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SOLUBILIZATION OF HUMAN PROSTATIC 5α-REDUCTASE

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Summary—A sensitive assay for 5α-reductase was introduced which is capable of detecting at least 0.2 U of activity per sample. The assay was used in developing a method for the solubilization of human prostatic 5α-reductase. Homogenisation conditions were devised under which 95% of the total prostatic 5α-reductase was released into the microsomal fraction. A combination of 0.1 M sodium citrate, 0.1 M KCl, 20% (v/v) glycerol, 0.5 mM NADPH and 1 μM testosterone was found to stabilise 5α-reductase in the presence of detergents. The effect of the presence of low concentrations of detergents in the assay on the activity of 5α-reductase was studied. Triton X-100, Lubrol PX and Nonidet P-40, caused a concentration-dependent inhibition of activity. The ability of several detergents (Triton X-100 MEGA-9, Tween 20, Tween 80, digitonin, Lubrol PX and Nonidet P-40) to solubilise 5α-reductase was studied. All detergents caused a concentration-dependent solubilization of 5α-reductase. Significant amounts of active solubilized enzyme were recovered only with Lubrol PX at concentrations less than 1.1 mg/ml. Seventy percent of the 5α-reductase was solubilized in an active form by extracting the membranes 3 times with 0.8 mg/ml Lubrol PX.

INTRODUCTION

In a number of androgen dependent tissues including the prostate, dihydrotestosterone (DHT) rather than testosterone, is the active androgen [1]. In these tissues DHT is produced from testosterone by the enzyme 5α-reductase (cholesterone 5α-reductase, 3-oxo-5α-steroid: $NADP^+$ 4-ene-oxidoreductase, EC 1.3.1.22). Much of the work on prostatic 5α-reductase has been directed towards its role in the aetiology of the two major pathological conditions of the prostate, benign prostatic hypertrophy (BPH) and carcinoma of the prostate (CAP). 5α-reductase activity is elevated 3–5-fold above normal levels in BPH tissue while in CAP there is a progressive decrease in 5α-reductase activity with the stage of the disease [2, 3].

In view of the importance of the enzyme in prostatic growth and function it is surprising to find that human prostatic 5α-reductase has not been characterised beyond the measurement of its kinetic parameters in crude subcellular fractions. 5α-Reductase is membrane-bound and is located in the nucleus and endoplasmic reticulum [4, 5]. A prerequisite to purification and further characterisation is therefore its solubilization from these membrane fractions. While methods have been described for the partial solubilization of 5α-reductase from rat prostate and epididymis [6, 7] there is no procedure for the solubilization of the enzyme from any human tissue. We report here the optimisation of conditions for the solubilization of human prostatic 5α-reductase.

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EXPERIMENTAL

Materials

Cholic acid, Lubrol PX, Nonidet P-40, Tween 20, Tween 80, glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase and unlabelled steroids were purchased from Sigma Chemicals Co. Poole, U.K. MEGA 9 was obtained from Cambridge Research Biochemicals, Harston, Cambridgeshire, U.K. 3,5,7,2′,4′,-Pentahydroxyflavone was supplied by BDH Chemicals Ltd, Poole, U.K. All other chemicals were supplied by Sigma Chemical Co. or Fisons Scientific Apparatus, Loughborough, U.K. Gelman ITLC plates were obtained from Mackay & Lynn, Edinburgh, U.K.

5α-Dihydro 4-[^{14}C]testosterone (100–150 Ci/mmol) and 1,2,6,7-[^{3}H]testosterone (100 Ci/mmol) were obtained from Amersham International, Amersham, U.K. [^{3}H]testosterone was purified as described below.

Purification of [^3H] *testosterone*

[^{3}H]Testosterone was adjusted to a specific radioactivity of 4.24 μCi/nmol by the addition of unlabelled testosterone and purified on ITLC plates (solvent system dichloromethane–ether 9:1 (v/v). The testosterone spot was cut from the plate and the steroid was eluted using ethanol. The purified testosterone was adjusted to 20 μM and stored at −20°C.

