Solubilization and partial characterization of rat epididymal Δ^4 -steroid 5 α -reductase (cholestenone 5 α -reductase)

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Epididymal Δ^4 -steroid 5 α -reductase (cholestenone 5 α -reductase), the enzyme that catalyses the conversion of testosterone into the biologically active metabolite dihydrotestosterone (17 β -hydroxy-5 α -androstane-3-one), is a membrane-bound enzyme found in both nuclear and microsomal subcellular fractions. In order to characterize epididymal Δ^4 -steroid 5 α -reductase, it was first necessary to solubilize the enzymic activity. Of the various treatments tested, a combination of 0.5% (w/v) Lubrol WX, 0.1 M-sodium citrate and 0.1 M-KCl maintained enzymic activity at control values and solubilized 66% of total epididymal Δ^4 -steroid 5 α -reductase activity in an active and stable form. The sedimentation coefficient of solubilized Δ^4 -steroid 5 α -reductase, as determined in continuous sucrose density gradients, was greater for the microsomal than for the nuclear enzyme (11.6S compared with 10.1S). Although the apparent K_m values of the enzyme for testosterone were similar in nuclear and microsomal subcellular fractions (range 1.75×10^{-7} – 4.52×10^{-7} M), the apparent K_m of the enzyme for NADPH was about 30-fold greater for the microsomal enzyme than for the nuclear enzyme. The apparent K_m of the enzyme for either substrate was not significantly altered after solubilization. The relative capacity of steroids to inhibit the enzymic activity, the pH optima and the effects of Ca^{2+} and Mg^{2+} were similar for membrane-bound and solubilized Δ^4 -steroid 5 α -reductase in both the nuclear and the microsomal fractions. The results reported demonstrate that epididymal Δ^4 -steroid 5 α -reductase can be solubilized in an active and stable form with no significant changes in the kinetic characteristics of the enzyme after solubilization; furthermore, kinetic and molecular-size differences observed for the nuclear and the microsomal forms of the enzyme suggest that there may exist at least two forms of epididymal Δ^4 -steroid 5 α -reductase.

Δ^4 -Steroid 5 α -reductase (cholestenone 5 α -reductase, 3-oxo-5 α -steroid:NADP⁺ Δ^4 -oxidoreductase, EC 1.3.1.22) catalyses the conversion of testosterone into the 5 α -reduced androgen dihydrotestosterone (17 β -hydroxy-5 α -androstane-3-one). The presence of Δ^4 -steroid 5 α -reductase activity in the epididymis is well established (Gloyne & Wilson, 1969; Inano *et al.*, 1969; Frankel & Eik-Nes, 1970; Blaquier, 1971; Djøseland, 1976; Robaire *et al.*, 1977a,b).

Spermatozoa mature in the caput–corpus epididymis and are subsequently stored in the cauda epididymis (Orgebin-Crist *et al.*, 1975; Bedford, 1975; Turner, 1979). Although the mechanism(s) by which the epididymis accomplishes these functions remains obscure, both maturation and storage of spermatozoa have been shown to be androgen-dependent (Orgebin-Crist *et al.*, 1975; Bedford, 1975). Importantly, the regulatory effects of testo-

sterone on these epididymal functions are mediated by 5 α -reduced androgens (Lubicz-Nawrocki, 1973; Podesta *et al.*, 1975; Orgebin-Crist *et al.*, 1975, 1976; Cohen *et al.*, 1981). Thus the physiological significance of epididymal Δ^4 -steroid 5 α -reductase activity lies in the formation of the biologically active metabolite dihydrotestosterone from testosterone.

In the epididymis, Δ^4 -steroid 5 α -reductase is a membrane-bound protein found in both nuclear and microsomal subcellular fractions of epididymal homogenates (Robaire *et al.*, 1977a; de Larminat & Blaquier, 1978; Kinoshita, 1981). Whereas hepatic Δ^4 -steroid 5 α -reductase activity has been solubilized and partially characterized (McGuire & Tomkins, 1960; Golf & Graef, 1978), little is known about this enzyme in androgen target tissues. Though some progress has been made toward solubilizing prostatic Δ^4 -steroid 5 α -reductase in the

past (Frederiksen & Wilson, 1971; Shimazaki *et al.*, 1971), these efforts have not been pursued. The lack of a method for solubilizing epididymal Δ^4 -steroid 5 α -reductase activity has limited the biochemical characterization of this enzyme. We now report that epididymal Δ^4 -steroid 5 α -reductase can be solubilized in an active and stable form from nuclear and microsomal fractions. Molecular-size estimates of solubilized Δ^4 -steroid 5 α -reductase as well as some of the kinetic properties of the enzyme, in both membrane-bound and solubilized forms, differ for the nuclear and the microsomal enzymes.

