

# The Intranuclear Binding of Testosterone and $5\alpha$ -Androstan- $17\beta$ -ol-3-one by Rat Prostate\*

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## SUMMARY

The intranuclear binding of radioactive hormone in prostate after the intravenous administration of testosterone- $1,2\text{-}^3\text{H}$  to rats has been studied. Nuclei obtained by sucrose density gradient centrifugation were extracted with buffer containing 0.6 M NaCl, and the soluble radioactivity was separated into bound and free fractions by gel filtration on Sephadex G-25 or G-200. As early as 15 min after testosterone administration, the radioactivity recovered in the bound form was predominantly dihydrotestosterone.

By gel filtration of nuclear extracts on Sephadex G-200 and by the use of digestive enzymes, it was shown that dihydrotestosterone was bound to an acidic nuclear protein. Finally, several characteristics of this binding phenomenon were defined; the binding was stable to freezing for as long as 8 days, stable to short term incubation at  $20^\circ$  but not at  $37^\circ$ , and partially stable to repeated gel filtration on Sephadex.

Several recent studies have shown that radioactivity originating from labeled testosterone is bound to the sites of active RNA synthesis within the nuclei of those sexual tissues that are responsive to androgen stimulation (2, 3). However, the possibility that such binding is not caused by testosterone itself must be considered in view of the rapid conversion of testosterone to  $5\alpha$ -androstan- $17\beta$ -ol-3-one (dihydrotestosterone),<sup>1</sup> that takes place in target cell nuclei (4). Because the enzyme responsible for this conversion appears to be associated with the nuclear chromatin (4), dihydrotestosterone too might well be present in chromatin in significant amounts. Such localization of dihydrotestosterone at a site where testosterone is thought to exert

its main action would support the view that dihydrotestosterone is an active form of the hormone in some tissues (1, 4).

The experiments described in this paper were undertaken to determine whether the hormone bound to the presumed active site in chromatin is indeed testosterone or dihydrotestosterone. In addition, several properties of the hormone receptor site were characterized.

## EXPERIMENTAL PROCEDURE

**Preparation of Animals**—Male, Sprague-Dawley rats, weighing 175 to 250 g, were used in these experiments. Three to six rats were castrated, eviscerated, and functionally hepatectomized under ether anesthesia. Immediately upon completion of the surgical procedure, each rat was given an injection of 250  $\mu\text{C}$  of testosterone- $1,2\text{-}^3\text{H}$ . From 1 to 2 hours later, these rats were killed by decapitation, and the ventral lobe of the prostate from each rat was removed. To this tissue was added prostatic tissue from 8 to 10 intact rats that had not received labeled testosterone.

**Homogenization of Tissue**—Nuclei were isolated by a modification of the method of Maggio, Siekevitz, and Palade (5) which has been described in detail in a previous paper (4). The prostatic tissue was rinsed in 0.25 M sucrose solution, allowed to drain on filter paper, and chopped with an automatic tissue slicer. The pulp was suspended in 0.88 M sucrose-1.5 mM  $\text{CaCl}_2$ , and the cells were ruptured in a Dounce homogenizer with 25 strokes of a loosely fitting plunger and 15 strokes of a tightly fitting plunger. The crude homogenate was then filtered through two layers of gauze and sedimented in a refrigerated centrifuge at  $800 \times g$  for 10 min. The supernatant was decanted, and the pellet was resuspended in 0.88 M sucrose containing 1.5 mM  $\text{CaCl}_2$ . Three strokes of the tightly 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  $\text{CaCl}_2$ . The tubes were centrifuged at 23,500 rpm (average,  $53,000 \times g$ ; minimum,  $33,000 \times g$ ) for 90 min with an SW 25.1 rotor in a Spinco model L ultracentrifuge at  $32^\circ\text{F}$ . The supernatant was decanted, and the pellet was resuspended in 10 ml of Tris-HCl buffer, pH 7.0 (0.01 M), containing EDTA ( $5.0 \times 10^{-5}$  M),  $\text{MgCl}_2$

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<sup>1</sup> The trivial names used are: testosterone,  $\Delta^4$ -androsten- $17\beta$ -ol-3-one; dihydrotestosterone,  $5\alpha$ -androstan- $17\beta$ -ol-3-one.

( $5.0 \times 10^{-3}$  M), 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M), and NaCl (0.05 M). This technique yielded highly purified nuclei as determined by light microscopy. An aliquot was diluted and counted in a Spencer counting chamber (American Optical Company, Buffalo, New York). Methyl blue was added to the buffer to stain the nuclei.

**Extraction of Nuclei**—Nuclei were allowed to swell for 10 min in 1 to 2 ml of hypotonic Tris-HCl buffer, pH 7.4 (0.01 M), containing EDTA ( $5.0 \times 10^{-5}$  M),  $MgCl_2$  ( $5.0 \times 10^{-3}$  M), 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M), and NaCl (0.05 M). The nuclei were then ruptured by exposing them to sonic waves at 20,000 cycles per sec for four 5-sec intervals with a 1-cm probe of a Bronwill Biosonik (Bronwill Scientific, Rochester, New York). The probe was stationed 1 cm from the bottom of the tube, and an energy input of 50 was used routinely. The tubes were kept immersed in ice during the entire procedure.

