High-Affinity Binding of 4-Androstene-3,17-dione in Rat Liver†

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ABSTRACT: Intraperitoneal administration of [1,2,6,7-^{3}H]testosterone to castrated male rats resulted in the appearance of an [^{3}H]androstenedione–protein complex ("complex A") in liver cytosol. When analyzed by sucrose density gradient centrifugation this complex had a sedimentation coefficient of 10 S. Complex A was stable toward treatment with dextran-coated charcoal, was unaffected by incubation with DNase and RNase, but was destroyed following incubation with trypsin, chymotrypsin, and protease. Complex A was also formed following incubation of liver cytosol from castrated male rats with [1,2,6,7-^{3}H]testosterone and after incubation of ABF (= the androgen-binding fraction collected at about 0.40 column volume during chromatography of liver cytosol from castrated male rats on Sephadex G-100) with [1,2-^{3}H]androstenedione. Incubation with ABF made it possible to study the binding of androstenedione in vitro without interfering metabolism of this substrate; saturation of the androstenedione-binding protein in ABF was obtained at a substrate concentration of about 1×10^{-7} *M* and the concentration of binding sites in ABF was about 8×10^{-16} mol/mg of protein (corresponding to 1.6×10^{-16} mol/mg of protein in unfractionated cytosol). When castrated male rats were injected intraperitoneally with [1,2,6,7-^{3}H]testosterone, liver nuclei specifically retained [^{3}H]androstenedione. In order to investigate whether androstenedione could be taken up from the blood into the liver cell, pulse-labeling experiments were performed where [1,2,6,7-^{3}H]testosterone was administered intravenously to intact male and female rats. The concentrations of [^{3}H]androstenedione and 5α-[^{3}H]dihydrotestosterone were higher in portal vein than in aortic blood indicating uptake of these metabolites in liver or lung tissue. When the ^{3}H-labeled unconjugated steroids in liver cytosol were measured in the same experiment, 5α-[^{3}H]dihydrotestosterone, 5α-[^{3}H]androstane-3α,17β-diol, and 5α-[^{3}H]androstane-3β,17β-diol were found to decrease rapidly in concentration just after injection of isotope whereas [^{3}H]androstenedione was present in very constant concentration in both male and female rats from about 2 min after administration of isotope. These results indicate the existence of specific mechanisms in liver cytosol protecting androstenedione from being metabolized. It is suggested that the hepatic androstenedione–protein complex—that presents several of the characteristics described for steroid–receptor protein complexes—is involved in androgen action in the liver.

During the neonatal period male rats are irreversibly programmed by testicular androgens to express a masculine type of liver enzyme activities in adult life (DeMoor and Denef, 1968; Einarsson et al., 1973; Gustafsson and Stenberg, 1973a). Also the degree of response to androgens in adult life—as measured by changed activities of sex-dependent hepatic enzymes after administration of androgens—is imprinted by the same mechanism leading to higher androgen responsiveness in male than in female rats (Gustafsson and Stenberg, 1973b). The changed androgen responsiveness of liver caused by neonatal testicular influence could be related to changed concentration of liver cytoplasmic receptor and/or nuclear acceptor proteins for androgens. The existence of androgen receptors in liver cytosol has been difficult to assess. Some authors have claimed that liver cytosol and nuclei are devoid of 5α-dihydrotestosterone-retaining mechanisms (Andersson and Liao, 1968) whereas others, on the basis of in vitro work, have described the occurrence of a receptor for 5α-dihydrotestosterone in liver (Milin and Roy, 1973). In view of these conflicting data in literature, it seemed essential to perform an investigation on the uptake and metabolism of testosterone in vivo in rat liver, with special reference to the binding of testosterone and its metabolites to proteins, and to combine this work with in vitro studies. The present investigation describes high-affinity binding of androstenedione to a protein in rat liver cytosol.

Materials and Methods

Steroids. [1,2,6,7-^{3}H]Testosterone (specific radioactivity, 84 Ci/mmol) and 4-[1,2-^{3}H]androstene-3,17-dione (specific radioactivity, 45.9 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. Unlabeled testosterone, 5α-dihydrotestosterone, 4-androstene-3,17-dione, 5α-androstane-3,17-dione, 3α-hydroxy-5α-androstan-17-one, 3β-hydroxy-5α-androstan-17-one, 5α-androstane-3α,17β-diol, and 5α-androstane-3β,17β-diol were generously supplied by Dr. J. Babcock, Upjohn Co., Kalamazoo, Mich.

Animal Experiments. Sprague-Dawley rats, 8 weeks old, were used for all experiments. In cases where castrated rats were used the operation was performed under ether anesthesia 14–16 hr before the experiment.

In one series of experiments two intact male animals were kept under ether anesthesia and 250 μCi of [1,2,6,7-^{3}H]testosterone was injected into the iliac vein in 200 μl of 1% (w/v) bovine serum albumin in distilled water. An intravenous injection of 500 IU of heparin was given just prior to the administration of isotope. The aorta and the portal vein were cannulated with thin needles and samples

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of 100 μl of blood were collected at different intervals from the vessels during 30 min after injection of isotope. The collected blood was hemolyzed by addition of 10 volumes of distilled water.

