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The Conversion of Testosterone to 5α-Androstan-17β-ol-3-one by Rat Prostate *in Vivo* and *in Vitro**

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SUMMARY

The identity of the nonpolar transformation products of testosterone-1,2-^{3}H, the time course of the appearance of these metabolites in various tissue compartments, and the subcellular distribution of testosterone and its metabolites in prostate have been investigated following the intravenous administration of the hormone to normal and to functionally hepatectomized rats. Evidence has been obtained in both types of animals that within 1 min following its administration testosterone is taken up by the prostate, and at least 90% is converted to three products, androstandiol, dihydrotestosterone, and androsterone. From prostatic nuclei, however, only testosterone-^{3}H and dihydrotestosterone-^{3}H were recovered for as long as 2 hours following testosterone injection.

Furthermore, it has been shown that in the presence of a $NADPH_2$-generating system prostatic nuclei convert testosterone to dihydrotestosterone, whereas prostatic cytoplasm, in addition, reduces dihydrotestosterone to androstandiol. The nuclear enzyme which performs this reaction has been partially characterized and appears to be located within the chromatin.

Finally, the tissue distribution of dihydrotestosterone-^{3}H has been investigated at a short time interval following testosterone-1,2-^{3}H injection; this metabolite was detected only in prostate, seminal vesicle, preputial gland, kidney, and plasma.

The synthesis of protein and nucleic acids in prostatic tissue of castrate rats is rapidly and markedly increased by the administration of testosterone (1–4). As early as 1 hour after such treatment significant enhancement of RNA polymerase activity has been detected in prostatic nuclei (5), and it is assumed that the increased protein synthesis is the consequence of the accelerated RNA synthesis. Although these changes have been attributed to the effects of testosterone itself, such an interpretation is obscured by the fact that testosterone is rapidly metabolized *in vivo* (6–8). While some of the testosterone metabolites have little biological activity, at least one, 5α-androstan-17β-ol-3-one (dihydrotestosterone),[1] is a potent androgenic agent (9). It is of importance, therefore, to determine whether the changes observed in prostate are due to testosterone itself or to a metabolic product of testosterone such as dihydrotestosterone.

The experiments described in this paper were undertaken to obtain further information on the identity of the testosterone metabolites in a target tissue, to analyze the time sequence of the appearance of these metabolites in various tissue compartments, and to investigate the subcellular distribution of testosterone and its metabolites following the intravenous administration of testosterone-1,2-^{3}H. In addition, the metabolism of testosterone has been studied *in vitro*.

EXPERIMENTAL PROCEDURE

Whole Animal Studies—Male, Sprague-Dawley rats, weighing 175 to 250 g, were used in these experiments. In preparation for the experiments *in vivo* some rats were castrated, eviscerated, and functionally hepatectomized under ether anesthesia (10). Either the operated animals or control rats were given 250 μC of radioactive testosterone intravenously. At intervals up to 2 hours following this administration, two or three rats were killed by decapitation. Blood was collected in heparin, pooled, and sedimented by centrifugation. An aliquot of the plasma was taken for determination of radioactivity and extraction of steroid compounds. The ventral lobe of the prostate gland from each rat was dissected free of its enveloping capsule and removed. The tissue from each group of two or three rats was combined, and to this was added prostatic tissue from eight to 10 normal rats to

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[1] The following trivial names are used: testosterone, (Δ^4-androsten-17β-ol-3-one); androstenedione (Δ^4-androsten-3,17-dione); dihydrotestosterone (5α-androstan-17β-ol-3-one); androsterone (5α-androstan-3α-ol-17-one); androstandiol (5α-androstan-3α,17β-diol); 5α-reductase, ($NADPH_2$:Δ^4-5α-3-ketosteroid oxidoreductase).

provide carrier material. To obtain tissue for experiments *in vitro*, normal rats of similar size were decapitated, and prostatic tissue was removed and homogenized.

Homogenization of Tissue—Nuclei were isolated by a modification of the method of Maggio, Siekevitz, and Palade (11). The prostatic tissue was rinsed in 0.25 M sucrose solution, allowed to drain on filter paper, and chopped with an automatic tissue slicer. Four passages of the sample through the slicer were required to give the tissue a pulpy texture. The pulp was suspended in 0.88 M sucrose-1.5 mM $CaCl_2$, and the cells were ruptured in a Dounce homogenizer. About 25 strokes of a loose fitting plunger and 15 strokes of a tight fitting plunger were required. The crude homogenate was then filtered through two layers of gauze and sedimented in a refrigerated centrifuge at 800 × *g* for 10 min. The supernatant was decanted and the pellet was resuspended in 0.88 M sucrose containing 1.5 mM $CaCl_2$. Three strokes of the tight fitting plunger in a Dounce homogenizer were used at this time to disperse the pellet. The volume was made up to 60 ml with the sucrose-calcium solution, and 20-ml aliquots were layered over double gradients of 5 ml of 2.2 M sucrose and 5 ml of 1.8 M sucrose, each containing 0.5 mM $CaCl_2$. The tubes were centrifuged at 33,000 × *g* for 90 min with an SW 25.1 rotor in a Spinco model L ultracentrifuge at a temperature setting of 32° F. The supernatant was decanted and the pellet was resuspended in 10 ml of Tris buffer, pH 7.0 (0.01 M), containing EDTA (5.0×10^{-5} M), $MgCl_2$ (5.0×10^{-3} M), mercaptoethanol (0.5×10^{-3} M), and NaCl (0.05 M).

Extraction of Steroids from Tissue—The method described by Folch, Lees, and Stanley (12) for the isolation of lipids from animal tissues was adapted for use in these experiments. Aliquots of different fractions of the tissue homogenate were shaken in a 5-fold greater volume of chloroform-methanol (2:1). The mixture was centrifuged at 1500 rpm for 10 min. The upper aqueous phase was removed and the lower phase was again shaken with a volume of upper phase solvent (chloroform-methanol-water, 3:48:47) equal to the volume of the aqueous phase removed. Following centrifugation the aqueous phase was again removed and the procedure repeated two more times. The aqueous phases were combined, and after the volume was reduced by boiling, an aliquot was taken for determination of radioactivity. The chloroform-methanol phase was taken to dryness and resuspended in chloroform. One aliquot was analyzed for radioactivity and another was removed for analysis by gas-liquid or thin layer chromatography.

Incubations in Vitro—Two fractions were routinely used in these experiments—the supernatant from the original 800 × *g* centrifugation (cytoplasm) and the pellet from the sucrose density centrifugation (nuclei). Approximate concentrations of these fractions and of testosterone-1,2-^{3}H and cofactors were as follows: cytoplasm, each milliliter contained 10 mg of protein corresponding to 200 mg of tissue; nuclei, 10^7 per ml containing 200 μg of protein; testosterone-1,2-^{3}H, 10^5 cpm per ml; glucose-6-P, 10^{-3} M; glucose-6-P dehydrogenase, 10^{-6} g per ml; $NADP^+$, 10^{-4} M. The final volume was made up to 1 or 2 ml with the Tris buffer described above. Incubations were carried out at 37° with constant shaking and were terminated by the addition of chloroform-methanol. Steroids were extracted and identified by thin layer or gas-liquid chromatography.

