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ANDROGENIC ACTIONS OF 5α-ANDROSTANE-3α,17β-DIOL IN RAT SUBMANDIBULAR GLAND

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Summary—To evaluate the action of 5α-androstane-3α,17β-diol(3α-diol) in rat submandibular gland, 5α-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD) and oxidative 3α-hydroxysteroid dehydrogenase (3α-HSDO) activities, and trypsin-like protease activities, were assayed in control, castrated and 3α-diol injected rats. 3α-Diol (1 mg/kg) was injected subcutaneously in castrated male rats daily for 7 days. A 47% decrease of 5α-reductase activity in the nuclei and a 30% decrease of 3α-HSD(O) activities in the cytosol were shown after castration. 3α-Diol restored the 5α-reductase and 3α-HSD(O) activities to 82 and 140% of the control submandibular gland, respectively. 3α-Diol raised the trypsin-like protease activity to near control values in the submandibular gland of castrated rats. Morphological observations also revealed a distinct effect of 3α-diol on the number of granules of granular duct cells. It is concluded that 3α-diol has an androgenic action in the rat submandibular gland. It stimulates the 3α-HSD(O). The 3α-HSD(O) in its turn may be responsible for DHT accumulation in the cells.

INTRODUCTION

Androgens are required for the development and maintenance of granular ducts and of the high concentration of their biologically-active polypeptides [1, 2]. An androgen-dependent increase in protease activity is associated with the development of granular tubules in mouse or rat submandibular gland [3–5]. In androgen target organs, testosterone undergoes 5α-reduction to form dihydrotestosterone (DHT), which is the active form of the androgen [6, 7]. Furthermore, DHT is reduced to 3α-diol by 3α-HSD [8]. Thus the DHT concentration is controlled by two testosterone metabolizing enzymes in androgen target cells. 3α-Diol is important as an endproduct of testosterone metabolism, and 3α-diol and DHT are readily interconvertible [9–12]. It has been reported that 3α-diol induces benign prostatic hyperplasia in young dogs [13, 14]. However, a 3α-diol binding protein has not been demonstrated in the cells. From these reports, it appears that 3α-diol might be an intracellular mediator of androgen actions, including the regulation of testosterone metabolism. To investigate the 3α-diol actions in the tissue, 5α-reductase, 3α-HSD and trypsin-like protease activities and morphological changes were examined in the submandibular gland of control, castrated and 3α-diol-treated rats. Also comparative studies of 5α-reductase and 3α-HSD activities in the submandibular gland and ventral prostate were performed to characterize the submandibular gland as an androgen-dependent organ.

MATERIALS AND METHODS

Animals

Adult male rats (7 weeks of age, 160–190 g) of the Wistar strain were used for this study. Fifteen rats were divided into 3 groups. They were housed at constant temperature (18°C) under artificial light for 12 h daily, with free access to food and water. Ten rats were castrated via the scrotal route and five castrated and five intact rats were untreated. The intact rats were used as controls. The remainder of the castrated animals received 7 daily injections of 1 mg/kg 3α-diol dissolved in sesame oil (1 mg/ml) starting 7 days after castration. The animals were sacrificed with ethyl ether 24 h after the last injection. The submandibular glands and ventral prostates were isolated, weighed and frozen at −70°C until assayed. Samples of the tissue were also taken for histological examination.

Chemicals

[4-^{14}C]Testosterone (SA 50 mCi/mmol), [4-^{14}C]dihydrotestosterone (SA 57 mCi/mmol) and [1,2-^{3}H]-5α-androstane-3α,17β-diol (SA 30.1 Ci/mmol) were purchased from New England Nuclear Corp.,

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Boston, Mass. Authentic steroid preparations, testosterone, DHT and 3α-diol were obtained from Sigma Chemical Co. (St Louis, Mo.) and from E. Merck, Darmstadt (Germany). *N*-benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride (BAPA), NADPH and $NADP^+$ were purchased from Sigma Chemical Co. All organic solvents were analytical grade.

