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METABOLISM OF TESTOSTERONE IN HYPOTHALAMUS OF MALE RAT

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SUMMARY

Metabolism of testosterone in the hypothalamus of the male adult rat was studied *in vitro*. The whole homogenate of the hypothalamus in the presence of $NADPH_2$ generating system converted testosterone mainly to dihydrotestosterone and 5α-androstan-3α-17β-diol. The reduction of testosterone to dihydrotestosterone was irreversible, while that of dihydrotestosterone to 5α-androstan-3α-17β-diol was reversible. Michaelis constant for the 5α-reductase in the hypothalamus was $7{\cdot}4 \times 10^{-7}$ M.

The administration of testosterone propionate caused no significant change of the 5α-reductase activity in the hypothalamus, in contrast to the marked induction of 5α-reductase activity in the prostate. Furthermore, the 5α-reductase appeared to be widely distributed in the subcellular particulate components in the hypothalamus, being located mainly in the nuclear fraction in the prostate.

These results suggest that there is some difference in the characteristics of the metabolism of testosterone in the hypothalamus and in the prostate.

INTRODUCTION

The hypothalamus has been considered to be a target tissue of androgens [1]. A steroid 5α-reductase has been also reported in the hypothalamus of the rat [2–4]. In recent years, studies *in vivo* have provided evidence that some 5α-reduced androgens are more potent than testosterone* with regard to the suppression of LH secretion [5–6]. These studies *in vivo* and *in vitro* suggest that 5α-reduction of testosterone in the hypothalamus has some physiological significance.

However, biological characteristics of androgen metabolism in the hypothalamus have not been adequately studied. The present experiments study testosterone metabolism in the hypothalamus of the male adult rat, in comparison with that in the prostate.

EXPERIMENTAL

Animals and preparation of tissues

Sprague–Dawley male rats weighing 200–300 g were used. All rats were housed under controlled lighting (light on 0500–2200). Animals were killed by decapitation between 1000 and 1100 h and the incubation was begun within 60 min after death. The preparation of the tissue was carried out at 0°C. The tissue block containing the hypothalamus was bounded anteriorly by the optic chiasma, posteriorly by the mammillary bodies, laterally by the hypothalamic sulci, and horizontally 2–3 mm under the basal surface. The block of hypothalamic tissue weighed approximately 25 mg. The rat ventral prostate was dissected free of capsules.

The conditions for incubation in vitro

The hypothalamic or prostatic tissues were homogenized in 19 vol. of 0·25 M sucrose containing 0·001 M EDTA in an all glass Potter–Elvehjem homogenizer at 0°C. One ml of the homogenate was added to a 25 ml Erlenmeyer flask containing 1 ml of 0·3 M potassium phosphate buffer, pH 7·4 supplemented with [1,2-^{3}H]-testosterone at various concentrations, NADP ($2{\cdot}6 \times 10^{-3}$ M), glucose (1 mg/ml), glucose 6 phosphate (2 mg/ml), and a crystal of glucose 6 phosphate dehydrogenase. Incubation was carried out at 37°C in a Dubnoff shaking water bath under an atmosphere of 95% O_2–5% CO_2 for various periods of time. The reaction was stopped by chilling on ice and by the addition of 5 vol. of chloroform–methanol (4:1 v/v). The metabolites were separated by column chromatography on partially esterified Amberlite-IRC 50 [7]. The mobile phase was ethanol–benzene–cyclohexane–water (50:350:200:2·8 by vol.) for the first chromatography, and ethanol–benzene–cyclohexane–water (25:50:225:1 by vol.) for the second chromatography. Identification of radioactive steroids was confirmed by recrystallization to constant specific radioactivity.

* Abbreviations: Androstenedione = 4-androstene-3,17-dione; Androstanedione = 5-androstane-3,17-dione; Dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one; Testosterone = 17β-hydroxy-4-androsten-3-one.

