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ANDROGEN METABOLISM BY AND BINDING TO MATURE RABBIT EPIDIDYMAL TISSUE: STUDIES ON CYTOSOL

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SUMMARY

The rabbit epididymis is capable of metabolizing [^{3}H]-testosterone to [^{3}H]-5α-androstan-17β-hyroxy-3-one (5αDHT) and to [^{3}H]-5α-androstan-3α, 17β-diol (3α-diol) under *in vitro* conditions. These reactions occurred more rapidly at 23°C than 0°C and were dependent upon time and substrate concentration in the linear manner characteristic of enzymatic reactions. Androgens were bound to two or more components in cytosol prepared from epididymal tissue. Androgens dissociated slowly from one of the components (the epididymal androgen receptor) and rapidly from the other component(s) (androgen binding protein (ABP) and/or testosterone binding globulin (TeBG). Differences in dissociation rates among these molecules were utilized to determine which androgens were bound to the androgen receptor. This was accomplished by adding unlabeled testosterone to pre-labeled cytosol to dissociate label that was bound to the rapidly dissociating moieties and separating the bound and free radioactivity by gel filtration chromatography. Androgens were extracted from the column eluates with diethyl ether, separated by thin layer chromatography, and identified by recrystallization to constant specific activity. The androgen receptor strongly favors the binding of 5αDHT since although 5αDHT constituted only 10-20% of the total pool of radioactive androgens, it comprised 50% of the androgens bound to the receptor. The remaining receptor-bound androgen consisted of testosterone and 3α-diol. The androgen receptor could be demonstrated in cytosol from both intact and castrated rabbits using these methods. However, the concentration of available receptor sites was more than twice as great in castrated animals than in intact ones. Total bound radioactivity in cytosol was examined by gel chromatography of labeled cytosol to which no unlabeled hormone had been added. Epididymal tissue from 3-day castrated rabbits produced significantly less 5αDHT ($P = 0.005$) than did epididymal tissue from intact rabbits.

INTRODUCTION

During the course of our studies on androgen binding to the rabbit epididymis, we have demonstrated that two high affinity binding components can be detected in cytosol prepared from epididymal tissue. One of the binding moieties (demonstrable in intact rabbits) is of testicular origin and enters the epididymis via testicular fluid [1]. Although this binding protein is distributed along the length of the epididymal duct in immature animals [2] and is found in high concentration in fluids collected from the cauda epididymidis of these animals [3], it is primarily confined to the caput region of adult rabbits [4]. It would appear that this protein, referred to as androgen binding protein (ABP), is compartmentalized within the lumen of the epididymis. The other androgen binding moiety can be demonstrated in cytosol prepared from the epididymis of castrated adult rabbits [5] and is capable of being transferred to epididymal nuclei [6]. This moiety appears to be the androgen receptor that is involved in mediating the action of androgens on the epididymis. In addition to these two binding moieties that are associated with epididymal tissue, rabbit plasma also contains a specific androgen binding protein (TeBG) [7].

The purpose of the studies reported here was: (a) to develop a rapid system for separating the androgen receptor from other androgen binding proteins that might be present in epididymal homogenates, (b) to determine favorable experimental conditions for examining androgen metabolism by the epididymis and (c) to examine the effects of these conditions on metabolism of testosterone by the epididymis and on the types of androgens bound to the epididymal cytoplasmic receptor.

MATERIALS AND METHODS

Chemicals. [1,2,6,7-^{3}H]-testosterone (85 Ci/mmol) was purchased from New England Nuclear Corp. and purified by thin layer chromatography (t.l.c.) prior to use. Unlabeled steroids were purchased from Steraloids. Cyproterone acetate (6-chloro-17-acetoxy-1α, 2α,-methylene-4,6-pregnadiene-3,20-dione) was donated by Schering A.G., Berlin. Ultra pure sucrose and buffer grade Tris (base) were purchased from Schwarz-Mann. Spectrofluor was purchased from Amersham-Searle. Pre-coated silica gel thin layer chromatography plates were purchased from EM Laboratories, Inc. and Sephadex was purchased from

Pharmacia Fine Chemicals. All chemicals used were reagent or analytical grade.

Animals and surgical procedures. Sexually mature (at least six months old) New Zealand White Rabbits were used in all the experiments reported here. Animals to be castrated were anesthetized with Sodium Nembutal (30 mg/kg). The scrotum was incised in the midline and a small incision was made in the tunica vaginalis testis through which the testis and epididymidis were withdrawn. The epididymis was dissected free of the testis and the testicular blood vessels were ligated. The testis was excised, the epididymis was returned to the scrotum, and the incisions were closed.