Experimental

Chemicals

Steroids were purchased from Steraloids (Wilton, NH, U.S.A.). Radiolabelled testosterone ([1,2,6,7- 3 H]testosterone, 98.2 Ci/mmol) was obtained from New England Nuclear (Lachine, Que., Canada) and purified by t.l.c. before use. All detergents and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade and purchased from Fisher Scientific Co. (Montreal, Que., Canada).

Tissues

Male Sprague-Dawley rats, obtained from the Charles River Breeding Farm (St. Constant, Que., Canada), were maintained on a 14 h-light/10 h-dark lighting schedule and given food and water *ad libitum*. Animals 10–11 weeks of age were decapitated, and epididymides were dissected free from surrounding tissues and rinsed with ice-cold 0.9% NaCl, weighed and then stored at -80°C until use.

Assay of Δ^4 -steroid 5 α -reductase activity

Δ^4 -Steroid 5 α -reductase activity was measured radiometrically by using previously described methods (Robaire *et al.*, 1977b; Robaire, 1979). Radiolabelled testosterone accounted for less than 2% of total testosterone present in all incubations. Conversion rates were maintained between 0.3% and 15% by using appropriate amounts of enzyme to ensure linearity. Under all conditions described, dihydrotestosterone accounted for more than 95% of all steroid metabolites formed from testosterone.

Solubilization of Δ^4 -steroid 5 α -reductase activity

Two different methods were used for the preparation of solubilized Δ^4 -steroid 5 α -reductase activity. In method A, a whole-membrane fraction was used to test for the effects of different solubilizing agents on Δ^4 -steroid 5 α -reductase activity. Method B describes the solubilization of enzymic activity from nuclear and microsomal fractions.

Method A. Epididymides were suspended in ice-cold Krebs–Ringer phosphate buffer containing

116 mM-NaCl, 4.5 mM-KCl, 2.5 mM-CaCl₂, 1.3 mM-MgCl₂, 0.1 mM-NADPH, 10 mM-sodium phosphate buffer and 684 mM-glycerol (pH 6.9). Unless otherwise stated, this buffer was used throughout. All operations were performed at 4°C . Tissues were homogenized in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.) at a setting of 5 for two 15 s periods. After filtration through a $93\ \mu\text{m}$ nylon mesh (Techo Co., Montreal, Que., Canada), the homogenate was centrifuged at 100 000 g for 1 h in a Beckman L2-65B ultracentrifuge with a type-65 rotor. The supernatant was discarded, and the pellet was resuspended in Krebs–Ringer phosphate buffer by five strokes in a Potter–Elvehjem Teflon/glass homogenizer (Sargent Welch, Toronto, Ont., Canada). Samples (250 μl) of the membrane preparation (13–16 mg of protein/ml) were added to solutions of the solubilizing agents dissolved in Krebs–Ringer phosphate buffer [total volume 1 ml containing 20% (v/v) glycerol; this concentration of glycerol was necessary for maintenance of membrane-bound Δ^4 -steroid 5 α -reductase activity]. The solutions were shaken for 1 h at 150 cycles/min. A sample from each solution was immediately assayed for Δ^4 -steroid 5 α -reductase activity, and the remainder was centrifuged at 150 000 g for 90 min; the supernatant was decanted off, and the pellet was resuspended in 1 ml of Krebs–Ringer phosphate buffer; both pellet (membrane fraction) and supernatant (solubilized fraction) were immediately assayed for Δ^4 -steroid 5 α -reductase activity.

Method B. Epididymides, dissected into caput–corpus and caudal segments, were suspended in Krebs–Ringer phosphate buffer, homogenized and filtered as described above. The resulting homogenate was centrifuged at 3000 g for 10 min in a Sorvall RC-5 centrifuge (Dupont, Newton, CT, U.S.A.). The supernatant was decanted into 10 ml ultracentrifuge tubes and centrifuged at 100 000 g for 60 min. The pellet of the 3×10^4 g-min centrifugation was washed three times and resuspended in Krebs–Ringer phosphate buffer (15–20 mg of protein/ml); this preparation was termed the nuclear fraction. The pellet of the 6×10^6 g-min centrifugation was resuspended in Krebs–Ringer phosphate buffer (15–18 mg of protein/ml); this preparation was termed the microsomal fraction; previous studies in our laboratory (Scheer & Robaire, 1983) and by others (de Larminat & Blaquier, 1978; Kinoshita *et al.*, 1980) indicate that Δ^4 -steroid 5 α -reductase is unlikely to be associated with other organelles in this fraction. Membrane-bound nuclear and microsomal Δ^4 -steroid 5 α -reductase activities were immediately assayed in a 5-fold dilution of the respective subcellular fractions. Samples of the nuclear and the microsomal fractions were then added to 10% (w/v) Lubrol WX, 2 M-sodium citrate, 2 M-KCl, glycerol and Krebs–Ringer phosphate buffer so that the final

