the concentration of dihydrotestosterone in the prostate was similar in the two groups.

## DISCUSSION

The findings in our study provide an explanation as to why the administration of  $3\alpha$ -androstanediol but not dihydrotestosterone induces the development of prostatic hyperplasia in the castrated dog (18, 19) whereas the androgen receptor in the dog prostate binds dihydrotestosterone and testosterone but not  $3\alpha$ -androstanediol (20). Dihydrotestosterone concentration in the prostate of the dogs treated with 0.5 mg  $3\alpha$ -androstanediol/kg body wt per d was, on average, three times that of dogs treated with the same dose of dihydrotestosterone and equivalent to that in naturally occurring prostatic hyperplasia. The concentration of  $3\alpha$ -androstanediol itself in the gland did not correlate with prostatic growth in castrated animals treated with different hormonal regimens. No evidence was found for the presence of another androgen that might serve as the intracellular mediator of growth. Importantly, when dihydrotestosterone was given in quantities sufficient to produce prostatic dihydrotestosterone levels equivalent to those resulting from treatment with  $3\alpha$ -androstanediol, prostatic hyperplasia was induced.

Considered together with previous findings from this and other laboratories, our study constitutes support for the concept that dihydrotestosterone accumulation

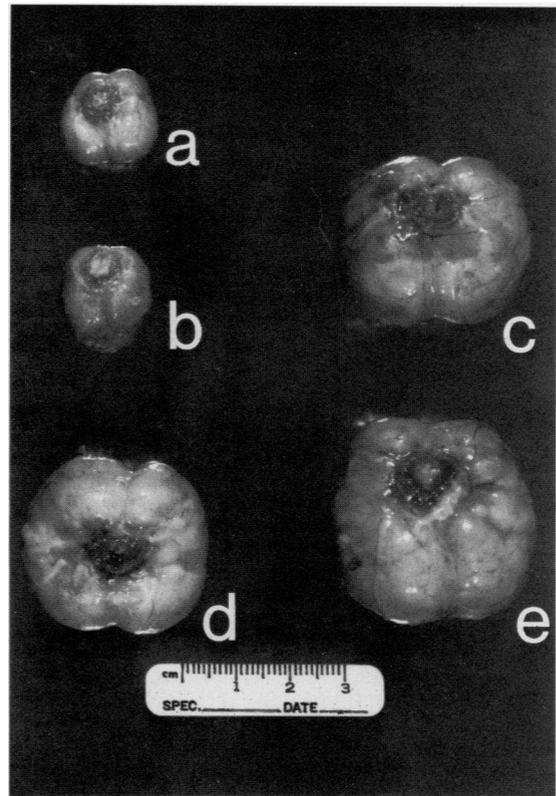


FIGURE 4 Photographs of prostates of castrated and control dogs and of dogs treated with supraphysiological doses of dihydrotestosterone and  $3\alpha$ -androstanediol. (a) Control, immature prostate, 3.2 g; (b) castrate prostate after 12 wk, 3.3 g; (c) spontaneous canine prostatic hyperplasia, 18.8 g; (d) castrated dog given fivefold doses of  $3\alpha$ -androstanediol (375 mg/wk) for 12 wk, 19.1 g; (e) castrate given fivefold doses of dihydrotestosterone (375 mg/wk) for 12 wk, 25 g.

in prostate is causally linked to the development of canine prostatic hyperplasia. Indeed, "Koch's postulates" have now been fulfilled in the sense that we predicted that the accumulation of dihydrotestosterone might be critical in its pathogenesis (10, 12), the dihydrotestosterone content is elevated in spontaneously occurring prostatic hyperplasia in man and dog (10–14), and the disorder has now been produced in the castrated dog by regimens that result in an increase in dihydrotestosterone concentration within the gland. Our present hypothesis is that dihydrotestosterone accumulation (probably together with increased estrogen formation) in aging man and dog results in the development of continued prostatic growth and, eventually, the syndrome of obstructive prostatic hyperplasia. The exact cause of the accumulation of the hormone within the gland remains unexplained.

Our study does not provide an explanation as to why the administration of  $3\alpha$ -androstanediol is more effective

tive than dihydrotestosterone in inducing the accumulation of the latter hormone in the gland and in causing the development of prostatic hyperplasia (22). Possibly, dihydrotestosterone is cleared more rapidly from plasma and tissues so that the steady-state concentration after its administration is low. For whatever reason, it appears that the conventional dose of dihydrotestosterone that we employed in previous studies is not sufficient to induce prostatic hyperplasia in dogs.

It is likewise not known how the high levels of dihydrotestosterone cause prostatic hyperplasia. The levels found in hyperplastic dog prostates are higher than those that would be expected to saturate the androgen receptors (20). Unfortunately, the tissue concentrations as measured in this study reflect the sum total of that in epithelial cells, stroma, and extracellular spaces (including the glandular acini), and it will be necessary to measure hormone concentrations in the various compartments before we can clarify the nature of this discrepancy.

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