In a second series of experiments intact male and female rats were given an intravenous injection of 250 μCi of [1,2,6,7-^{3}H]testosterone as described above. The animals were killed by a blow on the head 0.5, 2, 5, 10, 90, 180, and 360 min after administration of isotope. For each time interval 2–4 rats of each sex were used. When the experiment was terminated the liver was perfused in situ via the portal vein with 30–50 ml of ice-cold physiological saline. The liver was then taken out of the peritoneal cavity and kept in ice-cold TKE buffer.[1] All subsequent steps in the experimental procedure (see below) were performed at 0–4°.

In a third series of experiments castrated male rats were injected intraperitoneally with 250 μCi of [1,2,6,7-^{3}H]testosterone in 120 μl of acetone; 30 min after administration of isotope the animals were killed by a blow on the head. The liver was rapidly perfused in situ with ice-cold physiological saline, taken out, and kept in TKE buffer.

Preparation of Liver Cytosol. The saline was decanted and the liver was minced with scissors in 5–14 ml of TKE buffer (0.01 *M* KCl–0.001 *M* EDTA–0.01 *M* Tris-HCl (pH 7.4) (Puca and Brescianai, 1968)) and homogenized in the same medium with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 15 min at 20,000*g* in an MSE High Speed 18 centrifuge. The pellet was taken for preparation of nuclei (see below). The liver cytosol was obtained from the 20,000*g* supernatant after centrifugation for 70 min at 105,000*g* in a Type 40 Beckman-Spinco rotor.

Preparation of Liver Nuclei. The 20,000*g* sediment was resuspended in medium A (0.88 *M* sucrose–1.5 m*M* $CaCl_2$–1 m*M* $MgSO_4$–0.01 *M* Tris-HCl (pH 7.4) (Verhoeven and DeMoor, 1972)) and liver nuclei were prepared essentially as described before (Gustafsson and Pousette, 1974). An aliquot of the nuclear preparation was taken for determination of DNA (Burton, 1956) with calf thymus DNA (purchased from Sigma Chemical Co.) as standard.

Fractionation of Liver Cytosol Proteins on Sephadex G-25 and G-100. Lipid material on top of the liver cytosol fraction was sucked off and the cytosol was chromatographed on a Sephadex G-25 or G-100 column equilibrated with TKE buffer or, alternatively, with TKE buffer, 0.4 *M* with respect to KCl. Aliquots of the fractions collected were measured for radioactivity in a Packard liquid scintillation spectrometer, Model 4322. Protein was estimated as relative absorbance (Δ280–310 nm) or was measured according to Lowry (Lowry et al., 1951). Location of different fractions in the Sephadex G-100 chromatogram was expressed as the ratio between the elution volume and the column volume.

Density Gradient Centrifugation. Portions (0.2 ml) of the protein–steroid mixtures to be analyzed were layered on top of linear 5-ml 5–20% (w/v) sucrose density gradients in TKE buffer, 0.4 *M* with respect to KCl. The tubes were centrifuged at 40,000–50,000 rpm for 6, 14, or 20 hr at +2° in an SW 50.1 Beckman-Spinco rotor. At the end of the centrifugation the bottom of the tube was punctured and 3- or 4-drop fractions were collected and measured for radioactivity. The following markers were used: ovine serum albumin (3.7 S), bovine serum albumin (4.6 S), rabbit anti-androstenedione antibody (7 S), and catalase (11.2 S).

Dextran-Coated Charcoal Treatment. Experiments with dextran-coated charcoal were carried out as described by Beato and Feigelson (1972).

Extraction and Purification of Steroids. Suspensions of nuclei, cytosol, and hemolyzed blood were mixed with 10 volumes of acetone–ethanol, 1:1 (v/v), and kept in a shaking water bath at 37° overnight. The precipitate was filtered off and the extract was evaporated to dryness in vacuo. The residue was dissolved in a small volume of methanol and kept overnight at −20°; the precipitate formed was separated by centrifugation at 2000*g*. An aliquot of the supernatant was determined for radioactivity. Samples dissolved in methanol were then applied on thin-layer silica gel plates. Free steroids, steroid mono- and disulfates, and steroid glucuronides were separated in the solvent system ethyl acetate–96% (v/v) ethanol–15 *M* ammonium hydroxide, 5:5:1 (v/v) (Sarfaty and Lipsett, 1966). After drying, the thin-layer plates were scanned for radioactivity using a Berthold thin-layer scanner, Model II (Berthold, Wildbad, Germany). The relative amounts of free steroids and steroid conjugates were measured from the scanner chromatograms. No radioactive material was detected that chromatographed like steroid glucuronides. Zones corresponding to steroid mono- and disulfates were scraped off, and the conjugated steroids were extracted from the silica gel and solvolyzed. Thin-layer zones corresponding to unconjugated steroids were scraped off and extracted with methanol.

The unconjugated and solvolyzed steroids were rechromatographed on thin-layer silica gel plates in the solvent system ethyl acetate–chloroform, 1:4 (v/v). External steroid reference compounds were used. The relative amounts of steroid metabolites were measured from the scanner chromatograms. The zones containing radioactivity were scraped off and the steroids were eluted from the silica gel with methanol.

Radio-Gas Chromatography. Each of the methanol eluates were evaporated to dryness and (trimethyl)silylated. The silyl ethers were analyzed on a Hewlett-Packard Model 402 gas chromatograph equipped with a Barber-Colman Radioactivity Monitoring System, Model 5190. The stationary phases used were 1.5% SE-30 and 1% OV-17.

