# 3α-HYDROXYSTEROID DEHYDROGENASE IN THE CYTOSOL OF RAT SUBMANDIBULAR GLAND

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**Summary**—We examined the *in vitro* shuttle metabolism between dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-diol) by 3α-hydroxysteroid dehydrogenase (3α-HSD, E.C. 1.1.1.50) in rat submandibular gland (SMG) and ventral prostate (VP). The protein having molecular weight of 30 kDa, which was revealed by Sephacryl S-200 column chromatography, had 3α-HSD activity to produce 3α-diol from DHT, and also showed an oxidative 3α-HSD (3α-HSDO) ability to produce DHT from 3α-diol. From the kinetic studies, the apparent $K_m$ and $V_{max}$ values of 3α-HSD for DHT and NADPH were 6.4 μM, 1429 pmol/mg protein per min and 33.0 μM, 1205 pmol in SMG, and 9.3 μM, 377 pmol and 34.0 μM, 192 pmol in VP. The corresponding values of 3α-HSDO for 3α-diol and NADP⁺ were 18.0 μM, 714 pmol and 14.0 μM, 445 pmol in SMG, and 14.0 μM, 417 pmol and 36.0 μM, 77 pmol in VP. The affinities for DHT and 3α-diol and the cosubstrate requirements of this enzyme in SMG were similar to those in VP. However, higher capacities of 3α-HSD and 3α-HSDO in SMG than in VP were shown. This suggests that there may be more 3α-HSD in the SMG.

## INTRODUCTION

DHT is an active androgen in androgen target organs such as in VP, epididymis and seminal vesicles [1, 2]. Androgen target organs have the ability to convert testosterone to the potent androgen DHT by 5α-reductase and to further convert it to 3α-diol via 3α-HSD [2–4]. Intracellular DHT concentrations depend on the activities of 5α-reductase and 3α-HSD. It has been reported that the cytoplasm in SMG of rodents contains a specific DHT-binding protein, indicating that DHT is an active androgen in SMG and that the gland is a target for androgen [5, 6].

3α-Diol is important as a testosterone metabolism end-product in androgen target tissues [7, 8]. It has been reported that 3α-diol plays a role in the regulation of 5α-reductase and 3α-HSD, and interferes with oestradiol binding to receptors in the hypothalamus and/or hypophysis in immature female rats [9] and in mammary and myometrial tissues [10]. Also 3α-diol is rapidly reversed to DHT by 3α-HSD [11–13]. Walsh and Wilson[14], and DeKlerk *et al.*[15] reported the induction of benign prostatic hypertrophy by 3α-diol in young dogs. From a dose–response study of SMG epidermal growth factor concentrations in mice following the administration of a variety of androgenic steroids, Barthe *et al.*[16] found that 3α-diol generated a steep dose–response curve.

Not only 5α-reductase activities, but also 3α-HSD activities, determine androgen dependency of the tissue and characterize the tissue as an androgen target organ. Therefore, *in vitro* studies on 3α-HSD concerning DHT and 3α-diol metabolism were performed using [¹⁴C]DHT and [³H]3α-diol as substrates in SMG and VP.

## MATERIALS AND METHODS

### Animals

Male Wistar rats of 7 weeks of age, weighing 160–190 g, were used for this study. The rats were sacrificed under anesthesia using ether in order to obtain SMG and VP.

### Chemicals

[4-¹⁴C]DHT (SA 57 mCi/mmol) and [1,2-³H]3α-diol (SA 30.1 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Authentic steroid preparations, testosterone, DHT and 3α-diol, were obtained from Sigma Chemical Co. (St Louis, Mo.) and from E. Merk, Darmstadt (Germany). All organic solvents were of analytical grade. Cosubstrates, NADPH, NADP⁺, NADH and NAD⁺ were purchased from Sigma Chemical Co. Kieselgel 60/kieselgur F₂₅₄ were purchased from E. Merk, Darmstadt.