Assay of 5α-reductase

5α-Reductase was assayed at 37°C by following the conversion of [^{3}H] testosterone to [^{3}H] DHT. Each tube contained, in a final volume of 1 ml, 100 mM Tris–HCl pH 7.4, 100 mM sodium citrate, 100 mM

KCl, 20% (v/v) glycerol, 1 mM EDTA, 15 mM β-mercaptoethanol, 5 mM glucose-6-phosphate, 0.5 mM $NADP^+$, 0.25 U glucose-6-phosphate dehydrogenase and 1 μM-[^{3}H]testosterone. The tubes were preincubated for 15 min at 37°C to allow the complete conversion of $NADP^+$ and the assays were started by the addition of enzyme (5–50 μl). The reactions were allowed to proceed for 15 or 30 min and stopped by the addition of 1 ml diethyl ether followed by 100 μl ethanol containing approx 500 cpm [^{14}C]DHT and 25 μg each of unlabelled 3α-androstanediol, 3α-androstanediol, testosterone, DHT and androstenedione. Each tube was vortexed for 40 s and the phases were separated by brief centrifugation. The ether was transferred to a second tube and evaporated to dryness in a vacuum oven. Repeated extraction of the reaction mixture with ether did not increase the overall recovery of [^{3}H] DHT.

The steroids were taken up in 20 μl ethanol and applied to an ITLC plate. The plate was developed once in dichloromethane–ether, 9:1 (v/v). Using this solvent system DHT ($R_f = 0.60$) is well separated from testosterone ($R_f = 0.38$) and androstenedione ($R_f = 0.74$). The steroids were located by spraying the plates with methanol containing 0.5 mg/ml 3,5,7,2′,4′-pentahydroxyflavone and viewing under u.v. light (=300 nm). The DHT spots were then cut from the plate, placed in scintillation counter-vials and counted for ^{3}H and ^{14}C as described previously [8]. Blank tubes which contained either no enzyme or to which the enzyme was added after the addition of ether, were also run.

One unit of 5α-reductase activity is the amount of enzyme which catalyses the formation of 1 pmol of DHT/h at 37°C.

Tissue preparation

Human prostatic tissue was obtained from patients undergoing retropubic prostatectomy for BPH. The tissue was transported to the laboratory in ice-cold saline within 60 min. After the removal of excess blood the tissue was chopped into 1–3 g pieces, snap-frozen under liquid nitrogen and stored at −70°C until use. BPH was confirmed by histological examination.

The prostatic tissue was thawed, blotted dry and weighed. The remainder of the procedure was carried out at 4°C. The tissue (2–5 g) was chopped finely with scissors and then homogenised using one 30 s burst of a Ystral homogeniser (Scottish Scientific Instrument Centre Ltd, Edinburgh, Scotland [setting 10] in 5 vol of either 100 mM Tris–HCl, pH 7.4, 1 mM EDTA and 15 mM β-mercaptoethanol (Buffer A) (experiments reported in Fig. 3) or 100 mM Tris–HCl, pH 7.4, 20% (v/v) glycerol, 100 mM sodium citrate, 100 mM KCl, 1 mM EDTA, 15 mM β-mercaptoethanol (Buffer B). The homogenate was filtered through glass wool to remove cell debris and then centrifuged at 800 *g* for 10 min to provide a nuclear pellet and a post-nuclear supernatant. The post-nuclear supernatant was divided into 1 ml aliquots and these were centrifuged at 120,000 *g* for 45 min to give microsomal pellets. The pellets were either used immediately or could be stored at −70°C with no loss of activity.

Solubilization studies

Microsomal pellets were resuspended in 1 ml of buffer B containing 0.25 U glucose-6-phosphate dehydrogenase, 0.5 mM NADPH, 5 mM glucose-6-phosphate and 1 μM testosterone (Buffer C) in the presence or absence (control) of the following detergents: Triton X-100, Digitonin, MEGA 9, Tween 80, Tween 20, Lubrol PX and Nonidet P-40. The detergent concentrations used are given in Table 1. Resuspension was achieved by repeated passage through a 25/5 needle using a 1 ml syringe. The solubilized and membrane bound 5α-reductase activities were separated by immediately centrifuging the resuspended pellets at 120,000 *g* for 45 min. The supernatants (designated detergent-treated supernatants) were retained and the pellets (designated detergent-treated pellets) were resuspended in 1 ml of buffer C. Since there is considerable variation in the 5α-reductase activity of human prostatic tissue we have expressed the recovery of 5α-reductase activity in detergent treated pellets and supernatants as a percentage of the total recovery in control pellets and supernatants.

