Demonstration and Partial Characterization of Cytosol Receptors for Testosterone†

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ABSTRACT: Androgen uptake was investigated in several peripheral organs after administration of [1,2,6,7-^{3}H]testosterone to castrated male rats. The animals were killed after 30 min, the organs were taken out, and the radioactivity was determined after tissue combustion. A relatively high accumulation of androgen was found in pancreas, adrenals, spleen, thigh muscle, kidneys, and liver in addition to the classical androgen target organs coagulation glands, seminal vesicles, prostate, preputial glands, and harderian glands. In a second series of experiments, nuclear and cytosol fractions were prepared from prostate, seminal vesicles, coagulation glands, preputial glands, spleen, submaxillary glands, kidneys, and pancreas from castrated male rats given [1,2,6,7-^{3}H]testosterone, and these fractions were then characterized by thin-layer and radio-gas chromatography with respect to their patterns of labeled steroids. Only prostate and seminal vesicles were found to contain significant amounts of nuclear 5α-[^{3}H]dihydrotestosterone. The major nuclear androgen was [^{3}H]testosterone that was the only detectable labeled steroid in coagulation glands, preputial glands, and spleen and that constituted 70% or more of the nuclear radioactivity in seminal vesicles, submaxillary glands, kidneys, and pancreas. These results indicate that testosterone itself may be the predominant active androgen principle in vivo in most androgen target organs and that conversion to 5α-dihydrotestosterone is generally not a prerequisite for androgen activity. Using an ultrasensitive micromodification of isoelectric focusing (cf. M. Katsumata and A. S. Goldman (1974), *Biochem. Biophys. Acta 359*, 112. It was possible to show that cytosol from kidney, submaxillary gland, thigh muscle, and levator ani muscle and nuclei from kidney and submaxillary gland contained androgen-binding proteins with p*I*'s in the region 4.6–5.1 ("4.6–5.1 Complex"). This complex also formed in vitro after incubation of [1,2,6,7-^{3}H]testosterone with cytosol from kidney and submaxillary gland. [1,2,6,7-^{3}H]Testosterone was bound with high affinity to receptor proteins in cytosol from both kidney, submaxillary gland, and thigh muscle with dissociation constants of 5.0×10^{-12} *M* (kidney), 3.3×10^{-11} *M* and 4.1×10^{-10} *M* (two types of binding sites, submaxillary gland), 2.4×10^{-12} *M* (thigh muscle) and 1.9×10^{-12} *M* (levator ani muscle). The number of binding sites was in all cases between 1 and 20 fmol/mg of protein. On the basis of these results the hypothesis is presented that a common class of testosterone receptors is present in most organs and that these receptors can be detected both in vivo and in vitro provided methods sensitive enough are utilized.

Androgen action in the ventral prostate of the rat is thought to be mediated by a 5α-reduced metabolite of testosterone, 5α-dihydrotestosterone (Bruchovsky and Wilson, 1968a). Receptor proteins for 5α-dihydrotestosterone have been detected in cytosol and nuclei of rat ventral prostate (Bruchovsky and Wilson, 1968b; Liao and Fang, 1969) and certain other androgen-dependent target organs, e.g., kidney (Gehring et al., 1971). These initial results were often taken to indicate that testosterone action in target organs generally proceeded via the formation of 5α-dihydrotestosterone that was specifically bound to cytosol receptor proteins and transported into the cell nucleus. Some in vitro studies on the 5α-reductase activity in skin have, e.g., been carried out on the assumption that 5α-dihydrotestosterone is the physiologically active androgen in skin (Voigt and Hsia, 1973) although any receptor proteins for 5α-dihydrotestosterone in skin have never been demonstrated. In fact, some recent reports cast doubt upon the validity of the hypothesis that 5α-dihydrotestosterone is the general mediator of androgen action in target organs. Jung and Baulieu (1972) have shown that rat levator ani muscle contains a receptor protein with higher affinity for testosterone than for 5α-dihydrotestosterone and Bullock and Bardin (1974) have described a testosterone receptor protein in mouse kidney. Evidence has also been presented that rat liver contains a specific receptor active in intranuclear transport of 4-androstene-3,17-dione (Gustafsson et al., 1975) and in hypothalamus, androgen action may at least partially be mediated via aromatized metabolites (Reddy et al., 1974).

The best criterium for a steroid receptor is presumably that it should be able to bind the steroid specifically and then transport it into the cell nucleus. Despite the vast information on androgen receptor proteins that is presently accumulating, relatively few studies have been carried out in vivo. In view of the present confusion and uncertainty as to the nature of the androgen metabolites that are transported into the cell nuclei of various androgen target organs in vivo, we considered it of interest to perform a characterization of intranuclear metabolites of systematically administered [1,2,6,7-^{3}H]testosterone in several organs showing significant androgen uptake. Furthermore, isoelectric focusing was used as a sensitive method to detect androgen-binding proteins in cytosol and nuclear extracts from some of the androgen target organs. Previous investigations on androgen receptor proteins have mainly utilized centrifugation and gel filtration techniques. Mainwaring and Irving (1973) have recently used isoelectric focusing to characterize the 5α-dihydrotestosterone receptor protein in rat prostate and found a single peak at p*I* = 5.8 when analyzing a purified preparation. Using a micromethod for electrofocus-

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ing, Katsumata and Goldman (1974) have very recently described the occurrence of multiple receptors for 5α-dihydrotestosterone in unfractionated rat prostate cytosol. These authors pointed out the great advantages of the micromethod as an ultrasensitive method for detection of steroid receptor proteins allowing characterization of receptor components in tissue specimens containing less than 1 mg of protein. In the present study, the method of Katsumata and Goldman has been used as a tool to identify androgen receptor proteins in rat kidney, submaxillary gland, and skeletal muscle.