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*Tissue preparation and incubation*

Freshly prepared cytosol fractions from SMG and VP were used for all experiments. Immediately after removal, the tissues were homogenized in ice-cold TME buffer (50 mM Trishydroxymethylamino-methane, 5 mM $MgCl_2$ and 1 mM EDTA, pH = 7.4 at 20°C) using a Teflon glass homogenizer. The supernatant (800 $g$, 20 min) was centrifuged at 10,000 $g$ for 20 min. Then, the supernatant was centrifuged at 105,000 $g$ for 1 h to obtain a cytosol fraction as an enzyme source. All procedures were performed at 0–4°C.

The specific activity of the radio-labelled steroid substrate was diluted with an authentic non-radioactive preparation to saturate the enzyme. Unless otherwise specified, the incubation medium contained the enzyme preparation, the radioactive steroid substrate, 0.25 M sucrose, TME buffer, and 0.2 mM NADPH or $NADP^+$. The sequence for preparing the incubation medium was to dissolve the steroid substrate in a drop of propylene glycol, to dissolve the cosubstrate in TME buffer, then carry out the enzyme preparation. The final volume of the incubation medium was adjusted to 3 ml. Incubation was carried out at 37°C for 20 min in an atmosphere of 95% $O_2$ and 5% $CO_2$ with constant shaking. Other conditions for incubation are described under each experimental section.

*Extraction and separation of steroids*

Immediately after the incubation, enzymatic reactions were arrested by adding 10 ml of methylene chloride. Non-radioactive testosterone, DHT and 3α-diol (100 $\mu$g each/flask) were added as carriers and to estimate recovery. Extraction was carried out 3 times with 10 ml of methylene chloride while shaking vigorously. The combined extracts were dried at 40°C under reduced pressure, and the residue was dissolved in 100 $\mu$l chloroform. Ascending chromatography was performed in order to separate steroids on thin-layer plates precoated with kieselgel 60/kieselgur $F_{254}$ (20 × 20 cm) with a solvent system using benzene and acetone (4:1, v/v). The radioactive spots were detected autoradiographically by exposure to X-ray film (Kodak XRP-1). The carrier steroids were detected under an ultraviolet lamp or in iodine vapor. Only a 5–10% loss of carrier steroids was noted during the extraction step. Spots where steroids were detected autoradiographically were scraped off the thin-layer plate and extracted with ethylether from the absorbent. Definitive identification of metabolites was accomplished by demonstrating constant specific radioactivity during repeated recrystallization [17].

*Quantitation of steroids and protein*

Radioactivity of each steroid fraction was measured with a liquid scintillation spectrometer (Packard, Model 3390) in a toluene scintillator (toluene 667 ml, polyoxyethylene (10) octylphenyl ether

333 ml, omnifluor 4 g). Counting efficiency was approximately 70% for carbon-14, and 40% for tritium. The amount of each metabolite was calculated from its % yield and the specific radioactivity of the substrate. The protein content in each tissue preparation was determined according to the method of Lowry *et al.*[18] with bovine serum albumin (Boehringer–Mannheim) as a standard.

*Column chromatography*

Partial purification of the 3α-HSD in SMG cytosol was carried out by three kinds of column chromatography in a cold room at 4°C. 3α-HSD activity was detected by the incubation of each eluate and the substrate, DHT or 3α-diol, in the presence of the required cosubstrate. The incubation conditions were the same as described above. Cytosol (0.3 ml) was applied to a Bio-Gel A-1.5 m (Bio-Rad) column (0.9 × 16 cm) and then eluted with 1 ml of TME buffer per fraction at a flow rate of 5 ml/h. An aliquot (0.5 ml) of the eluate obtained from the fraction numbers 8 and 9 was applied to a DE-52 (Whatman) column (1 × 20 cm) using a linear gradient of 0–2.0 M NaCl in equilibration buffer and eluted, 2 ml per fraction, at a flow rate of 12 ml/h. The eluates of fraction number 7 and 8, which contained 3α-HSD activity, were combined. Then 2 ml of the DE-52 eluates was applied to the Sephacryl S-200 (Pharmacia Fine Chemicals) column (1.6 × 36 cm) and the elution was carried out with 5 ml of TME buffer per fraction at a flow rate of 15 ml/h to obtain fraction number 10, with 3α-HSD activity.