Protein estimation

Protein was estimated by the method of Bradford [9] using bovine serum albumin as standard. Appropriate blanks containing the detergent under study were used.

RESULTS

Assay of 5α-reductase

Preliminary experiments demonstrated that human prostatic 5α-reductase is saturated at a testosterone concentration of 1 μM and at an NADPH concentration of 0.5 mM (not shown). The production of DHT was linear with time up to 45 min (Fig. 1a). Figure 1b shows that DHT production is linear over the range 0–3 pmol DHT formed/15 min. The assay can detect as little as 0.05 pmol DHT/15 min which corresponds to 0.2 U per sample. Other experiments (not shown) demonstrated that the rate of DHT production is proportional to the amount of enzyme added up to at least 100 U of 5α-reductase/sample. Under the assay conditions described, the conversion of DHT to 3α(β)-androstanediol was negligible.

Homogenisation conditions

In order to maximise the 5α-reductase in the microsomal pellet the amounts and subcellular distribution of activity released by two different homogenisation methods were compared (Fig. 2). Homogenisation using high shear forces (Ystral) released 1.7

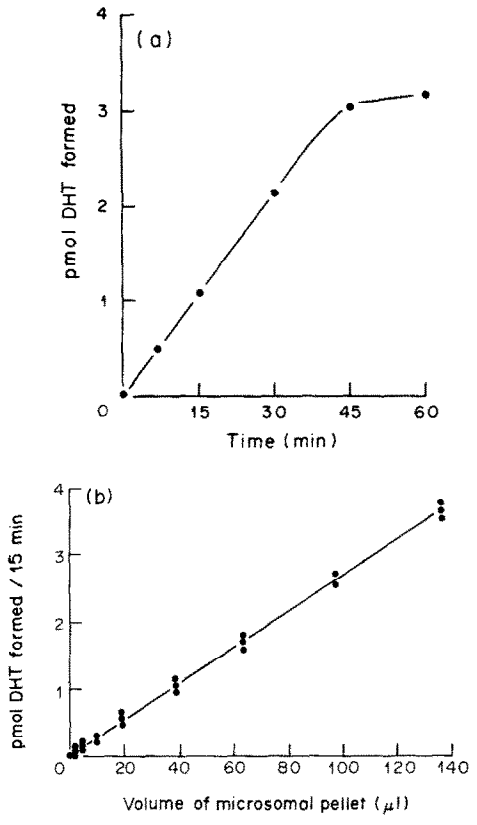


Fig. 1. Effect of time and sample volume on DHT production (a) 10 μl samples of a microsomal pellet were incubated at 37°C in the presence of 0.5 mM NADPH and 1 μM [^{3}H]testosterone (4.54 μCi/nmol) for the times indicated. (b) The indicated volume of a microsomal pellet was incubated (in triplicate) at 37°C for 15 min in the presence of 0.5 mM NADPH and 1 μM [^{3}H]testosterone (4.54 μC/nmol).

times as much protein into the homogenate as a low shear method (Dounce) [Fig. 2a]. Homogenisation using the Ystral released 5.2 times as much 5α-reductase activity as did the use of the Dounce homogeniser (Fig. 2b). Centrifugation at 800 *g* of the homogenate obtained by each method indicated that 96% of the 5α-reductase released by the Ystral homogeniser was located in the post-nuclear supernatant. All of the 5α-reductase activity in the post-nuclear supernatants was located in the microsomal pellet after centrifugation at 120,000 *g*.

Stability of 5α-reductase

In preliminary experiments, low concentrations of Lubrol PX, Triton X-100 and Nonidet P-40 were found to inactivate 5α-reductase. Conditions which prevented this inactivation were sought. The experiment described in Fig. 3a compared the effects of buffer A and Buffer C on the inactivation of 5α-reductase by Lubrol PX. In buffer A 5α-reductase was inactivated at Lubrol PX concentrations greater than 0.06 mg/ml and is completely inactivated at 0.8 mg/ml. In buffer C inactivation did not occur until the Lubrol PX concentration was greater than 0.8 mg/ml and was completely inactivated at 4.0 mg/ml. Similar results were obtained when the microsomal pellets were resuspended in buffer A or buffer C containing Tween 20 or Nonidet P-40 (not shown).

