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PROSTATE 3α-HYDROXYSTEROID DEHYDROGENASE: ITS PARTIAL PURIFICATION AND PROPERTIES

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SUMMARY

3α-Hydroxysteroid dehydrogenase (E.C. 1.1.1.50) has been purified from the cytosol fraction (supernatant fluid at 105,000 *g*) of rat prostate by precipitation with ammonium sulphate (40–100% saturation), followed by chromatography on DEAE-cellulose and Sephadex G-100. Electrophoretic analysis of the enzyme preparation at the final stage revealed two major protein bands which were closely located on the gel, and a few minor bands. The enzyme activity was exclusively localized in the major bands. The final preparation was purified about 190 fold in S.A., compared with the cytosol fraction precipitated at 40–100% of ammonium sulphate saturation. Molecular weight of the 3α-hydroxysteroid dehydrogenase was estimated as 34,000 daltons from the elution pattern of the activity through the Sephadex G-100 gel filtration. The final enzyme preparation was devoid of receptor component with high affinity for 5α-dihydrotestosterone. Optimal temperature of the final 3α-hydroxysteroid dehydrogenase preparation at pH 7.4 was about 45–47.5°C. The enzyme preferred NADP(H) to NAD(H), and reduction of 5α-dihydrotestosterone was more efficiently catalyzed by the enzyme than oxidation of 5α-androstane-3α,17β-diol.

INTRODUCTION

Testosterone was incubated with cell-free homogenates of rat prostate, and 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol were isolated and firmly identified as the metabolites [1]. 5α-Androstane-3α,17β-diol was formed from 5α-dihydrotestosterone by reduction of its 3-oxo group by 3α-hydroxysteroid dehydrogenase. Activity of the dehydrogenase was almost exclusively localized in the cytosol fraction or in the supernatant fluid at 105,000 *g* of the prostate [2], whereas the 5α-reductase which is involved in the conversion of testosterone to 5α-dihydrotestosterone was localized in the outer membrane of the nuclei and the rough- and smooth-surfaced endoplasmic reticula of the prostate [3]. The complex of 5α-dihydrotestosterone bound cytosol 9*s* receptor of the prostate was incubated with the 3α-hydroxysteroid dehydrogenase fraction extracted from the rat prostate cytosol fraction in the presence of NADPH. As a result, free 5α-androstane-3α,17β-diol which showed no significant affinity for the cytosol receptor was formed as the major metabolite, and this metabolite had far less androgenic activity in the castrated rat [4]. Therefore, the 3α-hydroxysteroid dehydrogenase appears to play an important role in the degradation of the receptor–androgen complex and catabolism of the true androgen. This paper deals with the partial purification of the 3α-hydroxysteroid dehydrogenase from the prostate cytosol fraction, and some properties of the purified enzyme are described.

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MATERIALS AND METHODS

Steroids. [4-^{14}C]-5α-Dihydrotestosterone (S.A. 50.6 mCi/mmol) and [1,2,4,5,6 and 7-^{3}H]-5α-dihydrotestosterone (S.A. 80 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Other non-radioactive steroids used for references were obtained from Steraloids (Wilton, NH).

Preparation of the cytosol fraction from rat prostates. Male rats of the Wistar strain (3 months of age) were sacrificed by decapitation, and immediately the ventral prostates were isolated. The prostates were homogenized in 0.25 M sucrose solution at pH 7.4 by a Polytron homogenizer (20 OD, Kinematica, Luzern, Switzerland) and then by a tight-fitting glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 105,000 *g* for 60 min at 2°C, and the cytosol fraction was obtained as the supernatant fluid.

Fractionation of the enzyme preparation by precipitation with ammonium sulphate. Protein in the cytosol fraction was precipitated at the concentrations of ammonium sulphate between 0 and 40%, and then between 40 and 100% saturation. The precipitate obtained between 40 and 100% saturation was dissolved in a very small amount of the buffer which consisted of 5 mM KH_2PO_4, 7 mM 2-mercaptoethanol 1 mM EDTA–Na_2 and glycerol 20% (v/v), adjusted to pH 7.5.