Subcellular fractionation

Approximately 150 mg aliquots of hypothalamic or prostatic tissues were homogenized in 5 ml of 0·88 M sucrose containing 1·5 mM $CaCl_2$ in a Potter all glass homogenizer. The homogenate was sedimented in a refrigerated centrifuge at 800 *g* for 10 min. The pellet was rehomogenized in 1 ml of this homogenization buffer in a teflon glass homogenizer and layered over a discontinuous sucrose gradient consisting of 2·5 ml of 2·2 M sucrose containing 0·5 mM $CaCl_2$ and 1·0 ml of 1·8 M sucrose containing 0·5 mM $CaCl_2$. It was sedimented at 39,000 *g* for 90 min in RP 40 A rotor of a Hitachi ultracentrifuge at 0–2°C, as described by Maggio, Siekevitz and Palade[8]. The resulting sediment, which was designated as the nuclear fraction, was resuspended in 3 ml of 0·88 M sucrose containing 1·5 mM $CaCl_2$. By light microscopy this nuclear preparation contained almost pure nuclei with a few cytoplasmic tags. For the preparation of cytoplasmic components, the supernatant of the 800 *g* centrifugation was centrifuged at 10,000 *g* for 10 min in a No 3 rotor in a Tominaga refrigerated centrifuge. The pellet designated as the mitochondrial fraction was washed twice with 2 ml of the homogenization buffer. The supernatant fraction was centrifuged at 105,000 *g* for 60 min in a PR-50-123 rotor in the Hitachi ultracentrifuge. The pellet was designated as the microsomal fraction and the supernatant as the cytosomal fraction, respectively. Each fraction was incubated with [1,2-^{3}H]-testosterone as described above.

Androgen treatment

Fourteen adult male rats were castrated under ether anesthesia, and a second group received daily injection of 2 mg of testosterone propionate subcutaneously. They were killed on the seventh day after treatment. Control intact rats were killed on the same day. Tissue from two animals were used for each incubation.

Assay for protein, RNA, and DNA

Protein was determined by the method of Lowry *et al.*, with BSA as standard [9], RNA was measured by the method of Munro *et al.*, with yeast RNA as standard [10]. DNA was determined by the method of Burton with calf thymus DNA as standard [11].

Radioactivity was measured in a Packard Tricard Scintillation Spectrometer Model 3320 using a toluene based solution containing 4 g PPO, 100 mg POPOP in 1000 ml toluene.

Chemicals

[1,2-^{3}H]-testosterone (S.A. 48 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. [1,2-^{3}H]-Dihydrotestosterone and [1,2-^{3}H]-5α-androstan-3α-17β-diol were obtained from the incubation of [1,2-^{3}H]-testosterone with the homogenate of rat ventral prostate and by chromatographic purification. Non-radioactive steroids were obtained from Steraloids, Inc. (Paulig, New York). NADP, glucose 6 phosphate, glucose 6 phosphate dehydrogenase, BSA, calf thymus DNA were purchased from Sigma (St. Louis, Missouri 63178, U.S.A.). All other chemicals were of analytical grade.

RESULTS

Analysis and identification of formed steroids

Figure 1 shows a typical elution pattern by column chromatography on partially esterified Amberlite IRC-50. Good separation was obtained of metabolites of testosterone formed after incubation with the hypothalamus or the prostate preparation. Three prominent peaks of radioactivity were consistently identified in the areas corresponding to dihydrotestosterone, testosterone, and 5α-androstan-3α,17β-diol on the first chromatography. The identity of these metabolites separated was confirmed by recrystallization experiments as shown in Table 1. The first chromatographic separation seemed to provide an adequate means of separation of dihydrotestosterone and 5α-androstan-3α-17β-diol. Furthermore, the elution volume of these 7 steroids in this chromatographic system was constant and the recovery of initial radioactivity averaged 83% (70–93%) after the first