To the solution of sonically ruptured nuclei was added an equal volume of Tris-HCl buffer, pH 7.4 (0.01 M), with EDTA ( $5.0 \times 10^{-5}$  M),  $MgCl_2$  ( $5.0 \times 10^{-3}$  M), 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M), and NaCl (1.15 M). The final NaCl concentration was thus 0.6 M. Exceptions are mentioned in the text. The solution was then sedimented in a refrigerated centrifuge at  $17,000 \times g$  for 20 min. The supernatant was withdrawn carefully with a Pasteur pipette. The pellet was extracted again with 0.5 ml of Tris-HCl buffer, pH 7.4 (0.01 M) containing EDTA ( $5.0 \times 10^{-5}$  M),  $MgCl_2$  ( $5.0 \times 10^{-3}$  M), 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M), and NaCl (0.6 M), and the supernatant was combined with the one obtained from the first extraction. The pooled supernatants were then used for gel filtration experiments. All the extraction procedures were performed in a  $4^\circ$  cold room; in each instance the pH of the buffers was measured on a Beckman pH meter at the temperature of extraction.

**Gel Filtration Procedures**—Sephadex G-200 and G-25 coarse (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey) were prepared according to the instructions provided by the manufacturer. For gel filtration with Sephadex G-25, the columns were  $40 \times 0.8$  cm and for gel filtration with G-200 Sephadex,  $100 \times 0.8$  cm. The columns were washed extensively with Tris-HCl buffer, pH 7.4 (0.01 M), containing EDTA ( $5.0 \times 10^{-5}$  M),  $MgCl_2$  ( $5.0 \times 10^{-3}$  M), 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M), and NaCl (0.6 M). Subsequent filtrations were all performed at  $4^\circ$ . The usual sample volume was 2 ml, and this represented an extract of  $2.0 \times 10^8$  nuclei at most. More concentrated extracts occasionally failed to pass through the Sephadex G-200. Samples were eluted with the same buffer used for equilibration of the columns. Flow rates were 1 ml per min for Sephadex G-25 and 2 ml per hour for Sephadex G-200. Usually, fractions of 2 ml each were collected.

**Recovery and Identification of Steroids**—The method of Folch, Lees, and Sloane Stanley (4, 6) was used to extract steroids from aqueous fractions. Samples thus obtained were analyzed by thin layer chromatography and gas-liquid chromatography as previously described (4).

**Analytical Procedures**—Nucleic acids were extracted according to the method of Maggio *et al.* (5). DNA was measured by the diphenylamine method (7) with calf thymus DNA as a standard. RNA was determined by the orcinol reaction (7) with yeast RNA as a standard. The method of Lowry *et al.* (8) was used for protein determinations; bovine serum albumin was used as the reference protein.

**Enzymes**—Deoxyribonuclease I was obtained from Sigma, ribonuclease from Mann, Pronase, B grade, from Calbiochem.

**Radioactive Materials**—Testosterone-1,2- $^3H$  (5.00 mC/0.03 mg) was purchased from New England Nuclear and prepared for use by adding 0.2 ml of a 1:400 aqueous solution of Tween 40 to each 1 ml of testosterone in ethanol-benzene. The ethanol-benzene was then evaporated, and the remaining aqueous solution was made up to the desired volume with water.

**Liquid Scintillation Counting**—Liquid scintillation counting was carried out either with Bray's mixture (9), diphenyloxazole-toluene solution (4 g of diphenyloxazole per liter in toluene), or a diphenyloxazole-toluene solution containing a solubilizer, BBS-3 (Beckman Instruments). The latter solution contained toluene, 1 liter; diphenyloxazole, 6 g;  $H_2O$ , 5 ml; and BBS-3, 100 ml. Bray's mixture or the diphenyloxazole-toluene-solubilizer solution was used for counting aqueous samples. 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 made where necessary. The samples were counted in automatic liquid scintillation counters of either the refrigerated or ambient temperature types.

## RESULTS

**Extraction of Nuclei**—Experiments were first performed to establish the conditions for extraction of bound radioactivity from the nucleus. Toward this end, the solubility of nuclear radioactive material, protein, DNA, and RNA was studied in solutions containing concentrations of NaCl varying from 0.05 to 2.0 M. The results of such studies are shown in Fig. 1. About 40% of the radioactivity appeared in the supernatant (soluble) fraction when the NaCl concentration in the buffer was 0.05 M (Fig. 1A). This percentage increased to 85 to 90% at NaCl concentrations of 0.6 M or greater. Inasmuch as no further increase in amount of radioactive material extracted took place beyond 0.6 M NaCl, this concentration was considered suitable for use in later experiments. As can be seen from Fig. 1, B, C, and D, about 85% of the protein, 50% of the DNA, and 50% of the RNA are in solution at this concentration of NaCl.

Because a portion of the radioactive material was insoluble in a 0.6 M NaCl solution, the effect of a second extraction was studied. Accordingly, the pellet remaining after the first extraction was resuspended in buffer containing 0.6 M NaCl, sonically ruptured as before, and centrifuged. As shown in Table I, the amount of radioactive material remaining in the  $17,000 \times g$  pellet after the second extraction was less than 1% of the amount in the original pellet. Thus, total extraction of nuclear radioactivity was assured with the use of the 2-fold extraction procedure in most experiments.

**Effects of pH on Extraction**—The effect of variations in pH on the recovery of radioactivity from labeled nuclei was then investigated. To test for a relation between pH and solubility, labeled nuclei were extracted once in the usual manner but at three different pH values. The results are shown in Table II. Equivalent amounts of radioactivity were present in the  $53,000 \times g$  pellets of all three samples. Approximately 25% less radioactive material was extracted into the  $17,000 \times g$  supernatant at pH 6.2 than at pH 7.4 and 8.5. The unextracted radioactivity remained associated with the  $17,000 \times g$  pellet. Thus, in order to obtain complete extraction of nuclei, the pH of

buffers used in subsequent experiments was always adjusted to 7.4.