## RESULTS

*Metabolites from the substrate and the expression of the enzyme activity*

[$^{14}$C]DHT (20 × $10^4$ dpm, 12.16 $\mu$M) and the cytosol fractions from rat SMG or VP were incubated with 0.2 mM NADPH. The major $C_{19}$-steroid metabolite obtained from DHT corresponded to authentic 3α-diol on thin-layer chromatography plates. No significant amounts of androstanedione were recognized under the conditions employed in the present study. The major metabolite was finally identified as 3α-diol using constant specific radioactivity with repeated recrystallization. Recrystallization data are described elsewhere [19]. Therefore, the product 3α-diol was defined as a 3α-HSD activity index for DHT, and the enzyme activity was expressed as the amount of product per min per mg protein. In the oxidative 3α-HSD assay, [$^3$H]3α-diol (20 × $10^4$ dpm, 22.79 $\mu$M) and the cytosol fractions from rat SMG and VP were incubated in the presence of 0.2 mM $NADP^+$. DHT was obtained as a metabolite of 3α-diol. No other metabolite was observed. The product DHT was defined as a 3α-HSDO activity index for 3α-diol. Among the subcellular fractions from SMG and VP tissues, 3α-HSD showed the
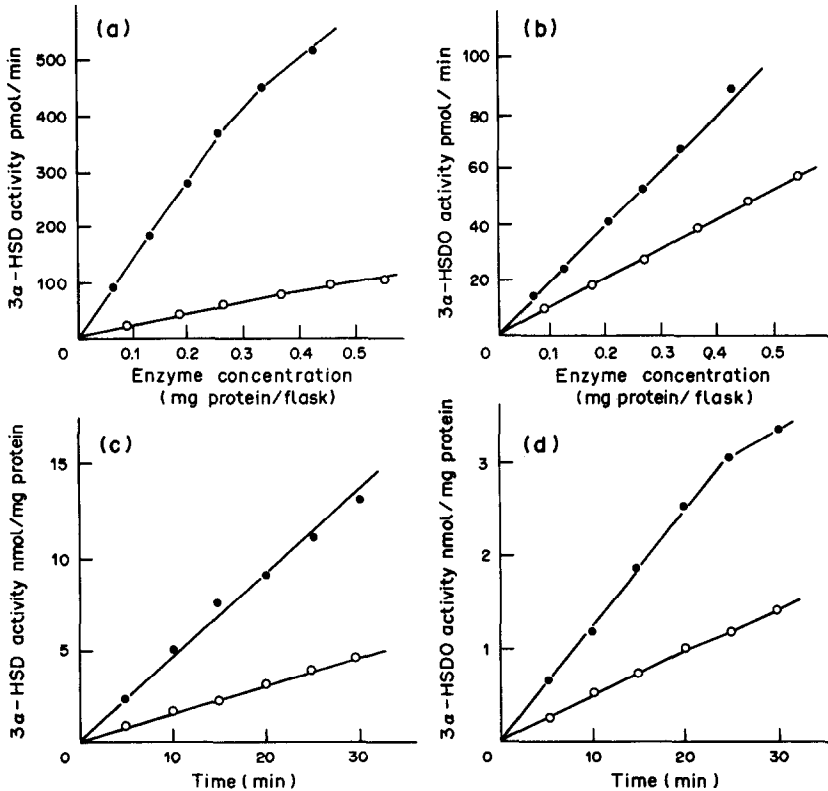
Fig. 1. Effect of enzyme concentration and incubation time on the 3α-hydroxysteroid dehydrogenase (3α-HSD) and oxidative 3α-hydroxysteroid dehydrogenase (3α-HSDO) activities. (a) and (b), effect of enzyme concentration. The indicated amounts of cytosolic protein from submandibular gland (●) and ventral prostate (○) were incubated with [$^{14}$C]dihydrotestosterone (6.4 $\mu$M) and NADPH (a) and with [$^3$H]5α-androstane-3α,17β-diol (5.7 $\mu$M) and NADP$^+$ (b) at 37°C for 20 min. C and D, effect of time of incubation. Cytosol from submandibular gland (●) and ventral prostate (○) were incubated with [$^{14}$C]dihydrotestosterone (6.4 $\mu$M) and [$^3$H]5α-androstane-3α,17β-diol (5.7 $\mu$M) in the presence of NADPH and NADP$^+$, respectively, at 37°C for indicated times.