Preparation and incubation of tissue. Three days following castration, the rabbits were killed by administering a lethal dose of Nembutal. The epididymides were removed from 2 animals, trimmed of fat and connective tissue, rinsed in TES buffer [10 mM Tris–HCl (pH 7.5), 1.0 mM EDTA, 0.25 M sucrose], and weighed. The weighed tissues were pooled, thoroughly minced with iridectomy scissors, and divided into aliquots of approximately 800 mg. The aliquots were then mixed with [^{3}H]-testosterone alone or in combination with unlabeled testosterone, and TES in a final volume of 2 ml. The samples were then incubated for various periods of time at 0°C and/or at 23°C.

Preparation and analysis of cytosol. At the conclusion of the incubation period, the minces were homogenized using a Polytron Pt-10. The homogenates were centrifuged for 15 min at 2020 *g*, the supernatants were decanted and further centrifuged for 30 min at 229,000 *g* to obtain the cytosol fraction. When indicated, unlabeled testosterone or cyproterone acetate was added to the cytosols after incubation, at a concentration of approximately 8.6×10^{-5} M to dissociate loosely bound hormone. This high concentration was used to decrease the statistical probability of reassociation of labeled hormone with rapidly dissociating binding proteins.

Unlabeled testosterone can inhibit the binding of [^{3}H]-androgens to the epididymal androgen receptor, to ABP and to TeBG [4, 6, 7]. Although cyproterone acetate can inhibit androgen binding to the receptor [6, 8–9], it is a poor competitor for androgen binding sites on ABP and TeBG [7–9]. Owing to the slow rate of dissociation of androgens from the epididymal androgen receptor [5, 8–9], only the rapidly dissociating binding moieties, ABP and TeBG [7–10] would be affected by the addition of competitors to the binding system. Since the statistical probability of [^{3}H]-androgens reassociating with ABP and/or TeBG would be higher in the presence of unlabeled cyproterone acetate than in the presence of unlabeled testosterone, the expectation would be that dissociation studies conducted using cyproterone acetate would reflect binding to all three binders, while studies conducted using unlabeled testosterone would reflect binding only to the androgen receptor.

After cytosol had been incubated with unlabeled hormone for at least 30 min on ice, a 1 ml aliquot of each cytosol was applied to 6 mm × 17 cm columns of Sephadex G-25 and eluted with TE buffer [10 mM Tris–HCl (pH 7.5) 1.0 mM EDTA]. The flow rate of the columns was approximately 30 ml/h. These columns were used to separate protein bound radioactivity from that which was not bound. The column eluates were either counted to localize the radioactive peaks or fractions known to correspond to the regions of bound and free radioactivity were pooled separately and extracted with ether.

Extraction and characterization of radioactive steroids. The pooled column fractions were extracted three times with anhydrous diethyl ether using 10 ml of ether for each extraction. The organic phases were combined and 50 μg each of testosterone, 5α-androstan-17β-hydroxy-3-one (5αDHT), 5α-androstan-3α, 17β-diol, and 5α-androstan-3β, 17β-diol were added. The ether was then evaporated to dryness. The walls of each tube were washed with 2 ml of ether. The ether was evaporated and 100 μl of absolute ethanol were added to each tube.

Fifty μl of each sample were then spotted on a silica gel plate as were unlabeled standards and labeled testosterone and 5αDHT. The plates were chromatographed in a solvent system containing chloroform–acetone (93:7, v/v). Testosterone was visualized using U.V. light and the other standards were detected after spraying the plates with a 10% solution of phosphomolybdic acid in acetone and heating at approximately 100°C for 5 min. The plates were then divided into columns perpendicular to the origin and then subdivided into 1/2 cm sections horizontal to, and beginning 1/2 cm below, the origin. Testosterone, 5αDHT, and the diols were well separated under these conditions. However, the 3α and 3β diols were not usually clearly separated from each other. The areas were then scraped and counted in 5 ml of a toluene-spectrafluor scintillation fluid.

We have monitored the losses of label at each step in the procedure. The 1.0 ml aliquots of cytosol added to the Sephadex columns usually contained about 26% of the total label in the incubation mixture. Loss on the column is 10–15% of the amount applied. Recovery from the thin layer plate is about 80%.