Preparation of tissue subfractions

The tissues were homogenized in ice-cold TME buffer using a Teflon glass homogenizer. The TME buffer consisted of 50 mM Trishydroxymethylaminomethane, 5 mM $MgCl_2$ and 1 mM EDTA (pH = 7.4 at 20°C). The homogenized tissue diluted in 10 volumes TME buffer was used for the estimation of trypsin-like protease activity. The supernatant (800 *g*, 20 min) was centrifuged at 105,000 *g* for 1 h to obtain a cytosol fraction as an enzyme source for 3α-HSD. The pellet (800 *g*, 20 min) was suspended in 20 ml of 0.88 M sucrose and this suspension was layered on top of 5 ml of 1.8 M sucrose solution. After centrifugation at 55,000 *g* for 90 min, the nuclear fraction was obtained as a pellet. The nuclear fraction was also used as an enzyme source for the estimation of 5α-reductase activity.

Morphological studies

Immediately after the removal, the submandibular glands and ventral prostates obtained from each group were diced and fixed in 10% neutral buffered formalin. The tissues were then dehydrated through graded ethanol and embedded in paraffin. Sections of 4 μm thickness were cut and stained with hematoxylin and eosin, and examined with a light microscope.

Determinations of 5α-reductase and 3α-HSD

The activities of 5α-reductase and 3α-HSD were determined in nuclear and cytosol fractions of the submandibular gland and ventral prostate, respectively. The specific radioactivity of the steroid substrate was diluted with an authentic non-radioactive preparation to saturate the enzyme. The incubation medium, for the 5α-reductase activity assay, consisted of 1 ml nuclear fraction in TME buffer, the radioactive testosterone substrate, 1 ml 0.25 M sucrose and 0.2 mM NADPH in 1 ml TME buffer. For the 3α-HSD assay, the labeled DHT and the cytosol fraction were incubated with 0.2 mM NADPH. In the oxidative 3α-HSD assay, [^{3}H]3α-diol and the cytosol fraction were incubated in the presence of 0.2 mM $NADP^+$. The incubations were carried out at 37°C for 20 min with constant shaking in an atmosphere of 95% O_2 and 5% CO_2. The metabolites were separated by thin-layer chromatography (kieselgel 60/kieselgur F_{254}, E. Merck, Darmstadt). Spots where steroids were detected autoradiographically by exposure to X-ray film (Kodak XRP-1) were scraped off the thin-layer plate and extracted with ethyl ether. Radioactivity was determined with a toluene base solution (toluene 667 ml, polyoxyethylene (10) octylphenyl ether 333 ml, omunifluor 4 g). The protein content was determined according to the method of Lowry *et al.*[15] with bovine serum albumin as a standard.

Determination of protease activity in the submandibular gland

BAPA was used as a substrate for the estimation of trypsin-like protease activity in the homogenate of the rat submandibular gland [16]. The incubation medium, consisting of 1 ml of 0.1 mM glycine–NaOH buffer (pH = 9.2 at 20°C), 0.5 ml of the homogenized tissue and 0.5 ml of 1 mM BAPA in glycine–NaOH buffer, was incubated for 15 min at 37°C. The reaction was terminated by the addition of 1 ml of 1 M sodium acetate–HCl (pH = 4.2 at 20°C). Enzyme activities were expressed as the amount of *p*-nitroaniline liberated from BAPA. The amount of *p*-nitroaniline was determined quantitatively by a spectrometer measuring the absorbance at 410 nm.

RESULTS

Changes of tissue weight by castration and 3α-diol treatment

The effects of castration and 3α-diol on submandibular gland and ventral prostate weight are shown in Fig. 1. Control, castrated and 3α-diol-treated rat submandibular glands were 0.30 ± 0.03, 0.28 ± 0.02 and 0.30 ± 0.02 g in weight, respectively. There were no significant differences in submandibular gland weight between the three groups. However, the ventral prostate weight was dramatically decreased (0.03 ± 0.002 g) by castration and restored to the 33% level (0.10 ± 0.01 g) of the control (0.30 ± 0.02 g) by 3α-diol injections.