highest activity in the cytosol fraction. Low 3α-HSD activity was found in microsomes and nuclear fractions [20]. To further elucidate the above findings, the 3α-HSD(O) activities were studied in relation to cytosolic protein concentration and incubation time. The enzyme activities in VP increased linearly with increasing amounts of cytosolic protein. The 3α-HSD(O) activities in SMG cytosol was linear with protein up to 0.42 mg/flask. Cytosolic 3α-HSD(O) in SMG and VP increased sequentially up to 40 min. Representative data are presented in Fig. 1.

## Cosubstrate requirements of 3α-HSD

The cosubstrate requirements of 3α-HSD and 3α-HSDO in cytosol were investigated. When [$^{14}$C]DHT or [$^3$H]3α-diol was incubated with cytosol of rat SMG in the presence of NADPH, NADP$^+$, NADH or NAD$^+$ as cosubstrates, 3α-diol production was greatest in the presence of NADPH and NADP$^+$ was the preferred cofactor in the conversion of 3α-diol to DHT (Table 1). The kinetic studies of 3α-HSD and 3α-HSDO activities for the cosubstrates were performed by incubating cytosol with various concentrations of NADPH and NADP$^+$. The Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) of the

enzyme were estimated by a Lineweaver–Burk plot [21]. The apparent $K_m$ value and $V_{max}$ of 3α-HSD for NADPH were estimated to be 33.0 $\mu$M and 1205 pmol/mg protein per min in SMG, and 34.0 $\mu$M and 192 pmol in VP (Fig. 2a). The corresponding 3α-HSDO values for NADP$^+$ were 14.0 $\mu$M and 445 pmol in SMG, and 36.0 $\mu$M and 77 pmol in VP (Fig. 2b).

Table 1. Requirement of cosubstrate for 3α-hydroxysteroid dehydrogenase activity

| Cosubstrate | 3α-HSD | 3α-HSDO |
|---|---|---|
| NADPH | 161.5 | 7.4 |
| NADP$^+$ | 46.7 | 120.6 |
| NADH | 39.0 | 5.8 |
| NAD$^+$ | 7.9 | 13.7 |