The identity of the steroids recovered from the plates was determined by recrystallization to constant specific activity. Recrystallization was performed by pooling extracts of the cytosol-bound fractions and the cytosol-free fractions from several experiments by washing of the extraction tubes with ether. The ether was then evaporated to dryness and 150 μl of ethanol were added to each tube. Aliquots (50 μl) of the ethanol solubilized steroids, in duplicate, were applied to t.l.c. plates, chromatographed as described above, and the steroids were localized using one of the duplicates which contained standards. Areas on the other duplicate, corresponding to the migration of the standards, were scraped into Soxhlet extraction thimbes

and refluxed over boiling chloroform for two hours. The chloroform was allowed to cool and then was transferred quantitatively to a conical centrifuge tube and evaporated to dryness under a stream of nitrogen over a steam bath. Chloroform was then added to the conical tube and the chloroform was transferred to a preweighed 1/2 dram vial. The conical tube was washed twice with chloroform. The chloroform was again evaporated using nitrogen and the sample was further dried under vacuum. The weight of the sample in the vial was determined and a measured amount of the appropriate unlabeled steroid was added to it. The samples were recrystallized using solvent systems methanol–water and acetone–water.

RESULTS

Separation of bound from free hormone

Since these studies were designed to examine both binding of androgens to the epididymal cytoplasmic receptor as well as metabolism of androgen by epididymal tissue, it was necessary to establish an effective mechanism for separating macromolecular bound from unbound hormone in the incubation mixture and for separating hormone bound to receptor from that bound to ABP and/or TeBG. This was accomplished by Sephadex G-25 chromatography of cytosol prepared from minces of epididymal tissue from 3-day castrated adult rabbits that had been preincubated with 2.8×10^{-8} M [^{3}H]-testosterone for 60 min at 0°C followed by an incubation at 23°C for 120 min. as described in Materials and Methods. Figure 1 shows elution profiles that were obtained. Two peaks of radioactivity were present, a sharp peak corresponding to the elution volume of proteins from the column and a broader peak corresponding to the elution volume of lower molecular weight moieties. Identification of the macromolecular binding component as the epididymal receptor is complicated by the fact that ABP and/or TeBG might also be present in this fraction. To compensate for the presence of non-receptor androgen binding components in the macromolecular peak, we took advantage of the fact that androgens dissociate slowly from their receptor [5, 8] while they dissociate rapidly from ABP [2, 8–10] and from TeBG [7]. To dissociate label that was loosely bound to ABP and/or TeBG, we added 8.6×10^{-5} M unlabeled testosterone to cytosol that had been prelabeled with [^{3}H]-testosterone. Following a 30 min. incubation on ice, the cytosol was applied to a Sephadex G-25 column and chromatographed. Figure 1 shows the results obtained when the same pool of cytosol was chromatographed prior to or after the addition of radioinert testosterone. It can be noted that, in the presence of unlabeled testosterone, a considerable reduction in binding to the macromolecular peak had occurred. This indicated that, although a large portion of the binding was to rapidly dissociable non-receptor components, a fraction of the binding was to the slowly dissociating epididymal androgen receptor.

The data presented in Table 1 show the absolute amount of total radioactivity associated with the bound and free fractions obtained from G-25 chromatography of cytosols. When no unlabeled hormone was added to the cytosol prior to chromatography, the amount of hormone present in the bound fraction represents that which is tightly bound to the androgen receptor and that which is loosely associated with ABP and/or TeBG. As can be noted (Table 1), a large amount of bound radioactivity was present in untreated cytosol from both castrated and intact rabbits. The addition of unlabeled testosterone or cyproterone acetate to the samples prior to chromatography lead to a great diminution in the amount of label present in the bound fraction and a corresponding increase in the amount of radioactivity in the free fraction. These effects are caused by the displacement of loosely bound ligands from ABP and/or TeBG. Furthermore, there were differences between castrated and intact rabbits in the amount of radioactivity that could be displaced by these agents (Table 1). Unlabeled testosterone caused about an 80% reduction in total binding to cytosol from castrated rabbits, but a 97% reduction in binding to cytosol from intact animals. Cyproterone acetate caused a 65% displacement of label from 3-day-castrate cytosol

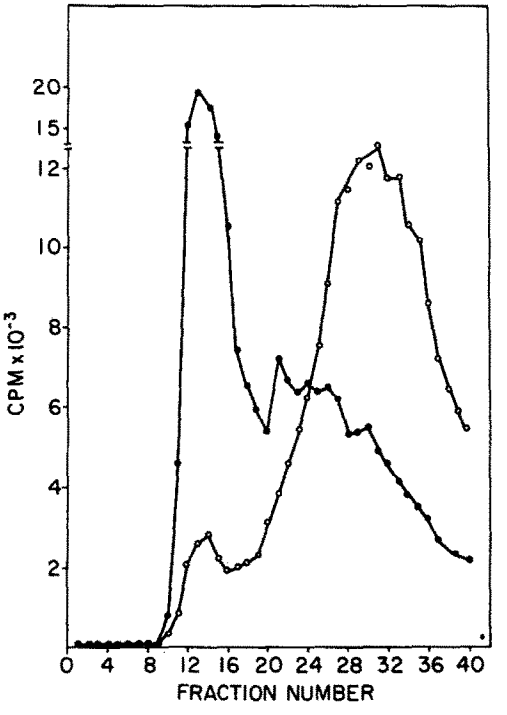

Fig. 1. Separation of bound from unbound steroids by chromatography on Sephadex G-25. Cytosols prepared from epididymal minces of 3-day castrated rabbits were chromatographed on G-25 columns either prior to (●——●) or after (○——○) the addition of unlabeled testosterone.