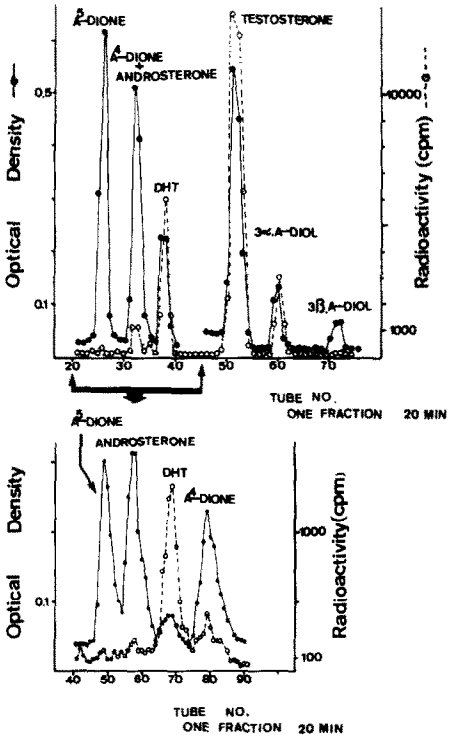

Fig. 1. Separation of metabolites of [^{3}H]-testosterone by column chromatography on partially esterified Amberlite IRC-50. The whole homogenate of the rat prostate was incubated with [^{3}H]-testosterone (5×10^{-7} M) for 30 min in the presence of $NADPH_2$ generating system. First chromatography: Eluent—EtOH–benzene–cyclohexane–H_2O (50:350:200:2·8 v/v). Second chromatography: Eluent—EtOH–benzene–cyclohexane–H_2O (25:50:225:1 v/v). A^5-DION: Androstanedione. A^4-DION: Androstenedione. 3α-A-diol: 5α-androstan-3α,17β-diol. 3β-A-diol: 5α-androstan-3β.17β-diol.

Table 1. Recrystallization of ^{3}H-labelled steroids identified by first chromatography as dihydrotestosterone, testosterone and 5α-androstan-3α-17β-diol

	Dihydrotestosterone S.A. d.p.m./μg		5α-Androstan-3α-17β-diol S.A. d.p.m./μg		Testosterone S.A. d.p.m./μg	
	mother liquid	crystal	mother liquid	crystal	mother liquid	crystal
Starting material	29·1		4·36		154	
Methanol-H_2O	29·4	27·9	4·81	4·45	153	157
Ethanol-H_2O	29·7	32·7	4·23	4·52	150	148
Acetone-H_2O	27·0	26·9	4·68	4·36	144	150

Materials tentatively identified by first chromatography as dihydrotestosterone, testosterone, and 5α-androstan-3α-17β-diol were obtained from the incubation of the whole homogenate of hypothalamus and added to the appropriate carrier steroids for recrystallization.

chromatographic separation. These results imply that our chromatographic procedures are suitable for the studies in androgen metabolism in the target tissues.

Metabolites identified after 120 min incubation of whole homogenate of the hypothalamus with testosterone

Radioactivity present in the products of incubation after 120 min incubation of the rat hypothalamus in the presence or absence of the $NADPH_2$ generating system is shown in Table 2 in terms of the percentage of the substrate radioactivity. In addition to dihydrotestosterone and 5α-androstan-3α-17β-diol as major products, a small amount of radioactivity was incorporated into androsterone and androst-4-ene-3,17-dione in the presence of the $NADPH_2$ generating system. Some formation of the 5α-reduced steroids was observed during the incubation in the absence of the $NADPH_2$ generating system. Boiled tissue of hypothalamus in the presence of the $NADPH_2$ generating system could not metabolize testosterone to other steroids. These results showed that the 5α-reductase in the hypothalamus is dependent on $NADPH_2$, as in the other target tissues previously described by several authors [12, 13].