Materials and Methods

Steroids. [1,2,6,7-^{3}H]Testosterone (specific radioactivity, 84 Ci/mmol), 4-[1,2,6,7-^{3}H]androstene-3,17-dione (specific radioactivity, 83 Ci/mmol), and 5α-[1,2,4,5,6,7-^{3}H]dihydrotestosterone (specific radioactivity, 100 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. Unlabeled testosterone, 5α-dihydrotestosterone, 4-androstene-3,17-dione, 5α-androstane-3α,17β-diol, progesterone, estradiol, and cortisol were generously supplied by Dr. J. Babcock, Upjohn Co., Kalamazoo, Mich. Cyproterone acetate was a gift from Dr. F. Neumann, Schering AG, Berlin, West Germany.

Animal Experiments. Sprague-Dawley rats, 8 weeks old, were used in 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 castrated male rats were given an intraperitoneal injection of 250 μCi of [1,2,6,7-^{3}H]testosterone in 120 μl of acetone; 30 min later the animals were killed by a blow on the head. A number of tissues and organs (see Table I) were rapidly removed. The wet weights of these specimens were determined and then the samples were combusted in a tissue combustion device (Oxymat, Intertechnique, Plaisir, France) prior to measurement of radioactivity using a Packard liquid scintillation spectrometer, Model 2425. Calculation of radioactivity in dpm was performed with a correction factor automatically determined using an external standard.

In a second series of experiments four to six castrated male rats were given 250 μCi of [1,2,6,7-^{3}H]testosterone as described above and were killed 30 min later. The prostate, seminal vesicles, coagulation glands, preputial glands, spleen, submaxillary glands, kidneys, pancreas, and a piece of skeletal muscle—organs that showed a significant uptake of radioactivity (cf. Table I)—were taken out and each organ was pooled with the same organ from the other rats. Cytosol and nuclei were prepared from all tissues except skeletal muscle using a method yielding highly pure nuclei (Gustafsson and Pousette, 1974). From skeletal muscle only cytosol was prepared.

Identification of Radioactive Metabolites. The nuclear sediment was resuspended in 2 ml of buffer. In order to extract the radioactivity, the nuclear suspension and the cytosol 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 distilled water and passed through a 10-g XAD-2 column as previously described (Eriksson and Gustafsson, 1970). Aliquots of the methanol eluate from the column were taken for measurement of radioactivity and for identification of radioactive metabolites by thin-layer and radio-gas chromatography.

Table I: Average Retention of Labeled Steroid in Various Organs after Administration of 250 μCi of [1,2,6,7-^{3}H]Testosterone to Castrated Male Rats 30 min before Death.

Tissue	Concn of Labeled Steroid (pmol/g of Tissue)
Pancreas	34.2 ± 22.7
Stomach	16.1 ± 11.7
Adrenals	15.4 ± 4.7
Prostate	12.0 ± 4.0
Liver	11.2 ± 6.7
Coagulation glands	10.4 ± 2.8
Skeletal muscle	9.4 ± 7.2
Spleen	8.3 ± 10.3
Seminal vesicles	8.2 ± 2.8
Harderian glands	6.9 ± 6.7
Kidneys	6.5 ± 4.2
Preputial glands	5.9 ± 1.0
Thyroid gland	3.9 ± 1.7
Cowper's glands	2.8 ± 1.5
Infraorbital lacrimal glands	2.8 ± 0.6
Exorbital lacrimal glands	2.8 ± 0.9
Submaxillary glands	2.0 ± 0.9
Lungs	1.9 ± 0.8
Heart muscle	1.8 ± 1.1
Thymus	1.5 ± 0.7
Blood	1.4 ± 0.5

Thin-layer chromatography was performed on precoated silica gel plates (250 μ, Merck AG, Darmstadt, West Germany). The samples were dissolved in methanol and applied on the chromatoplates which were developed in the solvent system ethyl acetate–96% (v/v) ethanol–15 *M* ammonium hydroxide, 5:5:1 (v/v). This solvent system enabled separation of free steroids, steroid monosulfates, disulfates, and glucuronides (Sarfaty and Lipsett, 1966). After drying, the thin-layer plates were scanned for radioactivity using a Berthold thin-layer scanner, Model II (Berthold, Wildbad, West Germany). The major part of the radioactive steroids extracted from cytosol and nuclei of the androgen target organs in question chromatographed like unconjugated steroids. Only very small amounts of radioactivity were found in zones corresponding to the mobilities of reference steroid monosulfates, disulfates, and glucuronides.

The thin-layer chromatographic zones corresponding to unconjugated steroids were scraped off and the silica gel was extracted with methanol. The extract was rechromatographed on thin-layer plates in the solvent system ethyl acetate–chloroform, 1:4 (v/v). External radioactive reference steroids were chromatographed on the same chromatoplates as the biological samples. The relative amounts of radioactive steroid metabolites were measured from scanner chromatograms. The zones containing radioactivity were scraped off and the silica gel was eluted with methanol.

Each methanol eluate was evaporated to dryness and the extract was trimethylsilylated. 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 time, relative to 5α-cholestane (t_R) on SE-30 and on OV-17, as the reference steroid.

Protein Binding of Androgen in Kidney, Submaxillary Gland, and Skeletal Muscle in Vivo. Cytosol from kidney, submaxillary gland, skeletal (thigh) muscle, and levator ani

muscle and nuclei from kidney and submaxillary gland were investigated for high-affinity protein binding of metabolites of intraperitoneally administered [1,2,6,7-^{3}H]testosterone (see above). Cytosol fractions were prepared yielding protein concentrations of about 0.6 (levator ani muscle), 5 (submaxillary glands and thigh muscle), and 20 (kidneys) mg/ml. In order to extract proteins from the nuclei, the nuclear pellet was suspended in 2 ml of 0.4 *M* KCl–0.001 *M* EDTA–0.01 *M* Tris-HCl (pH 7.4) and kept at 0–2° for 2 hr. Centrifugation was performed at 30,000*g* for 30 min. The cytosol preparations were liberated from nonbound and nonspecifically protein-bound steroids by treatment with dextran-coated charcoal according to Beato and Feigelson (1972).