5α-reductase and 3α-HSD activities

The 5α-reductase activities were expressed as the sum of DHT and 3α-diol produced from the substrate testosterone. 3α-Diol produced from the substrate DHT was used as an index of 3α-HSD activity and DHT from 3α-diol as 3α-HSDO activity. The above defined amount of product(s) from each substrate increased linearly with the amount of enzyme preparation added to the incubation medium under the conditions employed. Also, time study revealed a linear increase of the amount of product with elapsed time of incubation up to 40 min for 5α-reductase and 30 min for 3α-HSD(O) (Fig. 2).

Effect of castration and 3α-diol on 5α-reductase and 3α-HSD

The effects of castration and 3α-diol on the testosterone metabolizing enzymes, 5α-reductase and 3α-HSD, were studied *in vitro* in nuclei and cytosol of the submandibular gland and ventral prostate. The

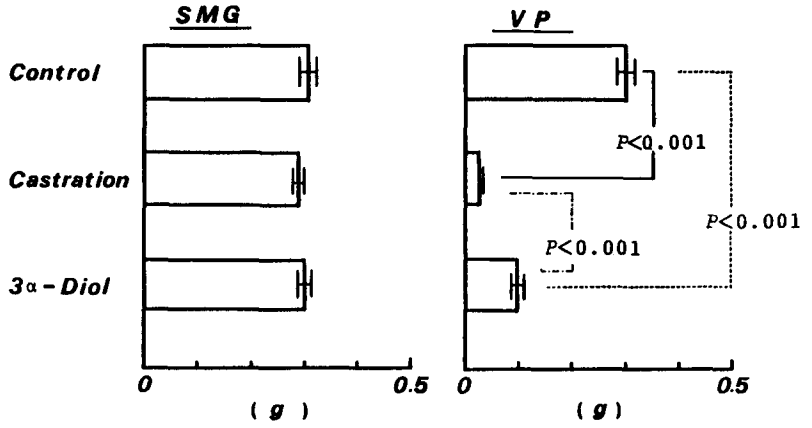

Fig. 1. The effects of 5α-androstane-3α,17β-diol (3α-Diol) on rat submandibular gland (SMG) and ventral prostate (VP). SMG and VP were obtained from nontreated rats (control), castrated rats (castration) and castrated rats receiving 3α-diol (1 mg/kg/day) for 7 days (3α-Diol). Each bar represents the mean ± SD ($n = 6$). Statistical analysis was carried out by Student's t-test.

results are summarized in Table 1. 5α-Reductase activities in submandibular glands were decreased by castration to about 52% of the control, and increased to about 80% by 3α-diol. The activities of 3α-HSD and 3α-HSDO decreased after castration, reaching 70 and 72% of the control submandibular gland. The weights of ventral prostate were too little to assay the 5α-reductase and 3α-HSD activities in castrated rats. An unexpected finding was the increase in 3α-HSD and/or 3α-HSDO activity in the submandibular gland induced by 3α-diol (about 140% of the control activity level). An even more important increase (approximately 225%) was observed for the 3α-HSD or 3α-HSDO activity in the ventral prostate.

Trypsin-like protease activity in the submandibular gland

For the studies of the effect of castration and 3α-diol treatment on the submandibular glands, trypsin-like protease activities were analyzed in the tissue homogenates. Figure 3 shows that trypsin-like protease activity in the submandibular glands of castrated rats was significantly reduced compared with that in control rats ($P < 0.001$). There was a 25% reduction in the protease. This decrease was largely corrected after 3α-diol injections for 7 days ($P < 0.1$).