The cytosol preparation from submandibular gland was incubated with [$^{14}$C]5α-dihydrotestosterone (1.86 $\mu$M) for 3α-hydroxysteroid dehydrogenase (3α-HSD) kinetics or with [$^3$H]5α-androstane-3α, 17β-diol (1.71 $\mu$M) for oxidative 3α-HSD (3α-HSDO) as a substrate in the presence of indicated cosubstrates, respectively. The final concentration of each cosubstrate was 0.2 mM. Incubation conditions and further details are described in Materials and Methods. Figures represent pmol/mg protein/min and are the average of intra-assay triplicates.
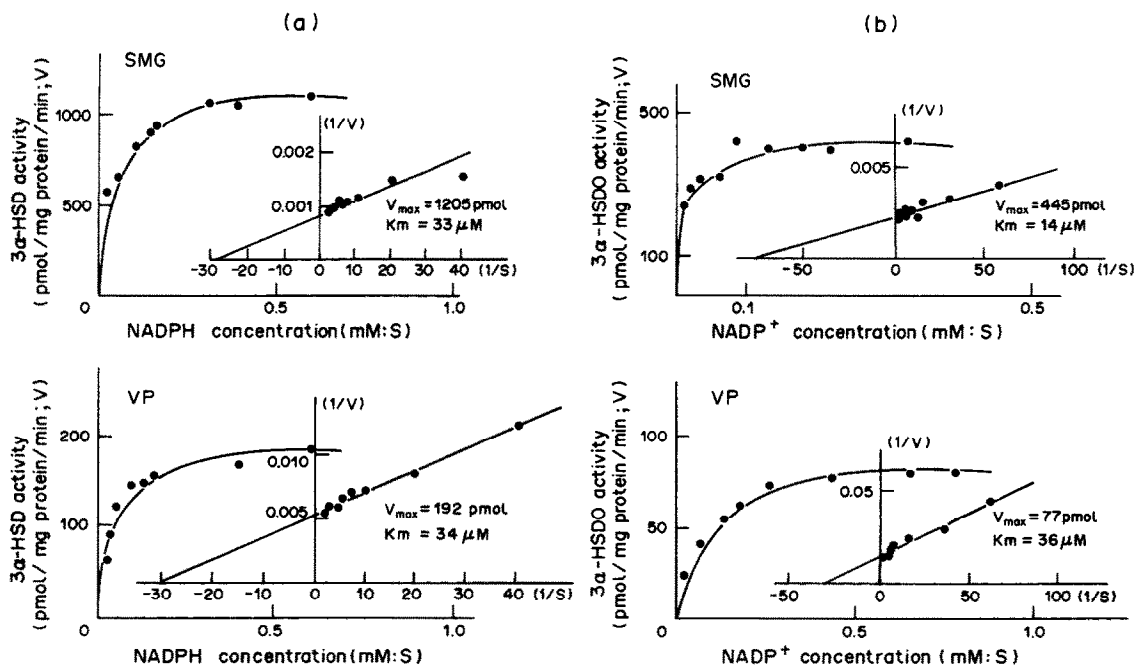
Fig. 2. Kinetic properties of 3α-hydroxysteroid dehydrogenase (3α-HSD) and oxidative 3α-hydroxysteroid dehydrogenase (3α-HSDO) in cytosol of rat submandibular gland and ventral prostate. (a) The saturation curve of 3α-HSD with various concentrations of NADPH ($0.013 \sim 0.600$ mM) as a cosubstrate and the constant concentration of [$^{14}$C]dihydrotestosterone ($23.80$ $\mu$M) as a substrate is shown. (b) The saturation curve of 3α-HSDO with various concentrations of NADP$^+$ ($0.008 \sim 0.784$ mM) and the constant concentration of [$^3$H]3α-diol ($22.80$ $\mu$M) is shown. The $K_m$ and $V_{max}$ of 3α-HSD for NADPH and 3α-HSDO for NADP$^+$ were estimated by a Lineweaver–Burk plot.

## *Kinetic parameters of 3α-HSD for DHT and 3α-diol*

Cytosol from SMG or VP was incubated in various concentrations of DHT, at a pH of 7.4 for 20 min in the presence of 0.2 mM NADPH. The apparent $K_m$s of 3α-HSD for DHT were 6.4 $\mu$M in SMG and 9.3 $\mu$M in VP (Fig. 3a). The apparent $V_{max}$ of the enzyme were 1429 pmol in SMG and 377 pmol in VP. When 3α-diol was incubated as substrate, the apparent $K_m$ of 3α-HSDO for 3α-diol were revealed as 18.0 $\mu$M in SMG and 14.0 $\mu$M in VP (Fig. 3b). The apparent $V_{max}$ of the enzyme were 714 pmol in SMG and 417 pmol in VP.

## *Estimation of the size of 3α-HSD*

The 3α-HSD in SMG cytosol was purified by 3 kinds of column chromatography, Bio-Gel A-1.5 m, DE-52 and Sephacryl S-200. The chromatographic elution profiles of 3α-HSD by the columns revealed only one distinct peak of 3α-HSD activity by each chromatographic method. The molecular weight of the 3α-HSD protein was estimated to be about 30 kDa by the retention time on the S-200 chromatograms (Fig. 4), using trypsin inhibitor (29 kDa) and ovalbumin (43 kDa) as standard proteins. This 30 kDa protein in SMG cytosol had the ability to convert DHT to 3α-diol and to reconvert 3α-diol to DHT..