Thin Layer Chromatography—Glass plates, 20 × 40 cm, were coated with a suspension of Silica Gel H (Brinkmann) in distilled water (30 g/72 ml) and activated in an oven for 1 hour at 100°. The thickness of the coating was 250 μ. A small quantity (50 μg each) of testosterone, dihydrotestosterone, and androstandiol was added to the samples of tissue extracts. These steroids served as standard reference points and as carrier material. After the tissue extracts were spotted, the plates were developed in ethyl ether-benzene (9:1). The solvent front was allowed to advance to the end of the plates over a period of 6 to 7 hours. After completion of chromatography the plates were dried, sprayed with a 0.005% rhodamine G solution, and examined under ultraviolet light. The position of the standards was marked, and the plate was divided into horizontal bands. These bands were scraped individually and transferred to counting vials. One milliliter of methanol and 10 ml of 0.4% diphenyloxazole in toluene were added to each vial, and the samples were assayed for radioactivity. Complete separation of dihydrotestosterone from testosterone and of androstandiol from testosterone was consistently obtained. However, androstenedione and androsterone were not always clearly separated from dihydrotestosterone and, when these metabolites were encountered, their identity was routinely confirmed by gas-liquid chromatography.

Gas-Liquid Chromatography—Gas-liquid chromatography of steroids was carried out in a Research Specialties Apparatus equipped with an ionization detector. The columns (6 feet × ¼ inch) were packed with 3% QF1 on Gas-Chrom Q 100 to 120 mesh (Applied Sciences, State College, Pennsylvania). The carrier gas was argon, and the flow rate was 100 ml per min. Column temperature was 220°. For radioactivity determination samples were collected directly into toluene containing the scintillating system. The recovery of radioactivity was 80 to 90% both for the thin layer plates and for the gas-liquid method.

Recrystallization Experiments—The identity of dihydrotestosterone-^{3}H and androstandiol-^{3}H as conversion products of testosterone was further confirmed in some experiments by adding appropriate carriers after they were isolated by thin layer chromatography and recrystallizing these materials to constant specific activity. For their crystallization, the method of Shikita and Hall (13) was utilized with the following solvent systems: methanol-H_2O; acetone-H_2O; chloroform-hexane (androstandiol); ether-hexane (dihydrotestosterone); ethyl acetate-cyclohexane; benzene-heptane.

Preparation of Dihydrotestosterone-^{3}H—Dihydrotestosterone-^{3}H was prepared enzymatically from testosterone-1,2-^{3}H in the following manner. Testosterone-1,2-^{3}H (5.0×10^5 cpm) was incubated with purified nuclei (5.0×10^7 per ml), glucose-6-P (2.5×10^{-3} M), glucose-6-P dehydrogenase (1.3×10^{-6} g per ml) and $NADP^+$ (1.3×10^{-4} M) for 1 hour at 37°. At the end of the incubation the radioactive material was extracted with chloroform-methanol, and dihydrotestosterone was isolated by thin layer chromatography. As determined by gas-liquid chromatography at least 95% of the radioactive material recovered was dihydrotestosterone. The amount of conversion of testosterone was usually 50% or more.

Counting of Nuclei—Nuclei were diluted in buffer and counted in a Spencer counting chamber (American Optical Company, Buffalo, New York). One drop of methyl blue was added to the buffer to stain the nuclei.

Analytical Procedures—For extraction of nucleic acids the procedure described by Maggio *et al.* (11) was followed. RNA was measured by the orcinol reaction (14) with yeast RNA as a standard. DNA was measured by the diphenylamine method

(14) with calf thymus DNA as a standard, and protein was determined by the method of Lowry *et al.* (15) utilizing bovine serum albumin as the reference protein.

Radioactive Materials—Testosterone-1,2-^{3}H (5.00 mC/0.03 mg) was purchased from New England Nuclear. To 5 ml of testosterone in ethanol-benzene was added 1 to 2 drops of Tween 40. The solution was taken to dryness and reconstituted with 5 ml of distilled water. Frequent tests of purity were made by thin layer chromatography or gas-liquid chromatography. In general at least 90% of the radioactive material had the same R_F value as testosterone when tested by thin layer chromatography or the same column retention time as testosterone when tested by gas-liquid chromatography. Occasionally androstenedione was detected and formed 5 to 7% of the total radioactivity. If testosterone-1,2-^{3}H was stored in aqueous solution for longer than 1 week this proportion was often much higher.

Liquid Scintillation Counting—Liquid scintillation counting was carried out with either Bray's mixture (16) or diphenyloxazole-toluene (4 g of diphenyloxazole per liter in toluene). Bray's mixture was used for counting aqueous samples, and the diphenyloxazole-toluene solution was used for counting samples obtained from thin layer chromatography or from gas-liquid chromatography. Internal standards were used to estimate quenching and corrections were applied where necessary. All samples were counted in an automatic refrigerated liquid scintillation counter.

Chemicals—Testosterone was purchased from Nutritional Biochemicals; androstenedione from Chemed, Inc. (White Plains, New York); dihydrotestosterone, androsterone, and androstandiol from Steraloids, Inc. (Pawling, New York).

$NADP^+$ and glucose-6-P (disodium) trihydrate were purchased from Nutritional Biochemicals. Glucose-6-P dehydrogenase was obtained from Sigma.

RESULTS

Distribution of Radioactivity, DNA, RNA, and Protein in Cell Fractions—The results of experiments to study the distribution of radioactivity in different cell fractions of prostate from functionally hepatectomized rats are shown in Table I. Animals were killed 1 to 2 hours after intravenous injection of testosterone-1,2-^{3}H. The results are expressed as total recovery of radioactivity, DNA, RNA, and protein in the whole homogenate and as a percentage recovery in the various subcellular fractions. Approximately two-thirds of the cellular RNA, protein, and radioactivity were recovered in the 800 × *g* supernatant, whereas more than 90% of the DNA was present in the 800 × *g* pellet. When the 800 × *g* pellet was resuspended and sedimented in a sucrose density gradient half of the cell DNA was recovered in the 33,000 × *g* pellet and an almost equal amount was recovered in the 33,000 × *g* supernatant. Thus the number of nuclei recovered in the 33,000 × *g* pellet (averaging 1.6 × 10^7 per prostate) represented only half of the total. Of the nuclei recovered in the 33,000 × *g* pellets, less than 5% were contaminated with cytoplasmic tags, as determined from examination by light microscopy. The DNA content per nucleus averaged 8.0 × 10^{-12} g, corresponding to values expected for mammalian cells (17). The 33,000 × *g* pellet contained 13% of the radioactivity, and since only 50% of the nuclei were recovered in this fraction the amount of radioactivity associated with nuclei was closer to 26% of the total. Thus, under the conditions used in these studies, the distribution of radioactivity in rat ventral prostate was approximately 3:1 between cytoplasm and nuclei 1 to 2 hours following the intravenous administration of testosterone-1,2-^{3}H, a distribution similar to that previously reported from this laboratory for the duck preen gland (18).