**Binding of Nuclear Radioactivity**—The next experiments were designed to distinguish between nuclear radioactive steroid in a bound form and that in free form. For this purpose, analysis of nuclear extracts was effected by gel filtration on Sephadex G-25 (10). The results of one such experiment are shown in Fig. 2. When a radioactive nuclear extract was eluted in successive 2-ml fractions, the radioactivity was recovered in two peaks (Fig. 2A). As shown in B, C, and D, the protein, DNA, and RNA eluted with the initial peak of radioactivity, and the second peak of radioactivity corresponded to the elution volume observed when free testosterone and dihydrotestosterone were passed through similar columns under the same conditions. On

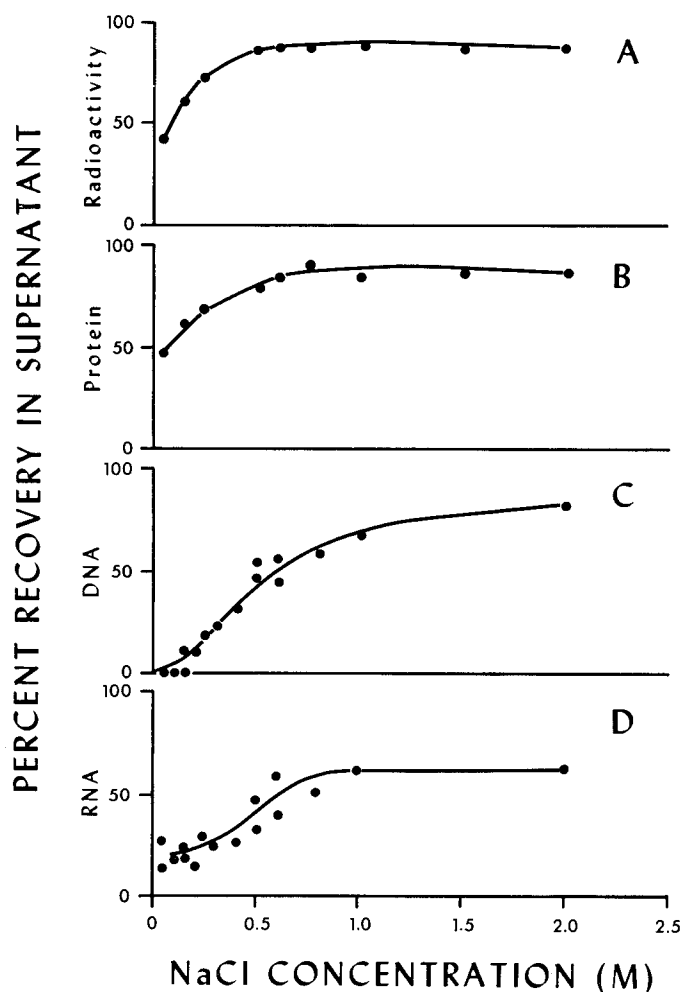


FIG. 1. Extraction of nuclei with different concentrations of NaCl. Sonically disrupted nuclei were divided into equal parts of 1 ml each containing approximately 1200 cpm of  $^3\text{H}$  radioactivity, 700  $\mu\text{g}$  of protein, 150  $\mu\text{g}$  of DNA, and 50  $\mu\text{g}$  of RNA. To each part was added an equal volume of Tris-HCl buffer, pH 7.4 (0.01 M), containing EDTA ( $5.0 \times 10^{-5}$  M),  $\text{MgCl}_2$  ( $5.0 \times 10^{-3}$  M), and 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M) and containing twice the concentration of NaCl required to give the final NaCl concentrations shown above. The fractions were centrifuged ( $17,000 \times g$ , 20 min) and divided into supernatants and pellets. The amount of radioactivity, protein, DNA, and RNA recovered in the supernatant is shown. The results of two experiments are combined. A, B, C, and D are described in the text.

TABLE I

## Repeat extraction of nuclei

Radioactive nuclei ( $1.5 \times 10^8$ ) mixed with unlabeled nuclei ( $0.9 \times 10^8$ ) were sonically disrupted and extracted with buffer containing 0.6 M NaCl as described under "Experimental Procedure." The pellet resulting from the first centrifugation was resuspended in buffer containing 0.6 M NaCl, sonically disrupted, and centrifuged ( $17,000 \times g$ , 20 min). The distribution of radioactivity in the various fractions obtained is shown.

Fraction	Recovery cpm
Whole nuclei	
53,000 $\times g$ pellet.....	211,280
Nuclear fractions	
17,000 $\times g$ Supernatant 1.....	197,184
17,000 $\times g$ Pellet 1.....	7,160
17,000 $\times g$ Supernatant 2.....	4,176
17,000 $\times g$ Pellet 2.....	840

TABLE II

## Effect of pH on extraction of nuclear-binding material

Labeled nuclei were sonically disrupted and extracted in the cold with buffer containing 0.6 M NaCl. The pH of each buffer was measured at the extraction temperature.

pH of buffer	Whole nuclei	Nuclear fractions	
	53,000 $\times g$ pellet	17,000 $\times g$ supernatant	17,000 $\times g$ pellet
	cpm	cpm	
6.2	40,500	23,300	14,200
7.4	40,700	32,560	4,300
8.5	40,500	36,200	4,300

the basis of such evidence, it was concluded that the first peak contains chromatin-bound, while the second peak consists of free radioactivity.