A steroid was considered identified if it had the same thin-layer chromatographic mobility and the same retention times, relative to testosterone (t_R), on SE-30 and on OV-17 as the reference steroid.

Enzymes. Trypsin and chymotrypsin were purchased from Worthington Biochemical Corporation (Freehold, N.J.) and protease Type VI (purified from *Streptomyces griseus*) was purchased from Sigma Chemical Company (St. Louis, Mo.).

Results

Studies on Testosterone Metabolites in Blood and Liver after Pulse Labeling with [1,2,6,7-^{3}H]Testosterone. Tables I and II show the findings in aortic and portal vein blood from a male rat at various intervals after intravenous administration of [1,2,6,7-^{3}H]testosterone. Similar results were obtained from another male rat. As can be seen from Table I, a relatively constant concentration of ^{3}H-labeled steroids was present in aortic blood about 13 min after injection of isotope. Most of the radioactivity was in the form of unconjugated metabolites. In portal vein blood unconjugated metabolites were slightly more predominant than in

[1] Abbreviations used are: TKE buffer, 0.01 *M* KCl–0.001 *M* EDTA–0.01 *M* Tris-HCl (pH 7.4); ABF, androgen-binding fraction.

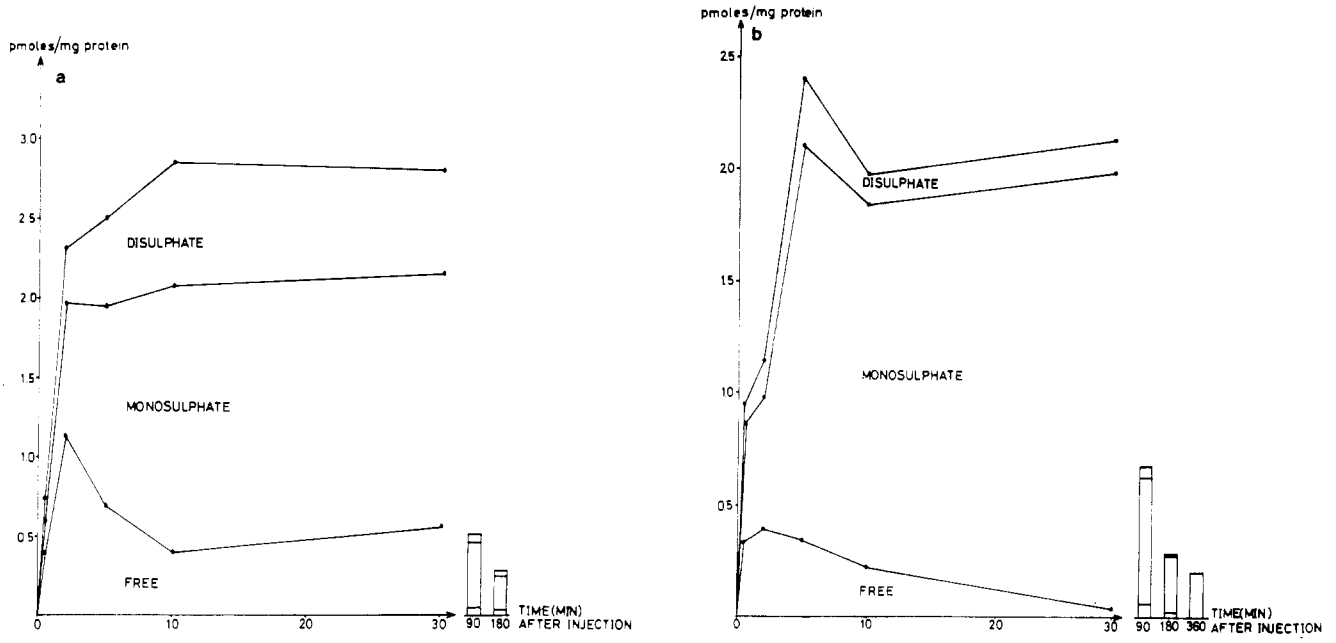

FIGURE 1: Distribution of radioactive metabolites in the free, monosulfate, and disulfate fractions of liver cytosol from intact male (a) and female (b) rats after intraperitoneal injection of [1,2,6,7-^{3}H]testosterone. Each point represents the mean value of 2–4 experiments. The figures have been drawn in a cumulative way for each time after injection so that the upper line represents the totally recovered radioactivity and the areas between the lines the radioactivity in the free, monosulfate, and disulfate fractions, respectively.

Table I: ^{3}H-Labeled Steroids Present in Aortic Blood Collected from a Male Rat (Same Animal as in Table II) at Various Time Intervals after Intravenous Administration of 250 μCi of [1,2,6,7-^{3}H] Testosterone.

	Amounts of Steroid Metabolites (nCi/ml of Blood)					
		Unconjugated				
Time after Injection (min)	Total	Testo-sterone	Andro-stene-dione	5α-Dihy-drotesto-sterone	Mono-sul-fates	Disul-fates
1.1	360	151	86	68	35	25
2.7	220	125	37	0	34	28
6.3	180	79	34	0	15	25
12.9	130	48	26	0	72	17
20.8	140	55	25	0	40	40
30.8	120	36	16	0	35	60

Table II: ^{3}H-Labeled Steroids Present in Portal Vein Blood Collected from a Male Rat (Same Animal as in Table I) at Various Time Intervals after Intravenous Administration of 250 μCi of [1,2,6,7-^{3}H] Testosterone.