Further purification of the 3α-hydroxysteroid dehydrogenase by column chromatography. After the columns were equilibrated with the above mentioned buffer, the protein solution was subjected to the column chromatography on DEAE-cellulose (Whatman Biochem. Ltd., Kent, England) and Sephadex G-100 (Pharmacia, Uppsala, Sweden). Details of the

elution are described with the results obtained. Void vol. in Sephadex G-100 column chromatography was measured by Blue Dextran 2000 (Pharmacia, Uppsala, Sweden). The eluates were assayed for the enzyme activity and protein content.

Enzyme assay and measurement of protein content. [4-^{14}C]-5α-Dihydrotestosterone (3 nmol, 3×10^4 c.p.m. per flask) was incubated at 37°C for 60 min with several fractions in the presence of NADPH (1.2 μmol per flask, Boehringer, Mannheim, Germany) in the 5 mM phosphate buffer containing 7 mM 2-mercaptoethanol, 1 mM EDTA-Na_2 and 20% (v/v) glycerol, after total vol. of incubation mixture was adjusted up to 2.5 ml. After the incubation, the enzyme reaction was terminated by adding methylene chloride (3 ml per flask) and the mixture was vigorously shaken to extract the steroids.

To the methylene chloride extract, non-radioactive marker steroids such as progesterone, 4-androstene-3,17-dione, 17α-hydroxyprogesterone, testosterone and 11-deoxycortisol were added. After the extract was concentrated under the reduced pressure, the mixture was analyzed by t.l.c. with silica gel (E. Merck, G and GF, 4:1, w/w, Darmstadt, Germany) in a system of benzene–acetone mixture (8:2, v/v). The marker steroids were visualized under U.V. light (254 nm). 5α-Androstane-3α,17β-diol was localized between the spots of testosterone and 11-deoxycortisol. By autoradiographic detection, the radioactive spot corresponding to 5α-androstane-3α,17β-diol was scraped from the plate and extracted from the silica gel with methylene chloride. Radioactivity in the extract was measured by a liquid scintillation spectrometer, after dissolving the extract in the following liquid scintillator (PPO 4 g, and POPOP 100 mg in 1000 ml of toluene). From the amount of radioactivity measured, the amount of the product in nmol was calculated for evaluation of 3α-hydroxysteroid dehydrogenase activity. Protein content in the eluate was measured by its absorption at 280 nm [5].

Expression of the enzyme activity. Tentatively, to compare the enzyme activities in fractions, one unit of the 3α-hydroxysteroid dehydrogenase activity was defined as the activity of forming 1 nmol of 5α-androstane-3α,17β-diol per min from 5α-dihydrotestosterone. Under the assay condition, the velocity of the enzyme reaction was confirmed as proportional to the concentration of the dehydrogenase.

Gel electrophoresis. Polyacrylamide gel electrophoresis of the enzyme preparation was carried out, according to the procedure previously reported [6]. Protein on the gel was stained by a solution of Coomassie Brilliant Blue 1.25 g, 454 ml of 50% aqueous methanolic solution (v/v) and glacial acetic acid 46 ml.

RESULTS

Separation of the 3α-hydroxysteroid dehydrogenase in the cytosol fraction by fractional precipitation with ammonium sulphate. The cytosol protein was precipitated by gradual addition of solid ammonium sulphate at the following concentrations, 0–40% and 40–100% saturation of the salt. As shown in Table 1, the major activity of the 3α-hydroxysteroid dehydrogenase was localized in the fraction precipitated by 40–100% saturation. Specific activity of the enzyme in the 40–100% saturation fraction was more than 2 times as much as that in the other fraction (Experiment I). The fraction precipitated by 40–100% saturation of the salt was separated into the following subfractions: 40–55%, 55–70% and 70–100% saturation of ammonium sulphate. The enzyme activity in these subfractions were analyzed by the same method as previously mentioned along with the fraction precipitated at 0–40% saturation. After incubation of these fraction with 5α-dihydrotestosterone in the presence of NADPH, 5α-androstane-3α,17β-diol was hardly detectable in the fraction precipitated by 0–40% saturation, but the three subfractions contained activities similar to that of the dehydrogenase (Experiment II). Therefore, the pooled fraction precipitated by 40–100% saturation was subjected to the following two different types of column chromatography.

