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Abstract

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BIOCHEMICAL STUDY OF TESTOSTERONE IN SKIN: *IN VIVO* AND *IN VITRO* METABOLISM OF TESTOSTERONE IN RAT SKIN

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Abstract. *In vitro* and *in vivo* testosterone metabolism was studied using rat skin. The *in vitro* stoichiometric studies strongly suggested the existence in the skin of 5α-reductase and other enzymes involved in testosterone metabolism. The predominant metabolites were 5α-dihydrotestosterone, androstandiol and $\triangle^4$-androstenedione. The rate of testosterone metabolism *in vitro* was higher in preputial skin than in dorsal skin. The main metabolites were androstandiol and $\triangle^4$-androstenedione *in vivo* whereas dihydrotestosterone and androstandiol were predominant *in vitro*. $\triangle^4$-Pathway is considered to be more active *in vivo* in adults than in newborns. The difference in the *in vivo* uptake increase of testosterone-^{3}H after castration suggests the possible existence in the organs and skin areas of androgen pools of different sizes. The *in vivo* uptake and metabolism of testosterone were examined in the nuclei of newborn rats. Dihydrotestosterone and androstandiol detected in the nuclei of preputial skin, dorsal skin and liver were 42.8%, 24.3% and 27.8%, of total radioactivity incorporated into the respective organs. The liver incorporated only a small amount of radioactivity compared with the skin.

The skin has been thought to be one of the target organs of testosterone (T). Several specific metabolites of T have been identified using the whole rat skin (1) and rat preputial gland (2).

Since dihydrotestosterone is the most potent androgen in the prostate, this suggests that the mode of action of T in the skin (3, 4) is related to 5α-reductase. However, there is no well established study on the relationship between 5α-reductase and the presence of T in the skin. Much additional work remains to be done to clarify not only the specific pathway of T metabolism but physiological significance of T and 5α-reductase in the skin.

MATERIALS AND METHODS

Chemicals. Testosterone-4-^{14}C (specific activity 52.8 μCi/μ mole) was purchased from New England Nuclear Corp., and checked for purity by thin layer chromatography in a ligroin-propylene glycol system. A methanolic stock solution containing 1.1×10^6 cpm (9.5 n moles) in each 0.1 ml was prepared and stored at -20°C. 5α-Dihydro-(1α, 2α (n)-^{3}H)-testosterone (40,000-60,000 mCi/m mole),

and (7 (n)-^{3}H)-testosterone (>5,000 mCi/m mole) were from the Radiochemical Centre, Amersham. Coenzymes, glucose-6-phosphate dehydrogenase and cold androgens were purchased from Sigma Chemical Company. Reagents were of analytical grade and were from Wako Pure Chemical Industries.

Animals. Four-week-old male Wistar rats were used. The dorsal hair was plucked 3 days before the experiment to stimulate the anagen hair cycle. Castration was performed when necessary by surgical manipulation 24 hr before each experiment, so that the endogeneous levels of T and its metabolites were significantly low.

Tissues for in vitro incubations. The rats were killed by decapitation. The dorsal and preputial skin was shaved with a safety razor and then swabbed with acetone. The skin was dissected with surgical scissors and the *panniculus carnosus* was removed. Tissues were minced with scissors into the smallest pieces in chilled Krebs-Ringer phosphate buffer (pH 7.4).

Incubations. Five hundred milligrams of minced tissue were added to the standard incubation mixture consisting of NAD 3.3 μmoles, NADP 3.3 μmoles, glucose 6-phosphate 12 μmoles, glucose-6-phosphate dehydrogenase 2 u, penicillin G 500 u and streptomycin 500 μg in a final volume of 5 ml of Krebs-Ringer phosphate buffer (pH 7.4). Prior to incubation, 0.1 ml of stock solution of radioactive substrate was evaporated to dryness under N_2 gas and was suspended in the reaction mixture. The mixtures were saturated with 95% O_2-5% CO_2 gas, capped and incubated at 37°C for 2 hr in a shaking incubator. The reaction was stopped by the addition of 4 volumes of chloroform-methanol (2 : 1 v/v). As controls, the incubation mixture without skin tissue or with added boiled skin tissue was treated in the same way.