Table 1. Total radioactivity eluted from G-25 column

Experimental conditions	Bound (c.p.m. × 10^{-3})*	Free (c.p.m. × 10^{-3})
3-day Castrate		
No unlabeled hormone	102.14 ± 10.13	102.46 ± 6.65
Unlabeled T	11.98 ± 0.56	182.96 ± 21.44
Unlabeled C.A.	35.48 ± 11.94	161.73 ± 7.95
Intact		
No unlabeled hormone	161.65 ± 22.16	76.88 ± 8.72
Unlabeled T	5.39 ± 0.09	173.67 ± 19.25
Unlabeled C.A.	22.38 ± 2.88	176.54 ± 7.91

Aliquots (1.0 ml) of cytosol prepared from epididymal minces that had been incubated with 8×10^{-9} M [^{3}H]-testosterone for 1 h at 0°C and 2 h at 23°C were layered on Sephadex G-25 columns prior to (no unlabeled hormone) or after the addition of unlabeled hormones (8.6×10^{-5} M). Fractions were collected and the amount of total radioactivity present in the bound and free peaks was determined.

* The c.p.m. represent the mean ± S.E.M. of 6 individual experiments for both castrated and intact animals when no unlabeled hormone was added prior to chromatography and 3 experiments each for both groups when labeled T (testosterone) or C.A. (cyproterone acetate) were added prior to chromatography.

and an 84% displacement from cytosol of intact rabbits. These results indicate that epididymal cytosol from castrated rabbits contains a larger percentage of slowly dissociating androgen receptor than does cytosol from intact rabbits. The presence of a slowly dissociating component in cytosol from intact rabbits provides evidence that the small amount of available androgen receptor that is present in these animals can be detected by the methods employed.

When column eluates were analyzed by t.l.c. to determine which androgens were present in the bound and free fractions, the results presented in Table 2 were obtained. Testosterone and 5αDHT were bound to epididymal cytosol from castrated and intact rabbits, whereas testosterone, 5αDHT and diols were present in the unbound (free) fraction. Prior to the addition of unlabeled hormones to cytosol from 3-day castrated rabbits, approximately 80% of the bound androgens were represented by testosterone. When unlabeled testosterone was added to cytosol from the animals prior to gel filtration, the androgens bound to the slowly dissociating component were equally distributed between testosterone and 5αDHT. When unlabeled cyproterone acetate was added to castrate cytosol, the same distribution of label was obtained as in cytosol to which no unlabeled hormone had been added (Table 2). These observations suggest that the slowly dissociating component in epididymal cytosol from castrated rabbits has a greater affinity for 5αDHT than do ABP and/or TeBG. The effect of unlabeled hormones on the types of androgens bound to cytosol from intact rabbits was similar to

Table 2. Androgens present in eluate from G-25 column

Experimental conditions	% Bound			% Free		
	T	DHT	Diols	T	DHT	Diols
3-day Castrate						
No unlabeled hormone	79.9 ± 1.2	20.1 ± 1.2	N.D.*	83.8 ± 1.8	9.5 ± 0.9†	6.7 ± 1.1
Unlabeled T	49.8 ± 1.6	50.2 ± 1.6	N.D.	89.8 ± 3.2	9.1 ± 1.1	3.7 ± 1.5
Unlabeled C.A.	79.4 ± 2.2	20.6 ± 2.2	N.D.	84.9 ± 2.3	7.5 ± 0.3	7.4 ± 2.5
Intact						
No unlabeled hormone	63.3 ± 2.3	36.8 ± 2.3	N.D.	75.8 ± 1.5	16.0 ± 1.8†	8.2 ± 0.6
Unlabeled T	50.7 ± 8.2	49.3 ± 8.2	N.D.	67.6 ± 6.2	24.7 ± 5.6	7.7 ± 1.3
Unlabeled C.A.	75.6 ± 6.3	24.4 ± 6.3	N.D.	65.3 ± 7.0	29.6 ± 6.9	5.1 ± 0.3

The experimental design and number of experiments is the same as described in Table 1. However, the peaks of bound and free radioactivity were extracted with ether and chromatographed on TLC to determine what androgens were present in each peak. * N.D. = not detectable. † The amount of DHT formed in incubations of epididymides from intact rabbits is significantly greater ($P = 0.005$) than that formed in incubations of epididymides from 3-day castrated rabbits ($n = 6$ for each group).