It has recently been observed in this laboratory that dihydrotestosterone, a reduction product of testosterone, is formed and localized in nuclei of male accessory glands of reproduction. Even as early as 5 min after the intravenous administration of testosterone-1,2- $^3\text{H}$ , the major fraction of prostatic radioactivity was recovered as dihydrotestosterone (4). For determination of whether there was preferential binding of one or the other steroid to chromatin, the fractions containing "bound" and "free" hormone were combined after gel filtration, and the radioactivity was extracted into chloroform-methanol and examined by gas-liquid chromatography. The results are presented in Table III. Of the radioactivity extracted from the fractions with bound radioactivity, 88% was identified as dihydrotestosterone. Of the free radioactivity, 56% was dihydrotestosterone. These results indicate that the steroid bound to chromatin is primarily dihydrotestosterone.

**Binding of Nuclear Radioactivity to Chromatin Protein**—In order to obtain further resolution of intranuclear binding sites, chromatography of labeled nuclear soluble material was performed on Sephadex G-200. The results of one such experiment are shown in Fig. 3. From Fig. 3A, it can be seen that the radioactivity has been resolved into three peaks. The first

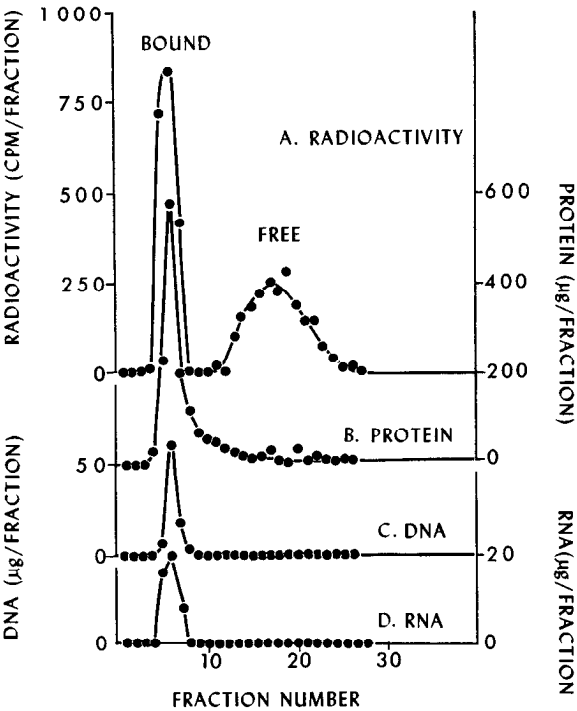


Fig. 2. Separation on Sephadex G-25 of testosterone-1,2-<sup>3</sup>H-labeled chromatin into bound and free fractions. Four functionally heptectomized rats were given injections of 250 µC of testosterone-1,2-<sup>3</sup>H each. These rats along with 26 normal rats were killed 2 hours later. From the pooled prostates,  $3.8 \times 10^8$  nuclei were recovered, and an aliquot containing  $1 \times 10^8$  nuclei was extracted as described under "Experimental Procedure" and analyzed by gel filtration.

TABLE III

Identification of steroid components of bound and free peaks

A sample of  $5.0 \times 10^7$  nuclei containing  $0.7 \times 10^7$  radioactive nuclei was extracted as described under "Experimental Procedure" and subjected to gel filtration on Sephadex G-25. Fractions containing bound and free radioactivity were pooled separately and then extracted into chloroform-methanol. The lipid extracts were analyzed by gas-liquid chromatography.

Source of radioactivity (peak)	Distribution of radioactivity		
	Total radioactivity	% recovered as:	
		Dihydro-testosterone	Testosterone
Bound.....	<i>cpm</i> 968	88	6
Free.....	1826	56	35

peak was eluted with the void volume and was associated with protein and DNA as indicated in Fig. 3, B and C. RNA (not shown) also emerged with the void volume. The second peak was associated with protein only, and the third peak consisted of free radioactivity. Thus, a large fraction of the intranuclear steroid was associated with a nuclear protein.

From this experiment, it was not clear whether the radioactivity forming the first peak was itself bound to nucleic acid or to protein. To distinguish between these possibilities, fractions forming Peak 1 were pooled, divided, and incubated with deoxy-

ribonuclease or Pronase following the procedure described in a later section of this paper. The incubated samples were then examined by gel filtration on Sephadex G-25, and the amount of binding was determined. The results obtained are listed in Table IV. In the control sample, 55% of the radioactivity remained bound following incubation at 26° for 90 min and gel filtration. The deoxyribonuclease caused a 5-fold reduction in

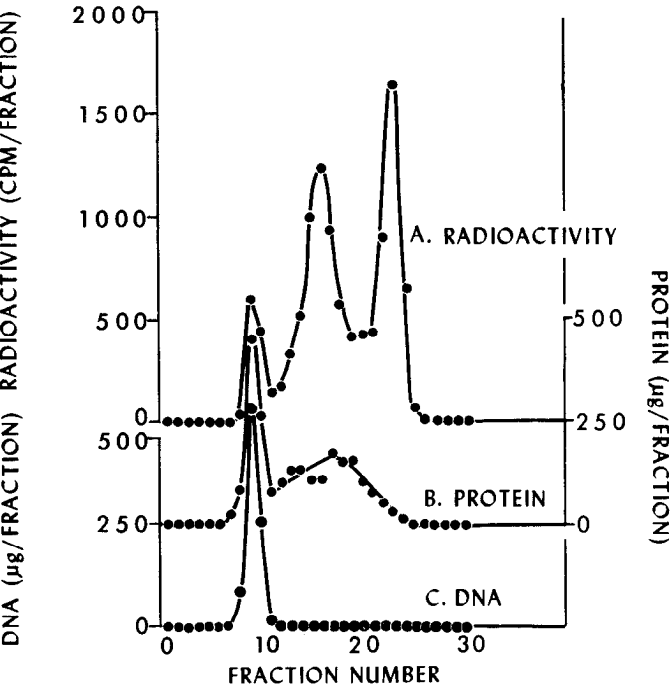


Fig. 3. Separation of testosterone-1,2-<sup>3</sup>H-labeled chromatin into DNA, protein, and free components on Sephadex G-200. Approximately  $1.6 \times 10^8$  nuclei containing  $0.3 \times 10^8$  radioactive nuclei were extracted as before. The nuclear-soluble material was then chromatographed on Sephadex G-200. Fractions of 2 ml each were collected and analyzed for radioactivity, protein, DNA, and RNA.