Histological observations

A marked decrease in the number of granules in granular duct cells of the submandibular gland were observed 7 days after castration (Fig. 4a, b). Treatment with 3α-diol resulted in reaccumulation of the granules in the cells to a level approximating that of the controls (Fig. 4c). Acinar cells, intercalated duct cells and intra-lobular duct cells, were not affected by castration or 3α-diol treatment.

Histological examination of the transverse section of the ventral prostate showed that their tubules were lined by columnar cells with a round or ovoid basal nucleus (Fig. 4d). All the tubules were formed by a single type of cell. Figure 4e shows that the size of duct cell in the ventral prostate was markedly decreased following castration. The administration of 3α-diol caused the duct cells to return to their control size and shape (Fig. 4f).

DISCUSSION

It is well known that DHT is the active metabolite of testosterone in the prostate. It is formed intracellularly from testosterone by the enzyme 5α-reductase, and it is degraded predominantly by the 3α-HSD yielding 3α-diol. Cytoplasm in target organs contains a specific DHT-binding protein. 3α-Diol has been regarded as a less active testosterone degradation product. However, 3α-diol is important as an end-product of testosterone metabolism. 3α-Diol in plasma is more active than DHT in suppressing gonadotropin secretion in the castrated rat and has a potent inhibitory effect on sexual maturation in the female rat [17–19]. 3α-Diol plays a role in the control of FSH secretion in the immature female rat [20] and castrated male rats [21, 22] and in LH secretion in ovariectomized rats [23, 24]. Several reports are available about the action of 3α-diol in tissues. 3α-Diol has been shown to maintain the prostate glands and seminal vesicle in castrated rats [25]. Walsh and Wilson[13] and DeKlerk *et al.*[14] have reported the induction of benign prostatic hyperplasia by 3α-diol in young dogs. It has also been reported that 3α-diol plays a role in the regulation of 5α-reductase and 3α-HSD and interferes with estradiol binding to receptors in the hypothalamus and/or hypophysis in the immature female rat and in mammary and myometrial tissues [26]. From the study of the dose–response of the submandibular gland epidermal growth factor concentrations, in mice following the administration of a variety of androgenic steroids, Barthe *et al.*[27] has described that 3α-diol and DHT