Effects of detergent on 5α-reductase activity

The effects of the presence of various detergents in the assay on 5α-reductase activity are shown in Fig. 3b. MEGA 9, Digitonin, Tween 20 and Tween 80 had no effect on 5α-reductase activity over the concentration range tested. Triton X-100, Lubrol PX and Nonidet P-40 inhibited 5α-reductase activity at concentrations greater than 15,20 and 10 μg/ml respectively.

Solubilization studies

Preliminary studies indicated that 5α-reductase activity was not solubilized by resuspending microsomes in buffer C containing a range of NaCl concentrations (0.1–2.0 M) This indicated that the enzyme was not a peripheral membrane protein and that solubilization using a detergent would be required.

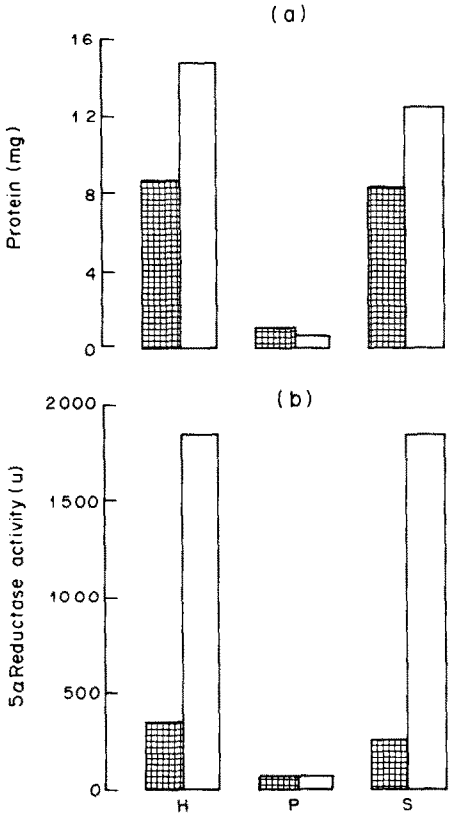
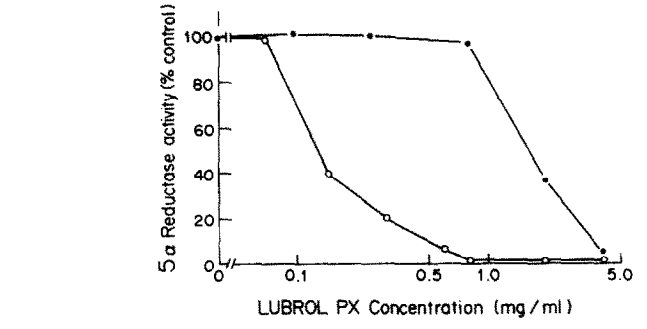
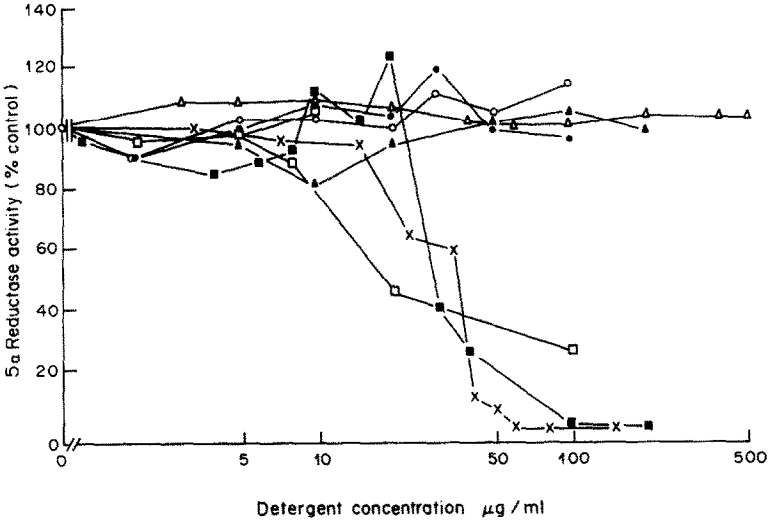


Fig. 2. Effect of homogenisation on amount and distribution of 5α-reductase activity in prostatic homogenates. Prostatic tissue was homogenised in 5 vol of buffer B using either a Dounce homogeniser (closed bars) or a Ystral homogeniser at setting 10 (open bars). The homogenates (H) were centrifuged at 800 *g* for 15 min to yield nuclear pellets (P) and post-nuclear supernatant (S). Samples were assayed for (a), protein and (b), 5α-reductase activity by incubation for 15 min at 37°C in the presence of 0.5 mM NADPH and 1 μM [^{3}H]testosterone (4.54 μCi/nmol).