## DISCUSSION

We measured 3α-HSD with DHT or 3α-diol as substrates, and with NADPH or NADP$^+$ as cosubstrates under optimized conditions in SMG and VP. The affinities of 3α-HSD for NADPH and 3α-HSDO for NADP$^+$ in SMG were similar to those in VP, but the ability of 3α-HSD(O) to produce 3α-diol from DHT or DHT from 3α-diol in SMG was higher than that in VP. The kinetic study of 3α-HSD for DHT obtained from SMG and VP cytosol had also revealed similar $K_m$ values. However, the apparent $V_{max}$ of the enzyme in SMG was about 3.5 times higher than that in VP. These kinetic studies suggest that cytosol 3α-HSD activity in SMG had a similar affinity for the substrate and cosubstrate to that in VP, and had a higher capacity than VP. There may be more enzymes in the SMG. Verhoeven *et al.*[22] have reported three 3α-hydroxysteroid oxidoreductases, which could interconvert DHT and 3α-diol in rat kidney. A soluble NADPH-dependent oxidoreductase, and microsomal NADPH- and NADH-dependent enzymes have been distinguished. The fact that the apparent $V_{max}$ values were significantly higher in SMG than in VP means that DHT degradation is greater in SMG than in VP. For this reason, VP possesses larger amounts of DHT in cells than SMG does. Not only 5α-reductase, which
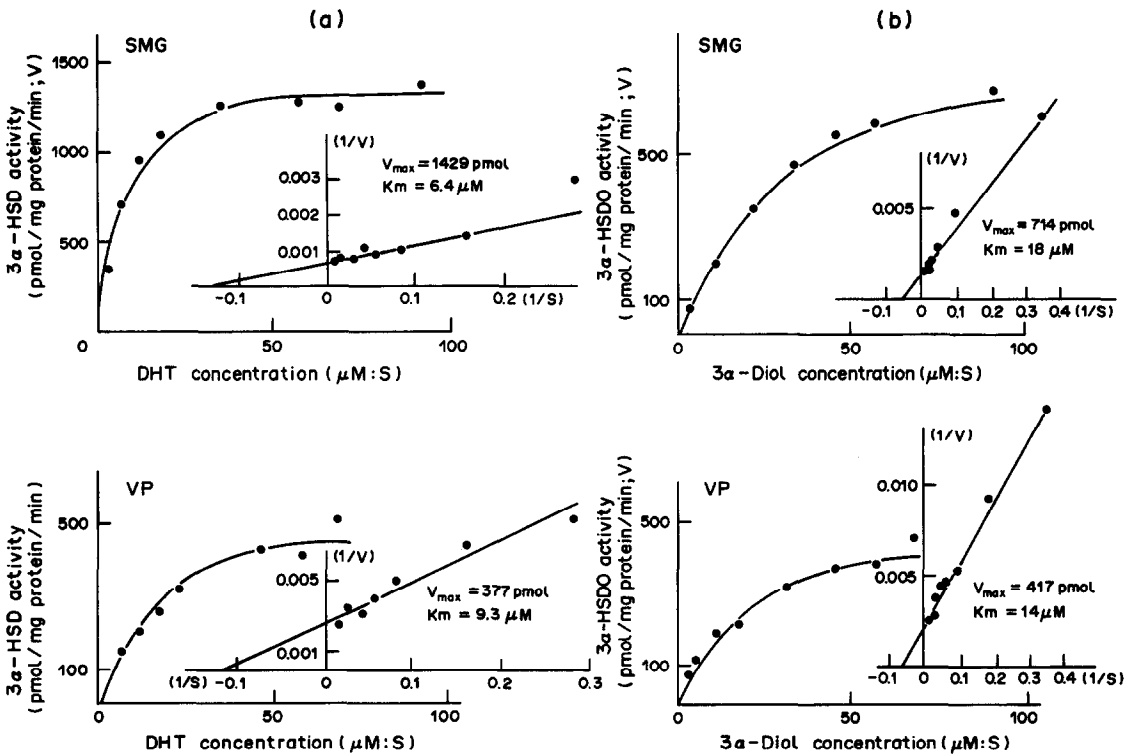
Fig. 3. Kinetic properties of 3α-hydroxysteroid dehydrogenase (3α-HSD) and oxidative 3α-hydroxysteroid dehydrogenase (3α-HSDO) in cytosol of rat submandibular gland and ventral prostate. (a) The saturation curve of 3α-HSD with various concentrations of dihydrotestosterone (3.20 ∼ 93.20 μM) and the constant concentration of 0.2 mM NADPH is shown. (b) The saturation curve of 3α-HSDO with various concentrations of 5α-androstane-3α,17β-diol (2.85 ∼ 91.17 μM) and the constant concentration of 0.2 mM NADP⁺ is shown.