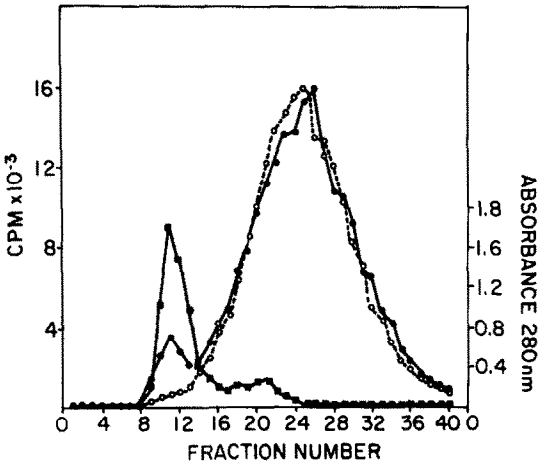

Fig. 2. Inhibition of bindings to the receptor peak caused by unlabeled testosterone. Epididymal minces from 3-day castrated rabbits were incubated with [^{3}H]-testosterone alone (●——●) or with [^{3}H]-testosterone and unlabeled testosterone (○——○). At the conclusion of the incubations, cytosol was prepared, unlabeled testosterone was added to dissociate loosely bound radioactivity, and the samples were chromatographed on Sephadex G-25. The absorbance of the fractions at 280 nm was determined (■——■) to locate proteins.

that obtained with 3-day castrated rabbits (Table 2) except for the fact that a higher percentage of the androgen bound in the absence of unlabeled hormone was 5αDHT. This finding may be accounted for by the fact that production of 5αDHT is significantly greater ($P = 0.005$) in intact than in castrated rabbits (Table 2). Additional data on this phenomenon will be provided in another communication. The presence of unlabeled hormone had no effect on the types of androgens present in the unbound fractions from either the castrated or intact rabbits (Table 2) as would be expected since most of the metabolism would have occurred prior to chromatography.

Binding to the macromolecular peak was further evaluated by incubating epididymal minces from 3-day castrated rabbits with 2.8×10^{-8} M [^{3}H]-testosterone alone or in combination with 8.5×10^{-5} M unlabeled testosterone for 120 min at 0°C and then for 60 min at 23°C. After the incubation has been concluded, 8.5×10^{-5} M unlabeled testosterone was added to the incubation tubes to dissociate loosely bound label, and the cytosols were chromatographed on Sephadex G-25. The elution profiles are presented in Fig. 2. Two peaks of radioactivity were obtained when the incubations were conducted with [^{3}H]-testosterone alone, the first representing hormone bound to the slowly dissociating macromolecular component and the other representing unbound hormone. When the incubation was performed in the presence of unlabeled testosterone, the macromolecular peak was eliminated, indicating that a limited number of high affinity androgen binding sites are present in the cytosol. In addition, these data indicate that less than 20% of the label present in the void volume of the column can be accounted for by spillover of unbound hormone.

Identification of steroids from t.l.c. plates

Tentative identification of testosterone and its metabolites was made by the fact that the compounds co-chromatographed with both labeled and unlabeled authentic standards. The steroids were further identified by extraction from the silica gel and recrystallization to constant specific activity as described in materials and methods. The data presented in Table 3 indicate that the [^{3}H]-androgens recovered from the plates do correspond to authentic, testosterone, 5αDHT, and to 3α-diol.

The effect of various incubation parameters on [^{3}H]-testosterone metabolism and binding by epididymal tissue

To determine the optimum incubation conditions for evaluating androgen metabolism by and binding to epididymal tissue, we incubated minces of epididymal tissue from 3-day castrated adult rabbits: (a) with various concentrations of [^{3}H]-testosterone, (b) at various temperatures and (c) for various lengths of time. At the conclusion of the incubations, receptor-bound androgens were separated from ABP and/or TeBG and unbound androgens, and the types of androgens in these fractions were determined as described above.

Table 3. Recrystallization of samples from t.l.c. plates

		c.p.m./mg		
Solvent	Fraction*	T	5αDHT	3αDiol
Methanol—H_2O	CB*	2819	503	—†
Acetone—H_2O		2713	424	—
Methanol—H_2O	CF	22545	1283	2361
Acetone—H_2O		24408	1311	2496

* CB = Steroid bound to cytosol, CF—Unbound steroid in cytosol. † —"detectable amounts of this compound were not recovered from t.l.c. plates, therefore, recrystallization could not be performed.