(a)



(b)

Fig. 3. (a) Comparison of buffer components on stability of 5α-reductase in Lubrol PX. Microsomal pellets were resuspended in 1 ml of either buffer A (○) or buffer C (●) containing Lubrol PX at the concentrations indicated. The resuspended pellets were assayed immediately for 5α-reductase activity by incubation for 15 min at 37°C in the presence of 0.5 mM NADPH and 1 μM [^{3}H]testosterone (4.54 μCi/nmol). (b) Effect of detergent concentration on 5α-reductase activity. Microsomal pellets were prepared and resuspended in 1 ml of buffer B. 20 μl samples were assayed by incubation for 15 min at 37°C in the presence of 0.5 mM NADPH and 1 μM [^{3}H]testosterone (4.54 μCi/nmol) and in the presence of various detergents at the concentrations shown. (○), Tween 80; (●), Tween 20; (□), Nonidet P-40; (■), Lubrol PX; (△), MEGA 9; (▲), digitonin; (×), Triton X-100. Note that in (a) and (b) the detergent concentration is plotted on a non-linear scale.

The ability of several detergents to solubilize 5α-reductase activity was studied by resuspending microsomal pellets in the presence of increasing concentrations of the detergents as described under Experimental. The results of these experiments are shown in Table 1. All detergents caused a concentration-dependent decrease in the 5α-reductase activity remaining in the detergent-treated pellets. This was accompanied by a decrease in the protein content of the pellets and also by a decrease in the size of the pellets (not shown). There was no parallel increase in the amount of 5α-reductase activity present in the detergent-treated supernatants. With MEGA 9 and Triton X-100 no activity was recovered in any of the detergent-treated supernatants. With digitonin, Tween 80, Tween 20 and Nonidet P-40 the maximum recovery of 5α-reductase activity was 10–20%, even though under these conditions up to 90% of the activity had been lost from the pellet. With Lubrol PX the concentration-dependent decrease in the 5α-reductase activity of the detergent-treated pellets was accompanied by a parallel increase in the activity in the supernatant up to a Lubrol PX concentration of 1.1. At this concentration some 36% of the control activity was recovered in the supernatant. As the Lubrol PX concentration was increased above 1.1 the 5α-reductase activity recovered in the supernatant decreased to zero. Similar results were obtained in four other experiments.

The data presented in Table 1 indicate that Lubrol PX (1 mg/ml) affords the highest recovery of 5α-reductase in the detergent-treated supernatant. This detergent was therefore selected for further study. When prepared by the method described above the mean recovery of 5α-reductase in the Lubrol

Table 1. Recovery of 5α-reductase in detergent treated microsomal pellets and supernatants

Detergent	(mg/ml)	% Of 5α-reductase activity recovered in Detergent treated pellet	Detergent treated supernatant
None		96	4
Mega 9	0.5	105	0
	2.0	101	0
	5.6	101	0
	10.0	25	0
	35.0	0	0
	50.0	0	0
Digitonin	0.5	96	2
	1.0	90	2
	2.0	94	3
	5.0	22	16
	10.0	21	11
	20.0	15	12
Triton X-100	0.1	82	5
	0.2	85	4
	0.6	15	3
	1.2	3	1
	2.5	1	2
Lubrol PX	0.25	92	10
	0.8	65	27
	1.1	42	36
	2.0	20	21
	4.0	0	0
Tween 80	1.0	91	4.0
	2.0	87	2.5
	5.0	83	9.0
	10.0	70	7.0
	25.0	66	8.0
	50.0	51	7.0
Tween 20	1.0	97	3
	2.0	93	5
	5.0	77	6
	10.0	67	11
	25.0	59	13
	50.0	44	12
Nonidet P40	0.2	74	10
	0.5	32	12
	1.0	9	4

Microsomal pellets were prepared as described under Experimental and resuspended in 1 ml of buffer C (control) or buffer C containing detergents at the indicated concentrations.