The nuclear extracts and cytosol preparations were then chromatographed on Sephadex G-25 columns equilibrated with TKE buffer (0.01 *M* KCl–0.001 *M* EDTA–0.01 *M* Tris-HCl (pH 7.4)) (Puca and Brescianai, 1968) and the void volumes were taken for analysis by isoelectric focusing, essentially using the micromethod described by Katsumata and Goldman (1974). Three milliliters of sample was distributed in the gradient; 10-ml columns were used and focusing was performed at 1200 V for 15–20 hr. The columns were fractionated into counting vials or test tubes (30–60 fractions) and 0.5 ml of double-distilled water was added prior to measurement of pH. Radioactivity measurements were performed using Instagel^R (Packard Instrument Co., Inc., Warrenville, Downess Grove, Ill.) as scintillator liquid. Ferritin (p*I* = 5.0) and hemoglobin (p*I* = 7.2 and 7.6) were purchased from Sigma Chemical Co. (St. Louis, Mo.) and used as standards.

In selected cases, aliquots of the focused radioactive peaks were taken for identification of radioactivity. Twenty volumes of acetone–ethanol, 1:1 (v/v), was added to the samples in question. After filtration, the extracts were evaporated to dryness and the residues were dissolved in double-distilled water and passed through an Amberlite XAD-2 column. The steroids were extracted with methanol. Identification of the radioactive metabolites was performed by thin-layer chromatography and radio-gas chromatography as described above.

Nuclear Uptake in Vitro of Protein-Bound Androgen. Unlabeled nuclei were prepared from kidney from male animals castrated 14–16 hr prior to killing. This nuclear fraction was resuspended in ^{3}H-labeled cytosol from kidney obtained from castrated male rats given 250 μCi of [1,2,6,7-^{3}H]testosterone 30 min before death. The nuclear–cytosol preparation was incubated for 30 min at 37°. Control incubations of ^{3}H-labeled cytosol from kidney without added nuclei were also carried out. After incubation, the samples were cooled and centrifuged at 30,000*g* for 20 min. The supernatants were treated with dextran-coated charcoal and analyzed by isoelectric focusing as described above. The nuclear sediment was resuspended in buffer and an aliquot was taken off for determination of DNA according to Burton (1956).

Protein Binding of Androgen in Kidney, Submaxillary Gland, and Skeletal Muscle in Vitro. Cytosol was prepared from kidney, submaxillary gland, and skeletal muscle of four male rats castrated 14–16 hr before death; 1-ml portions of the cytosol preparations were added to test tubes containing ^{3}H-labeled steroids in different amounts. The steroids had been added to the test tubes in organic solvents that had been taken to dryness under nitrogen. It was ascertained that the steroids were completely dissolved in the added cytosol. The steroid–cytosol mixture was left on ice for 2 hr and was then treated with dextran-coated charcoal twice. Aliquots of the supernatants were taken for measurement of radioactivity and for protein determination according to Lowry et al. (1951). Dissociation constants and number of binding sites for androgen receptor proteins were determined according to Scatchard (1949). Time studies showed that no further specific binding of substrate occurred after 2 hr on ice. In selected cases, supernatants were pooled to provide enough amount of material, concentrated using a vacuum-concentrator with collodium bags (Sartorius-Membran-filter, Göttingen, West Germany), and then analyzed by isoelectric focusing. In separate experiments the ligand specificity of the androgen receptors was investigated in vitro by incubating cytosol with [1,2,6,7-^{3}H]testosterone in a concentration equal to K_d plus a competing unlabeled steroid in a concentration about 100 times that of [1,2,6,7-^{3}H]testosterone.

Table II: Relative Distribution of ^{3}H-Labeled Testosterone (T), 5α-Dihydrotestosterone (5αdHT), 4-Androstene-3,17-dione (A^4), and Polar Steroids in Nuclei and Cytosol from Various Organs of Castrated Male Rats Given an Intraperitoneal Injection of [1,2,6,7-^{3}H]Testosterone.[a]

		T (%)	5αdHT (%)	A^4 (%)	Polar Steroids (%)
Prostate	Nuclei[c]	30–45	55–70	0	0
	Cytosol[b]	15–70	15–20	10–40	10–35
Seminal vesicles	Nuclei	80	20	20	0
	Cytosol	10–20	15–30	45–70	10–30
Coagulation glands	Nuclei	100	0	0	0
	Cytosol	15–40	15–30	10–60	15–30
Preputial glands	Nuclei	100	0	0	0
	Cytosol	20–50	20–30	10–30	10–70
Adrenals	Cytosol	20–25	5–10	5–25	30–70
Spleen	Nuclei	100	0	0	0
	Cytosol	40–80	0	10–25	10–35
Submaxillary glands	Nuclei[e]	70	0	30	0
	Cytosol[d]	30–70	0	10–30	30–50
Kidneys	Nuclei	90	5	5	0
	Cytosol	20–40	10–15	5–15	50–60
Pancreas	Nuclei	70–90	0	10–30	0
	Cytosol	75–90	0	5–15	5–20
Skeletal muscle	Cytosol	60–70	5–10	10–15	10–15

[a] Five sets of experiments were carried out and in each experiment organs were pooled from four to six rats. [b] See Figure 1a. [c] See Figure 1b. [d] See Figure 2a. [e] See Figure 2b.

Results

Uptake of Androgen in Different Tissues. Table I shows the average retention of radioactivity (four experiments) found in various organs after administration of [1,2,6,7-^{3}H]testosterone to castrated male rats. The use of the tissue oxidizing technique allows safe comparisons between the uptake of radioactivity in various organs by eliminating the risk of different extractability of steroids from different tissues. A high uptake of radioactivity was found in the prostate, seminal vesicles, harderian glands, coagulation glands, and preputial glands, organs classically recognized as androgen target organs. A relatively high uptake, however, was also found in the pancreas, adrenals, kidneys, liver, thigh muscle, spleen, and submaxillary glands. A low uptake of radioactivity was found in heart muscle, thymus, and blood.