concentrations were 0.5% (w/v) Lubrol WX, 0.1 M-sodium citrate, 0.1 M-KCl and 20% (v/v) glycerol (7–10 mg of protein/ml). The suspension was then vigorously stirred with Teflon magnetic bars for 1 h. The activity remaining in the supernatant after a 1.2×10^7 g-min centrifugation was defined as the solubilized enzyme preparation.

Determination of sedimentation coefficients

Linear 5–15% (w/v) sucrose gradients in homogenization medium were prepared, by using a linear-sucrose-density-gradient mixer (Beckman Instruments, Palo Alto, CA, U.S.A.), in cellulose nitrate tubes (Beckman, no. 328906). The solubilized enzyme preparations were first concentrated by a 2 h dialysis against Ficoll 40 (Pharmacia, Montreal, Que., Canada) and subsequently layered on the gradients. The gradients were centrifuged at 175 000 g for 18 h in an SW-56 rotor in a Beckman L2-65B ultracentrifuge. At the end of the run, 150 μ l samples were collected from the perforated bottom of the tube by application of positive air pressure to the top of the tube. The fractions were immediately assayed for Δ^4 -steroid 5 α -reductase activity. Enzyme markers were run in parallel gradients. Enzyme activities were measured spectrophotometrically: catalase was measured by the method of Beers & Sizer (1952), fructose bisphosphate aldolase by the method of Jagannathan *et al.* (1956) and β -glucuronidase by the method of Fishman *et al.* (1948).

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. For determination of protein in solubilized enzyme preparations, standard curves were prepared in the presence of equivalent amounts of solubilization medium. All samples were centrifuged at 1500 g for 10 min just before reading of absorbance; the resulting standard curves were similar in the presence and in the absence of solubilization medium.

Data analysis

Statistical analysis was performed by the Mann-Whitney U-test ($\alpha = 0.05$) (Snedecor & Cochran, 1967). K_m and V_{max} values were determined by regression analysis as described by Wilkinson (1963). Intercept and slope values for Lineweaver-Burk, Dixon and Cornish-Bowden plots (Segel, 1975; Dixon & Webb, 1979) were obtained by linear regression (Snedecor & Cochran, 1967).

Results

Solubilization of epididymal Δ^4 -steroid 5 α -reductase activity

Various agents were tested for their capacity to

maintain and solubilize epididymal Δ^4 -steroid 5 α -reductase activity (Table 1). Of the compounds tested, only sodium citrate, Tween 80 and Lubrol WX did not decrease enzymic activity before centrifugation. The highest percentage of Δ^4 -steroid 5 α -reductase activity solubilized was obtained with 0.05% Emulgen 911 or with 0.5% Triton X-100; however, these detergents markedly decreased Δ^4 -steroid 5 α -reductase activity (solubilized enzyme activity was less than 10% of the original activity). As Lubrol WX and sodium citrate both maintained and solubilized enzymic activity, their use as solubilizing agents was further investigated.

To test whether the combined presence of solubilizing agents increased the percentage of Δ^4 -steroid 5 α -reductase activity solubilized, experiments were done with 0.5% Lubrol WX, 0.1 M-sodium citrate and 0.1 M-KCl in combination (Fig. 1). All six possible combinations maintained Δ^4 -steroid 5 α -reductase activity at control values. The single most

Table 1. Capacity of various agents to maintain and to solubilize epididymal Δ^4 -steroid 5 α -reductase activity
For experimental details see the text. The percentage of Δ^4 -steroid 5 α -reductase activity solubilized is expressed as the activity in the supernatant after a 1.2×10^7 g-min centrifugation divided by the total activity (pellet plus supernatant).