produces DHT from testosterone, but also 3α-HSD is important in testosterone metabolism, regulating the accumulation of DHT in cells.

From the results of $V_{max}$ values for the corresponding substrates, the 3α-HSD/3α-HSDO ratio was calculated and the ratios of 2.0 for SMG and 0.9 for VP were obtained. These values mean that half of the DHT in SMG was converted to 3α-diol while 3α-diol was readily converted to DHT, and that a constant concentration of DHT was retained in VP. These results also support the fact that VP possesses more DHT in cells than SMG does. Rat SMG does not need as much DHT as VP does. It has been revealed that testosterone was easily converted to DHT by 5α-reductase, while more than 90% of the DHT was not converted back to testosterone [12]. 3α-Diol was



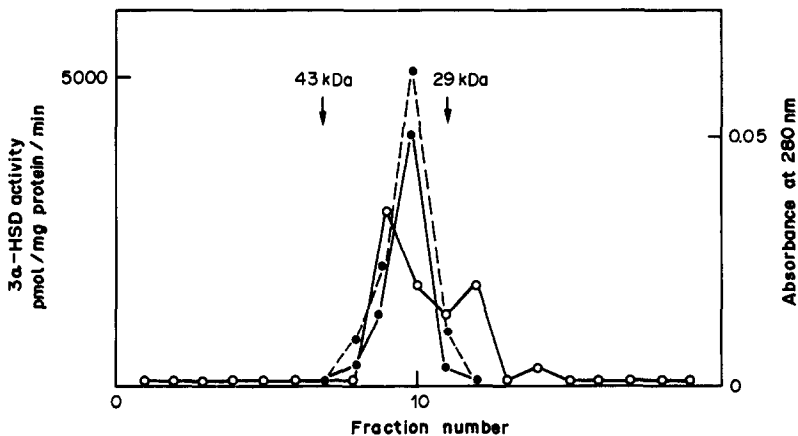Fig. 4. Elution patterns of 3α-hydroxysteroid dehydrogenase (—●—) and oxidative 3α-hydroxysteroid dehydrogenase (--●--) by Sephacryl S-200 column chromatography. Incubation procedures and chromatographic conditions are described in Materials and Methods. Protein (—○—) was measured optically at a wave length of 280 nm.

produced from DHT by 3α-HSD and the metabolite 3α-diol was converted back to DHT by 3α-HSDO *in vivo* while exhibiting high affinity [12, 23]. NADPH and $NADP^+$ were the preferred cofactors for 3α-HSD and 3α-HSDO, respectively. NADP, however, is present mainly in the reduced form in the tissues [24]. The fact that 3α-HSD/3α-HSDO ratio was estimated as nearly 1 in VP indicates that the NADP-dependent 3α-HSD itself has a role of controlling the steady-state levels of $NADP^+/NADPH$ *in vivo*. The 3α-HSD property which allows for the reversible conversion of DHT and 3α-diol is an actual and effective mechanism regulating a constant DHT level in cells.

We have demonstrated DHT degradation and production by 3α-HSD(O) having molecular weight of about 30 kDa in the cytosol of rat SMG and compared with VP. The possibility is suggested that 3α-HSD is a key enzyme in testosterone metabolism in androgen target organ.

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