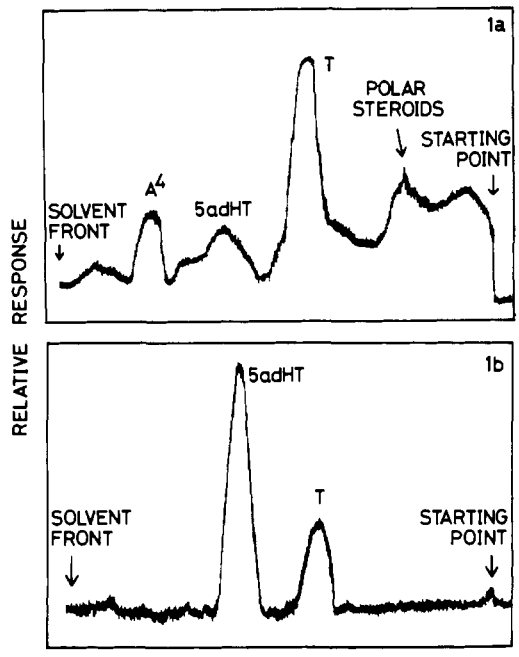

FIGURE 1: Radioactivity scanner chromatograms of extracts from the cytosol (a) and nuclear (b) fractions from the prostate gland of castrated male rats given 250 μCi of [1,2,6,7-^{3}H]testosterone 30 min before death. The metabolites were identified as follows: T, [^{3}H]testosterone; 5αdHT, 5α-[^{3}H]dihydrotestosterone; and A^4, 4-[^{3}H]androstene-3,17-dione.

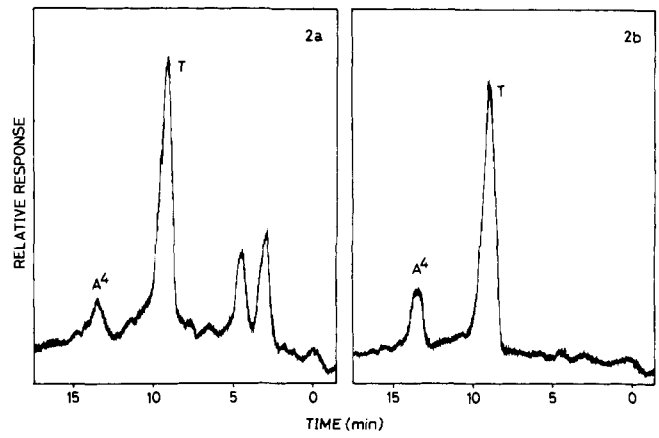

FIGURE 2: Radio-gas chromatograms of extracts from the cytosol (a) and nuclear (b) fractions from the submaxillary glands of castrated male rats given 250 μCi of [1,2,6,7-^{3}H]testosterone 30 min before death. The metabolites were identified as follows: T, [^{3}H]testosterone; and A^4, 4-[^{3}H]androstene-3,17-dione.

Characterization of Metabolites of [1,2,6,7-^{3}H]Testosterone in Tissues with a High and a Relatively High Uptake of Androgen. Table II summarizes the identifications and relative distributions of radioactive metabolites of [1,2,6,7-^{3}H]testosterone in cytosol and nuclei from prostate, seminal vesicles, coagulation glands, preputial glands, adrenals, spleen, submaxillary glands, kidneys, and pancreas. The data were obtained from five sets of experiments, each of which was carried out with four to six castrated male rats.

The major ^{3}H-labeled steroids found in nuclei and cytosol from all organs investigated were testosterone, 5α-dihydrotestosterone, and 4-androstene-3,17-dione. These compounds were shown to have the same mobility on thin-layer plates as the corresponding reference steroids. Furthermore, they chromatographed as the reference compounds also when analyzed by gas–liquid chromatography, using both SE-30 and OV-17 as the stationary phase. In addition to these major metabolites of [1,2,6,7-^{3}H]testosterone, the cytosol of all organs also contained polar steroids. Since these metabolites were never recovered from the nuclear fraction they were not investigated in detail. Their thin-layer and gas–liquid chromatographic behavior indicated that they mostly consisted of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol.

Prostate cytosol contained both ^{3}H-labeled testosterone, 5α-dihydrotestosterone, 4-androstene-3,17-dione, and polar steroids (Table II); in all experiments but one testosterone was the major component (Figure 1a). In contrast, 5α-dihydrotestosterone was the predominant metabolite in prostate nuclei even if a certain amount of testosterone was constantly present (Figure 1b). In cytosol from most other organs the same ^{3}H-labeled compounds were identified as in prostate cytosol. The only exceptions were spleen, submaxillary glands, and pancreas, the cytosol preparations of which did not contain any 5α-dihydrotestosterone (see Figure 2a). In both seminal vesicles coagulation glands, preputial glands, spleen, submaxillary glands, kidneys, and pancreas, testosterone was the major ^{3}H-labeled steroid in the nuclear fraction (see Figure 2b). The only organs besides prostate that contained detectable levels of nuclear 5α-dihydrotestosterone were the seminal vesicles. On the other hand, 4-androstene-3,17-dione was a significant nuclear constituent in seminal vesicles, submaxillary glands, kidneys, and pancreas. Androgens more polar than testosterone were never found in the nuclear fraction.

The findings described prompted us to investigate the occurrence of receptor proteins specific for testosterone in some of the organs listed in Table II. Kidney, submaxillary gland, and skeletal muscle were selected for further studies.

Protein Binding of Androgen in Kidney in Vivo and in Vitro. Isoelectric focusing of dextran-coated, charcoal-treated kidney cytosol prepared from castrated male rats given [1,2,6,7-^{3}H]testosterone 30 min before death yielded a reproducible pattern of peaks with a major complex with p*I*'s of 5.1 (larger peak) and 4.8 (smaller peak) ("4.8–5.1 Complex") and with minor peaks at pH 2.0, 4.5, 6.1, and 8.2 (see Figure 3a). When the cytosol receptor sites were saturated by intraperitoneal injection of 1 mg of testosterone 10 min before injection of [1,2,6,7-^{3}H]testosterone, isoelectric focusing showed that the "4.8–5.1 Complex" had disappeared and that the peaks at pH 2.0, 4.5, 6.1, and 8.2 had decreased significantly. Radio-gas chromatographic analysis of the "4.8–5.1 Complex" showed that it only contained [^{3}H]testosterone.

Isoelectric focusing of the nuclear extract obtained from kidneys of rats given [1,2,6,7-^{3}H]testosterone also yielded a reproducible pattern of peaks with a predominating "4.8–5.1 Complex". In some experiments small peaks were also found at pH 2.0 and 6.1 (Figure 3b).