Level of Radioactivity in Plasma Cytoplasm and Nuclei—A time sequence analysis of the concentration and composition of plasma and prostatic radioactivity following testosterone-1,2-^{3}H injection was then undertaken. Plasma and intracellular radioactivity was compared in normal animals and in functionally hepatectomized animals. Functional hepatectomy was carried out to determine whether by removal of viscera the loss of testosterone could be reduced and the incorporation of testosterone by prostate increased. The results of experiments in which plasma, cytoplasmic, and nuclear radioactivity was measured are shown in Fig. 1*A*. At the *top* is plotted plasma radioactivity as a function of time after an intravenous injection of testosterone-1,2-^{3}H. One minute after injection the plasma from functionally hepatectomized animals (*solid line*) contained 8.5 × 10^5 cpm per ml while plasma from normal animals (*broken line*) contained 5.3 × 10^5 cpm per ml. Within 5 min this level had fallen to 2.8 × 10^5 cpm per ml in normal animals and then declined more slowly to a level of 0.5 × 10^5 cpm per ml 2 hours after injection. In the surgically treated animals the initial disappearance of

TABLE I

Subcellular distribution of radioactivity in prostate 1 to 2 hours following intravenous administration of testosterone-1,2-^{3}H to functionally hepatectomized rats

In 16 experiments the total number of nuclei recovered in the 33,000 × *g* pellet from each rat averaged 1.6 × 10^7 (0.3 to 2.9 × 10^7). The average DNA per nucleus was 8.0 × 10^{-12} g.

Analysis	Experiments	Total recovery	Distribution in subcellular fractions			
			800 × *g* supernatant	800 × *g* pellet	33,000 × *g* supernatant	33,000 × *g* pellet
		cpm or mg	%	%	%	%
Radioactivity	6	290,000 (90,000–540,000)	58 (49–70)	38 (28–51)	22 (8–42)	13 (9–17)
DNA	5	5.6 (2.0–11.7)	8 (6–17)	91 (82–100)	42 (26–58)	50 (36–61)
RNA	3	8.5 (6.6–11.0)	58 (57–58)	39 (31–43)	20 (13–25)	7 (5–9)
Protein	6	112.0 (25–180)	70 (62–80)	33 (26–46)	21 (10–27)	4 (3–6)

radioactivity was slower than in normal animals. The level of radioactivity fell to 4.5 × 10^5 cpm per ml during the first 25 min and then declined slowly to a level of 3.0 × 10^5 cpm per ml at 90 min. These results suggest that the level of circulating radioactivity was approximately 2-fold greater in these animals than in normal animals during the 90-min interval following injection of testosterone-1,2-^{3}H. This difference may have arisen in part from a different volume of distribution and in part from a diminished turnover rate following functional hepatectomy.

In the *middle panel* of *Graph A* is a plot of the level of radioactivity in the 800 × *g* supernatant fraction of prostatic tissue (cytoplasm) as a function of time after the injection of testosterone-1,2-^{3}H. The results are expressed as radioactivity in the 800 × *g* supernatant per nucleus isolated. In the study of the functionally hepatectomized animals (*solid line*) each *point* represents a mean value obtained from three to seven separate experiments, whereas the curve for normal animals (*broken line*) represents a single study in which three animals were studied at each time point. It can be seen that radioactivity appeared in the cytoplasm of normal and functionally hepatectomized animals within 1 min (*initial point*). The labeling of cytoplasm in prostate from the latter group increased to a maximum between 30 and 60 min following injection. However, in normal animals the level of labeled material decreased during the initial 30 min and then was constant. At 30 min there was approximately 20 times more radioactivity in the cytoplasm of prostate cells taken from eviscerated animals. This difference became less after 60 min.

In the *lower panel* of graphs is a plot of radioactivity in the 33,000 × *g* pellet (nuclei) as a function of time after the injection of testosterone-1,2-^{3}H expressed as counts per min per nucleus isolated. Radioactivity was detected in the nuclei within 1 min following injection of testosterone-1,2-^{3}H. The amount of label in prostatic nuclei taken from functionally hepatectomized animals increased about 10 times to a maximum level between 60 and 90 min. The amount of label in nuclei from normal animals increased only slightly during the same interval. At 60 min there was a 4-fold difference in the amount of label in prostatic nuclei from the two groups of animals. Thus, while the uptake by rat prostate of testosterone-1,2-^{3}H may be increased if the animals are functionally hepatectomized prior to experimental use, there is rapid uptake of the label by both nucleus and cytoplasm in both types of animals.

Identification of Components Contributing to Radioactivity in Plasma Cytoplasm and Nuclei after Injection of Testosterone-1,2-^{3}H—The identity of the radioactivity in plasma and prostate was then studied (Fig. 1*B*). The proportion of the total radioactivity in different tissue samples that could be extracted in chloroform-methanol was first determined. In normal rats it was expected that this proportion might change with time since steroids are conjugated in the liver to water soluble compounds, and consequently the tissue samples described in Fig. 1*A* were extracted with chloroform-methanol. Aliquots were taken from the chloroform-methanol phase and the aqueous phase for estimation of radioactivity. It can be seen that the amount of radioactivity recovered in the chloroform-methanol phase of plasma and cytoplasm from normal animals (*top* and *middle panels, broken line*) decreased with time. On the other hand, the intranuclear radioactivity from the normal rats (*lower panel, broken line*) was completely extracted in chloroform-methanol at all times. In similar experiments with functionally hepatectomized rats (*solid lines*) all of the radioactivity was recovered in the chloroform-methanol phase in each of the tissues and at each time interval examined. These results indicate that a large percentage of the testosterone-1,2-^{3}H injected into normal animals was converted to polar derivatives within 1 hour. These polar derivatives were not identified.