Whole animal studies. The four-week-old castrated male rats and three-day-old male newborn rats were used. The hair of the experimental sites of the adult rat skin was plucked as in the *in vitro* studies. In each experiment three animals were used. At 60 min after the administration of the corresponding androgen, the animals were sacrificed by decapitation and the tissues were collected. The dorsal and preputial skin was shaved with a safety razor and swabbed with acetone. A sample of 200mg of tissue was used in each experiment. Tissues were minced into the smallest pieces in 10 ml of chloroform-methanol (2 : 1 v/v), and the extraction of the lipid fraction was done as described below. Nuclei were prepared following the method outlined in Fig. 1, and the lipid extraction was done by the addition of 4 volumes of chloroform-methanol (2 : 1 v/v).

A 5 μCi of T-7-^{3}H or DHT-(1α, 2α-^{3}H) dissolved in 0.1 ml of ethanol was injected intraperitoneally, and the dorsal skin, preputial skin and preputial gland were removed for the uptake study of the corresponding labeled androgen. The analysis of ^{3}H-labeled androgen metabolites was performed on the dorsal skin of male adult and newborn rats sacrificed after T-7-^{3}H administration. The uptake of T-7-^{3}H into the nuclei was examined in the dorsal skin, preputial skin and the liver of newborn rats.

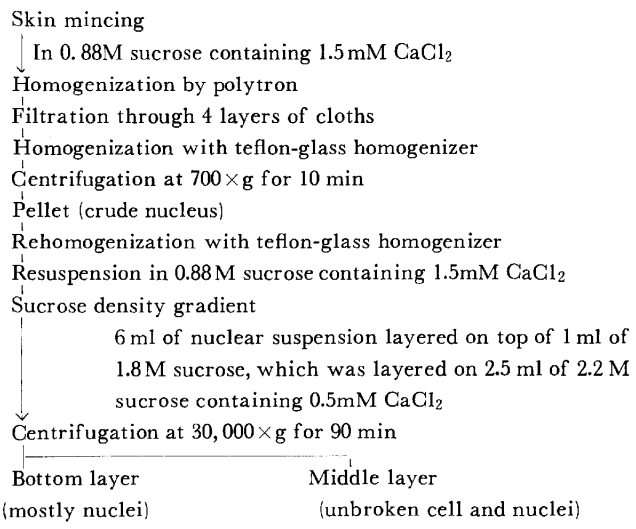

Fig. 1. Preparation procedure of nuclei from newborn rat skin.

Extraction and separation of androgens. The lipids were extracted and backwashed for neutrality by the procedure described by Folchi *et al* (5). The extract was evaporated to dryness under N_2 and the residue was taken up in 2 ml of chloroform and stored under N_2 at −20°C. Reference experiments were performed for zero time incubation. Aliquots of lipid fraction were divided by the n-hexane-methanol (4 : 1 v/v) system. The methanol layer was evaporated to dryness. The residue was redissolved in 0.5 ml of ethanol for the thin layer chromatograph.

Chromatographic procedure. Aliquots of the radioactive extracts (containing 10,000–20,000 dpm of ^{14}C) in ethanol were chromatographed on the thin layer plates of silica gel with a mixture containing 20 μg each of T, dihydrotestosterone, androsterone, androstandiol, epiandrosterone, androstenedione and androstandione as cold carriers. The plates were developed by ascending chromatography in chloroform-methanol (99 : 1, v/v) system (6).

After development the spots of the standard carriers were identified under ultraviolet light or with I_2 gas. In one experiment the spots were located on x-ray film exposed to the plate, as shown in Fig. 2.

DHT was separated from androsterone and epiandrosterone by the following method. The area corresponding to DHT migration was eluted and rechromatographed in dichlorethane-ether (9 : 1, v/v) for three successive migrations. These two systems permitted the complete separation of DHT from T, androsterone and epiandrosterone.

The spots were scraped individually and transferred to counting vials. The vials were filled with 1 ml of methanol and 10 ml of 0.4% diphenyloxazol and 0.01% dimethyl-POPOP in toluene and assayed for radioactivity in a liquid scintillation spectrometer.