The relatively high background of radioactivity found in the electrofocusing experiments was probably due to steroids that had dissociated from the macromolecules during the analysis.

Heating of ^{3}H-labeled kidney cytosol at 37° for 30 min resulted in a significant decrease of the size of the "4.8–5.1 Complex" whereas the peak at pH 2.0 was not reduced in size. When ^{3}H-labeled kidney cytosol was reconstituted with unlabeled kidney nuclei prepared from castrated male rats, the size of the "4.8–5.1 Complex" decreased significantly whereas no reduction was observed in the size of the other peaks (Figure 4).

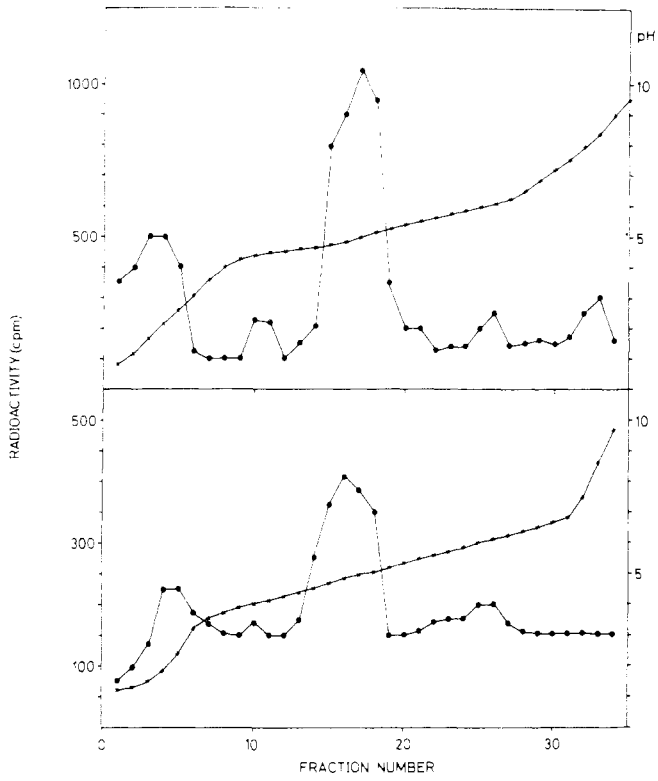

FIGURE 3: Isoelectric focusing of dextran-coated, charcoal-treated cytosol (a) and 0.4 M KCl extract of nuclei (b) from kidneys of a castrated male rat given 250 μCi of [1,2,6,7-^{3}H]testosterone 30 min before death. Each fraction contained 0.3 ml. (●) Radioactivity; (x) pH.

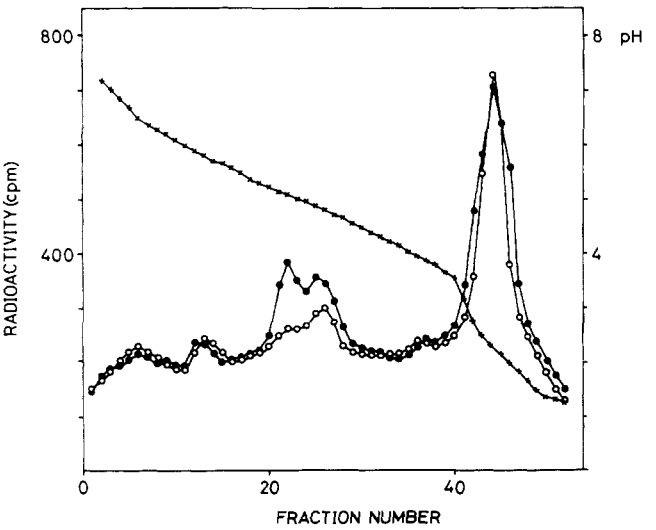

FIGURE 4: Effect of nuclear uptake of high affinity androgen-receptor complexes in kidney cytosol. (●) Radioactivity after isoelectric focusing of dextran-coated, charcoal-treated kidney cytosol heated at 37° for 30 min; (○) radioactivity after isoelectric focusing of dextran-coated, charcoal-treated kidney cytosol ihcubated with unlabeled kidney nuclei at 37° for 30 min (the concentration of DNA was 1 mg/ml of incubation medium). (x) pH.

After incubation of kidney cytosol with [1,2,6,7-^{3}H]testosterone at 0–2° for 2 hr it was not possible to detect any metabolism of the steroid. Based on several determinations, the K_d with [1,2,6,7-^{3}H]testosterone as ligand was calculated to be 5.0×10^{-12} M and the number of binding sites 1.5×10^{-15} mol/mg of protein, as determined from the Scatchard plots. When [1,2,4,5,6,7-^{3}H]dihydrotestosterone was used as ligand the K_d was found to be 1.0×10^{-11} M and the number of binding sites 1.7×10^{-15} mol/mg of protein. When 4-[1,2,6,7-^{3}H]androstene-3,17-dione was incubated with kidney cytosol, no high-affinity binding was observed. The [^{3}H]testosterone–protein complexes formed in vitro were also analyzed by isoelectric focusing. When samples from the "steep" part of the Scatchard plot (= samples containing high-affinity binding protein) were electrofocused, the "4.8–5.1 Complex" appeared. Analysis of samples from the "shallow" part of the plot (= samples containing low-affinity binding proteins) showed the presence of a major peak at pH 7.8.

Table III: Effect of Various Unlabeled Steroids on High-Affinity Binding of [1,2,6,7-^{3}H]Testosterone to Cytosol Protein of Kidney, Submaxillary Gland, and Thigh Muscle from Castrated Male Rats.[a]

Nonradioactive Steroid	Bound Radioactivity (% of control)		
	Kidney	Submax-illary Gland	Thigh Muscle
None	100	100	100
Progesterone	100	56.3	74
Cortisol	94.3	66.9	*b*
5α-Androstane-3α,17β-diol	52.6	33.1	*b*
Cyproterone acetate	48.1	2.3	100
5α-Dihydrotes-tosterone	20.3	8.0	94
Estradiol	11.0	0	100
4-Androstene-3,17-dione	8.6	2.3	0
Testosterone	0	0	0

[a] Aliquots of cytosol preparations were incubated with 5.0×10^{-12} M (kidney), 1×10^{-11} M (submaxillary gland), or 2.4×10^{-12} M (thigh muscle) [1,2,6,7-^{3}H]testosterone alone or with 5.0×10^{-10} M, 1×10^{-9} M, or 2.4×10^{-10} M, respectively, of nonradioactive steroids. [b] Determination not carried out.