Table 2. Optimisation of solubilization procedure using 0.8 mg/ml Lubrol PX

Modification to protocol	% Of 5α-reductase recovered in Pellet	Supernatant	Number of experiments
No modification	62.3 ± 13	30.6 ± 11	11
a. Incubation for 1 h, 4°C	65.0 ± 5.6	28.1 ± 4.4	2
Incubation for 2 h, 4°C	64.4 ± 4.0	27.8 ± 4.2	2
b. Sonication for 1 min, 4°C	50	25	1
Sonication for 3 min, 4°C	12	16	1
Sonication for 5 min, 4°C	8.3	10	1
c. Repeated extraction 2 x	31 ± 6.9	56.5 ± 8.7	2
d. Repeated extraction 3 x	8.6 ± 6.3	71.3 ± 14.9	6

The protocol described in materials and method was followed with additional modifications: (a), the resuspended microsomes were incubated, with shaking, for the times indicated; (b), the resuspended microsomes were sonicated in an MSE sonicater for the times indicated; (c), the initial Lubrol PX-treated pellet was resuspended once more in 1 ml of buffer C + 0.8 mg/ml Lubrol PX and then centrifuged at 120,000 *g* for 45 min. The supernatants were combined; (d), as in (c) with the addition of a third resuspension of the Lubrol PX-treated pellet followed by centrifugation at 120,000 *g* for 45 min. The supernatants were combined.

PX-treated supernatants was 30.6 ± 11%. We therefore studied the effect of varying this experimental protocol in order to improve upon this recovery. The variations in the protocol studied consisted of repeated extraction of the microsomal pellet, prolonged incubation after the initial resuspension and sonication. The results of these studies are displayed in Table 2. Incubation of the resuspended pellet for 1 or 2 h in the presence of 0.8 mg/ml Lubrol PX did not increase the recovery of 5α-reductase in the detergent-treated supernatant beyond 30%. Sonication of the resuspended pellets caused a decrease in the 5α-reductase activity recovered in the detergent-treated supernatant. Repeated extraction of the microsomal pellet with 0.8 mg/ml Lubrol PX increased the amount of 5α-reductase in the Lubrol-PX-treated supernatant to approx 70%. This was achieved using three extraction cycles. No increase in

the recovery was noted when a fourth extraction cycle was performed. Thus, a maximum of 70% of the 5α-reductase activity can be recovered by extracting the microsomal pellet 3 times with buffer C + 0.8 mg/ml Lubrol PX.

The 5α-reductase activity in the detergent-treated supernatant could not be pelleted by a further centrifugation at 120,000 *g* for 45 min. It therefore meets one of the criteria of a solubilized enzyme defined by Razin [10]. The solubilized enzyme could be stored overnight (20 h) at 4°C with only a 10% decrease in activity. Storage at 4°C for longer periods resulted in a considerable decrease in activity; only 35% of the initial activity remained after 72 h. The solubilized enzyme could also be stored at −20°C in the presence of 40% (v/v) glycerol for at least 7 days with no loss of activity (results not shown).

DISCUSSION

In order to solubilize 5α-reductase at Triton X-100, Lubrol PX and Nonidet P-40 concentrations of up to 4 mg/ml and also avoid any inhibition by these detergents in the assay, it was necessary to assay small sample volumes (5–20 μl). As the detergent-treated supernatants prepared in these studies frequently contained as little as 20 U/ml 5α-reductase, we clearly required an assay capable of detecting 0.2 U 5α-reductase/sample. Our previously published procedure [11] and those of other workers [2, 5, 12, 13] did not have the required sensitivity. Figure 1b shows that our revised assay procedure can reliably detect at least 0.2 U 5α-reductase/sample. This degree of reproducibility and sensitivity was achieved by using saturating concentrations of both substrates, and by the use of [^{3}H]testosterone with a high specific radioactivity. The entire procedure is rapid since 30 samples can be processed in 3 h.