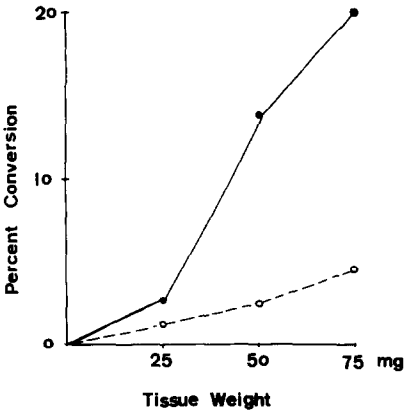

Fig. 2. Formation of 5α-reduced steroids from [^{3}H]-testosterone (5×10^{-8} M) by the rat hypothalamus as a function of tissue weight. Varying amounts of the hypothalamus homogenate were incubated for 2 h in the presence of $NADPH_2$ generating system. Dihydrotestosterone: ---O---, 5α-androstan-3α,17β-diol: ——●——.

Formation of dihydrotestosterone and 5α-androstan-3α-17β-diol from incubation of the hypothalamus with testosterone as a function of tissue weight and incubation time

The rate of formation of the sum of dihydrotestosterone and 5α-androstan-3α-17β-diol was nearly proportional to the tissue weight within the range of 25 to 75 mg as shown in Fig. 2. Time course study of the formation of dihydrotestosterone and 5α-androstan-3α-17β-diol from testosterone using 50 mg of whole homogenate of hypothalamus revealed that the total formation of 5α-reduced steroids was proportional to time up to 120 min, and formed dihydrotestosterone was reduced to 5α-androstan-3α-17β-diol (Fig. 3). A comparative study of time course using 50 mg of whole homogenate of the prostate was also performed (Fig. 3). A rapid increase in labelled dihydrotestosterone occurred early in the incubation with the prostate preparation. However, the total formation of 5α-reduced steroids was proportional to time only up to 60 min under the incubation conditions although 5α-androstan-3α-17β-diol was steadily formed.

Table 2. Metabolites isolated from incubation of rat hypothalamus for 2 h under various conditions

Metabolites	% Conversion of testosterone in intact homogenate		% Conversion of testosterone in boiled homogenate
	$NADPH_2$ generating system (+)	$NADPH_2$ generating system (−)	$NADPH_2$ generating system (+)
Dihydrotestosterone	2·77	1·60	0·01
5α-Androstan-3α-17β-diol	14·70	n.d.	0·01
5α-Androstan-3β-17β-diol	0·01	n.d.	n.d.
Androstenedione	0·72	0·3	n.d.
Androsterone	0·01	n.d.	n.d.
Androstanedione	0·01	n.d.	n.d.
Testosterone (unmetabolized)	61·00	70·0	81·10

[^{3}H]-testosterone ($2{\cdot}5 \times 10^{-8}$ M) was incubated with the whole homogenate of the hypothalamus in the presence or absence of a $NADPH_2$ generating system. Boiled homogenate was also used for experiment in the presence of a $NADPH_2$ generating system.