TABLE IV

Stability of Peak 1 radioactivity following enzymatic digestion of DNA and protein

A sample of  $2.4 \times 10^8$  nuclei containing  $1.0 \times 10^8$  radioactive nuclei was extracted in the usual manner, and the extract was passed through Sephadex G-200. Tris-HCl buffer, pH 7.4 (0.01 M), containing EDTA ( $5.0 \times 10^{-5}$  M),  $MgCl_2$  ( $5.0 \times 10^{-3}$  M), 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M), and NaCl (0.6 M) was used for the elution. Peak 1 fractions thus obtained were pooled and divided into three equal samples. Deoxyribonuclease or Pronase was added to two of the samples so that the concentration was 200 µg per ml. The final volume was 2.6 ml. All three samples were incubated at 26° for 90 min, after which each was chromatographed on Sephadex G-25. The amount of residual binding was then determined from the profiles obtained.

	Total radio-activity	DNA		Protein		% Bound
		0 min	90 min	0 min	90 min	
	<i>cpm</i>	<i>μg</i>		<i>μg</i>		
Control.....	3223					55
Deoxyribonuclease.....	2809	530	106			57
Pronase.....	3121			3700	1700	7



DNA content but did not affect the amount of binding, which remained equal to control. However, the Pronase, which reduced protein content by approximately 50%, caused an 8-fold reduction in the quantity of bound radioactivity. These results indicated that the major portion of the bound hormone in Peak 1 which remains bound after the second gel filtration is in fact bound to protein. Taken together, the results of the two latter experiments (Fig. 3 and Table III) suggest that intranuclear radioactivity originating from labeled testosterone is virtually all bound to nuclear protein. This conclusion is further substantiated in later experiments.

In an earlier experiment (Table II), it was observed that the steroid bound to nuclei was primarily dihydrotestosterone. Thus, it was expected that the ratio of dihydrotestosterone to testosterone in Peak 1 would equal the ratio in Peak 2 (Fig. 3) if the two peaks contained the same nuclear proteins. To test the correctness of this view, analysis of the steroid components of Peaks 1, 2, and 3 obtained by gel filtration of radioactive nuclear-soluble material on Sephadex G-200 was carried out. The results are listed in Table V. The ratio of dihydrotestosterone to testosterone in Peak 1 was 11:1, and in Peak 2, it was 13:1. This result is in accordance with expectation and lends support to the idea that the steroid-binding properties of the proteins in Peak 2 are similar to those of the proteins in Peak 1. The similarity in the degree of hormone binding by proteins of both peaks suggests that the same proteins may be responsible for the binding observed in the two peaks, that in Peak 1 being associated with nucleic acid.

**Time Sequence of Binding and Reduction of Testosterone-1,2-<sup>3</sup>H in Prostate**—All experiments described in previous sections of this paper were performed with nuclei obtained 1 to 2 hours after administration of testosterone-1,2-<sup>3</sup>H to rats. Since dihydrotestosterone was the predominant steroid bound during this time interval, it was of interest to learn whether similar binding would be observed at earlier time intervals. Accordingly, studies were carried out to determine the time sequence of binding and reduction of testosterone-1,2-<sup>3</sup>H in prostatic nuclei following its intravenous administration to rats. The results are shown in Fig. 4. The upper half of the figure is an illustration of the radioactivity profiles obtained when nuclear-soluble material from 1.5 g of prostate at 5, 15, and 60 min was chromatographed on Sephadex G-25. During the experiment the total nuclear radioactivity increased about 10-fold, and this radioactivity was distributed equally between bound and free fractions at the different time intervals. The distribution of radioactivity between testosterone and dihydrotestosterone in each peak is shown in the bar graph forming the lower half of Fig. 4. At 5 min, 300 counts or 40% of the bound radioactivity were dihydrotestosterone, but at 15 min this value increased to 1300 counts or 60% and at 60 min to 5000 counts or 80% of the bound hormone. By contrast, in the free fraction, testosterone was the predominant steroid at all times. Thus, as the conversion of testosterone to dihydrotestosterone increased, the amount of binding of dihydrotestosterone to nuclei also increased, whereas the small amount of testosterone bound to nuclei remained almost constant at all time intervals sampled.

**Susceptibility of Binding to Pronase Incubation**—Gel filtration of nuclear-soluble material on Sephadex G-200 indicated that intranuclear steroid was bound to a nuclear protein. Further evidence to support this conclusion was obtained when the susceptibility of steroid binding to various digestive enzymes was

TABLE V

*Steroid components of radioactive peaks obtained by gel filtration of nuclear-soluble material on Sephadex G-200*

Gel filtration of nuclear-soluble material was performed in the usual manner. Fractions contributing to Peaks 1, 2, and 3 were pooled separately, and the lipids were extracted and analyzed by thin layer chromatography for steroid content.