The average recovery of radioactivity was 85% on the thin layer plates. To estimate the amount of each product formed in these experiments, the amount of T used as substrate was multiplied by the ratio of radioactivity in the product to that in all metabolites.

Recrystallization. The identities of conversion products were confirmed in one experiment by adding the corresponding cold carriers after they were isolated by thin layer chromatography, and recrystallizing these materials in four different systems (methanol, acetone, ethylether-hexane and dichloromethane-hexane.) (Table 1).

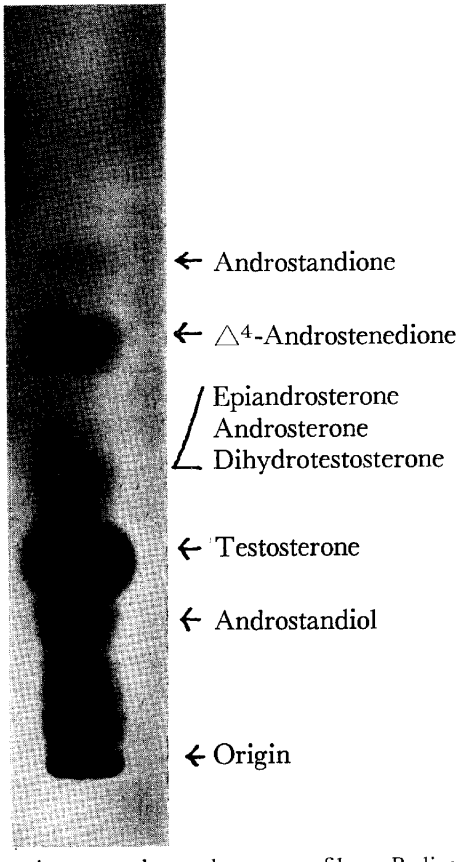

Fig. 2. Radioactive spots detected on x-ray film. Radioactive metabolites of testosterone were separated on thin layer chromatography. X-ray film was exposed to a chromatogram for 24 hr.

RESULTS

Recrystallization of the conversion products in the four different solvent systems produced almost similar results (Table 1), and this confirmed the identity of the androgens isolated by thin layer chromatography.

TABLE 1. IDENTIFICATION OF THE METABOLITES OF TESTOSTERONE

Crystallization	Solvent	Specific activity (dpm/mg of metabolite)			
		Androstandiol	Dihydrotestosterone	$\triangle^4$-Androstenedione	Androstandione
1	Methanol	30.2	39.5	143.4	10.0
2	Acetone	19.1	37.4	120.6	7.9
3	Ethylether-hexane	29.6	39.5	110.5	8.3
4	Dichlormethane-hexane	28.6	38.7	109.0	12.2

The metabolites isolated by thin layer chromatography from minced rat skin incubated with ^{14}C-testosterone were confirmed by recrystallization. Each radioactive metabolite was added to 200 mg of the corresponding carrier steroid and recrystallized.

The results of the chromatographic identification and conversion rate (%) of T-^{14}C metabolites in the *in vitro* experiments (number of animals used: 5) are shown in Table 2. The main *in vitro* metabolites of T were DHT and androstandiol in both dorsal and preputial skins, but the conversion rate of T was higher in preputial skin.

In vitro DHT formation from T-^{14}C was observed in the rat skin at various anatomical sites (number of animals used: 3) (Table 3). The prepuce showed a higher formation of DHT than that of the dorsum and the sole. The sole, where

TABLE 2. CONVERSION RATES OF TESTOSTERONE TO OTHER ANDROGENS

Skin area	No. of animals	Androstandiol (%)	Testosterone (%)	Dihydrotestosterone (%)	$\triangle^4$-Androstenedione (%)	Androstandione (%)
Dorsal	1	3.98	86.03	3.90	2.30	0.32
	2	2.47	88.86	5.19	2.20	1.09
	3	1.94	91.21	3.77	2.53	0.49
	4	2.20	89.01	5.94	1.39	0.83
	5	3.84	85.28	7.07	2.19	0.77
	Mean±SD	2.88±0.95	88.07±2.41	5.17±1.39	2.12±0.43	0.70±0.30
Prepuce	1	20.38	66.23	4.02	2.18	0.38
	2	2.13	85.04	7.78	3.57	2.13
	3	8.91	77.01	10.10	3.50	0.10
	4	7.32	57.59	19.71	7.93	2.50
	5	17.00	62.31	15.41	3.21	1.84
	Mean±SD	11.14±7.42	69.63±11.20	11.40±6.21	4.07±2.22	1.39±1.08