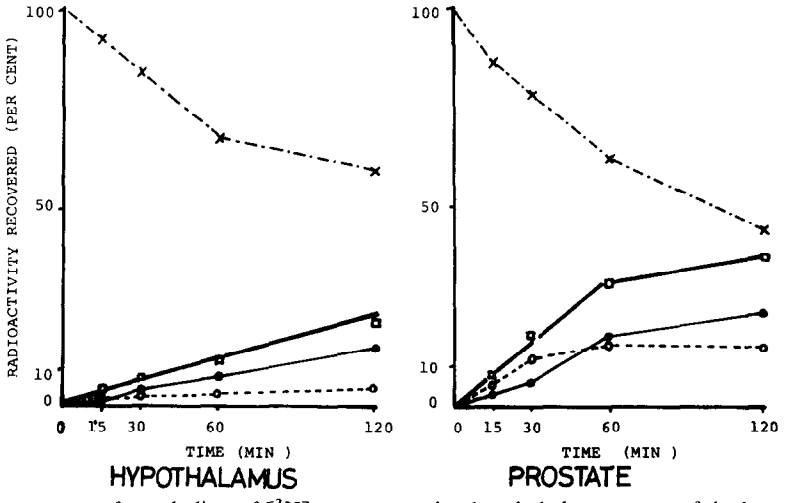

Fig. 3. Time course of metabolism of [^{3}H]-testosterone by the whole homogenate of the hypothalamus and prostate. Fifty mg of the homogenate of hypothalamus and the prostate were incubated with [^{3}H]-testosterone ($6{\cdot}8 \times 10^{-8}$ M) for various time. Testosterone: -—×—-, Dihydrotestosterone: ——O——. 5α-androstan-3α,17β-diol: ——●——. The sum of the formed 5α-reduced steroids: ——□——.

Effect of testosterone concentration in the hypothalamus incubation

Figure 4 shows the effect of testosterone concentration on the formation of 5α-reduced steroids between $2{\cdot}5 \times 10^{-8}$ M and $1{\cdot}25 \times 10^{-6}$ M. Michaelis constant for 5α-reductase in the hypothalamus derived from Lineweaver–Burk plot was $7{\cdot}4 \times 10^{-7}$ M. This value is similar to that in other androgen dependent tissues, such as rat prostate [14] and human skin [13].

Metabolites identified after incubation of whole homogenate of the hypothalamus with dihydrotestosterone or 5α-androstan-3α-17β-diol as a substrate

To ascertain whether reduction of testosterone to 5α-reduced steroids was reversible or not, experiments using dihydrotestosterone or 5α-androstan-3α-17β-diol as a substrate were performed (Table 3). Neither of these 5α-reduced steroids were converted to testosterone. 5α-androstan-3α-17β-diol was converted to dihydrotestosterone to some extent. These results indicate that 5α-reduction is irreversible, while reduction at C-3 is reversible.

Subcellular distributions of 5α-reductase in the hypothalamus or the prostate

The results of subcellular localization of the 5α-reductase of the hypothalamus and the prostate are shown in Table 4. In the hypothalamus the value of the 5α-reductase activity, expressed as the amount of the 5α-reduced steroids formed per mg protein/h, was highest in the microsomal fraction. The nuclear fraction also possessed a higher enzyme activity than whole homogenate. Furthermore, the 5α-reductase activity relative to mg protein increased during purification of the nuclear fraction.

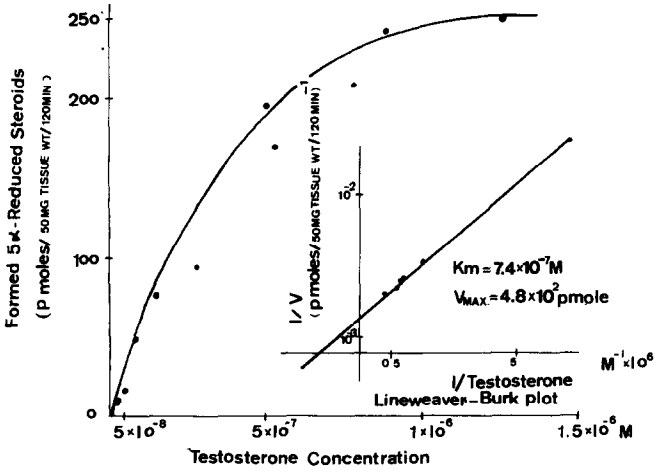

Fig. 4. Effect of concentration of testosterone on the formation of 5α-reduced steroids by the hypothalamus homogenate. Fifty mg of the homogenate of the hypothalamus was incubated as described under "Materials and Methods" with varying amounts of [^{3}H]-testosterone. The estimated K_m value derived from Lineweaver–Burk Plot was $7{\cdot}4 \times 10^{-7}$ M.