	Amounts of Steroid Metabolites (nCi/ml)					
		Unconjugated				
Time after Injection (min)	Total	Testo-sterone	Andro-stene-dione	5α-Dihy-drotesto-sterone	Mono-sul-fates	Disul-fates
2.0	380	144	91	95	25	10
4.8	330	119	165	46	10	10
8.6	190	57	67	49	50	10
16.0	160	53	30	75	42	11
22.7	160	53	43	64	63	12
30.5	160	30	75	30	97	13

aortic blood (Table II). Similar concentrations of labeled unconjugated testosterone were present in aortic and in portal vein blood. The concentration of [^{3}H]androstenedione was higher in portal vein blood than in aortic blood during the whole experiment. In addition, 5α-[^{3}H]dihydrotestosterone was present in relatively high concentration in portal vein blood but was undetectable in aortic blood.

In the liver cytosol the ^{3}H-labeled androgens were rapidly converted into conjugated steroids, both in male and female rats (Figure 1a and b). Thin-layer chromatographic analysis of the free steroids and the steroids liberated by solvolysis revealed three zones of radioactivity, the composition of which is shown in Table III. The composition of the steroid monosulfate fraction in male and female animals is shown in Figure 2a and b. In the unconjugated steroid fraction of liver cytosol, ring A saturated metabolites of [^{3}H]testosterone dominated by far in both male and female rats immediately after injection of the isotope (Figure 3a and b). Between about 2 and 10 min post-injection the concentrations of these steroids decreased rapidly down to fairly constant levels. In contrast to this, [^{3}H]androstenedione was present in very constant concentration in both male and female rats already from about 2 min after administration of the isotope.

Figure 4 shows the uptake of radioactivity in liver nuclei of intact male and female rats, respectively, after injection of [1,2,6,7-^{3}H]testosterone. In male rats, nuclear radioactivity was detected already about 30 sec after isotope administration; this level of radioactivity remained relatively constant even up to 360 min after injection of isotope. In female rats, the radioactivity recovered from liver nuclei increased at least up to about 30 min after injection; as in the male rats, detectable levels of radioactive metabolites were present at least until 360 min after injection. The metabolites present in nuclei from both male and female rats were identified as unconjugated [^{3}H]testosterone and [^{3}H]androstenedione.

When castrated male rats were given [1,2,6,7-^{3}H]testosterone intraperitoneally the only radioactive metabolite present in liver nuclei 30, 60, and 90 min after isotope administration was unconjugated androstenedione, as determined by thin-layer and radio-gas chromatography. The

Table III: Free Steroids and Steroids Liberated by Solvolysis Identified in Liver Cytosol after Pulse-Labeling with [1,2,6,7-^{3}H]-Testosterone.[a]

Thin-layer Chromatographic Zone	t_R SE-30	t_R OV-17	Identification	Remarks
I	0.83	1.35	[^{3}H]Androstenedione	♂, ♀
II	0.82	0.70	5α-[^{3}H]Dihydrotestosterone	♂, ♀
	1.00	1.00	[^{3}H]Testosterone	♂, ♀ (trace amounts found in both sexes)
	0.71	0.56	Unknown	♀ (major component in zone II)
III	0.97	0.50	5α-[^{3}H]Androstane-3β, 17β-diol	♂
	0.74	0.37	5α-[^{3}H]Androstane-3α, 17β-diol	♀

[a] ♂, ♀: metabolite found in livers from male and female rats, respectively. Retention times are given relative to testosterone (t_R of testosterone = 1.00).

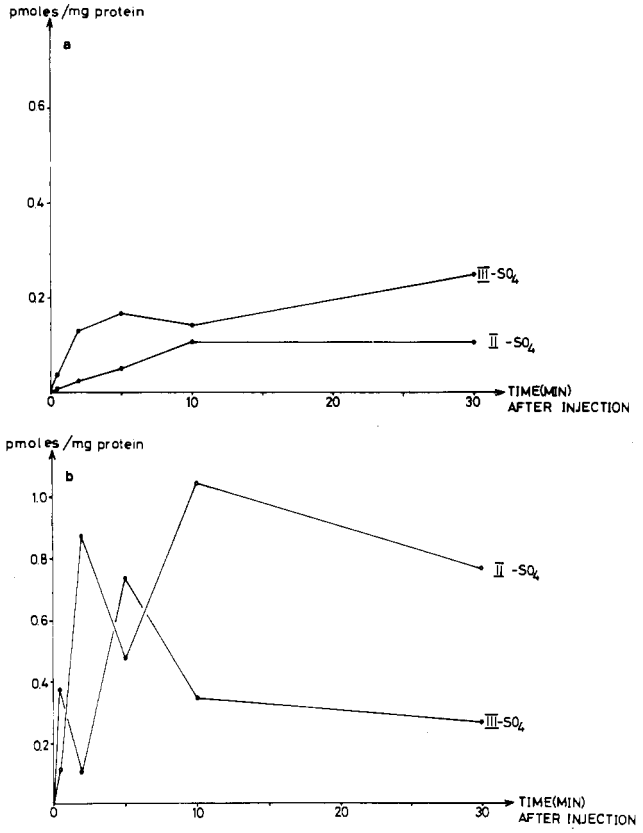

FIGURE 2: Labeled steroid monosulfates in liver cytosol from intact male (a) and female (b) rats after intraperitoneal injection of [1,2,6,7-^{3}H]testosterone. Each point represents the mean value of 2-4 experiments. Abbreviations: II-SO_4 and III-SO_4, steroid metabolites recovered from thin-layer chromatographic zones II and III (cf. Table I).

amount of radioactivity in liver nuclei from castrated male rats was about the same, when calculated in relation to DNA, as that recovered from liver nuclei from intact male rats under otherwise identical experimental conditions (about 2.3 × 10^{-2} pmol/mg of DNA).