Treatment		% of Δ^4 -steroid 5 α -reductase activity before centrifugation	% of Δ^4 -steroid 5 α -reductase activity solubilized
None (control)		100	0
KCl	0.5 M	92	0
	1.0 M	74	12
	2.0 M	70	21
EDTA	0.1 M	31	6
Sodium citrate	0.1 M	105	31
Emulgen 911	0.05% (v/v)	54	50
	0.20% (v/v)	0	Undefined*
	0.05% (v/v)	0	Undefined*
Triton X-100	0.10% (v/v)	18	0
	0.50% (v/v)	11	100
	1.00% (v/v)	0	Undefined*
Triton CF-54	0.10% (v/v)	9	Undefined*
	1.00% (v/v)	0	Undefined*
Tween 80	0.10% (v/v)	98	9
	0.50% (v/v)	109	11
	1.00% (v/v)	127	6
Lubrol WX	0.10% (w/v)	129	18
	0.50% (w/v)	103	25
	1.00% (w/v)	114	22

* Undefined ratio (no activity detectable in pellet or supernatant).

Fig. 1. Effects of the combined presence of solubilizing agents on epididymal Δ^4 -steroid 5 α -reductase activity. For experimental details see the text. ▨, Percentage of Δ^4 -steroid 5 α -reductase remaining before centrifugation; untreated control preparation is defined as 100% and is equal to 321 ± 19 pmol/h per mg of protein. ▩, Percentage of Δ^4 -steroid 5 α -reductase activity solubilized; solubilized activity is expressed as activity in supernatant after a 1.2×10^7 g-min centrifugation divided by the activity in the pellet plus that in the supernatant. + indicates the presence of solubilizing agent, - indicates the absence of solubilizing agent. Values are expressed as means $\pm$ S.E.M. ($n = 3$).

effective solubilizing agent was sodium citrate (25% of Δ^4 -steroid 5 α -reductase activity solubilized); Lubrol WX was 60% as effective as sodium citrate, and KCl was 36% as effective. The combined presence of Lubrol WX and sodium citrate solubilized 25% more Δ^4 -steroid 5 α -reductase activity than either agent alone. The addition of KCl increased solubilized Δ^4 -steroid 5 α -reductase activity by about 10% in the presence of either Lubrol WX or sodium citrate or both. Increasing the exposure time (3 h), temperature (22°C) or the concentration of the solubilizing agents (2% Lubrol WX, 0.4 M-sodium citrate and 0.4 M-KCl) before centrifugation did not further augment the percentage of enzymic activity solubilized (results not shown). Thus maximal solubilization of Δ^4 -steroid 5 α -reductase activity (66% of Δ^4 -steroid 5 α -reductase activity solubilized) was obtained by the exposure of membrane-bound Δ^4 -steroid 5 α -reductase to 0.5% Lubrol WX, 0.1 M-sodium citrate and 0.1 M-KCl for 1 h at 4°C.

Δ^4 -Steroid 5 α -reductase activity solubilized by 0.5% Lubrol WX, 0.1 M-sodium citrate and 0.1 M-KCl was not pelleted by a further 4.2×10^7 g-min centrifugation. When the solubilized enzyme preparation was dialyzed for 24 h against a medium consisting of 2.74 M-glycerol in Krebs-Ringer phosphate buffer, Δ^4 -steroid 5 α -reductase activity was maintained and could not be pelleted by a 4.2×10^7 g-min centrifugation. The solubilized enzyme preparation was stable for a period of at least 1 week when stored at -20°C.

Kinetic parameters

The kinetic parameters of caput-corpus epididymal Δ^4 -steroid 5 α -reductase activity were determined for the membrane-bound and solubilized forms of the nuclear and the microsomal enzyme (Table 2; for representative graph see Fig. 2). The apparent K_m for testosterone did not change on solubilization, nor were differences between nuclear and microsomal subcellular fractions evident. The apparent K_m for NADPH was also unaffected by solubilization, but was significantly lower (Mann-Whitney U-test) in the nuclear than in the microsomal fraction. The V_{max} of membrane-bound Δ^4 -steroid 5 α -reductase was 40% greater in the microsomal than in the nuclear fraction. Solubilization increased the V_{max} of the nuclear and the microsomal Δ^4 -steroid 5 α -reductase by 50% and 20% respectively; the V_{max} of solubilized nuclear and solubilized microsomal Δ^4 -steroid 5 α -reductase were not significantly different (Mann-Whitney U-test).