Purification of the 3α-hydroxysteroid dehydrogenase by a DEAE-cellulose column chromatography from the cytosol fraction precipitated by ammonium sulphate at 40–100% saturation. A DEAE-cellulose column (1.6 × 37 cm) was previously equilibrated with 5 mM phosphate buffer. The enzyme solution was dialyzed against distilled water and the contents of the cellophane bag were applied to the column. After elution with the buffer 350 ml, the concentration of sodium

Table 1. Fractionation of 3α-hydroxysteroid dehydrogenase by precipitation with ammonium sulphate from the cytosol fraction of rat prostate

Fraction (Saturation in % of ammonium sulphate)	Enzyme activity (Unit)	Protein (mg)	Specific activity (Unit/mg protein)
Experiment I			
0–40	4.6	633	0.0072
40–100	35.6	2,147	0.0166
Experiment II			
0–40	0.0397	5.5	0.0072
40–55	0.0985	7.5	0.0131
55–70	0.1653	16.7	0.0099
70–100	0.1190	9.0	0.0132

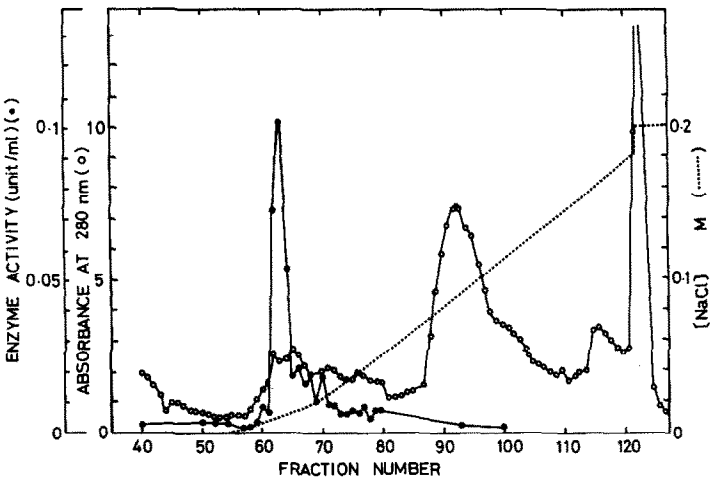

Fig. 1. DEAE-cellulose chromatography of the 3α-hydroxysteroid dehydrogenase of rat prostate cytosol. The enzyme preparation was applied to a DEAE-cellulose column. For elution of enzyme, concentration of sodium chloride was linearly increased from Fraction No. 55 as shown in broken line. open circle: absorbance at 280 nm. closed circle: 3α-hydroxysteroid dehydrogenase activity.

chloride was increased approximately linearly from 0 to about 0.2 M by gradually mixing 250 ml of 5 mM phosphate buffer with 250 ml of the same concentration of the phosphate buffer containing 0.2 M sodium chloride in a conventional gradient maker (Pharmacia, Uppsala, Sweden). The eluate was collected in 7 ml fractions. As shown in Fig. 1, a single peak of the dehydrogenase activity was observed in the eluate from the column at 0.01 M NaCl concentration. Therefore, fractions No. 62–No. 64 were pooled (21 ml).