Table III summarizes the effect of various unlabeled steroids on high affinity binding of [1,2,6,7-^{3}H]testosterone to kidney cytosol. Progesterone and cortisol showed none or weak competition for the binding sites whereas 4-androstene-3,17-dione, estradiol, and 5α-dihydrotestosterone competed more efficiently. The competition experiment with 5α-dihydrotestosterone further supports the observation described above that the androgen receptor in rat kidney binds testosterone with somewhat higher affinity than 5α-dihydrotestosterone.

Protein Binding of Androgen in Submaxillary Glands in Vivo and in Vitro. Figure 5 shows an isoelectric focusing of dextran-coated charcoal from submaxillary glands of a castrated rat given [1,2,6,7-^{3}H]testosterone. The pattern of peaks was more complex than that obtained when labeled kidney cytosol was electrofocused; steroid-binding proteins with the following pI's were observed: pI = 2.8, 4.6 (major protein), 5.1, 6.1, 7.3, and 8.7. A less complex pattern of peaks was found upon isoelectric focusing of the extract obtained from nuclei of submaxillary glands. In this case peaks were present at pH 4.6, 5.1 ("4.6–5.1 Complex"), and 6.1, indicating that the steroid-binding proteins in cytosol with pI's of 2.8, 7.3, and 8.7 did not participate in intranuclear transport of testosterone.

Scatchard analysis of binding studies in vitro under conditions where no metabolism of substrate occurred demonstrated high-affinity binding of [1,2,6,7-^{3}H]testosterone to protein in submaxillary gland cytosol (Figure 6). The exis-

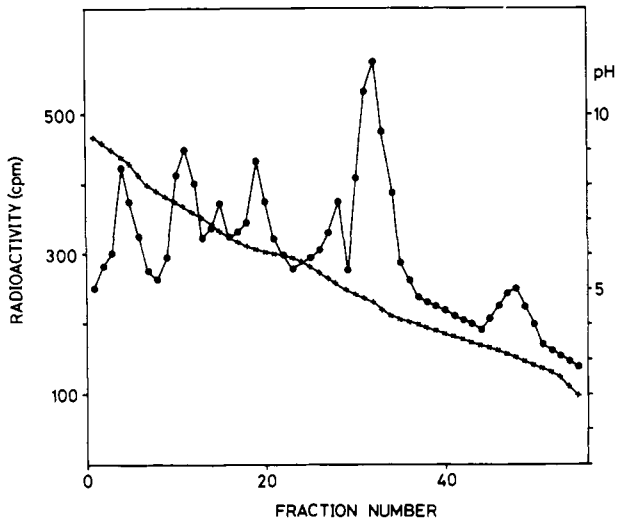

FIGURE 5: Isoelectric focusing of dextran-coated, charcoal-treated cytosol from submaxillary glands of a castrated male rat given 250 μCi of [1,2,6,7-^{3}H]testosterone 30 min before death. (O) Radioactivity; (x) pH.

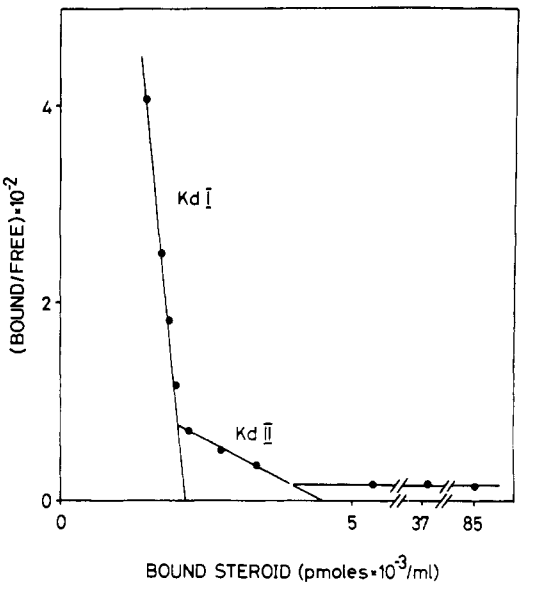

FIGURE 6: Scatchard plot of [1,2,6,7-^{3}H]testosterone binding in cytosol from submaxillary gland. Cytosol from submaxillary gland of castrated male rats was incubated with [1,2,6,7-^{3}H]testosterone for 2 hr. Bound radioactivity was determined using dextran-coated charcoal as described under Materials and Methods. Two different types of binding sites were found; the dissociation constants were: K_{d_I}, 3.3 × 10^{-11} M (number of binding sites 0.9 × 10^{-14} mol/mg of protein) and $K_{d_{II}}$, 4.1 × 10^{-10} M (number of binding sites 2.2 × 10^{-14} mol/mg of protein).

tence of two different types of binding sites was indicated, one with a K_d of 3.3 × 10^{-11} M (number of binding sites 0.9 × 10^{-14} mol/mg of protein) and the other with a K_d of 4.1 × 10^{-10} M (number of binding sites 2.2 × 10^{-14} mol/mg of protein). In vitro labeled cytosol was also analyzed by isoelectric focusing. Samples with specifically (= high affinity) bound [^{3}H]testosterone gave radioactive peaks at pH 4.6, 5.1 ("4.6–5.1 Complex"), and 6.1 whereas samples also containing nonspecifically (= low affinity) bound [^{3}H]testosterone also yielded peaks at pH 5.5 and 8.5.

The relative affinity of various steroid ligands for the high-affinity binding sites in submaxillary gland cytosol was estimated in competition experiments (see Table III). Whereas C_{21} steroids like cortisol and progesterone were relatively inefficient in competing with [1,2,6,7-^{3}H]testosterone for binding sites, estradiol, 4-androstene-3,17-dione, and cyproterone acetate were potent competitors. 5α-Dihydrotestosterone seemed to bind somewhat less well than testosterone.