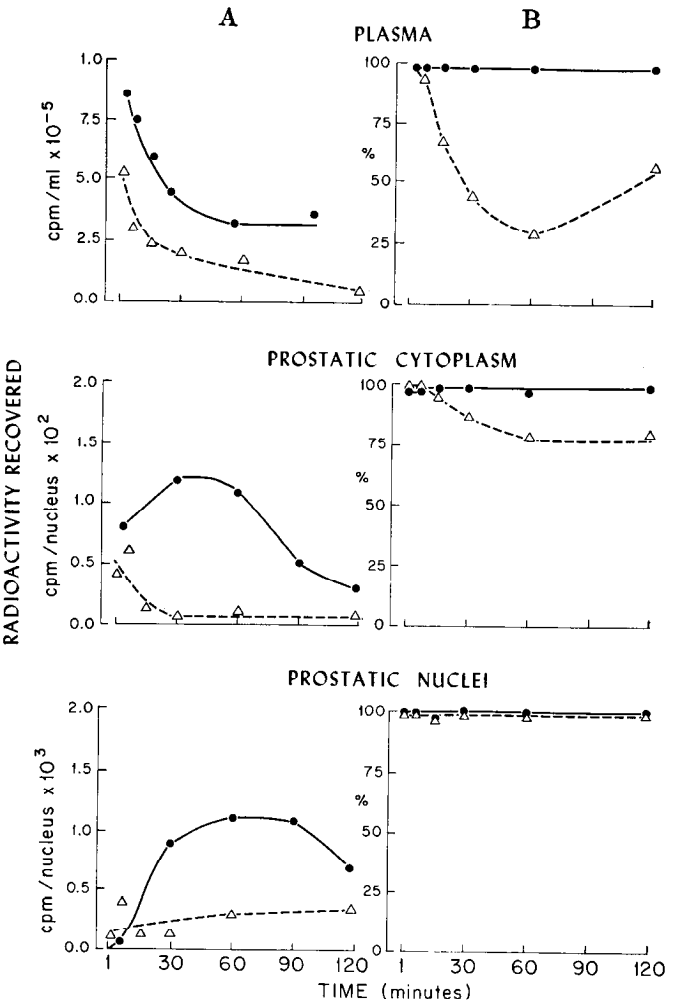

FIG. 1. Influence of functional hepatectomy on the levels of radioactivity in plasma, prostatic cytoplasm, and prostatic nuclei with time following the intravenous administration of testosterone-1,2-^{3}H. *A*, total radioactivity recovered; *B*, percentage of the recovered radioactivity extracted into chloroform-methanol at each time interval. △- - -△, normal rats; ●——●, functionally hepatectomized rats.

A further study of the identity of the steroid compounds in the chloroform-methanol extract was then carried out with thin layer and gas-liquid chromatography. The chromatographic techniques used for this study are illustrated in Fig. 2. Utilizing 40-cm plates developed in ethyl ether-benzene (9:1) it was possible to separate a variety of testosterone analogues, as shown on the *left side* of Fig. 2. When the chloroform-methanol extracts of prostate were added to the mixtures of carrier steroids and chromatographed in this way, it was apparent that only a fraction of the radioactivity recovered both from cytoplasm and nuclei was present in the testosterone area; in the nucleus, the major metabolite appeared to be dihydrotestosterone, whereas in the cytoplasm the areas corresponding to dihydrotestosterone

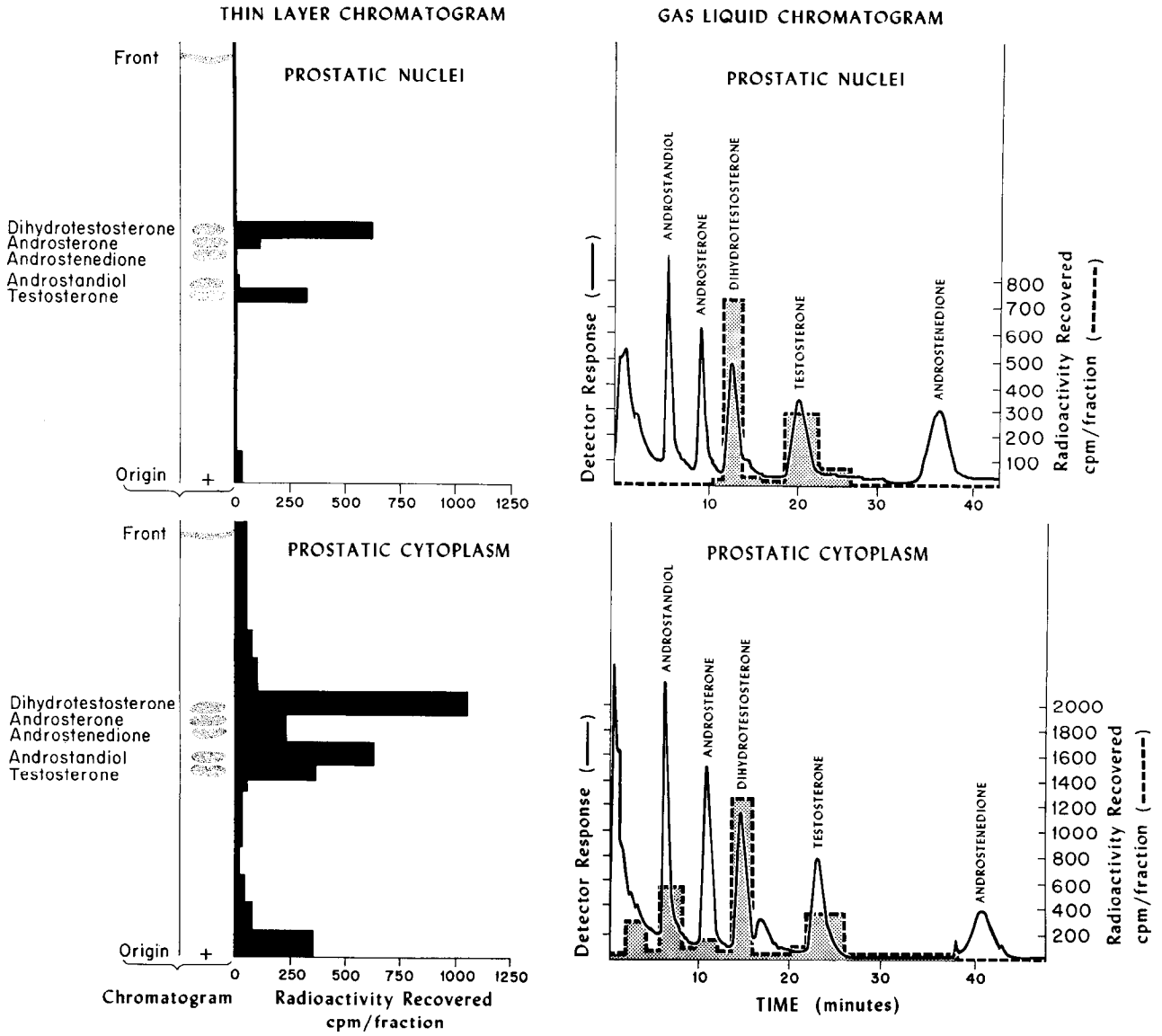

FIG. 2. Analysis of chloroform-methanol-extractable radioactivity from prostatic nuclei and cytoplasm by thin layer and gas-liquid chromatography following the intravenous administration of testosterone-1,2-^{3}H to rats. *Upper*, prostatic nuclei; *lower*, prostatic cytoplasm.

and androstandiol contained the major fraction of the radioactivity. When the same extracts were examined by gas-liquid chromatography (*right portion* of Fig. 2) similar results were obtained; dihydrotestosterone was the principal radioactive metabolite in the nucleus, whereas dihydrotestosterone, androstandiol, and small amounts of androsterone were the radioactive metabolites in the cytoplasm. Since these two chromatographic techniques produced similar results the thin layer method was used routinely for the time sequence studies. However, almost all time points in the studies *in vivo* were confirmed by gas-liquid techniques while at least one time point was confirmed by this method in each study *in vitro*.