Further purification of the enzyme by a Sephadex gel filtration. The enzyme preparation which was purified by a DEAE-cellulose chromatography was concentrated to 0.5 ml by adding ammonium sulphate, and then subjected to a Sephadex gel filtration. The column (1.6 × 92.5 cm) of Sephadex G-100 was equilibrated with the buffer without glycerol. After application of the enzyme preparation to the column, the enzyme preparation was eluted with the buffer without glycerol at a rate of 30 ml/60 min. The effluent was collected as 5 ml fractions. The enzyme activity was eluted around 110 ml of the buffer as shown in Fig. 2, where the void volume was around 75 ml. Then, the fractions from No. 21–24 were pooled (20 ml) as the final enzyme preparation. Data of purification of the enzyme at the three stages were summarized in Table 2, which showed about 190-fold purification in terms of S.A. at the final stage, compared with the fraction precipitated by ammonium sulphate at 40–100% saturation.

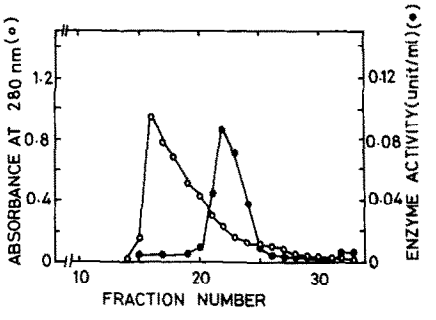

Fig. 2. Sephadex G-100 gel filtration of the 3α-hydroxysteroid dehydrogenase. The enzyme preparation was applied to a Sephadex G-100 column and developed with the 5 mM KH_2PO_4 buffer containing 1 mM EDTA-Na_2 and 7 mM 2-mercaptoethanol. open circle: absorbance at 280 nm. closed circle: the enzyme activity.

Electrophoretic analysis of the enzyme preparation. The enzyme preparation partially purified by the above mentioned procedures (about 30 μg protein in 5 μl) was subjected to a polyacrylamide gel electrophoretic analysis. After staining the developed gel with Coomassie Brilliant Blue, the two major protein bands which were closely located and a few minor bands were observed on the gel, as shown in Fig.

Table 2. Summarized results of purification of 3α-hydroxysteroid dehydrogenase from the cytosol fraction of rat prostate

Fraction	Total enzyme activity (Unit)	Total protein (mg)	Specific activity (Unit/mg protein)	Purification (Fold)
Precipitate at 40–100% saturation with ammonium sulphate	3.079	1,654	0.00186	1
DEAE-cellulose eluate (Fraction No. 62–64)	1.450	30	0.0483	26
Sephadex G-100 eluate (Fraction No. 21–24)	1.146	3.2	0.358	192

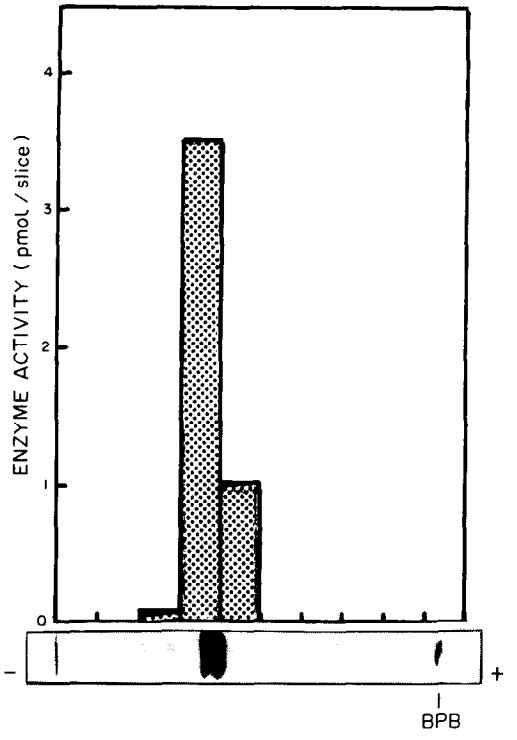

Fig. 3. Polyacrylamide gel electrophoresis of the 3α-hydroxysteroid dehydrogenase preparation obtained at the final step. The upper part represents distribution of 3α-hydroxysteroid dehydrogenase activities over the gel, and the lower part shows location of the stained protein bands by Coomassie Brilliant Blue. BPB: Bromphenol Blue.