In the cauda epididymis the apparent K_m of Δ^4 -steroid 5 α -reductase for testosterone did not differ significantly from the apparent K_m of any of the four enzyme preparations from the caput-corpus epididymis (range $1.75 \times 10^{-7} \pm 0.40 \times 10^{-7}$ M to $4.52 \times 10^{-7} \pm 0.20 \times 10^{-7}$ M; results not shown). The V_{max} of membrane-bound nuclear (121 ± 10 pmol of dihydrotestosterone formed/h per mg of protein) and microsomal Δ^4 -steroid 5 α -reductase (144 ± 21 pmol of dihydrotestosterone formed/h per mg of protein) was increased by 30% and by 45% in the respective

Table 2. *Kinetic parameters for various forms of caput–corpus-epididymal Δ^4 -steroid 5 α -reductase*

For experimental details see the text. $V_{\max}$ was determined with either testosterone or NADPH as the variable substrate. All values are expressed as means $\pm$ S.E.M. ($n = 3$).

	$10^7 \times K_m$ for testosterone (M)	$10^6 \times K_m$ for NADPH (M)	$V_{\max}$ (pmol of dihydrotestosterone formed/h per mg of protein)
Nuclear fraction			
Membrane-bound	1.76 ± 0.30	1.02 ± 0.43	419 ± 32
Solubilized	2.12 ± 0.22	1.19 ± 0.20	633 ± 20
Microsomal fraction			
Membrane-bound	2.50 ± 0.40	37.6 ± 4.10	586 ± 79
Solubilized	2.22 ± 0.28	29.8 ± 6.20	701 ± 66

Fig. 2. *Kinetic properties of epididymal Δ^4 -steroid 5 α -reductase*

For experimental details see the text. The saturation curve of Δ^4 -steroid 5 α -reductase with testosterone as the variable substrate and excess NADPH is shown. The curve was analysed by the method of Wilkinson (1963). The inset is a Lineweaver–Burk plot of the same curve. The K_m and $V_{\max}$ values obtained by the two methods were similar (K_m $2.05 \times 10^{-7} \pm 0.02 \times 10^{-7}$ M and 2.11×10^{-7} M respectively; $V_{\max}$ 629 ± 0.5 and 653 pmol of dihydrotestosterone formed/h per mg of protein respectively). This curve was generated by using the nuclear membrane-bound caput–corpus-epididymal preparation; similar curves were obtained for all other forms of the enzyme (see the text).

Fig. 3. *Inhibition of Δ^4 -steroid 5 α -reductase activity*

For experimental details see the text. The vertical axis represents the percentage of membrane-bound Δ^4 -steroid 5 α -reductase activity in the nuclear fraction in the presence of inhibitors. The horizontal axis represents the ratio of inhibitor to substrate concentration. For steroid inhibitor studies, the concentration of testosterone was $0.2 \mu\text{M}$ and the concentration of NADPH was 0.5 mM ; the inhibitor concentration ranged from 2 nM to 0.2 mM . For the NADPH inhibitor studies, the concentrations of testosterone and NADPH were $0.5 \mu\text{M}$ and $50 \mu\text{M}$ respectively; the NADPH concentration ranged from $0.4 \mu\text{M}$ to 10 mM . The relative inhibition by steroids was similar for all forms of the enzyme (see the text). ▲, Dihydrotestosterone, 5 α -androstane-3 α ,17 β -diol, 5 α -androstane-3 β ,17 β -diol, 5 α -dihydroprogesterone, 11 β -hydroxyandrost-4-ene-3,17-dione or -androst-5-ene-3,17-dione; ■, progesterone; ●, 17 α -hydroxyprogesterone; △, androst-4-enedione; ○, NADPH; □, NADPH.

subcellular fractions after solubilization. The $V_{\max}$ of cauda-epididymal Δ^4 -steroid 5 α -reductase activity ranged from 25% to 30% of that of the caput–corpus-epididymal enzyme for both the nuclear and the microsomal fractions in either the membrane-bound or solubilized forms.