^{14}C-Testosterone was incubated with minced rat skin (500 mg). Conversion rates were expressed as percent of radioactivity of metabolites to that of total incorporated radioactivity.

TABLE 3. ^{14}C-TESTOSTERONE METABOLISM *in vitro* IN MINCED MALE RAT SKIN FROM VARIOUS SITES

Site	dpm recovered in dihydrotestosterone fraction/mg tissue	Conversion rate (%) (dpm in dihydrotestosterone fraction/total dpm recovered)
Back	6.42	2.49
Prepuce	47.98	7.78
Sole	30.00	2.36

Each value represents the mean of 3 experiments (3 rats).

no pilosebaceous unit is present, metabolized T to the same extent as the dorsum.

The *in vitro* incubation of T-^{14}C with boiled skin tissue or without skin produced only negligible amounts of ^{14}C-labeled T metabolites. The time course of the *in vitro* formation of T metabolites (number of animals used : 1, mean of three determinations) during the incubation of dorsal skin with T-^{14}C appeared to be linear for 2 hr. The relationship between tissue weight and the rates of conversion of T-^{14}C to various metabolites are shown in Fig. 3 (number of animals used : 1, mean of three determinations). The formation of T metabolites as a function of T-^{14}C added was as shown in Fig. 4 (number of animals used : 1, mean of three determinations). The concentration of the substrate T-^{14}C was varied from 1×10^{-7} M to 40×10^{-7} M (number of animals used : 1, mean of three determinations).

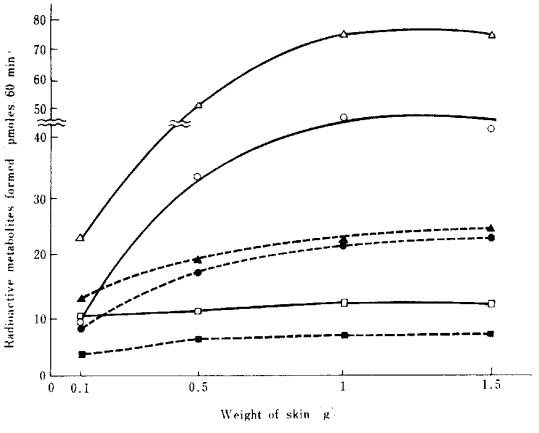

Fig. 3. The rate of formation of testosterone metabolite increased in proportion to the amount of tissue used. ^{14}C-Testosterone was incubated at 1.1×10^6 cpm (9.5 n moles). ○——○, Dihydrotestosterone; △——△, Sum of DHT, androstandiol, androstandione; ●·····●, Androstandiol; □——□, Δ^4-Androstenedione; ▲——▲, Sum of Δ^4-androstenedione, androstandione; ■·····■, Androstandione.

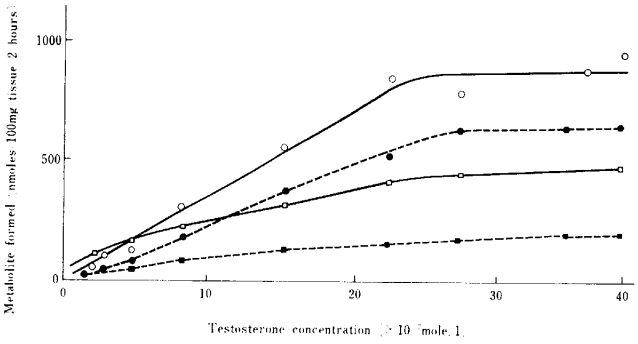

Fig. 4. The rate of formation of testosterone metabolites increased in proportion to the concentration of the substrate. ^{14}C-Testosterone was incubated with 500 mg of minced skin. ○——○, Dihydrotestosterone; ●——●, Androstandiol; □——□; △^{4}-Androstenedione; ■·····■ Androstandione.