3. The other gel which was prepared with the increased amount of the same preparation (about 300 μg of protein in 50 μl) under the identical condition was frozen and cut into serial sections 6 mm in thickness. Each section was homogenized in 0.5 ml of 5 mM phosphate buffer with a glass–Teflon homogenizer and was incubated with [^{14}C]-labeled 5α-dihydrotestosterone (0.438 nmol, 35,000 c.p.m.) in the presence of NADPH (1.2 μmol per flask) for 60 min at 45°C, which was the optimal temperature for the dehydrogenase, as described at the end of Results. After incubation, the radioactivity of 5α-androstane-3α,17β-diol was measured, and the amount of the product was calculated from radioactivity recovered. As shown in Fig. 3, the major activity of the 3α-hydroxysteroid dehydrogenase was found at the position of the major bands of the protein.

Detection of 5α-dihydrotestosterone-receptor in the final enzyme preparation. The prostatic cytosol fraction (18 mg protein/ml, supernatant fluid at 105,000 ***g***) and the final dehydrogenase preparation (0.5 mg protein/ml) were separately incubated with [^{3}H]-5α-dihydrotestosterone at 37°C in the absence of NADPH or other cofactors for 20 min. The mixture was then subjected to gel filtration on Sephadex G-100 (2.5 × 50 cm, void vol. 55 ml). The column chromatography was carried out by eluting with 10 mM Tris–HCl solution (pH 7.4), containing 0.05 mM EDTA-Na_2 and 0.5 mM 2-mercaptoethanol.

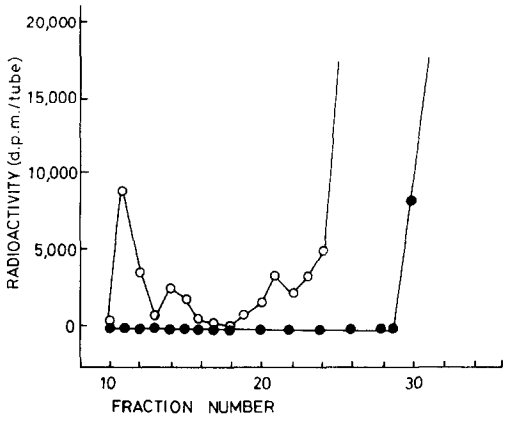

Fig. 4. Detection of androgen receptors in the final 3α-hydroxysteroid dehydrogenase preparation and in the cytosol fraction. The cytosol and the final enzyme fractions were incubated with high-specific active [^{3}H]-dihydrotestosterone (1.0 μCi) at 37°C for 20 min, and then the incubation mixtures were subjected to gel filtration of Sephadex G-100. open circle: the cytosol fraction. closed circle: the final enzyme preparation.

Every 5 ml of the effluent was collected and the tritium content in the effluent was measured in a liquid scintillation spectrometer (Mark II, Nuclear Chicago, Des Plaines, Ill.), after addition of the mixture of Triton X-100 and the regular liquid scintillator (1:3, v/v, 11 ml) to 0.5 ml of the effluent. As shown in Fig. 4, the cytosol fraction showed two significant peaks of tritium which bound the macromolecules, corresponding to position of 9*s* and 5*s* receptors [7]. In the case of the final preparation of the 3α-hydroxysteroid dehydrogenase, however, no detectable receptor which bound the tritiated 5α-dihydrotestosterone was observed. Furthermore, free steroids in fraction No. 33 of the final preparation of the dehydrogenase was analyzed by t.l.c. on silica gel developed with a solvent system (benzene–acetone = 8:2, v/v).