A time sequence study of the appearance of metabolites in the chloroform-methanol extracts of plasma and prostate following the intravenous administration of testosterone-1,2-^{3}H to normal and functionally hepatectomized rats is shown in Fig. 3. In the normal animals (*A*) the percentage of testosterone in plasma (*upper panel*) fell from 75 at 1 min (*initial point*) to 10 at 1 hour. Between 5 and 10% of the radioactivity was recovered in the form of androstandiol. No radioactivity was present as dihydrotestosterone. In the *middle panel* are shown the results of studies on cytoplasm. At 1 min 35% of the radioactivity was in the form of dihydrotestosterone, and another 35% was recovered as androstandiol. About 10% was testosterone. The level of dihydrotestosterone remained constant while the levels of androstandiol and testosterone fell from their initial values. In both normal plasma and normal cytoplasm the radioactive material not identified was largely material which failed to migrate and remained at the origin when thin layer chromatography was done. This material was probably composed of polar derivatives of testosterone, soluble in chloroform-methanol but less soluble in the ethyl ether-benzene solvent used for thin layer chromatography. The results of studies on nuclei are shown in the *lower panel*. Only testosterone and dihydrotestosterone were found to be associated with this fraction. One minute after the injection of testosterone-1,2-^{3}H 50% of the total radioactivity was

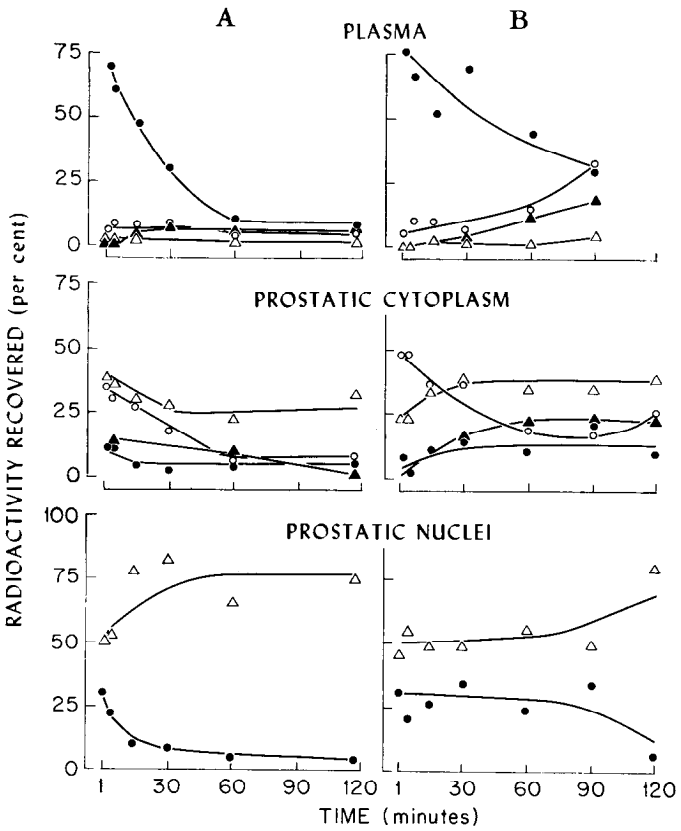

FIG. 3. Appearance of testosterone and metabolites in chloroform-methanol extracts of plasma and prostate with time following the intravenous administration of testosterone-1,2-^{3}H to rats. The chloroform-methanol extracts described in Fig. 1*B* were analyzed by thin layer and gas-liquid chromatography as described in the text. *A*, normal rats; *B*, functionally hepatectomized rats. ●——●, testosterone; △——△, dihydrotestosterone; ○——○, androstandiol; ▲——▲, androsterone.

identified as dihydrotestosterone and 35% was testosterone. With increasing time more dihydrotestosterone appeared, forming 75% of the total radioactivity at 0.5 hour. A high level was then maintained for the duration of the experiment. The proportion of testosterone fell to 10% at 0.5 hour and continued to decline slowly. By 2 hours less than 5% of the radioactivity was present as testosterone. The results of these experiments indicate that testosterone was rapidly metabolized both to polar and to nonpolar derivatives which appeared in the plasma and cytoplasm of prostate. In contrast, the only radioactive metabolite of testosterone identified in nuclei was dihydrotestosterone. The high proportion of this compound in the absence of other metabolites suggests that the localization of dihydrotestosterone in the nucleus is specific.

The results of experiments to identify the steroid components in chloroform-methanol extracts of plasma, cytoplasm, and nuclei taken from functionally hepatectomized rats are shown in Fig. 3*B*. It can be seen that the proportion of testosterone in plasma fell from 75% at 1 min to about 35% between 1 and 2 hours. Androstandiol appeared at 1 min and rose to 35% after 1 hour. Androsterone was detected at significant levels after 0.5 hour. Dihydrotestosterone was not detected until after 1 hour had elapsed. As shown in the *middle panel* from 1 min onward the principal radioactive steroid components of the cytoplasm were dihydrotestosterone, androstandiol, and androsterone, while testosterone accounted for little more than 10% of the total radioactivity. The absence of polar derivatives from the plasma and cytoplasmic fractions facilitated the identification of testosterone and metabolites recovered from functionally hepatectomized rats. As shown in the *lower panel* only dihydrotestosterone and testosterone were recovered from nuclei. Dihydrotestosterone was present at 1 min and formed 50% of the total radioactivity. This proportion increased with time while that of testosterone decreased. Thus, in eviscerated animals as in normal animals, testosterone was converted to dihydrotestosterone and androstandiol, and there appeared to be a specific association of dihydrotestosterone with prostatic nuclei. Two effects of functional hepatectomy were shown in these experiments: a decreased turnover of total radioactivity and a reduced conversion of testosterone to polar metabolites.

Dihydrotestosterone in Other Tissues—The presence of dihydrotestosterone, a potent androgen, in rat prostate suggests that this steroid may be functionally important in governing hormone-sensitive biochemical processes. The presence of dihydrotestosterone in other target organs and its absence from nontarget organs would be consistent with such a role. Accordingly, several tissues were examined for radioactive dihydrotestosterone 5 min after intravenous administration of testosterone-1,2-^{3}H to normal rats. From the results, as shown in Table II, it is evident that significant amounts of radioactive dihydrotestosterone were formed in prostate, seminal vesicles, and preputial gland. A small amount was recovered from kidney and plasma while none was recovered from other tissues tested. Thus dihydrotestosterone was found in large quantities only in the accessory sex tissues of the male rat.

Conversion in Vitro of Testosterone to Dihydrotestosterone—It was not possible to be certain whether the metabolites recovered from prostate arose from conversion of testosterone within the

TABLE II

Distribution of dihydrotestosterone-^{3}H in tissues 5 min after intravenous administration of testosterone-1,2-^{3}H

Three normal rats were injected with 250 μC of testosterone-1,2-^{3}H and killed 5 min later. Tissue extracts were assayed for steroid compounds either by thin layer or gas-liquid chromatography, and some samples were assayed by both methods. The percentage of the total counts per min in the aliquots identified as dihydrotestosterone-^{3}H by the different methods is shown.