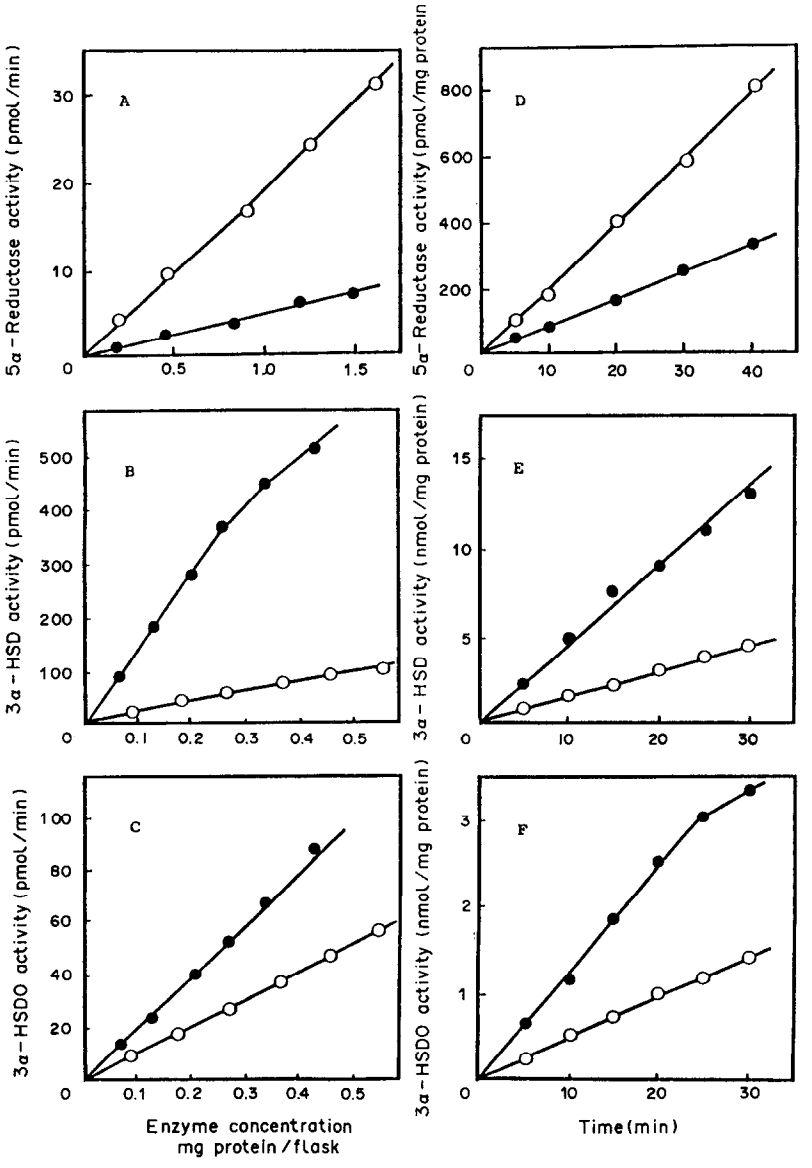

Fig. 2. Effect of enzyme concentration and incubation time on the 5α-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD) and oxidative 3α-hydroxysteroid dehydrogenase (3α-HSDO) activities. A, B and C, effect of enzyme concentration. The indicated amounts of nuclear protein from submandibular gland (●) and ventral prostate (○) were incubated with [^{14}C]testosterone (6.7 μM) at 37°C for 20 min in the presence of NADPH (A). The indicated amounts of cytosolic protein from submandibular gland (●) and ventral prostate (○) were incubated with [^{14}C]dihydrotestosterone (6.4 μM) and NADPH (B) and with [^{3}H]5α-androstane-3α,17β-diol (5.7 μM) and $NADP^+$ (C) at 37°C for 20 min. D, E and F, effect of time of incubation. Nuclei from submandibular gland (●) and ventral prostate (○) were incubated with [^{14}C]testosterone (6.7 μM) in the presence of NADPH at 37°C for indicated times (D). Cytosol from submandibular gland (●) and ventral prostate (○) were incubated with [^{14}C]dihydrotestosterone (6.4 μM) and [^{3}H]5α-androstane-3α,17β-diol (5.7 μM) in the presence of NADPH and $NADP^+$, respectively, at 37°C for indicated times. Other incubation condition and expression of the enzyme are described in the sections concerning Materials and Methods, and Results.

Table 1. Effect of castration and 5α-androstane-3α,17β-diol (3α-diol) on 5α-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD) and oxidative 3α-hydroxysteroid dehydrogenase (3α-HSDO) in submandibular gland (SMG) and ventral prostate (VP)

	5α-Reductase	3α-HSD	3α-HSDO
SMG			
Control	8.2 ± 0.18	529.4 ± 56.5	255.8 ± 11.7
Castration	4.4 ± 0.45	369.0 ± 33.0	184.5 ± 28.7
3α-Diol	6.7 ± 0.52	733.5 ± 30.1	359.4 ± 39.1
VP			
Control	71.5 ± 16.2	139.0 ± 18.3	119.1 ± 9.8
3α-Diol	50.7 ± 10.1	241.9 ± 23.0	379.0 ± 46.0
		Students's *t*-test, *P*	
SMG			
Control vs castration	<0.001	<0.001	<0.01
Control vs 3α-diol	<0.05	<0.001	<0.02
Castration vs 3α-diol	<0.001	<0.001	<0.001
VP			
Control vs 3α-diol	<0.05	<0.02	<0.001