Inhibition of Δ^4 -steroid 5 α -reductase activity

Various analogues of the substrates and products for Δ^4 -steroid 5 α -reductase were tested for their capacity to inhibit caput–corpus-epididymal Δ^4 -steroid 5 α -reductase activity (Fig. 3). Dihydro-

Fig. 4. Dixon plots of inhibitors of Δ^4 -steroid 5 α -reductase

For experimental details see the text. The enzyme source was membrane-bound Δ^4 -steroid 5 α -reductase from the nuclear fraction. (a) Inhibition by androst-4-enedione. The concentrations of testosterone were 0.066 μM ($\blacktriangledown$), 0.2 μM ($\square$) and 0.5 μM ($\bullet$). The NADPH concentration was 0.5 mM. The concentration of androst-4-enedione varied from 0.1 μM to 6.0 μM . (b) Inhibition by progesterone. The concentrations of testosterone were 0.066 μM ($\blacktriangledown$), 0.2 μM ($\square$) and 0.5 μM ($\bullet$). The NADPH concentration was 0.5 mM. The concentration of progesterone varied from 0.1 μM to 6 μM . (c) Inhibition by NADP $^+$. The concentration of testosterone was 0.8 μM . The concentrations of NADPH were 0.01 mM ($\blacktriangledown$) and 0.05 mM ($\bullet$). The concentrations of NADP $^+$ ranged from 0.001 mM to 0.1 mM. The insets show Cornish-Bowden plots of the same data (inhibitor concentrations are as described above).

testosterone, 5 α -androstane-3 α ,17 β -diol, 5 α -dihydroprogesterone, 11 β -hydroxyandrost-4-ene-3,17-dione, androst-5-ene-3,17-dione and NADP $^+$ did not inhibit nuclear or microsomal Δ^4 -steroid 5 α -reduc-

tase activity in either the membrane-bound or the solubilized state. Progesterone, 17 α -hydroxyprogesterone, androst-4-enedione and NADP $^+$ were the only compounds tested that inhibited Δ^4 -steroid

5 α -reductase activity. The I_{50} values for progesterone, 17 α -hydroxyprogesterone and androst-4-enedione were similar for the nuclear and the microsomal Δ^4 -steroid 5 α -reductase in both the membrane-bound and the solubilized state (Table 3). The I_{50} for NADP⁺ was 5-fold greater in the microsomal than in the nuclear fraction; it was not affected by solubilization.

In order to determine the kinetic nature of inhibition by progesterone, androst-4-enedione and NADP⁺, Dixon-plot and Cornish-Bowden plot analyses were done for all four forms of Δ^4 -steroid 5 α -reductase activity (Fig. 4). Since all four forms of the enzyme exhibited nearly identical kinetic profiles, only the profile for one of the forms is shown in Fig. 4. Androst-4-enedione was a competitive

inhibitor (Fig. 4a) of the nuclear and the microsomal Δ^4 -steroid 5 α -reductase in both the membrane-bound and the solubilized state. Even though the K_i of progesterone could not be determined accurately owing to the presence of multiple intercepts above the abscissa, the inhibition of Δ^4 -steroid 5 α -reductase activity by progesterone appeared to be mixed competitive (Fig. 4b). The cofactor NADP⁺ also appeared to be a competitive inhibitor of the reaction (Fig. 4c).

Estimates of molecular size

In order to estimate the molecular size of the solubilized forms of caput-corpus-epididymal Δ^4 -steroid 5 α -reductase, the mobility of enzymic activity in a 5–15% (w/v) linear sucrose density gradient in solubilization medium was determined (Fig. 5). The sedimentation coefficient was significantly greater for the microsomal form of the enzyme than for the nuclear enzyme (11.6 ± 0.1 S compared with 10.2 ± 0.2 S; $n = 3$; $\alpha = 0.05$).

Effects of Ca²⁺, Mg²⁺ and pH on Δ^4 -steroid 5 α -reductase activity

The presence of the bivalent cations Ca²⁺ and Mg²⁺ in the assay medium (concentration range

Fig. 5. Sedimentation coefficients of solubilized nuclear (a) and solubilized microsomal (b) Δ^4 -steroid 5 α -reductase activity

For experimental details see the text. The vertical axis represents pmol of dihydrotestosterone formed/h per fraction. The bottom of the gradient is on the left, the top of the gradient is on the right of the horizontal axis. For the graphed gradient, the calculated sedimentation coefficient for microsomal Δ^4 -steroid 5 α -reductase was 11.8S and that for nuclear Δ^4 -steroid 5 α -reductase was 10.4S.

Fig. 6. Effects of pH on Δ^4 -steroid 5 α -reductase activity

For experimental details see the text. The vertical axis represents the percentage of Δ^4 -steroid 5 α -reductase activity remaining; 100% is the enzyme activity at pH 6.9. The horizontal axis represents the pH of the assay solution. ■, Membrane-bound nuclear Δ^4 -steroid 5 α -reductase; ●, solubilized nuclear Δ^4 -steroid 5 α -reductase; ○, membrane-bound microsomal Δ^4 -steroid 5 α -reductase; □, solubilized microsomal Δ^4 -steroid 5 α -reductase. The concentration of each of the three buffers was 10mM.