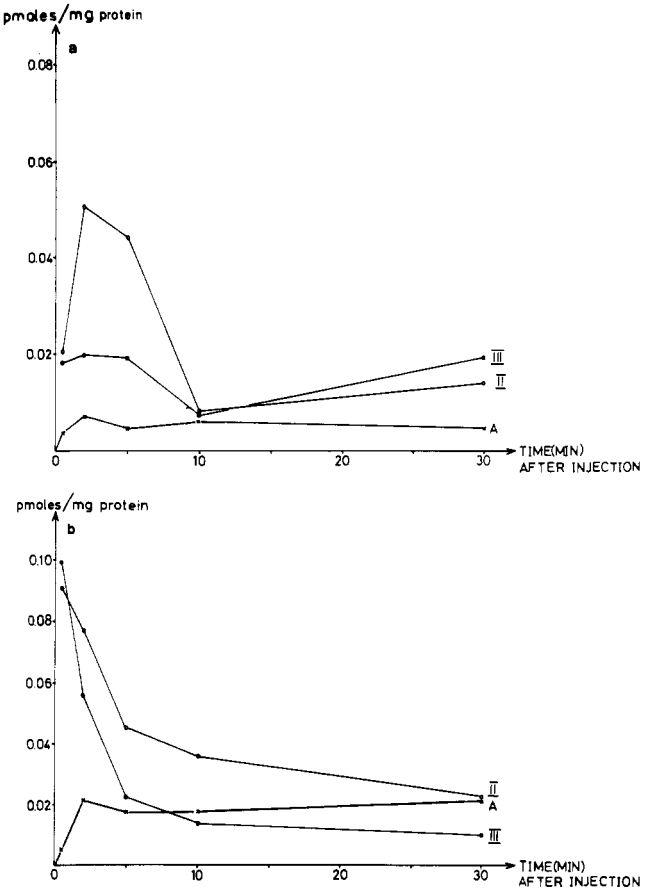

FIGURE 3: Labeled free steroids in liver cytosol from intact male (a) and female (b) rats after intraperitoneal injection of [1,2,6,7-^{3}H]testosterone. Each point represents the mean value of 2-4 experiments. Abbreviations: A = [^{3}H]androstenedione; II and III, steroid metabolites recovered from thin-layer chromatographic zones II and III (cf. Table I).

Studies on Androgen-Binding Proteins in Liver after Pulse-Labeling with [1,2,6,7-^{3}H]Testosterone. When the ^{3}H-labeled cytosol obtained from castrated male rats was fractionated on a Sephadex G-25 column equilibrated with TKE buffer usually about 5-10% of the radioactivity was recovered in the void volume. When the ^{3}H-labeled cytosol was fractionated on Sephadex G-100 equilibrated with TKE buffer, three main fractions of radioactivity were eluted (Figure 5). Peak C represented the unbound metabolites. Peak B consisted of protein-bound [^{3}H]testosterone and monosulfates and was eluted between 0.52 and 0.64 column volumes. Peak A consisted of protein-bound [^{3}H]androstenedione ("complex A") only and was eluted at about 0.40 column volume. The void volume was obtained at 0.32 column volume under the experimental conditions used. Treatment of peaks A, B, and C with dextran-coated charcoal (one treatment) resulted in elimination of 100% of peak C, 80% of peak B, and less than 8% of peak A from the supernatant after centrifugation.

In order to study the characteristics of complex A further, peak A from the Sephadex G-100 chromatography of ^{3}H-labeled cytosol from castrated male rats was concentrated about fivefold using a vacuum concentrator with collodium bags and analyzed by sucrose density gradient centrifugation. Complex A was found to have a sedimentation coefficient of 10 S (Figure 6). This mobility of complex A did not change when the KCl concentration of the gradient was changed from the standard value 0.4 *M* KCl to 0.01 or

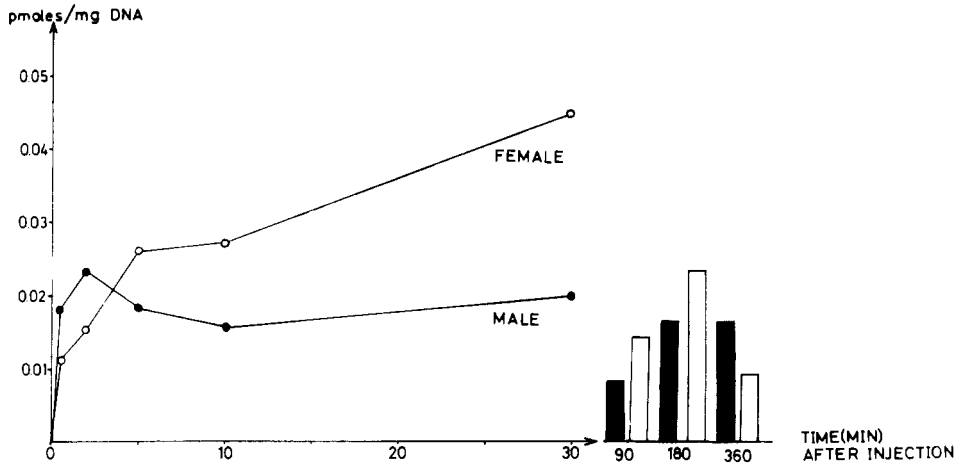

FIGURE 4: Radioactivity recovered from liver nuclei from intact male and female rats after intraperitoneal injection of [1,2,6,7-^{3}H]testosterone. Each point represents the mean value of 2-4 experiments. Filled bars and open bars represent male and female animals, respectively.