Rats were castrated for 7 days (Castration). The castrated rats were treated for 7 days with 1 mg/kg/day 3α-diol (3α-Diol). 5α-Reductase activity was assayed with nuclear fraction by incubating for 20 min at 37°C in the presence of [^{14}C]testosterone (6.6 μM) and NADPH. 3α-HSD activity or 3α-HSDO activity was assayed with cytosol fraction by incubating for 20 min at 37°C in the presence of [^{14}C]5α-dihydrotestosterone (DHT) (6.0 μM) or ^{3}H-3α-diol (6.3 μM) and NADPH (for DHT) or $NADP^+$ (for 3α-diol). Data expressed in pmol/mg protein per min (mean ± SD; $n = 6$).

generated a steep dose–response curve of some peptides in the rat submandibular glands. It has been reported that in the human [28] and the rat [29] prostate the increase in tissue DHT results not only from reduced metabolism but also from active back-conversion of 3α-diol.

The activity of 3α-HSD in submandibular gland was effectively decreased by 7 days castration. The restorations of 3α-HSD and 3α-HSDO by 3α-diol in submandibular glands were effective, showing more than an approximately 40% increase than control. Verhoeven and De Moor[30] demonstrated that the microsomal 3α-HSD activity in the male rat kidney was reduced by gonadectomy and that the activity was restored by not only DHT and testosterone, but 3α-diol. When the ratio of 3α-HSD/3α-HSDO were calculated from the mean values shown in Table 1, the ratio of 2.07 for control, 2.00 for castrated and 2.04 for 3α-diol injected groups were obtained. The fact that the ratio is firmly retained and was not affected by castration and/or 3α-diol means that NADPH dependent 3α-HSD systems are relatively irreversible and that the steady-state levels of NADPH/$NADP^+$ are controlled by 3α-HSD(O). The NADPH concentrations are considerably higher than $NADP^+$ concentrations in the tissues [31].

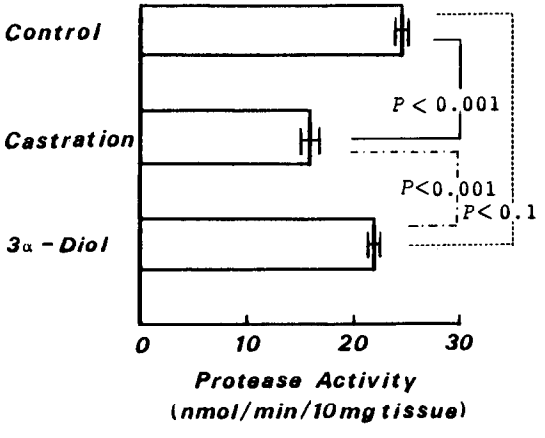

Fig. 3. The effects of 3α-diol on protease activities in rat submandibular gland homogenates. The homogenized tissue (0.5 ml) were incubated with 1 mM BAPA (0.5 ml) in 1 ml of 0.1 mM glycine–NaOH buffer for 15 min at 37°C. The amount of *p*-nitroaniline liberated from the substrate BAPA was used as an index of the enzyme activities and expressed as nmol/min/10 mg tissue. Each bar represents the mean ± SD ($n = 6$).

The activities of 5α-reductase in the nuclei of ventral prostate obtained from 3α-diol-treated rats was shown to be about 71% of the control, and 3α-HSD and 3α-HSDO activities were increased to the level of 174 and 318% of the control, respectively. The 3α-HSD(O) activity was more effectively restored by 3α-diol than 5α-reductase. The 3α-HSD/3α-HSDO ratio was calculated as 1.16 for control and 0.63 for the 3α-diol treated group. These results suggest that 3α-diol is a precursor for DHT in the ventral prostate. 5α-Reductase activity was not completely restored to its control activity by 3α-diol. 5α-Reductase in the nuclei and 3α-HSD in the cytosol obtained from the castrated rat ventral prostates were unassayable because of too little tissue weight. However, the enzyme activities of the castrated rats may have decreased, judging from the significantly decreased weight of the ventral prostate.