Protein Binding of Androgen in Muscle in Vivo and in Vitro. Electrofocusing of dextran-coated, charcoal-treated thigh muscle cytosol recovered from rats given [1,2,6,7-^{3}H]testosterone revealed the presence of androgen-binding proteins with pI's of 4.8 (major component), 5.1 ("4.8–5.1 Complex"), and 5.5 (minor component) (see Figure 7). Radio-gas chromatographic analysis of the "4.8–5.1 Complex" showed that [^{3}H]testosterone was the only steroid present. When in vivo labeled cytosol from levator ani muscle was analyzed under the same experimental conditions, only one androgen-binding protein (pI = 5.1) was observed. When labeled thigh muscle cytosol was treated with dextran-coated charcoal and analyzed by thin-layer and radio-gas chromatography, the only labeled steroid detected was [^{3}H]testosterone. Cytosol from levator ani muscle contained too small amounts of radioactivity to permit analysis of labeled steroid metabolites.

The presence of high-affinity binding sites for testosterone in thigh muscle cytosol was also demonstrated in vitro under conditions where no metabolism of substrate occurred. The K_d with respect to [1,2,6,7-^{3}H]testosterone was calculated to be 2.4 × 10^{-12} M and the number of binding sites 1.0 × 10^{-15} mol/mg of protein. Cytosol from levator ani muscle also displayed high-affinity binding of [1,2,6,7-^{3}H]testosterone. The K_d was calculated to be 1.9 × 10^{-12} M and the number of binding sites 5.5 × 10^{-15} mol/mg of protein. Competition experiments in vitro showed that the thigh muscle receptor for testosterone did not bind estradiol or cyproterone acetate (cf. Table III). Neither did 5α-dihydrotestosterone displace testosterone from the muscle receptor to any significant extent. 4-Androstene-3,17-dione efficiently displaced testosterone but when 4-[1,2,6,7-^{3}H]androstene-3,17-dione was incubated with thigh muscle cytosol in repeated experiments, it was not possible to demonstrate any high-affinity binding.

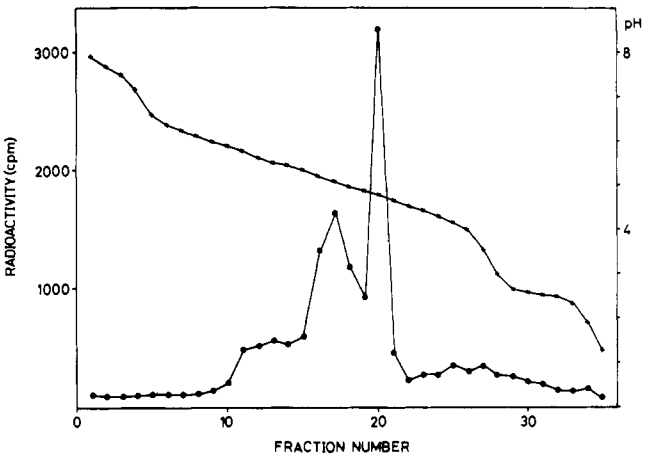

FIGURE 7: Isoelectric focusing of dextran-coated, charcoal-treated cytosol from thigh muscle of a castrated male rat given 250 μCi of [1,2,6,7-^{3}H]testosterone 30 min before death. (O) Radioactivity; (x) pH.

Discussion

In the present study, androgen uptake was investigated in

several organs after administration of [1,2,6,7-^{3}H]testosterone to castrated male rats. The method used to estimate the amount of labeled steroid taken up by the organ was tissue combustion followed by liquid scintillation. This method, which should be more reliable than conventional extraction methods employing organic solvent mixtures, showed a relatively high androgen accumulation in pancreas, adrenals, spleen, thigh muscle, kidneys, and liver in addition to the classical androgen target organs coagulation glands, seminal vesicles, prostate, preputial glands, and harderian glands. In all of the organs where practical circumstances permitted, nuclear and cytosol fractions were isolated and the nature of the labeled steroids was determined. Only prostate and seminal vesicles were found to contain significant amounts of nuclear 5α-[^{3}H]dihydrotestosterone; trace amounts were found in kidney nuclei. The major nuclear androgen, however, was [^{3}H]testosterone that constituted the only detectable nuclear androgen in coagulation glands, preputial glands, and spleen and 70% or more of the nuclear radioactivity in seminal vesicles, submaxillary glands, and kidneys. 4-[^{3}H]Androstene-3,17-dione has previously been found to be the major androgen in liver nuclei (Gustafsson et al., 1975) and in the present study this metabolite was found in nuclei from seminal vesicles, submaxillary glands, kidneys, and pancreas. These results indicate that testosterone itself may be the predominant active androgen principle in vivo in most androgen target organs and that conversion to 5α-dihydrotestosterone is generally not a prerequisite for androgen activity.

The following studies were concentrated upon detection and partial characterization of testosterone receptors in three of the organs shown to concentrate androgen in the initial studies, namely kidneys, submaxillary glands, and skeletal (thigh) muscle. Kidneys and submaxillary glands have previously been studied as androgen target organs in mice. As mentioned above, Bullock and Bardin (1974) have described a receptor for testosterone in mouse kidney and Dunn et al. (1973) have reported the presence of a receptor protein for 5α-dihydrotestosterone in mouse submaxillary gland. Skeletal muscle has hitherto resisted all attempts aimed at finding a specific androgen receptor protein.