Homogenisation using a Ystral homogeniser released 5 times as much 5α-reductase activity as did the use of Dounce homogeniser. The Ystral homogeniser was therefore used throughout this work. It is obvious from Fig. 2 that the 5α-reductase released by the Ystral homogeniser has approximately twice the specific activity of that released by the Dounce homogeniser. This suggests that the Ystral homogeniser released a pool of 5α-reductase with a higher specific activity than that released by the Dounce homogeniser. One possible explanation of this is that the Dounce homogeniser releases activity only from the epithelial tissue whereas a higher shear force is necessary to release activity from the stromal tissue. It has been shown that the specific activity of 5α-reductase is higher in stromal tissue than that in epithelial tissue [14]. Given that other workers find that the bulk of 5α-reductase activity is located in the nuclear membrane [5], our observation that 96% of the 5α-reductase remained in the post-nuclear supernatant may seem unusual. We believe that this is a consequence of nuclear disruption caused by the high shear force used in the homogenisation.

Scheer and Robaire [7] found that 25% of the 5α-reductase activity in rat epididymal membranes could be solubilized by incubation in the presence of 0.1 M KCl and 0.1 M citrate in the absence of detergent. In our hands the presence of KCl and citrate alone did not result in any solubilization of 5α-reductase activity. It may be that the results of Scheer and Robaire [7] are due to the stabilization of the small amount of 5α-reductase released simply by resuspending the membranes. A similar stabilization of 5α-reductase by KCl, NADPH and glycerol was observed by Moore and Wilson [6]. The components used in buffer C are a combination of those used by Scheer and Robaire [7] and Moore and Wilson [6] together with the substrates of 5α-reductase. The main effect of buffer C is to increase the concentration range of detergents that can be used in attempting to solubilize the 5α-reductase and so this buffer was used throughout the solubilization studies.

It is important to study the effects of low concentrations of detergents on enzyme activity prior to attempting to solubilize a membrane-bound enzyme, as small amounts of detergent will be carried over into the assay. Of the detergents used in these studies only Triton X-100, Lubrol PX and Nonidet P-40 caused any inhibition of 5α-reductase. In the solubilization studies reported in this paper both the concentrations of these detergents and the sample volumes used were chosen to ensure that no inhibition occurred. The effect of detergents on 5α-reductase activity has not been considered by other workers [6, 7] and this may have led to the underestimation of the recovery of solubilized 5α-reductase.

It is clear from Table 1 that not all of the activity which was lost from the pellets was recovered in the detergent-treated supernatant. For example, at the lowest Lubrol PX concentration used (0.25 mg/ml) greater than 100% of the activity lost from the pellet was recovered in the supernatant. With 1.1 mg/ml Lubrol PX only 62% of the activity lost from the pellet was recovered in the supernatant while with 4 mg/ml Lubrol PX none of the activity lost from the pellet was recovered. This suggests that there is a concentration-dependent inactivation of the solubilized 5α-reductase by Lubrol PX. Thus, the amount of 5α-reductase activity recovered at a given Lubrol PX concentration is a function of both the amount of enzyme solubilized at that detergent concentration and the degree of inactivation occurring at that concentration. Detergent inactivation presumably accounts for the poor recoveries of solubilized 5α-reductase obtained using other detergents [15].

In view of the above findings a low concentration of Lubrol PX (0.8 mg/ml) was used in further studies. At this Lubrol PX concentration 80% of the solubilized activity was recovered. However Table 1 shows that 65% of the 5α-reductase activity remains in the pellet. Further studies revealed that two additional cycles of resuspension and centrifugation with

0.8 mg/ml Lubrol PX removed all of the remaining activity from the pellet, of which approx 70% was recovered in the combined supernatants.

The results presented in this paper are the first demonstration of the solubilization of human prostatic 5α-reductase. Our method compares favourably with those described for the solubilization of 5α-reductase from rat tissues. Our overall recovery of solubilized enzyme exceeds that of Moore and Wilson [6] and is similar to that of Scheer and Robaire [7] who used a related detergent, Lubrol WX. Our procedure is fairly rapid: if frozen microsomes are used the solubilization can be completed within 3 h. The ability to prepare solubilized 5α-reductase will allow the further purification and characterisation of this enzyme. Although the solubilized 5α-reductase is fairly stable at 4°C, activity is lost during prolonged storage. This indicates that the purification should be performed as rapidly as possible. Studies in the purification of 5α-reductase are currently underway in this laboratory.

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