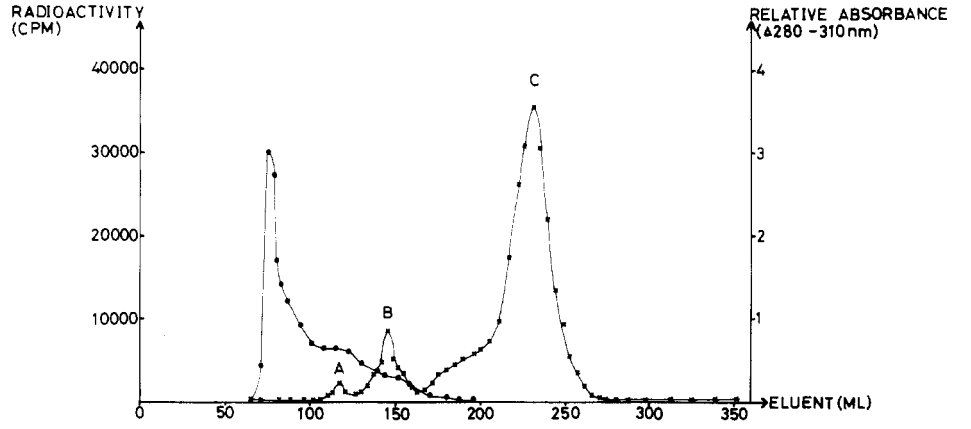

FIGURE 5: Chromatography on Sephadex G-100 (total volume: 250 ml) of liver cytosol from castrated male rats injected with [1,2,6,7-^{3}H]testosterone. The column was eluted with TKE buffer (0.01 *M* KCl-0.001 *M* EDTA-0.01 *M* Tris-HCl (pH 7.4)). (O) Relative absorbance (Δ280-310 nm); (x) radioactivity (cpm). For further explanations, see text.

2 *M*. The addition of 10% (v/v) glycerol, 3 m*M* thioglycerol, or 1 m*M* dithiothreitol to the gradient did not either change the sedimentation characteristics of complex A. Furthermore, a sedimentation value of 10 S was obtained for complex A even in the absence of EDTA or at a pH of 6.2 (using 0.05 *M* phosphate buffer). As shown in Table IV, complex A was destroyed by incubation with protease and by combined incubation with trypsin and chymotrypsin whereas DNase and RNase were without effect. Complex A was stable at 37° for at least 60 min but was destroyed when kept at 65° for 10 min. These data indicate that the androstenedione-binding component of complex A is a protein.

Comparative Studies on Androgen-Binding Proteins in Blood after Pulse Labeling with [1,2,6,7-^{3}H]Testosterone. In order to exclude the possibility that complex A originated from blood some comparative investigations were made of the androgen-binding proteins in this tissue. An aliquot (0.2 ml) of aortic serum recovered from a castrated male rat 30 min after intravenous injection of [1,2,6,7-^{3}H]testosterone was analyzed by sucrose density gradient centrifugation. The only radioactive peak detected had a sedimentation coefficient of 4.6 S corresponding to albumin. Furthermore, an aliquot of labeled rat serum was chromatographed on Sephadex G-100 using the same conditions as for analysis of hepatic androgen-binding proteins. No radioactivity could be detected at about 0.40 column volume; most radioactivity appeared at about 0.57 column volume and at about one column volume (= protein-unbound material). Based on these results it was concluded that complex A was formed in the liver itself and was not transported into the hepatocytes from blood.

Studies on Binding in Vitro of [1,2,6,7-^{3}H]Testosterone and [1,2-^{3}H]Androstenedione to Proteins in Liver Cytosol. When [1,2,6,7-^{3}H]testosterone was incubated with unlabeled cytosol from castrated male rats at 37° for different lengths of time (10 sec to 30 min) and then chromatographed on a Sephadex G-100 column, a similar pattern of peaks was eluted (peaks A, B, and C) as when liver cytosol labeled in vivo with [1,2,6,7-^{3}H]testosterone (cf. above) was analyzed in the same way. The material in peak A exclusively consisted of [^{3}H]androstenedione and sedimented as a single, homogeneous peak on sucrose gradients with a sedimentation coefficient of 10 S (complex A) (Figure 7). Peak B was shown to consist of about 50% steroid monosulfates and 50% unconjugated steroids in several experiments. The unconjugated steroid fraction mainly contained [^{3}H]testosterone. Peak C consisted of protein-unbound material.