Table 4. The affect of [^{3}H]-testosterone concentration on androgens unbound in the cytosol

[^{3}H]-Testosterone concentration in incubation (M × 10^{-9})	Androgen concentration in cytosol (M × 10^{-11})*	
	Testosterone	5αDHT
2.06	57.0 ± 7.3	4.1 ± 1.2
4.12	120.0 ± 22.5	8.9 ± 1.5
8.25	310.4 ± 39.0	17.8 ± 1.6
16.50	531.5 ± 37.2	36.6 ± 6.0

* Epididymal tissue (≅ 400 mg/ml) from 3-day castrated rabbits was preincubated with increasing concentrations of [^{3}H]-testosterone for 2 h at 0°C followed by an incubation at 23°C for 1 h. Aliquots of cytosol were chromatographed on Sephadex G-25, extracted with ether, and run on t.l.c. plates. The numbers represent the mean ± S.E.M. of three experiments at each [^{3}H]-testosterone concentration.

a. *The effect of [^{3}H]-testosterone concentration.* When a fixed amount of epididymal tissue (≅ 400 mg/ml) from 3 day castrated rabbits was preincubated with various concentrations of [^{3}H]-testosterone for 2 h at 0°C and for 1 h at 23°C, we noted that there was a concentration-dependent linear increase in the amount of [^{3}H]-5αDHT formed (Table 4). When the types of androgens bound to the cytoplasmic receptor were determined, it was noted that both testosterone and 5αDHT were present. The available 5αDHT binding sites appeared to be saturated; while saturation of the available testosterone binding sites was approached, but not achieved (Fig. 3).

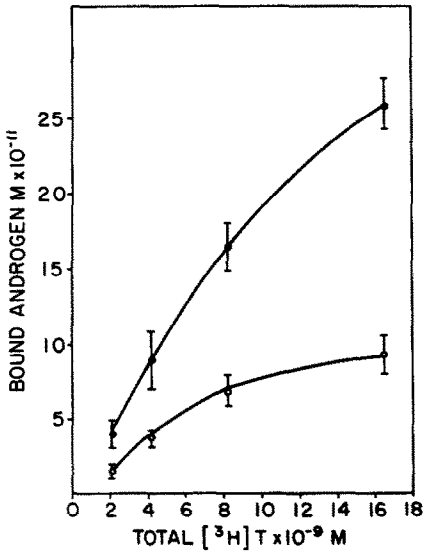

Fig. 3. The effect of hormone concentration on androgen binding to epididymal cytosol. Epididymal minces from 3-day castrated rabbits were incubated with various concentrations of [^{3}H]-testosterone, cytosol was prepared, unlabeled testosterone was added to dissociate loosely bound label, the samples were chromatographed on Sephadex G-25, the bound fractions were extracted with ether and chromatographed on t.l.c. plates. Testosterone (●——●), 5αDHT (○——○). The means ± S.E.M. of 3 separate experiments are plotted.

b. *The effect of duration and temperature of incubation.* Testosterone metabolism was evaluated using 8 × 10^{-9} M [^{3}H]-testosterone in the incubation medium and by incubating the minces at 0°C for various lengths of time. Table 5 presents data indicating that little conversion of testosterone to metabolites occurred over a 4 h incubation at this temperature. The only metabolite detected was 5αDHT and this represented less than 5% of the total radioactivity in the cytosol. On the other hand, when binding to the cytoplasmic receptor was examined (Fig. 4), it was noted that there was an increase in the amount of bound 5αDHT with time; by 4 h approximately 20% of the bound label was 5αDHT.

When incubations were performed at 23°C, there was a time-dependent linear decrease in the amount of unbound [^{3}H]-testosterone in the cytosol (Fig. 5) and a concomitant linear increase in 5αDHT and diol formation. Both of these metabolites could be detected at the earliest time point examined. The effect of incubation at 23°C on the types of androgens bound to the cytosol receptor are presented in Fig. 6. As incubation time was increased, there was a rapid decline in the percentage of testosterone bound to the receptor and a corresponding increase in the percentage of 5αDHT that was bound. After 2 h of incu-

Table 5. Unbound androgens in cytosol (0°C)

Incubation time (h)	% of total*	
	T	5αDHT
0.5	96.8 ± 3.3	3.3 ± 0.6
1.0	94.0 ± 1.3	6.0 ± 1.3
2.0	94.8 ± 1.3	5.3 ± 1.3
4.0	95.3 ± 1.0	3.8 ± 0.5

Minced epididymal tissue from 3-day castrated rabbits was incubated with 8 × 10^{-9} M [^{3}H]-testosterone at 0°C for the time intervals indicated. The tissues were homogenized, cytosol was prepared and bound steroids were separated from unbound on G-25 columns.

* The values represent the mean ± S.E.M. of 4 separate experiments.