Tissue	Amount analyzed	Thin layer chromatography		Gas-liquid chromatography	
		Total activity	Dihydrotestosterone	Total activity	Dihydrotestosterone
	g or ml	*cpm*	%	*cpm*	%
Plasma	3.0	8,190	1.8		
Gut	1.4			37,234	<1.0
Liver	1.5	18,900	<1.0	4,136	<1.0
Heart	1.0	9,767	<1.0		
Lungs	0.9	13,359	<1.0		
Levator ani muscle	0.3	1,257	<1.0		
Testis	1.7	13,531	<1.0		
Kidney	1.0	18,019	5.3	13,694	3.3
Prostate	0.5	3,165	29.5	3,305	39.3
Seminal vesicles	0.5	4,000	29.1	2,341	44.2
Preputial gland	0.5	1,952	27.8	2,391	19.8

TABLE III

Testosterone-1,2-^{3}H reduction by prostatic nuclei

The complete mixture contained nuclei (0.5×10^8), glucose-6-PO_4 (12.5×0^{-3} M), glucose-6-P dehydrogenase (0.8×10^{-6} g), $NADP^+$ (0.4×10^{-3} M), testosterone-1,2-^{3}H (1.8×10^5 cpm), Tris buffer, pH 7.0 (0.01 M), EDTA (5.0×10^{-5} M), $MgCl_2$ (5.0×10^{-3} M), mercaptoethanol (0.5×10^{-3} M), and NaCl (0.05 M). Two milliliters were incubated. After 90 min at 37°, the lipids were extracted and analyzed by thin layer chromatography as described in the text.

Incubation mixture	^{3}H Chromatography	Amount recovered	
		Testosterone	Dihydrotestosterone
	cpm	%	%
Complete mixture	3924	34	54
Complete mixture minus nuclei	2699	94	0
Complete mixture minus $NADP^+$	4140	83	4
Complete mixture minus glucose-6-P + glucose-6-P-dehydrogenase	3697	75	1
Complete mixture minus $NADP^+$ + glucose-6-P + glucose-6-P-dehydrogenase	4252	91	0

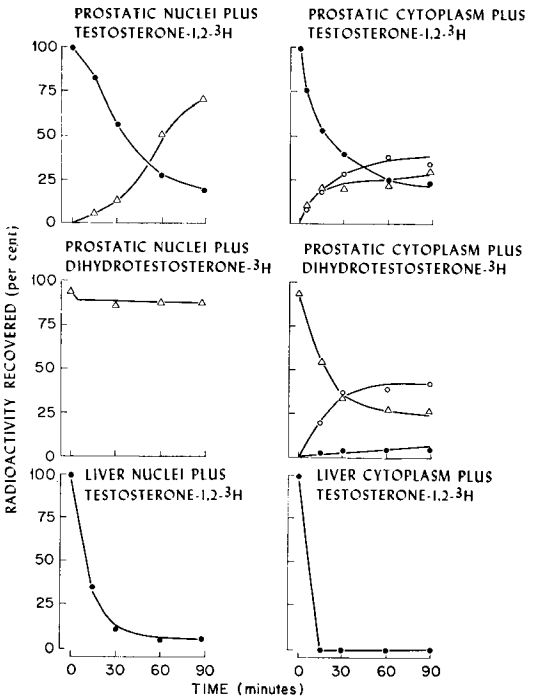

FIG. 4. Metabolism of testosterone-1,2-^{3}H and dihydrotestosterone-^{3}H by homogenates of prostate and liver. Each incubation flask contained glucose-6-PO_4 (2.5×10^{-3} M), glucose-6-PO_4 dehydrogenase (1.3×10^{-6} g per ml), $NADP^+$ (1.3×10^{-4} M), either testosterone-1,2-^{3}H (1.5×10^5 cpm per ml) or dihydrotestosterone-^{3}H (5.0×10^4 cpm per ml), homogenate as indicated, and Tris buffer, pH 7.0 (0.01 M), containing EDTA (5.0×10^{-5} M), mercaptoethanol (0.5×10^{-3} M), $MgCl_2$ (5.0×10^{-3} M) and NaCl (0.05 M). The final volume was 2 ml. The four homogenates consisted of either prostatic nuclei (1.0×10^8) or liver nuclei (1.4×10^8) obtained from 3 g of tissue or of $800 \times g$ supernatant corresponding to 0.2 g, wet weight, of prostate or liver. At the time indicated aliquots were removed for analysis as described in the text. ●——●, testosterone; △——△, dihydrotestosterone; ○——○, androstandiol.

prostate itself or whether they originated from plasma after conversion by other tissues. In order to test for the presence in prostate of reducing enzymes that might be responsible for the conversions observed, several experiments *in vitro* were performed. The results of a study of the conditions required for such a demonstration are shown in Table III. In the presence of $NADP^+$ and a $NADPH_2$-generating system prostatic nuclei actively reduce testosterone to dihydrotestosterone, whereas, in the absence either of $NADP^+$ or the $NADPH_2$-generating system, virtually no conversion was demonstrable. The results of a study in which the ability of prostatic cytoplasm and nuclei to perform these reductions was compared to similar fractions from liver are shown in Fig. 4. During a 90-min incubation with prostatic nuclei the percentage of testosterone fell from 100 to 25 while the percentage of dihydrotestosterone increased from 0 to 75. In the cytoplasmic fraction both dihydrotestosterone and androstandiol were formed from testosterone-1,2-^{3}H. These experiments confirmed the presence of the enzyme $NADPH_2$: Δ^4-5α-3-ketosteroid oxidoreductase in nuclei and cytoplasm obtained from rat prostate.

These experiments did not distinguish between two possible pathways by which androstandiol synthesis could take place. Androstandiol could either be formed from testosterone through a sequential reaction in which dihydrotestosterone is an obligatory intermediate or via a reaction in which dihydrotestosterone is not an intermediate. Evidence to support the first mechanism could be obtained if the conversion of dihydrotestosterone to androstandiol could be shown. Accordingly, prostatic cytoplasm and nuclei were incubated with dihydrotestosterone-^{3}H under conditions identical with those utilized for testosterone-1,2-^{3}H. As shown in the *middle panels* of Fig. 5, the concentration of dihydrotestosterone remained constant during a 90-min incubation with nuclei. No androstandiol was formed. When dihydrotestosterone was incubated with prostatic cytoplasm, however, there was a marked decrease in the percentage of dihydrotestosterone with a concomitant increase in the percentage of androstandiol up to 45% at 90 min. These results suggest that the pathway for metabolism of testosterone to androstandiol

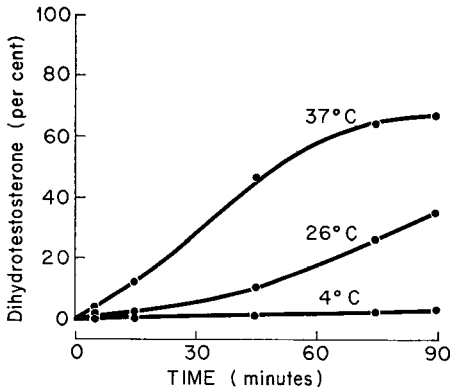

FIG. 5. The effect of incubation temperature on the conversion of testosterone to dihydrotestosterone by prostatic nuclei. Each incubation flask contained prostatic nuclei (0.3×10^8 per ml), glucose-6-PO_4 (2.5×10^{-3} M), glucose 6-phosphate dehydrogenase (1.3×10^{-6} g per ml), $NADP^+$ (2.5×10^{-4} M), testosterone-1,2-^{3}H (1.3×10^5 cpm per ml), Tris buffer, pH 7.0 (0.01 M), EDTA (5.0×10^{-5} M), $MgCl_2$ (5.0×10^{-3} M), mercaptoethanol (0.5×10^{-3} M), and NaCl (0.05 M) in a final volume of 5 ml. At the times indicated 1-ml aliquots were removed and analyzed as described in the text.