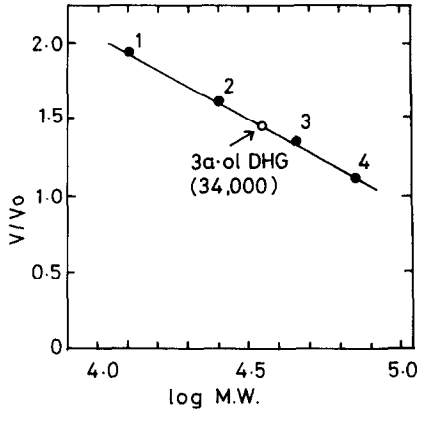

Fig. 5. Estimation of molecular weight of the 3α-hydroxysteroid dehydrogenase by the gel filtration of Sephadex G-100. Standard proteins used as the references. 1. cytochrome *c* 2. α-chymotrypsinogen A 3. ovalbumin 4. bovine serum albumin. open circle: 3α-hydroxysteroid dehydrogenase. V/V_0: ratio of the vol. of effluent to the void vol. log M.W.: log molecular weight.

Table 3. Cofactor preference of the 3α-hydroxysteroid dehydrogenase in oxido-reduction between 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol

Substrate	Cofactor (μmol/flask)	Product (nmol/flask)
5α-Dihydrotestosterone	None	0.018
	NADH (1.4)	0.180
	NADPH (1.2)	0.666
5α-Androstane-3α,17β-diol	None	0.012
	NAD^+ (1.4)	0.066
	$NADP^+$ (1.2)	0.213

Enzyme preparation (0.01 unit in 0.3 ml per flask) and substrates (3 nmol) were incubated with the cofactors at 37°C for 60 min in the 5 mM phosphate buffer (final volume 2.5 ml/flask, pH 7.5), containing 7 mM 2-mercaptoethanol, 1 mM EDTA–Na_2 and 20% (v/v) glycerol.

Almost all of the tritium was found on the spot corresponding to 5α-dihydrotestosterone, but no activity on the spot of 5α-androstane-3α,17β-diol.

Estimation of molecular weight of the 3α-hydroxysteroid dehydrogenase by gel filtration. As shown in the elution pattern from the Sephadex G-100 column, the enzyme activity was eluted in a symmetrical peak at fraction No. 22 (Fig. 2), and the vol. of total eluate up to this fraction was 110 ml. Simultaneously, molecular weight marker proteins such as cytochrome *c*, α-chymotrypsinogen A, bovine serum albumin and ovalbumin were examined to ascertain their elution patterns under identical conditions. From the relationship between the molecular weights of the marker proteins and the elution vols relative to the void vol. (75 ml), the molecular weight of the 3α-hydroxysteroid dehydrogenase was estimated as 34,000 daltons, according to the measurement illustrated in Fig. 5.

Oxidoreduction between 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol in the presence of several cofactors. By employing the final preparation of the 3α-hydroxysteroid dehydrogenase, reduction of 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol in the presence of NADPH and NADH, and oxidation of 5α-androstane-3α,17β-diol in the presence of $NADP^+$ and NAD^+ were compared. As shown in Table 3, neither 5α-dihydrotestosterone nor 5α-androstane-3α,17β-diol were oxido-reduced by the enzyme in the absence of the factors. For reduction of 5α-dihydrotestosterone by the enzyme, NADPH was preferable to NADH as hydrogen donor. In the case of oxidation of 5α-androstane-3α,17β-diol to 5α-dihydrotestosterone by the dehydrogenase, $NADP^+$ was preferred to NAD^+. On the other hand, with the same time of incubation (60 min), the amount of the product obtained by the enzymic oxidation was almost one third of the reduced product by the same enzyme.

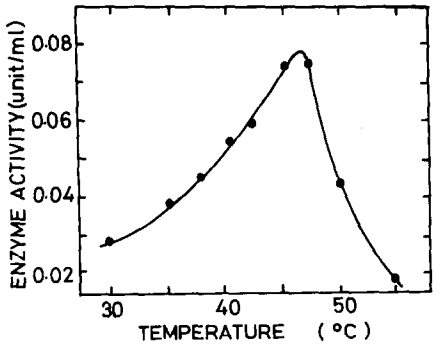

Fig. 6. Optimal temperature of the 3α-hydroxysteroid dehydrogenase at pH 7.4. 5α-Dihydrotestosterone (3 nmol, 3×10^4 c.p.m.) was incubated with the partially purified enzyme preparation (8.5×10^{-3} unit) and NADPH (1.2 μmol) per flask for 60 min at the various temperatures.