Table 3. I_{50} values for inhibitors for Δ^4 -steroid 5 α -reductase

For experimental details see the text. For androstenedione, progesterone and 17 α -hydroxyprogesterone the testosterone concentration was 0.2 μ M and the NADPH concentration was 0.5 mM. The inhibitor concentration range was 1.0 nM–0.1 mM. For NADP⁺, the testosterone concentration was 0.8 μ M and the NADPH concentration was 0.05 mM; NADP⁺ concentration ranged from 0.2 μ M to 0.1 mM.

	$10^{-7} \times I_{50}$ (M)			
	Nuclear fraction		Microsomal fraction	
	Membrane-bound	Solubilized	Membrane-bound	Solubilized
Androst-4-enedione	8.0	4.0	8.1	10.0
Progesterone	13.2	10.0	9.6	10.3
17 α -Hydroxyprogesterone	11.1	9.8	13.7	11.1
NADP ⁺	81	70	386	333

0.01–10 mM for both cations) did not affect nuclear or microsomal Δ^4 -steroid 5 α -reductase activity in either the membrane-bound or the solubilized form (results not shown). The pH profiles of enzymic activity were similar for all four forms of the enzyme (Fig. 6). Maximal enzymic activity was found to be in the range pH 5.8–7.5; half-maximal enzymic activity was evident at about pH 5 and pH 8.5. Preincubation for 1 h of each of the four enzyme preparations at pH values ranging between 4 and 9, followed by return to pH 6.9 for enzyme assay (standard conditions), resulted in a pH–enzyme activity curve having the same profile as that shown in Fig. 6. This would indicate that the effect of alterations in pH on enzyme activity is a reflection of changes in the stability of the enzyme rather than in the affinity of the active site of the enzyme for the substrate (Dixon & Webb, 1964).

Discussion

The results reported in the present paper demonstrate that epididymal Δ^4 -steroid 5 α -reductase can be solubilized in an active and stable form by using Lubrol WX, sodium citrate and KCl. The ultracentrifugation characteristics of the enzyme preparation in the solubilization medium demonstrate that a basic requirement for solubilization of membrane-bound proteins is indeed fulfilled (Razin, 1972). As the kinetic properties of solubilized Δ^4 -steroid 5 α -reductase were very similar to those of the membrane-bound enzyme, solubilization of the enzyme does not result in major alterations of the active site of the enzyme.

Although previous experiments had demonstrated that Δ^4 -steroid 5 α -reductase activity is markedly greater in the caput–corpus epididymis than in the cauda epididymis (Robaire *et al.*, 1977a; Pujol & Bayard, 1978; Robaire, 1979; Kumari *et al.*, 1980), the underlying reasons for this difference between the two tissue segments were

unknown. As the $V_{\max}$ of the enzyme is about 5-fold greater in the caput–corpus epididymis than in the cauda epididymis, and as the apparent K_m of Δ^4 -steroid 5 α -reductase does not differ, it appears that the difference in enzymic activity in the two tissue segments may be due to a greater number of catalytically similar enzyme molecules in the caput–corpus epididymis.

Similar apparent K_m values of Δ^4 -steroid 5 α -reductase for testosterone as demonstrated in the present study have also been reported for the nuclear and the microsomal Δ^4 -steroid 5 α -reductase in the human (Kinoshita, 1981) and rhesus-monkey epididymis (de Larminat & Blaquier, 1978) and rat ventral prostate (Moore & Wilson, 1974; Nozu & Tamaoki, 1974). Hepatic Δ^4 -steroid 5 α -reductase differs from the enzyme in the epididymis and prostate as the enzyme (a) is predominantly microsomal (Roy, 1971; Moore & Wilson, 1972) and (b) has a K_m for testosterone that is greater by three orders of magnitude (Nozu & Tamaoki, 1974). The presence of Δ^4 -steroid 5 α -reductase with low apparent K_m values (i.e. approx. 10^{-7} M) in both nuclear and microsomal subcellular fractions, however, is not limited to classical androgen-target tissues, since similar enzyme characteristics are found in brain (Cheng & Karavolas, 1975), kidney (Verhoeven & De Moor, 1971) and lung (Verhoeven *et al.*, 1974).