TABLE IV

Confirmation by crystallization of identity of dihydrotestosterone-^{3}H and androstandiol-^{3}H isolated by thin layer chromatography from homogenates of rat prostate incubated with testosterone-1,2-^{3}H

Material tentatively identified by thin layer chromatography and gas-liquid chromatography as either androstandiol-^{3}H or dihydrotestosterone-^{3}H was pooled from two incubations of prostatic homogenates and added to the appropriate carrier steroid for recrystallization.

Crystallization	Dihydrotestosterone-^{3}H plus dihydrotestosterone (100 mg)		Androstandiol-^{3}H plus androstandiol (90 mg)	
	Solvent	Specific activity	Solvent	Specific activity
		cpm/mg		*cpm/mg*
1	Methanol-H_2O	366	Methanol-H_2O	758
2	Acetone-H_2O	360	Acetone-H_2O	680
3	Ether-hexane	369	Chloroform-hexane	797
4	Ethylacetate-cyclohexane	343	Ethylacetate-cyclohexane	722
5	Benzene-heptane	329	Benzene-heptane	703

may involve dihydrotestosterone as an intermediate. It is clear that, while the enzyme NADPH$_2$:Δ^4-5α-3 ketosteroid oxidoreductase was found to be associated both with the nuclear and cytoplasmic fractions, the enzyme NADPH$_2$:3α-ketosteroid oxidoreductase was present only in the cytoplasmic fraction. These studies support the idea, as do studies described earlier in the paper, that the presence of dihydrotestosterone in the nucleus might have special functional significance.

If the presence of dihydrotestosterone were a unique property of a target organ, then the absence of this substance from a tissue that is not a target organ would be predicted. To test this prediction, testosterone-1,2-^{3}H was incubated with cytoplasmic and nuclear fractions from liver in the presence of NADP$^+$, glucose-6-P, and glucose-6-P dehydrogenase. The results are shown as the *lower panels* of Fig. 4. In both cases there was rapid conversion of testosterone to products other than dihydrotestosterone or androstandiol. The products formed had the characteristics of polar compounds, as judged from their failure to migrate from the origin when tested by thin layer chromatography. Since the enzyme which reduces testosterone to dihydrotestosterone is known to be present in rat liver (19), the failure to detect dihydrotestosterone in these experiments must mean that the formation of polar metabolites is so rapid that substrate for the reaction is depleted or that any dihydrotestosterone formed is immediately converted to a polar derivative.

Crystallization Studies—Although the dihydrotestosterone and androstandiol isolated in the previous experiment were identified with the use of thin layer and gas-liquid chromatography, crystallization studies were carried out as further means of establishing their identity. Dihydrotestosterone-^{3}H was prepared by conversion *in vitro* of testosterone-1,2-^{3}H. Androstandiol-^{3}H was similarly prepared from dihydrotestosterone-^{3}H. The compounds were first isolated on thin layer plates, and the purity of these radioactive steroids as judged from gas-liquid chromatography was greater than 90%. The compounds were then mixed with 90 or 100 mg of the appropriate standard compounds and carried through five successive crystallizations in a series of solvent systems utilizing the methods of Shikita and Hall (13). The results as shown in Table IV indicate that crystals of constant specific activity were obtained. These findings are consistent with the previous identification of these metabolites by gas-liquid and thin layer chromatography.

Characterization of Steroid Reduction Reaction in Nuclei—A series of studies of the characteristics of the reaction for the reduction of testosterone to dihydrotestosterone by prostatic nuclei was then performed. The influence of incubation temperatures on the reduction is shown in Fig. 5; approximately twice as much dihydrotestosterone was formed at 37° as at 26°, and virtually none was formed in the cold. The relation of the

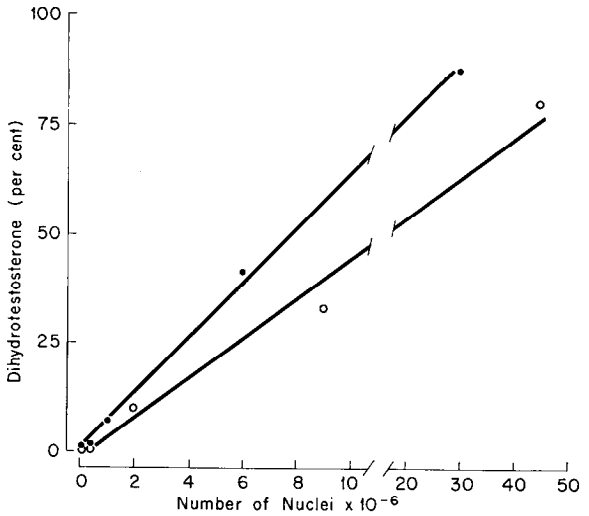

FIG. 6. Influence of the number of nuclei on the conversion of testosterone to dihydrotestosterone. Each incubation flask contained nuclei as indicated, glucose-6-PO$_4$ (3.2×10^{-3} M), glucose 6-phosphate dehydrogenase (1.6×10^{-6} g per ml), NADP$^+$ (3.1×10^{-4} M), testosterone-1,2-^{3}H (3.0×10^5 cpm per ml), Tris buffer, pH 7.0 (0.01 M), EDTA (5.0×10^{-5} M), MgCl$_2$ (5.0×10^{-3} M), mercaptoethanol (0.5×10^{-3} M), and NaCl (0.05 M) to make a final volume of 2.0 ml. After incubation for 90 min at 37° the lipids were extracted and analyzed as described in the text. ●, 200-g rats; ○, 400-g rats.

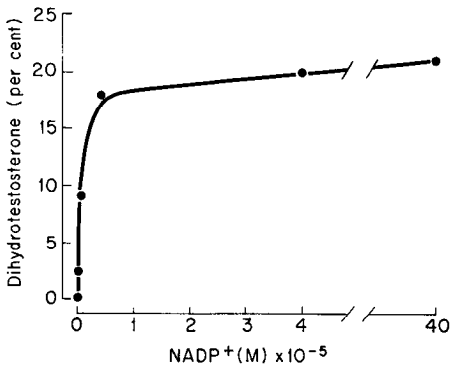

FIG. 7. Influence of NADP$^+$ concentration on the conversion of testosterone-1,2-^{3}H to dihydrotestosterone-^{3}H by prostatic nuclei. Each incubation flask contained nuclei (3.0×10^7), glucose-6-PO$_4$ (4.2×10^{-3} M), NADP$^+$ as indicated, glucose-6-PO$_4$ dehydrogenase (1.0×10^{-6} g per ml), testosterone-1,2-^{3}H (3.0×10^5 cpm per ml), Tris buffer, pH 7.0 (0.01 M), EDTA (5.0×10^{-5} M), MgCl$_2$ (5.0×10^{-3} M), mercaptoethanol (0.5×10^{-3} M), and NaCl (0.05 M), making a final volume of 1.5 ml. After incubation at 37° for 30 min, the lipids were extracted and analyzed by thin layer chromatography as described in the text.