Optimal temperature of the 3α-hydroxysteroid dehydrogenase. When the incubation medium was fixed at pH 7.4, the optimal temperature for the partially purified dehydrogenase preparation was found around 45–47.5°C, and beyond 50°C, the enzyme activity was severely denatured, as shown in Fig. 6.

DISCUSSION

Formation of 5α-androstane-3α,17β-diol from 5α-dihydrotestosterone and testosterone has been reported in homogenates and subcellular fractions of ventral prostate [1, 8, 9, 10], seminal vesicle [11], kidney [12], submaxillary gland [12], skin [15] and epididymis [16]. These are tissue responsive to androgen, but the conversion also occurs in liver [17]. In testicular tissue which is the major site of androgen formation, 3α-reduction of 5α-dihydrotestosterone occurs [18], and the site of the reduction is thought most likely to be the Sertoli cell [19].

Several characteristics of the 3α-hydroxysteroid dehydrogenase in the cytosol fraction of rat ventral prostate had been revealed without purification of the enzyme; optimal pH 6.4, and linear relationships between 3α-reduction and incubation time, or enzyme concentration, etc. [9]. In addition to those results, the molecular weight of the partially purified preparation of the enzyme was estimated as 34,000 daltons, on the basis of the dehydrogenase activity, and optimal temperature at pH 7.4 was about 45–47.5°C in the present paper. In this regard, Unhjem [20] has reported that the molecular weight of the dehydrogenase in the same organ is about 40,000–50,000 dal-

tons by a Sephadex gel filtration, without any previous purification of the enzyme from the cytosol fraction. The molecular weight of 3α-hydroxysteroid dehydrogenase of mouse kidney was reported as 28,700 and 38,700 daltons [21], and 20,000–40,000 daltons for rat ovarian cytosol [22]. When purified from *Pseudomonas testosteroni*, the molecule of the 3α-hydroxysteroid dehydrogenase consisted of two subunits with molecular weight 25,000 daltons [23].

As 5α-dihydrotestosterone was employed as the substrate for assay of 3α-hydroxysteroid dehydrogenase in the present study, 5α-androstane-3α,17β-diol was obtained as the major product. No significant amount of other metabolites such as its 3β-epimer or its further metabolites were found. Previously, the 3β-epimer was reported as one of the active androgens upon the cultured prostate cell [24], but the cytosol fraction was practically devoid of the 3β-hydroxysteroid dehydrogenase activity under the condition employed for the enzyme assay.

By fractional precipitation with ammonium sulphate, the precipitate at 0–40% saturation contained receptor component [7], and other factor(s) which competitively inhibited the 5α-reduction of testosterone by the prostate microsomal fraction [1]. The same inhibiting factor was reported in the case of seminal vesicle [11]. On the other hand, almost 90% of the total enzyme activity in the cytosol fraction was concentrated in the precipitate with 40–100% saturation, which was practically free from the receptor, which is in agreement with our previous result [7].

We tried to stain the enzyme activity on the gel by a histochemical principle of Formazan formation, which was effective in demonstration of porcine testicular 17β-hydroxysteroid dehydrogenase in the gel [25]. Due to the relatively low activity in the direction of oxidation of 5α-androstane-3α,17β-diol by the 3α-hydroxysteroid dehydrogenase in comparison with its reducing activity, however, location of the dehydrogenase activity on the gel could not be determined by this procedure. Therefore, the thin sections of the frozen gel were serially prepared, and each section was assayed on the basis of the reducing activity of the enzyme. In the final step, enzyme activity was easily lost in the absence of glycerol. The electrophoretic analysis and further purification of the enzyme may be carried out in the near future, after a method of stabilizing the purified enzyme has been established.

Recently, 3α-hydroxysteroid dehydrogenase has been solubilized from the hepatic microsomal fraction of rat, and purified by a double affinity chromatography to yield the enzyme preparation with a S.A. 100 fold that of the microsomal activity [26].

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