A previously unreported observation for Δ^4 -steroid 5 α -reductase in any tissue is that the enzyme in the nuclear fraction has a K_m for NADPH about 30-fold lower than that of the microsomal enzyme. This difference indicates that the nuclear and the microsomal Δ^4 -steroid 5 α -reductase may have significant catalytic differences with regard to their cofactor, NADPH. The possibility is also raised that differential regulation of nuclear and microsomal enzymic activities may be achieved by cofactor availability. The potential importance of the apparent K_m of Δ^4 -steroid 5 α -reductase for NADPH is further underlined by data that indicate that, in

certain pseudo-hermaphroditic syndromes associated with Δ^4 -steroid 5 α -reductase abnormalities, the enzyme has a normal apparent K_m value for testosterone but abnormal values for NADPH (Griffin & Wilson, 1980).

The lack of inhibition of Δ^4 -steroid 5 α -reductase activity by Δ^5 -steroids and by 11-hydroxy steroids is similar to that observed with prostatic (Frederiksen & Wilson, 1971) and ovarian Δ^4 -steroid 5 α -reductase (Koninckx *et al.*, 1979). The absence of inhibition of Δ^4 -steroid 5 α -reductase by 5 α -reduced steroids indicates that the enzyme in the rat epididymis is not likely to be directly regulated by the steroid products of the reaction. Similar observations have been reported for Δ^4 -steroid 5 α -reductase in the human epididymis (Kinoshita, 1981), ventral prostate (Frederiksen & Wilson, 1971), kidney (Verhoeven & De Moor, 1971) and ovary (Koninckx *et al.*, 1979).

As in the rat prostate (Frederiksen & Wilson, 1971), the ovary (Koninckx *et al.*, 1979) and human epididymis (Kinoshita, 1981), Δ^4 -reduced steroids are potent inhibitors of Δ^4 -steroid 5 α -reductase activity. In contrast with the rat epididymal enzyme, progesterone has been reported to be more effective than androst-4-enedione in inhibiting Δ^4 -steroid 5 α -reductase activity in these tissues. Therefore the active site of the rat epididymal enzyme recognizes 17-substituted steroids in a different manner from that of the enzymes of other species and other tissues. The inhibition of epididymal Δ^4 -steroid 5 α -reductase activity by 17-substituted steroids is of particular biological significance, as progesterone has been shown to diminish the fertilizing capacity of epididymal spermatozoa (Lubicz-Nawrocki & Chang, 1973). As this effect was reversible by 5 α -reduced androgens, it appears that progesterone inhibition of Δ^4 -steroid 5 α -reductase activity decreases the functional capacities of the tissue.

The lack of inhibition by NAD⁺ in either subcellular fraction is consistent with evidence from the human (Kinoshita, 1981) and monkey epididymis (de Larminat & Blaquier, 1978), which indicates that NADH is an ineffective cofactor for both the nuclear and the microsomal Δ^4 -steroid 5 α -reductase. Thus Δ^4 -steroid 5 α -reductase preferentially recognizes the phosphorylated form of the nicotinamide-adenine dinucleotide cofactor both as an inhibitor and as a substrate.

The difference in sedimentation coefficients between the nuclear and the microsomal Δ^4 -steroid 5 α -reductase suggests that the molecular size and/or shape of nuclear enzyme is less than that of the microsomal enzyme. A similar dependence of the sedimentation coefficient on the subcellular localization of Δ^4 -steroid 5 α -reductase activity has also been reported for the ventral-prostatic enzyme (Frederiksen & Wilson, 1971), although the sedimentation

coefficients were considerably higher (range 13.5–15S) than for the epididymal Δ^4 -steroid 5 α -reductase. Thus, in the androgen-dependent tissues in which the molecular size of the nuclear and the microsomal Δ^4 -steroid 5 α -reductase has been investigated, it appears that molecular-size differences exist for the enzyme depending on the subcellular localization.

As nuclear and microsomal Δ^4 -steroid 5 α -reductase activity appear to be differentially regulated as a function of age and tissue segment (Scheer & Robaire, 1983), the investigations into the biochemical properties of the enzyme in different subcellular localizations are of importance to determine the functional significance of intracellular distribution of the enzyme activity. The present data suggest that there may indeed be major differences between the nuclear and the microsomal Δ^4 -steroid 5 α -reductase. The capacity to prepare solubilized Δ^4 -steroid 5 α -reductase in an active and stable form without major alterations in the kinetic properties of the enzyme in either subcellular fraction will allow the further investigation of the biochemical properties of the nuclear and the microsomal Δ^4 -steroid 5 α -reductase in the epididymis.

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