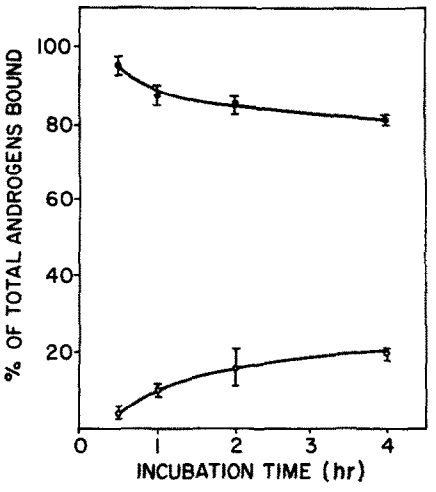

Fig. 4. The effect of temperature and duration of incubation on androgens bound to epididymal cytosol. Epididymal minces from 3-day castrated rabbits were incubated with [^{3}H]-testosterone for 0.5 to 4 h at 0°C. Procedures described above were utilized to determine which androgens were bound to the epididymal androgen receptor. Testosterone (●——●), 5αDHT (○——○). The means ± S.E.M. of 4 separate experiments are plotted.

bation, binding of 5α-androstan-diol to the receptor could be detected; since relatively little of this steroid was produced (Fig. 5), it must have considerable affinity for the receptor. These studies indicated that incubation of the epididymal minces for 1–2 h at 23°C with labeled testosterone would be sufficient conditions for examining androgen binding to and metabolism by epididymal tissue.

DISCUSSION

The studies of several investigators [7–10] have demonstrated that the dissociation of androgens from ABP and TeBG is very rapid (a matter of minutes), while dissociation from receptors in general [11] and the epididymal androgen receptor in particular [5, 8–10] is quite slow (a matter of at least several hours). We took advantage of these observations to devise a method of examining androgen binding to the epididymal androgen receptor in the presence of the other androgen binding components. This was accomplished by adding a large excess of unlabeled androgen to the labeled cytosols (at 0–4°C) to dissociate label that was bound to ABP and/or TeBG. The samples were then chromatographed on Sephadex G-25 to separate macromolecular-bound label from that which was not bound. Our presumption was that most, if not all, of the remaining macromolecular bound androgens would represent that associated with the slowly dissociating androgen receptor.

In the dissociation studies (which were conducted at 0–4°C), both unlabeled testosterone and unlabeled cyproterone acetate were used. It has been shown [8–9] that while cyproterone acetate is an effective inhibitor of androgen binding to receptors at a 100-fold molar excess, it is unable to inhibit androgen binding to ABP [8–9] or TeBG [7] at this concentration. In contrast, unlabeled testosterone at a 100-fold molar excess, causes complete inhibition of [^{3}H]-5αDHT binding to all three binding moieties [4, 6–7]. In the present studies, cyproterone acetate at a 3,000-fold molar excess was shown to be less effective than the same concentration of testosterone in preventing the reassociation of [^{3}H]-androgens with the rapidly dissociating androgen binding components in epididymal cytosol. The differences in the relative affinities of these agents for the rapidly dissociating moieties were exploited as a device for emphasizing the differences between androgen binding to receptor, and androgen binding to the aggre-

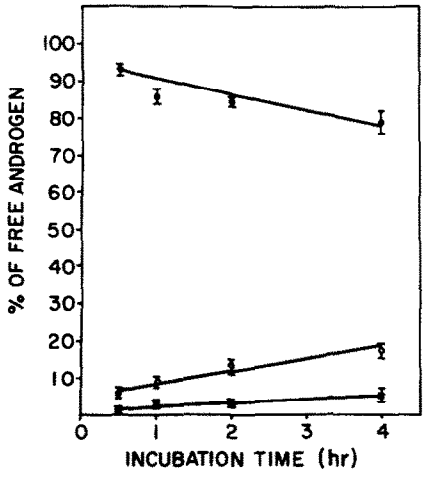

Fig. 5. The effect of incubation at 23°C on unbound androgens present in cytosol. Testosterone (●——●), DHT (○——○), diols (■——■). The means ± S.E.M. of 4 separate experiments are plotted.