In the present investigation, indications were obtained both in vivo and in vitro for the existence of receptor proteins for testosterone in kidney, submaxillary gland, and thigh muscle from male rats. Several initial experiments where sucrose gradient centrifugation was employed as the method to detect receptor proteins in these organs after labeling in vivo were unsuccessful, probably due to the limited sensitivity of this technique. However, the micromodification of isoelectric focusing described by Katsumata and Goldman proved to be an excellent method for detection of steroid receptors. Cytosol from kidney, submaxillary gland and thigh muscle and nuclei from kidney and submaxillary gland were all shown to contain major androgen-binding proteins with pI's in the region 4.6–5.1. Usually it was possible to resolve the "4.6–5.1 Complex" into two components with one peak at pH 4.6 or 4.8 and one peak at pH 5.1. Further indications for the participation of the "4.6–5.1 Complex" in intramuscular transport of androgens were obtained from reconstitution experiments with kidney cytosol labeled in vivo and unlabeled kidney nuclei which showed a specific uptake of this complex. Finally, the labeled complex disappeared from cytosol if large amounts of unlabeled testosterone were injected prior to administration of [1,2,6,7-^{3}H]testosterone, showing that it was possible to saturate the binding sites for testosterone on the receptor molecules in vivo. In addition to the "4.6–5.1 Complex", steroid-binding proteins were also found that focused in other pH regions. In kidney and thigh muscle cytosol these proteins were of minor importance but in cytosol from submaxillary glands the electrofocusing pattern was quite complex. However, the extracts from nuclei of both kidneys and submaxillary glands mainly contained the "4.6–5.1 Complex" proteins.

Also in vitro it was possible to detect formation of the "4.6–5.1 Complex" after incubation of [1,2,6,7-^{3}H]testosterone with cytosol from kidney and submaxillary gland. Testosterone was bound with high-affinity to receptor proteins in cytosol from both kidney, submaxillary gland, and thigh muscle. The different K_d's between kidney and thigh muscle receptors on one hand and submaxillary gland receptors on the other need not necessarily mean that different types of receptors are present in these organs. As shown by isoelectric focusing, other components than the "4.6–5.1 Complex" were also responsible for the high-affinity binding of [1,2,6,7-^{3}H]testosterone in vitro and varying quantitative relationships between the "4.6–5.1 Complex" and these components may well explain the different K_d's observed for different organs.

It is of special interest to note that the receptors for testosterone in thigh and levator ani muscle showed similar K_d values (about 2×10^{-12} M). Furthermore, both muscles contained androgen-binding proteins with a pI of 5.1. However, the number of binding sites in levator ani muscle was about five times higher than in thigh muscle. These results indicate that levator ani and thigh muscle contain similar or identical receptor proteins for testosterone and that the different androgen responsiveness between the two types of muscle is due to different concentration of these androgen receptor proteins in the muscle cytosol.

In addition to the "4.6–5.1 Complex" isoelectric focusing revealed androgen-binding components with other pI values, especially in submaxillary glands. The implications of this apparent multiplicity of androgen-binding proteins are not quite clear at the present time. The electrofocusing data may imply the existence of more than a single species of receptor molecule for testosterone in the organs investigated. On the other hand, the multiple peaks may represent various modifications of a single macromolecule. It is evident that further studies involving analysis of partially purified receptor preparations are needed to clarify this point.

In conclusion, the present study has shown that the majority of peripheral organs showing significant androgen uptake after intraperitoneal administration of [1,2,6,7-^{3}H]testosterone to castrated male rats concentrate [^{3}H]testosterone as the major intranuclear androgen. Isoelectric focusing of nuclear extracts from kidney and submaxillary gland and of cytosol from kidney, submaxillary gland, thigh muscle, and levator ani muscle have shown the occurrence of major androgen-binding proteins with pI's between 4.6 and 5.1. High-affinity testosterone binding was also observed in vitro in cytosol from all of these organs. In view of these data it is tempting to speculate that a common class of testosterone receptors is present in most organs and that these receptors can be detected in both in vitro and in vivo experiments provided methods sensitive enough are utilized. A similar situation seems to exist for glucocorticoid receptor proteins that have also recently been shown to be present in several more tissues than was originally believed (Ballard et al., 1974).

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The Macromolecular Composition of *Xenopus laevis* Egg Jelly Coat†

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ABSTRACT: The three morphologically and functionally distinct jelly coat layers of *Xenopus laevis* eggs, J_1, J_2, and J_3, were separated by manual dissection, solubilized with dithiothreitol, and physicochemically analyzed. The chemical composition of the three jelly layers varied from 37 to 48% protein and 63 to 52% carbohydrate. The carbohydrate consisted of hexosamines, galactose, and fucose. Some of the carbohydrate in each of the jelly layers was covalently linked to protein through O-glycosidic bonds as β elimination of the carbohydrate moiety in the presence of alkali was observed. In agreement with a previous finding, covalently attached sulfate was localized within the innermost jelly coat layer, J_1. Cellulose acetate electrophoresis at pH 8.0 resolved a total of nine macromolecular components from the three jelly coat layers differentially staining for protein and carbohydrate: J_1 yielded two anodically migrating components; the middle layer J_2 yielded two cathodically migrating macromolecular components; the outermost layer J_3 contained five species, three anodic and two cathodic. Sodium dodecyl sulfate agarose gel electrophoresis analysis yielded nine unique species, six of which stained coincidently for protein and carbohydrate. Immunoelectrophoresis and Ouchterlony double diffusion analyses using antiserum to total jelly components resolved nine different antigenic species with cross-reactivity between one or two macromolecules in layers J_1 and J_3. Analytical sedimentation velocity centrifugation revealed eight distinct species all of which exhibited hypersharp schlieren patterns and whose $s_{20,w}$ values were highly concentration dependent. On the basis of these analyses, *Xenopus laevis* egg jelly layers are composed of at least 8–9 distinct macromolecular species. The majority of these macromolecules are uniquely associated with different jelly coat layers.

A characteristic feature of amphibian eggs is the presence of a water-insoluble gelatinous matrix, termed the jelly coat, surrounding the egg and usually composed of several distinct layers. The egg jelly coat layers are secretory products of the tubular gland cells lining the oviduct and are deposited around the eggs as they traverse the oviduct following their release from the ovary. As determined by light microscopy studies, the jelly coats are morphologically simple or without any peculiar or distinguishing features. Histochemical studies and chemical analyses of several amphibian egg jelly coats have indicated the presence of protein and carbohydrate (for review of work published before 1966, see Monroy, 1965, and Metz, 1967; Lee, 1967; Freeman, 1968; Shivers and James, 1970a; Steinke and Benson, 1970). Immunological analyses of egg jelly coats have indi-

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