In a separate experiment a castrated male rat was injected intramuscularly with 1 mg of testosterone dissolved in 0.5 ml of propylene glycol 2 hr prior to sacrifice. Liver cytosol from this rat was incubated with [1,2,6,7-^{3}H]testost-

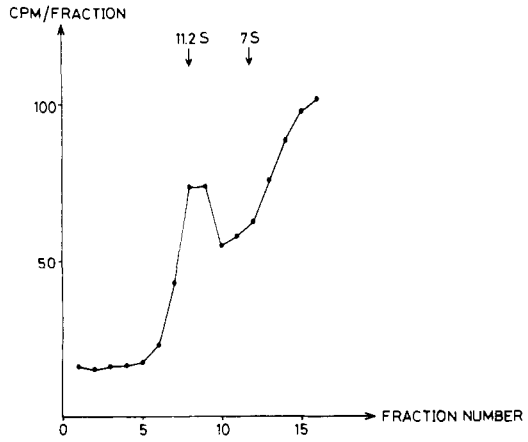

FIGURE 6: Sucrose density gradient centrifugation of peak A recovered after chromatography on Sephadex G-100 of liver cytosol from castrated male rats injected with [1,2,6,7-^{3}H]testosterone (cf. Figure 5). Centrifugation was performed at 50,000 rpm for 6 hr at +2° in an SW 50.1 rotor. The mobilities of rabbit anti-androstenedione antibody (7 S) and catalase (11.2 S) are indicated. For further explanations, see text.

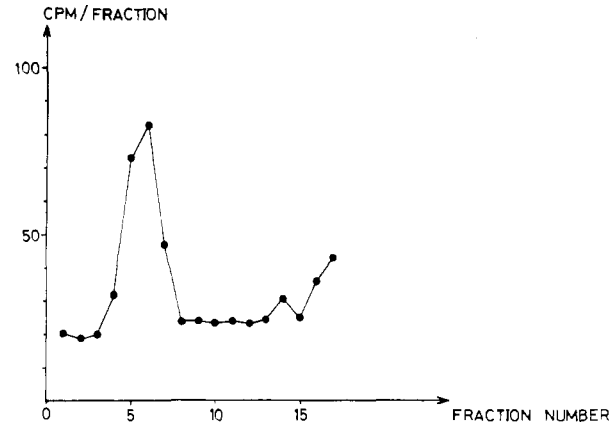

FIGURE 7: Sucrose density gradient centrifugation of peak A recovered after chromatography on Sephadex G-100 of liver cytosol from castrated male rats incubated with [1,2,6,7-^{3}H]testosterone. For conditions of incubation, see text. Centrifugation was performed at 40,000 rpm for 13 hr at +2° in an SW 50.1 rotor. The radioactive peak corresponds to a protein with a sedimentation coefficient of 10 S.

Table IV: Effect of Various Enzymes on Complex A.

Treatment	Bound Radioactivity[a] (% of Control)
Control 0°	100
37°, 60 min	104
37°, 60 min, 14 m*M* $CaCl_2$	103
Trypsin + chymotrypsin (1 mg of each, 37°, 60 min, 14 m*M* $CaCl_2$)	3
Protease (1 mg, 37°, 60 min)	5
RNase (100 μg, 37°, 60 min)	100
DNase (100 μg, 37°, 60 min, 14 m*M* $MgCl_2$)	100
65°, 10 min	0

[a] Complex A was isolated by Sephadex G-100 chromatography of liver cytosol from castrated male rats given [1,2,6,7-^{3}H]testosterone as described in Materials and Methods. Prior to incubation, complex A was chromatographed on a Sephadex G-25 column equilibrated in 1 m*M* EDTA–0.02 *M* Tris-HCl (pH 7.4). The total incubation volume was 0.35 ml corresponding to 4.3 OD 280–310 units of protein. When the incubations had been carried out, the remaining amount of complex A was measured by sucrose density gradient centrifugation as described in Materials and Methods.

erone at 37° for 15 min and then chromatographed on a Sephadex G-100 column. Radioactivity appeared as usual at about 0.6 column volume (peak B) and at the column volume (peak C) whereas no radioactivity was found at 0.40 column volume. Thus it was possible to prevent the formation of labeled complex A by excess amounts of unlabeled androgen indicating the presence of a limited amount of steroid-binding sites in the protein component of complex A.

Several experiments were then carried out in order to study the binding of [1,2,6,7-^{3}H]testosterone and [1,2-^{3}H]androstenedione to proteins in liver cytosol under conditions where precautions were taken to minimize the normally extensive metabolism of steroids in this tissue. Even at 0° the metabolism of labeled testosterone and androstenedione in liver cytosol was found to be extremely rapid and extensive. It was found convenient to fractionate liver cytosol on Sephadex G-100 prior to binding studies. Routinely, 5 ml of liver cytosol was chromatographed on a 250-ml column of Sephadex G-100 equilibrated in TKE buffer, 0.4 *M* with respect to KCl and 1 m*M* with respect to dithiothreitol. The eluate between 0.38 and 0.42 column volume ("ABF" = androgen-binding fraction) was collected and was used for in vitro binding studies. When [1,2-^{3}H]androstenedione was incubated with ABF for 12 hr at 0° no metabolism could be detected.