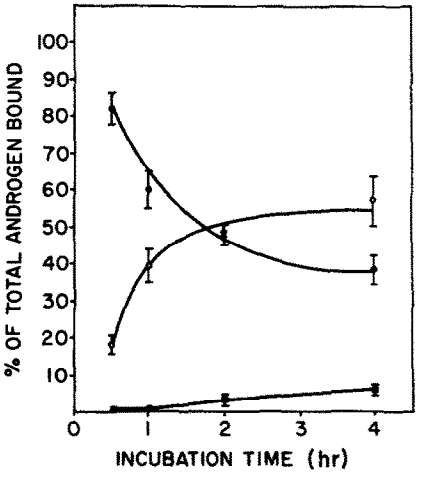

Fig. 6. The effect of incubation at 23°C on androgens bound to the epididymal cytosplasmic receptor. Testosterone (●——●), DHT (○——○), diols (■——■). The means ± S.E.M. of 4 separate experiments are plotted.

gate of all the androgen binding systems potentially present in the cytosol. When testosterone was used to prevent reassociation of [^{3}H]-androgens with ABP and/or TeBG, the only binding remaining should represent that to the receptor. Under these circumstances, 5αDHT represented about 50% of the bound androgens. When cyproterone acetate was used to prevent reassociation, binding to all three binding components should be present. This mixture of binding proteins is reflected by the "dilution" of bound 5αDHT to approximately 20% of the bound androgens.

Using the dissociation technique described above, we were able to demonstrate the presence of the androgen receptor in epididymal cytosol from both 3-day castrated rabbits and from intact rabbits. However, the amount of available receptor sites present in castrated rabbits was at least twice that present in intact animals, suggesting the increased availability of binding sites for [^{3}H]-androgens following the decrease in endogenous androgen levels.

The metabolism of [^{3}H]-testosterone by the rabbit epididymis is dependent on substrate concentration, duration, and temperature of incubation in a manner characteristic of enzymatic reactions. The data presented here indicate that the adult rabbit epididymis possesses the enzymes 4-ene-3 ketosteroid 5α-oxidioreductase and 5α-androstane-3α(3β)-hydroxysteroid dehydrogenase that are necessary for the conversion of testosterone to 5αDHT and 5αDHT to androstanediols, respectively. Testosterone, 5αDHT, and 3αdiol are all capable of binding to the epididymal androgen receptor. The affinity of the receptor for these steroids appears to be 5αDHT > 3αdiol > testosterone. The binding of 3α-diol to the epididymal androgen receptor has previously been reported by other investigators [12]. Testosterone metabolism by and binding to epididymal tissue appears to be an independent phenomenon since binding of androgens to the receptor can occur at 0°C in the virtual absence of metabolism.

Previous work on androgen metabolism by the intact rat epididymis has been conducted following *in vivo* injection of [^{3}H]-testosterone [13] and following *in vitro* incubation of epididymal slices with [^{3}H]-testosterone [14]. These investigators examined testosterone metabolites present in homogenates of epididymal tissue and found that 5αDHT represented 40–50% of the metabolites and that 5α-androstan-3α, 17β-diol represented approximately 20% of the metabolites. They also were able to detect 5α-androstan-3β, 17β-diol; 4-androstene-3,17,dione; 5α-androstan-3, 17-dione and 5α-androstan-3α-hydroxy-17-one. None of the latter compounds comprised more than 10% of the total androgens present in the incubation mixture. In our system which consisted of minced epididymal tissue for 3-day castrated rabbits, the only metabolites present in sufficient quantity to characterize were 5αDHT and 5α-androstan-3α, 17β-diol. It is likely that our inability to detect the other compounds described by Djøseland and his colleagues is in part attributable to the decreased metabolism that would occur at the lower incubation temperature (23°C) that we used and is in part attributable to the decrease in epididymal androgen metabolism following castration. Since we were interested in examining not only steroid metabolism by epididymal tissue, but also the binding of steroids to macromolecular components in the incubation mixture, the lower incubation temperature was required to prevent denaturation of these components (particularly the epididymal androgen receptor).

Tindall *et al.*[15] performed studies on androgen uptake and metabolism by epididymal tissue obtained from 18-h castrated rats at various time intervals after the intravenous injection of [^{3}H]-testosterone. These investigators showed that there was a time-dependent decrease in the percentage of testosterone in epididymal supernatant, and a concomitant increase in dihydrotestosterone and "other metabolites."

Neither the studies of Djøseland nor those of Tindall were designed to distinguish between androgens bound to macromolecular components from those that were not bound, let alone to distinguish between androgens bound to receptor from androgens bound to ABP. In contrast, our studies do investigate all of these parameters and show that striking differences exist in the types of metabolites present in the various compartments. A higher percentage of 5αDHT is present in the bound compartment than in the free owing to a high affinity of androgen binding proteins for this metabolite [4, 5, 7, 10]. Furthermore, there appears to be a subdistribution of androgen metabolites within the bound compartment such that a higher percentage of 5αDHT is bound to the androgen receptor than to the rapidly dissociating moieties, i.e., ABP and TeBG. Differences in the distribution of androgens among the binding proteins may be of consequence in the delivery of androgens to the epididymis and the subsequent uptake and utilization of them by the tissue.

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