Source of radioactivity	Distribution of radioactivity		Ratio of dihydrotestosterone to testosterone
	Dihydrotestosterone	Testosterone	
	cpm		
Peak 1.....	265	25	11:1
Peak 2.....	1052	82	13:1
Peak 3.....	547	364	2:1

tested. Steroid-labeled chromatin was incubated for 90 min at 26° either with no enzymes or with ribonuclease, deoxyribonuclease, or Pronase, and the amount of binding was determined by chromatography on Sephadex G-25. Ribonuclease treatment reduced the RNA content from 1680 to 930 µg but, as shown in Fig. 5, did not affect binding compared to control. Deoxyribonuclease treatment reduced the DNA content from 480 to 66 µg. There is a slight decrease in binding apparent in the profile shown in Fig. 5. However, during the incubation with deoxyribonuclease a small amount of protein precipitate formed. When the radioactivity precipitated with this protein was added to the bound radioactivity in the soluble fraction, the total amount of binding did not differ from control. Following Pronase treatment the protein content fell from 390 to 250 µg, and almost all the radioactivity was transferred from the bound to the free peak. These results are in agreement with the previous conclusion that the principal binding of steroid is to nuclear protein.

**Effects of Freezing, Temperature, and Repeated Gel Filtration**—Several experiments were carried out to test the stability of steroid binding under various conditions. Labeled nuclei were frozen for different periods of time, thawed, sonically disrupted, extracted, and chromatographed on Sephadex G-25 to examine the effects of freezing. The results are shown in Fig. 6A. It is evident from the profiles obtained that freezing did not destroy or enhance binding during the 8-day experimental period.

The effects of temperature on steroid binding are shown in Fig. 6B. In this set of experiments, labeled nuclei were sonically ruptured and then incubated at 4°, 20°, or 37° for 90 min. At the end of the incubation period, the samples were extracted, and the extracts were analyzed by gel filtration on Sephadex G-25. Binding was not affected by incubations at 4° or 20° but was almost completely destroyed by incubation at 37°.

The stability of binding to repeated gel filtration was also studied, and the results shown in Fig. 6C were obtained. In these experiments, radioactive nuclear soluble material was passed through a Sephadex G-25 column, and the fractions containing bound radioactivity were pooled. This sample was then passed through Sephadex G-25 again or alternately through Sephadex G-200. After the first passage of nuclear soluble material, about 55% of the radioactivity was recovered in the bound fractions. On the second passage of this material through Sephadex G-25, 63% was recovered as bound. When the second passage was through Sephadex G-200 rather than Sephadex G-25 only 45%