When the pH of the medium was varied from 6.0 to 9.0 (Krebs-Ringer phosphate buffer between pH 6.0 and 8.0, and Krebs-Ringer-0.05 M tris-HCl between pH 8.0 to 9.0). The rate of DHT and androstenedione formation *in vitro* was maximum at pH 7.5 (number of animals used : 1, mean of three determinations) (Fig. 5).

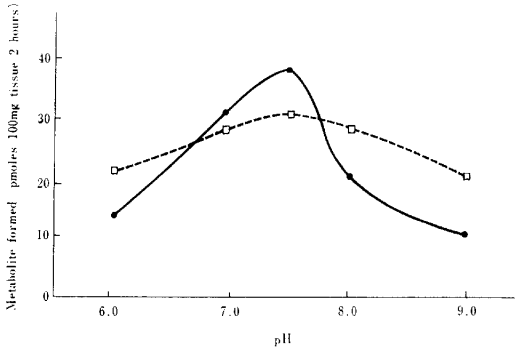

Fig. 5. pH dependence of the formation of testosterone metabolites. Krebs-Ringer-0.1 M phosphate buffer (pH 6.0-8.0) and Krebs-Ringer 0.05 M tris-HCl buffer (pH 8.0-9.0) were used. ●——●, Dihydrotestosterone; □·····□, △^{4}-Androstenedione.

Fig. 6 shows the results of the *in vivo* uptake of T-^{3}H by rats (number of animals used : 3) sacrificed at 60 min after intraperitoneal T-^{3}H injection. T-^{3}H uptake in normal male rats was 10.3 by dorsal skin, 27.3 by preputial skin, 37.9 by the liver, 37.2 by the preputial gland and 8.6 by blood, whereas the corresponding T-^{3}H uptake values of the castrated rats were 64.8, 191.7, 987.2, 457.3 and 51.1 (dpm/mg dry tissue weight), respectively.

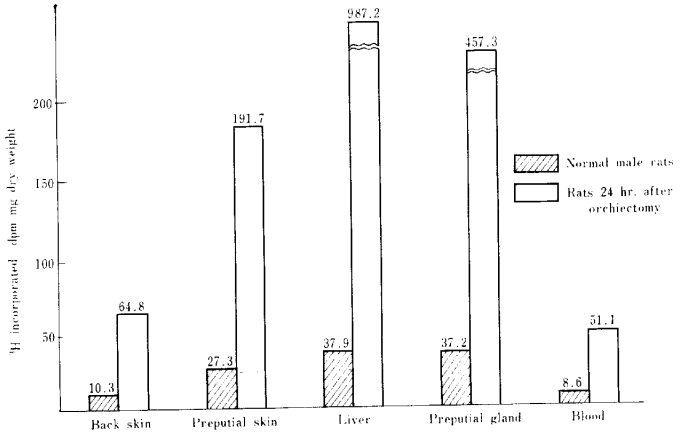

Fig. 6. *In vivo* uptake of ^{3}H-testosterone into the skin and other tissues of intact and castrated rats.

As shown in Table 4, the *in vivo* uptake of DHT-^{3}H in the end organ was significantly lower than that of T-^{3}H.

The main metabolites in the adult rat skin *in vivo* were androstandiol and $\triangle^4$-androstenedione, but in newborn rats DHT was the predominant metabolite *in vivo*. The total *in vivo* uptake of T-^{3}H in adult rat skin was 3 times higher than that in newborn rats (Table 5).