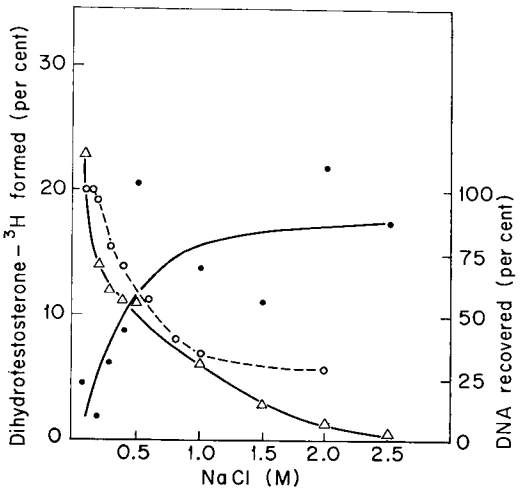

FIG. 8. The reduction of testosterone-1,2-^{3}H by NaCl-soluble and insoluble fractions isolated from nuclei. Sonically disrupted nuclei (4.0×10^8) were divided into 10 equal parts of 1 ml each. To each part was added an equal volume of Tris buffer, pH 7.0 (0.01 M), containing EDTA (5.0×10^{-5} M), $MgCl_2$ (5.0×10^{-3} M), and mercaptoethanol (0.5×10^{-3} M) containing twice the concentration of NaCl required to give the final NaCl concentration shown above. The fractions were centrifuged (17,000 × *g*, 10 min) and divided into supernatants and precipitates. The precipitates were resuspended in 2 ml of Tris buffer. Glucose 6-phosphate (3.0×10^{-3} M), glucose 6-phosphate dehydrogenase (0.8×10^{-6} g per ml), NADP (0.3×10^{-4} M), and testosterone-1,2-^{3}H (3.0×10^5 cpm per ml) were added to each sample. These were then incubated for 90 min and extracted with chloroform-methanol (2:1). In a similar experiment DNA determinations were carried out in place of assays for enzyme activity. △——△, dihydrotestosterone formation by pellet; ●——●, dihydrotestosterone formation by supernatant; ○- - -○, DNA recovered in pellet.

number of nuclei to the rate of conversion is shown in Fig. 6; at concentrations below 10^6 nuclei virtually no conversion could be shown, whereas active reduction of testosterone occurred at higher concentrations. The $NADP^+$ requirement is shown in Fig. 7; the minimal concentration of $NADP^+$ at which a near maximal rate of reaction was observed was 0.5×10^{-5} M.

The activity of the 5α-reductase in different nuclear soluble fractions as measured by conversion of testosterone-1,2-^{3}H is illustrated in Fig. 8. A curve for the recovery of DNA is also shown. It can be seen that the enzyme activity in the nuclear insoluble fractions parallels the amount of DNA recovered in the same fractions between NaCl concentrations of 0.1 and 1.0 M. For example, when the NaCl concentration reaches 1.0 M, 75% of the enzyme is soluble, whereas approximately 25% of both enzyme activity and DNA are insoluble. The fact that the enzyme is insoluble in dilute salt solutions and shows solubility characteristics similar to DNA is consistent with the possibility that the enzyme is associated with nuclear chromatin.

DISCUSSION

Several conclusions appear to be warranted from these studies. First, as has previously been shown in rabbit and dog (20, 21), radioactive testosterone disappears very quickly from the circulation of the rat. This rapid metabolism is the result of at least two processes, formation of polar metabolites and conversion to a variety of neutral transformation products. Second, the rapid disappearance of circulating testosterone is markedly retarded by functional hepatectomy; this effect of hepatectomy may be due in part to a diminished circulating blood volume and consequently a decreased volume of distribution for the administered hormone. In addition, however, it is quite clear that the functional hepatectomy (or evisceration, or both) results in an abolition of the appearance of the polar metabolites in the circulation, whereas the appearance of the neutral metabolites is either enhanced or unchanged by this treatment. Thus the formation of the neutral circulating metabolites of testosterone must take place principally in extraabdominal sites.

Third, as has previously been implied from the work of Wilson and Loeb (18), testosterone-1,2-^{3}H is rapidly taken up by accessory sex tissue, and as previously reported by Farnsworth and Brown (8) the hormone thus taken up is quickly metabolized to dihydrotestosterone, androstandiol, and androsterone by the prostate. The fact that at each time interval examined more than 90% of the radioactivity recovered from prostate was in the form of metabolites and that these metabolites were detectable at times at which trivial levels were detectable in blood suggests that their formation does in fact take place within the prostate itself in intact animals.

Fourth, as in the case of the duck preen gland, following the administration of testosterone-1,2-^{3}H there is selective localization of the isotope in the nuclei of the prostate (18). The nuclear radioactivity has been shown to consist of testosterone (25 to 50%) and a single testosterone metabolite, dihydrotestosterone (50 to 75%). Because of the parallelism of the appearance of this metabolite in cytoplasm and nucleus, it was not possible to determine from the time sequence studies *in vivo* whether the dihydrotestosterone was formed in the cytoplasm alone or both in nucleus and cytoplasm. When the metabolism of testosterone was investigated in preparations *in vitro*, however, it became clear that both prostatic cytoplasm and prostatic nuclei convert testosterone to dihydrotestosterone in the presence of the appropriate cofactors. Thus nuclear dihydrotestosterone can arise both from the cytoplasm and from formation within the nuclei themselves. While the enzyme that performs this conversion has been detected in a variety of tissues (19, 22, 23), including prostate (24, 25), this is the first report of its presence in nuclei.

Fifth, while only one enzyme for testosterone metabolism was detected in prostatic nuclei, the enzyme for the further conversion of dihydrotestosterone to androstandiol is present in cytoplasm. Thus, while the nuclei of prostate convert testosterone to a very potent androgen, the cytoplasm reduces it to less active androgen (9). In this regard the prostatic cytoplasm, like liver, serves to inactivate that hormone which has not reached the nuclei.

Finally, the fact that dihydrotestosterone-^{3}H is detectable in significant quantities only in organs known to be responsive to testosterone suggests that its presence might have potential explanatory value for testosterone action in these tissues. The previous demonstration by Dorfman and Shipley that this substance is indeed a potent androgen (9) and the fact that it is the predominant form of the hormone within prostatic nuclei, a presumed site for testosterone action, are in keeping with this possibility.

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The Conversion of Testosterone to 5α-Androstan-17β-ol-3-one by Rat Prostate *in Vivo* and *in Vitro*

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