To further evaluate the effect of 3α-diol on the physiological function in the rat submandibular gland, trypsin-like protease activities were measured in the homogenate of the tissue. A 37% decrease of the protease activity was caused by castration, which almost restored to a control level by 3α-diol. The decrease and recovery of the enzyme activities were

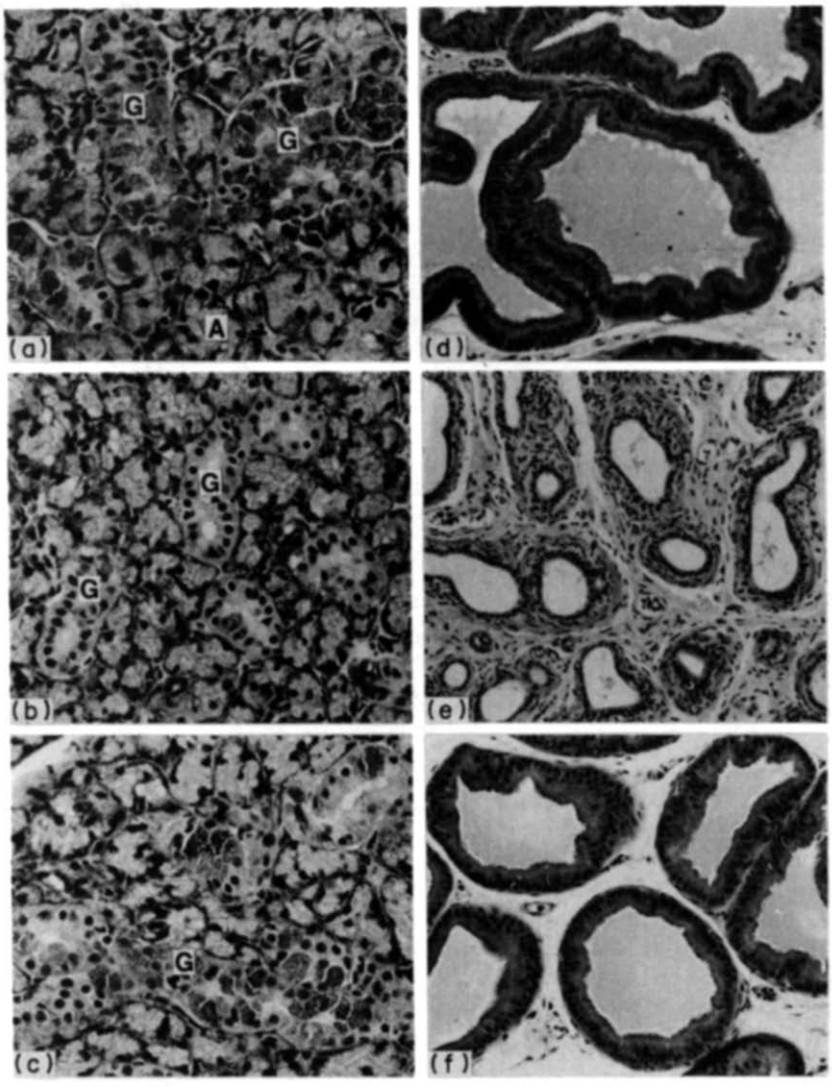

Fig. 4. (a) Control submandibular gland showing acinar cell (A) and granular duct (G). Granular ducts contain numerous secretory granules. ×250. (b) Castrated submandibular gland. The number of granules in granular duct cells (G) are significantly decreased. ×250. (c) 3α-Diol treated submandibular gland. The granules in granular duct cell (G) reaccumulate to nearly the control level. ×250. (d) Control ventral prostate. Their tubules are lined by columnar cells. ×150. (e) Castrated ventral prostate. Epithelial cells become cuboidal. ×150. (f) 3α-Diol treated ventral prostate. The duct cells return to their control size. ×150.

similar to the changes of 5α-reductase activities. The restoration of the number of the granules in the granular duct cells supports the view that 3α-diol has an androgenic effect in the submandibular gland. 3α-Diol is therefore an important androgen in the rat submandibular gland.

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