In the experiments where the binding of [1,2-^{3}H]androstenedione to ABF was investigated the incubations were usually performed for 3–5 hr at 0°. When analyzed by sucrose density gradient centrifugation the incubation mixture yielded a peak with a sedimentation coefficient of 10 S. This sucrose gradient peak was shown to be resistant toward treatment with dextran-coated charcoal and the incubation mixtures were always treated with dextran-coated charcoal prior to analysis by sucrose density gradient centrifugation. Using this analytical design, experiments were made to saturate ABF with increasing amounts of unlabeled androstenedione. In these assays the steroid was added in 20 μl of ethanol to 0.5 ml of ABF and the mixture was incubated at 0° for 3 hr. Parallel incubations with 100-fold excess of unlabeled androstenedione were also carried out. High-affinity binding of androstenedione was then calculated from the difference in the 10S peak between the total quantity bound and the residual binding in the presence of excess of unlabeled androstenedione. Saturation of an androstenedione-binding protein in ABF was obtained at a substrate concentration of about 1×10^{-7} *M* and the concentration of binding sites was found to be in the range of 8×10^{-16} mol/mg of protein in ABF (corresponding to 1.6×10^{-16} mol/mg of protein in unfractionated cytosol).

Discussion

The present investigation has shown that castrated male rats specifically concentrate [^{3}H]androstenedione in liver nuclei after intraperitoneal administration of [1,2,6,7-^{3}H]testosterone. This indicates the existence of specific mechanisms for the uptake of androstenedione into liver cytosol from blood or, alternatively, for the formation of androstenedione in liver cytosol and, furthermore, for the protection of androstenedione from metabolism in liver cytosol and for the uptake of androstenedione into liver nuclei.

Pulse-labeling studies with [1,2,6,7-^{3}H]testosterone showed a higher concentration of [^{3}H]androstenedione in portal vein blood than in aortic blood indicating uptake of androstenedione into liver or lung tissue. When the andro-

gen pool in the liver was pulse labeled with [1,2,6,7-^{3}H]testosterone the concentration of [^{3}H]androstenedione was relatively constant throughout the experiment, even up to several hours after administration of the isotope. In view of the rapid and extensive metabolism of androstenedione in liver homogenate and cellular subfractions in vitro (Einarsson et al., 1973; Gustafsson and Stenberg, 1973a; Pearlman and Pearlman, 1961) these results indicate the existence of specific mechanisms in liver cytosol protecting androstenedione from being metabolized.

The most probable mechanism is the presence of specific macromolecule(s) in liver cytosol that bind androstenedione with a high affinity and that participate in the intranuclear uptake of androstenedione. In experiments where castrated male rats were given [1,2,6,7-^{3}H]testosterone it was possible to demonstrate the formation of a [^{3}H]androstenedione–protein complex in liver cytosol ("complex A"). It was shown that complex A did not originate from blood. Binding of [1,2-^{3}H]androstenedione to a partially purified liver cytosol protein was accomplished in vitro and the resulting steroid–protein complex was found to behave like complex A when analyzed by Sephadex G-100 chromatography and by sucrose density gradient centrifugation (sedimentation coefficient 10 S). It was possible to saturate the ligand binding sites in the complex A protein by excess unlabeled androgen both in vivo and in vitro. Saturation in vitro was obtained at a substrate concentration of about 1×10^{-7} *M* which is similar to what has been found for androgen receptor proteins (Bullock and Bardin, 1974; Danzo et al., 1973). Finally, also in agreement with what has previously been reported for androgen receptors (Bullock and Bardin, 1974; Danzo et al., 1973), complex A was relatively stable toward treatment with dextran-coated charcoal.

When castrated male rats were injected with [1,2,6,7-^{3}H]testosterone also other metabolites besides [^{3}H]androstenedione were bound to proteins in liver cytosol. The steroid monosulfates bound were essentially removed from the proteins by treatment with dextran-coated charcoal indicating a binding of low affinity. Sulfate-binding proteins have been described in connection with hepatic disposition of glucocorticoids (Litwack et al., 1973) and it is possible that similar or identical proteins are involved in binding of androgen metabolites in liver cytosol. Whatever their function may be they are obviously not involved in intranuclear transfer of steroid hormones. Indications were also obtained for the existence of a testosterone-binding protein in liver cytosol. This protein was not studied in the present investigation. It may play some role in the intranuclear uptake of [^{3}H]testosterone observed when intact male and female rats were injected with [1,2,6,7-^{3}H]testosterone. However, no indications were obtained for the existence of a 5α-dihydrotestosterone-binding receptor in liver cytosol as reported by Milin and Roy (1973). Even if specific binding of 5α-dihydrotestosterone does occur in liver cytosol in vitro this phenomenon seems to be unrelated to intranuclear uptake of androgens in vivo since 5α-[^{3}H]dihydrotestosterone was never found in liver nuclei after pulse labeling with [1,2,6,7-^{3}H]testosterone.

In conclusion, the present investigation has demonstrated the binding of androstenedione to a protein in rat liver cytosol with a limited number of binding sites and with a high affinity for androstenedione. The possible role of this androstenedione–protein complex in androgenic regulation of liver enzyme activities is not clear at the present time. Recent findings indicate that the hypothalamo–hypophyseal axis exerts a superior control of liver enzyme levels (Gustafsson et al., 1974; Gustafsson and Stenberg, 1974) and it is possible that the hepatic androgen-binding protein described in this investigation primarily participates in the control of other processes than the regulation of hepatic steroid metabolism. Thus, androstenedione has been found to have a growth-stimulating and an anabolic effect similar to that of testosterone (Kochakian, 1964). On the other hand, lack of activity of androstenedione in other biological tests does not necessarily exclude the participation of an androstenedione–receptor protein complex in the mechanism of action of androgens in these biological systems. It may be that the androgen has to enter the cell as testosterone, rather than as androstenedione, as a prerequiste for the final formation of an androstenedione–receptor protein complex.

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