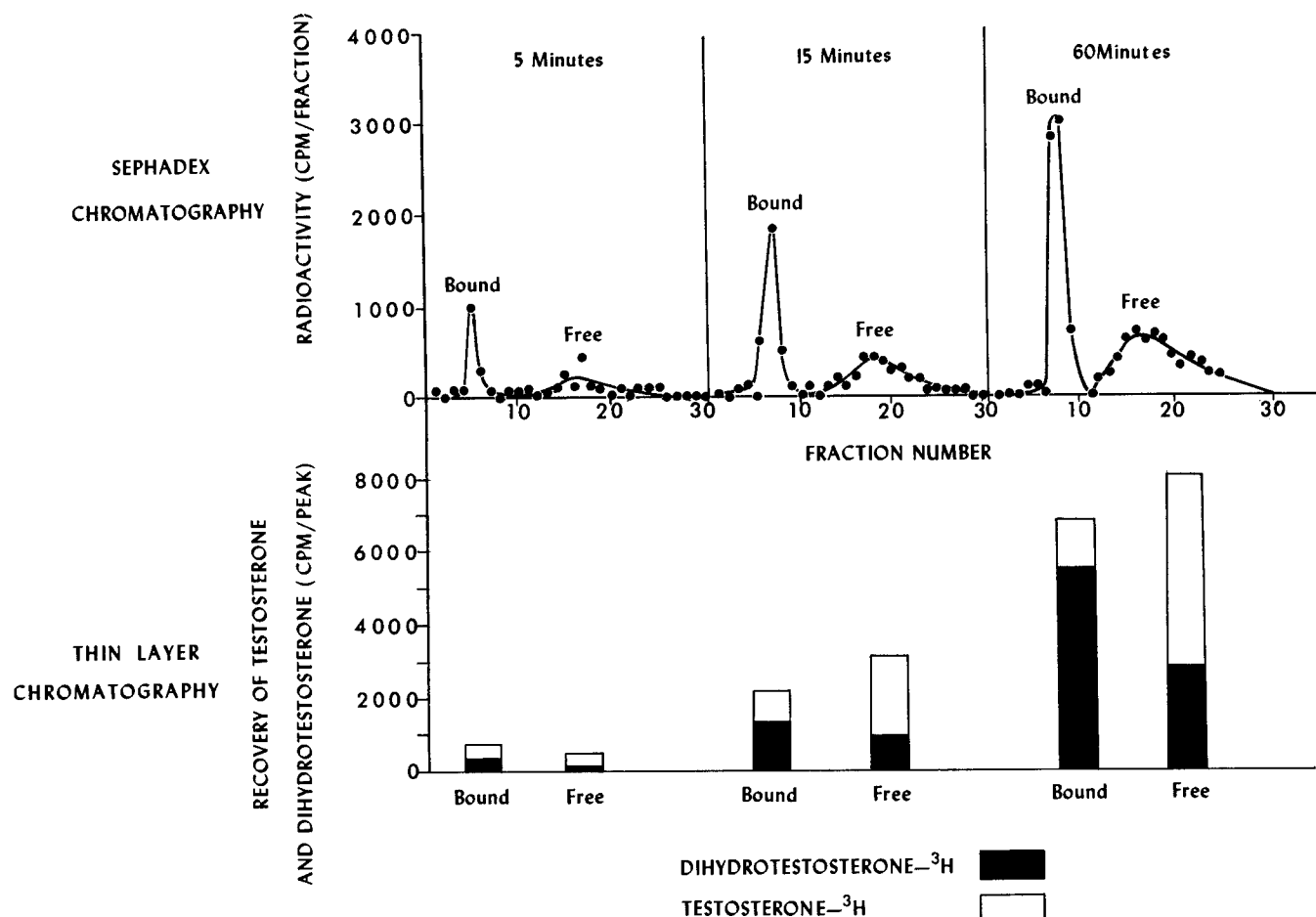


FIG. 4. Time sequence of binding and reduction of testosterone-1,2-<sup>3</sup>H in prostate. Three functionally hepatectomized rats were killed 5, 15, and 60 min after receiving an intravenous injection of 200  $\mu$ C of testosterone-1,2-<sup>3</sup>H. Prostatic tissue obtained weighed 1.3, 1.5, and 1.5 g, respectively. Nuclei were purified and extracted in the usual manner. Extracts from samples containing  $2.4$  to  $3.6 \times 10^7$  nuclei were analyzed by gel filtration on Sephadex G-25. The bound and free fractions were pooled, and radioactivity was extracted with chloroform-methanol (2:1). These samples were then examined for steroid content by thin layer chromatography.

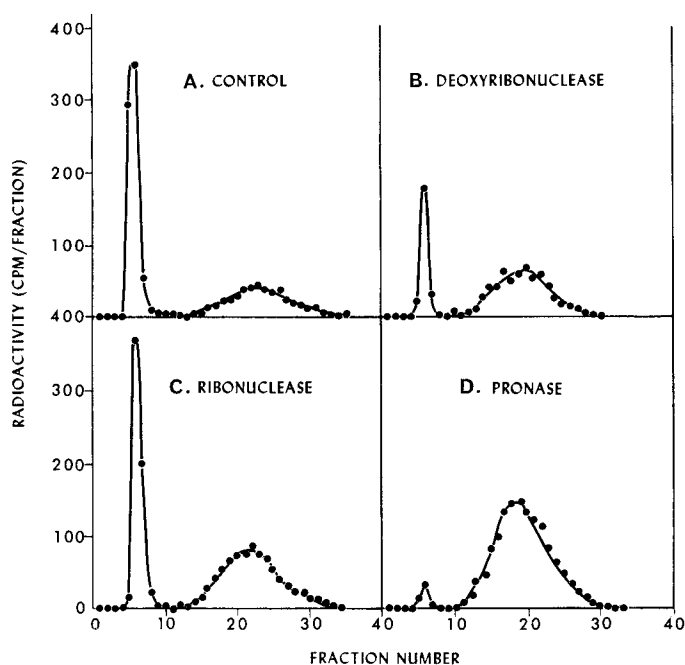


FIG. 5. Susceptibility of binding of Pronase incubation. For each sample,  $0.9 \times 10^8$  nuclei from treated rats and  $4.5 \times 10^7$  nuclei from nontreated rats were used. Nuclei were suspended in Tris-HCl buffer, pH 7.4 ( $0.01$  M), containing EDTA ( $5.0 \times 10^{-5}$  M),  $MgCl_2$  ( $5.0 \times 10^{-3}$  M), 2-mercaptoethanol ( $0.5 \times 10^{-3}$  M), and NaCl ( $0.05$  M). Following sonic disruption, each aliquot was treated with ribonuclease ( $100$   $\mu$ g per ml), deoxyribonuclease ( $100$   $\mu$ g per ml), or Pronase ( $100$   $\mu$ g per ml). One sample acted as control. The total volume of the incubation mixture was  $1.65$  ml. The samples were incubated at  $26^\circ$  for  $90$  min after which they were extracted as described under "Experimental Procedure" and analyzed for bound radioactivity by gel filtration on Sephadex G-25.