TABLE 4. *In vivo* UPTAKE OF ^{3}H-TESTOSTERONE AND ^{3}H-DIHYDROTESTOSTERONE INTO THE RAT SKIN AT VARIOUS SITES. (dpm/mg DRY TISSUE WEIGHT)

Androgens	Back skin		Prepuce		Preputial gland	
	Normal	Castrated	Normal	Castrated	Normal	Castrated
^{3}H-Testosterone	10.3±0.53	64.8±1.50	27.3±3.1	191.7±20.3	37.2±5.10	457.3±28.1
^{3}H-Dihydrotesto-sterone	0.59±0.29	0.13±0.10	0.40±0.12	34.60±44.8	0.31±0.03	41.17±49.0

Each value represents the mean and its standard deviation of 3 experiments (3 rats).

TABLE 5. COMPARISON OF *in vivo* UPTAKE OF ^{3}H-TESTOSTERONE INTO THE DORSAL WHOLE SKIN BETWEEN ADULT AND NEWBORN RATS

Androgens	Newborn rats (dpm/mg protein)	%	Adult rats (dpm/mg protein)	%
Androstandiol	49.96	17.3	116.53	15.8
Testosterone	119.67	42.9	464.42	62.5
Dihydrotestosterone	76.01	26.9	26.08	3.5
$\triangle^4$-Androstendione	27.04	8.8	105.86	14.2
Androstandione	11.56	4.0	30.43	4.1
Total	284.24		743.32	

The total *in vivo* uptake of T-^{3}H by nuclei of newborn rat tissues was 13,230 dpm/mg protein in preputial skin, 9,053 dpm/mg protein in dorsal skin and 640 dpm/mg protein in the liver. The nuclei of the skin showed a higher uptake than those of the liver. The fraction of DHT was 11.8% in preputial skin and 6.4% in dorsal skin and 11.4% in the liver. DHT + androstandiol was 42.8% in preputial skin, 24.3% in dorsal skin and 27.8% in the liver (Table 6).

TABLE 6. UPTAKE OF ^{3}H-TESTOSTERONE INTO THE NUCLEI OF NEWBORN RAT SKIN

Androgens	Prepuce		Back		Liver	
	dpm/mg protein	%	dpm/mg protein	%	dpm/mg protein	%
Androstandiol	4115	31.0	1715	17.9	105.3	16.4
Testosterone	6354	48.1	5515	57.6	400.0	62.4
Dihydrotestosterone	1553	11.8	615	6.4	73.3	11.4
Androstenedione	708	5.4	908	9.5	42.7	6.7
Androstandione	500	3.8	400	4.2	20.0	3.1

Nuclei were prepared at 60 min after the intraperitoneal administration of ^{3}H-testosterone.

DISCUSSION

The present *in vitro* stoichiometric studies strongly suggested the involvement of 5α-reductase and other enzymes in the metabolism of T in rat skin in the anagen hair cycle (Figs. 3, 4, 5). The main metabolic events were 5α-reduction of the $\triangle^4$-double bond, oxidation of the 17β-hydroxyl group of T and its metabolites and the reduction of 3-keto group of the 5α-steroids. Recently Rampini *et al.* (1) reported that the metabolism of T fluctuated during the hair cycle in the dorsal skin of rats. The present study (Table 2) confirmed the presence of the three main metabolites in the anagen hair cycle of the rat dorsal skin.

5α-Androstan-17β-ol-3-one (DHT) has been recognized as physiologically potent androgen in rat prostate by Bruchovsky and Wilson (2) and Anderson and Liao (7). In this study DHT was found in various areas of the rat skin.

The 5α-reductase activity of the whole skin might be attributed to the epidermis, hair follicles, sebaceous gland, fibroblasts and other dermal cellular elements. The significance of 5α-reductase in the epidermis was evident in the results obtained from the plantar skin (Table 3) where neither sebaceous gland nor hair follicle is present and which exhibited the same activity as dorsal skin. Androgen-dependent skin, such as preputial skin showed a higher activity of 5α-reductase *in vitro* than dorsal skin. Some reservations must be taken into account as this study was performed with a much higher concentration of T than in normal male rats (5.5 ng/ml blood) (8) for the convenience of the experiment.

Unhjem, Tveter and Aakvaag (9) has reported that among various androgens in the blood, T was selectively taken up into the prostate gland. The results

of the present experiment indicate the selective uptake of T by dorsal and preputial skins, as well as by the preputial gland (Table 4). It is of interest that the skin took up T selectively rather than DHT as is true in the prostate gland which is a typical androgen-dependent organ.

The metabolic pattern was different *in vitro* (Table 2) and *in vivo* (Table 5) in adult rats. The main metabolites were androstandiol and $\triangle^4$-androstenedione *in vivo* whereas DHT and androstandiol were predominant *in vitro*. In the newborn rats DHT was the predominant metabolite *in vivo* (Table 5). Whether the lower levels *in vivo* of DHT (Table 5) in adult rat skin reflect the active catabolism of DHT in adults requires further investigations. $\triangle^4$-Pathway is considered to be more active *in vivo* in adults than in newborns. We suppose, therefore, that the hormonal milieu, to which the experiment sites were exposed, might influence T metabolism in skin.

Bruchovsky and Wilson (10) have reported that the percentages of radioactivity recovered in DHT fraction after injection of radioactive T into rats were 20 to 30% in the preputial gland, seminal vesicles and prostate, but less than 1% in the gut, liver, lungs and muscles. On the other hand, DHT was the only metabolite identified in cell nuclei and constituted 75% of radioactivity in the nuclear fraction at 30 min after injection of T-^{3}H.

In the present study newborn rats were used for examining DHT formation by nuclei because the nuclei of the skin were far more easily prepared in newborns than in adult rats. The combined DHT and androstandiol percentage detected in newborn nuclei was 42.8% in preputial skin, 24.3% in dorsal skin and 27.8% in the liver (Table 6). Only a small amount of radioactivity was incorporated in the liver nuclei. The accumulation of DHT in the nuclei after the *in vivo* T-^{3}H administration was higher in androgen-dependent areas though at a lower concentration than in the accessory organs.

The increased uptake of ^{3}H-T after castration (Fig. 6) seems to suggest the possible existence of an androgen pool in each organ. The size of the pool might differ from organ to organ and from area to area. Further investigations are also required on the dynamic *in vivo* fate of testosterone administered exogeneously.

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REFERENCES

1. Rampini, E., Voigt, W., Davis, B. P., Moretti, G. and Hsia, S. L.: Metabolism of testosterone-4-^{14}C by rat skin. Variations during the hair cycle. *Endocrinology* **89**, 1507–1514, 1971.
2. Bruchovsky, N. and Wilson, J. D.: The conversion of testosterone to 5α-androstan-17β-

ol-3-one by rat prostate '*in vivo*' and '*in vitro*'. *J. Biol. Chem.* **243**, 2012-2021, 1968.
3. Sansone, G. and Reisner, R. M.: Differential rates of testosterone to dihydrotestosterone in acne and in normal human skin—a possible pathogenic factor in acne. *J. Invest. Dermatol.* **56**, 366-372, 1971.
4. Adachi, K., Takayasu, S., Takashima, I, Kano, M. and Kondo, S.: Human hair follicles. *J. Soc. Cosmet. Chem.* **21**, 901-924, 1970.
5. Folchi, J., Lees, M. and Stanley, G. H. S.: A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497-509, 1957.
6. Gomez, E. C. and Hsia, S. L.: *In vitro* metabolism of testosterone-4-^{14}C and $\triangle^4$-androstene-3, 17-dione-4-^{14}C in human skin. *Biochemistry* **7**, 24-32, 1968.
7. Anderson, K. M., and Liao, S.: Selective retention of dihydrotestosterone by prostatic nuclei. *Nature* **219**, 277-279, 1968.
8. Coyotupa, J., Paylow, A. F. and Kovacic, N.: Serum testosterone and dihydrotestosterone levels following orchiectomy in the adult rat. *Endocrinology* **92**, 1579-1581, 1973.
9. Unhjem, O., Tveter, K. J. and Aakvaag, A.: Preliminary characterization of an androgen-macromolecular complex from the rat ventral prostate. *Acta Endocrinol.* **62**, 153-164, 1969.
10. Bruchovsky, N. and Wilson, J. D.: The intranuclear binding of testosterone and 5α-androstan-17β-ol-3-one by rat prostate. *J. Biol. Chem.* **243**, 5953-5956, 1968.