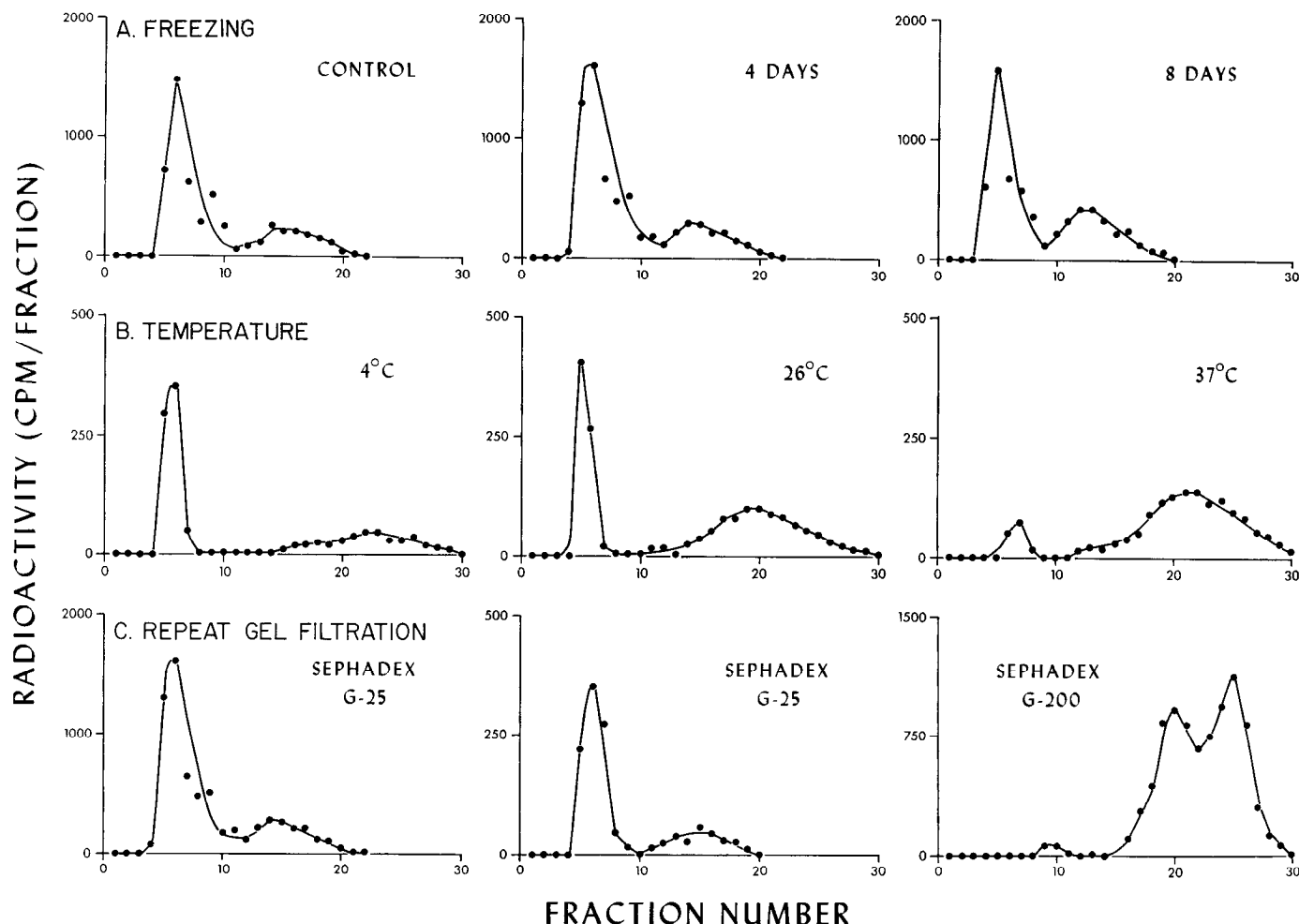


FIG. 6. Effects of freezing, temperature, and rechromatography on binding. In each experiment, labeled nuclear-soluble material was analyzed by gel filtration for amount of binding after various treatments (see text). Following gel filtration on Sephadex G-25, the bound peak was chromatographed again either on Sephadex G-25 or on Sephadex G-200.

or less of the radioactivity was recovered as bound. Thus, with successive passages of labeled nuclear soluble material through Sephadex there was further loss of steroid binding. The loss was greater on gel filtration through Sephadex G-200 than through Sephadex G-25. The reason for this disparity is not as yet clear; it is possible that it is the consequence of variations in the length of the columns and the rates of flow.

#### DISCUSSION

These experiments clearly indicate that intranuclear radioactivity originating from labeled testosterone and bound to nuclei consists of two steroids, testosterone and dihydrotestosterone. After intravenous administration of testosterone-1,2- $^3\text{H}$ , the intranuclear binding of dihydrotestosterone increased strikingly with time in comparison with the binding of testosterone (17- as opposed to 3-fold) (Fig. 4). Since it has previously been shown that the  $5\alpha$ -reductase which converts testosterone to dihydrotestosterone within prostatic nuclei is also located in chromatin (4), it is possible that the bulk of the testosterone binding is to the reductase site whereas dihydrotestosterone, which does not require reduction *in situ* for its action (11), may bind to other nuclear sites. As early as 15 min after the administration of testosterone-1,2- $^3\text{H}$ , the amount of dihydro-

testosterone bound exceeded the amount of testosterone bound. After 1 hour, the ratio of dihydrotestosterone to testosterone varied between 4:1 and 13:1 in different experiments. The preponderance of dihydrotestosterone bound at all but the earliest time intervals suggests that, under steady state conditions, the form of the hormone bound to chromatin is primarily dihydrotestosterone.

The data presented in this paper also indicate that steroid binding in the nucleus takes place to nuclear protein. This result is in agreement with the report of Wilson and Loeb that radioactive testosterone is bound to nuclear protein in duck preen gland (2). These observations differ from those of Mangun, Neal, and Williams (3), who reported binding of the steroid to DNA of prostate nuclei taken from castrate rats. However, in the latter studies similar binding was not observed in intact rats. Moreover, the mode of administration of testosterone-1,2- $^3\text{H}$  in the latter investigations was by a direct injection of hormone into prostate. By contrast, the labeling of nuclei in experiments reported in this paper was by the intravenous route. It is not clear whether the different treatments would result in the differences of binding observed or whether another variable is responsible. The results presented in this paper strongly suggest that binding of steroid is to nuclear protein. However, the

possibility that DNA may act to stabilize the binding of steroid and protein has not been excluded.

The binding protein appears to be partly insoluble in weakly acid buffers (Table II). Since acidic nuclear proteins are relatively insoluble in weak acids, it is possible that the binding protein is itself an acidic protein.

Taken together, the bioassays by Dorfman and Shipley (11) indicating that dihydrotestosterone is a more potent androgen in the rat than is testosterone itself, the previous observation in this laboratory that dihydrotestosterone is the principal metabolite of testosterone in the nuclei of male accessory glands of reproduction in the rat (4), and the additional observation reported here that the binding of dihydrotestosterone to prostatic nuclei, a presumed site of steroid action, markedly exceeds that of testosterone strengthens the view that dihydrotestosterone may indeed be an active form of testosterone in this tissue.

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