Table 3. Metabolites formed after incubation of the whole homogenate of the hypothalamus with dihydrotestosterone or 5α-androstan-3α-17β-diol as a substrate

	Dihydrotestosterone	5α-Androstan-3α-17β-diol
Testosterone	n.d.*	n.d.
Dihydrotestosterone	17·3	3·5
5α-Androstan-3α-17β-diol	79·3	44·2
5α-Androstan-3α-17β-diol	n.d.	n.d.
Androstanedione	0·1	7·2
Androsterone	0·1	10·6
Androstenedione	n.d.	n.d.
Undetermined steroid†		15·3

[^{3}H]-Dihydrotestosterone ($1{\cdot}3 \times 10^{-8}$ M) and [^{3}H]-5α-androstan-3α-17β-diol were each incubated with 50 mg of homogenated hypothalamus tissue in the presence of $NADPH_2$ generating system. Each value was expressed in term of per cent conversion from substrate into metabolites.

* n.d.: not detected.

† This metabolite had a mobility on column chromatography identical with that of 5β-androstan-3α-17β-diol, but did not reveal constant radioactivity on recrystallization.

It was concluded that the 5α-reductase in the hypothalamus was localized not only in the microsomal fraction but also in the nuclear fraction. Considerable amounts of the 5α-reductase were recovered in the mitochondrial fraction. However, in this fraction, RNA to protein ratio as a characteristic parameter for contamination of the ribosome pellets was fairly high. Furthermore, the specific activity of the enzyme in this fraction was lower than that in the whole homogenate. Therefore, it can not be concluded that the 5α-reductase is present in the mitochondria itself. On the other hand, in the case of the prostate, nearly half the 5α-reductase is localized in the purified nuclear fraction, which possessed the highest relative specific activity. These results were in agreement with the observation by Shimazaki[15] and Wilson[14].

Effect of testosterone propionate on the 5α-reductase of the hypothalamus and of the prostate. The effect of pharmacological dose of testosterone propionate on the activity of the 5α-reductase of the hypothalamus and of the prostate was investigated. The results are summarized in Table 5. The activity of the 5α-reductase of the male rat hypothalamus did not significantly change by castration or testosterone replacement. In the prostate, the castration at 7 days resulted in a decrease in the 5α-reductase activity expressed in terms of formed 5α-reduced steroids/g of the prostate. However, the 5α-reductase activity relative per mg protein did not significantly change because of concomitant decrease in tissue weight. After testosterone replacement, the activity of the 5α-reductase was increased to a markedly greater level than the control value. These comparative studies suggest that there is some difference between the hypothalamus and the prostate in the regulation of the 5α-reductase activity by androgen.

Table 4. Intracellular distribution of 5α-reductase in the hypothalamus and prostate

	Whole homogenate	800 *g* Pellet	2·2 M Sucrose pellet	10,000 *g* Pellet	105,000 *g* Pellet	105,000 *g* Supernatant
Hypothalamus						
5α-reductase pmol:mg prot^{-1}.hr^{-1}	13·6	13·7(17·3%)	19·2(9·6%)	12·3(21·0)	28·9(22·4%)	nil
DNA μg.mg prot^{-1}	17·2	100·0(40·0%)	246·2(10·5%)	7·2(11·0%)	11·4(9·0%)	2·2(2·5%)
RNA μg.mg prot^{-1}	9·3	35·0(14·3%)	92·3(3·6%)	7·6(21·4%)	33·3(36·2%)	10·0(19·2%)
Prostate						
5α-reductase pmol.mg prot^{-1}.hr^{-1}	32·5	86·3(80·4%)	190·5(28·1%)	67·6(1·2%)	16·1(0·9%)	1·2(1·2%)
DNA μg.mg prot^{-1}	10·3	32·9(76·1%)	214·9(58·0%)	4·3(3·3%)	nil	nil
RNA μg.mg prot^{-1}	5·7	10·1(42·2%)	9·4(4·6%)	16·9(25·0%)	4·1(1·2%)	0·9(5·0%)

The subcellular fractions prepared as described under "Materials and Methods" were incubated with testosterone (5×10^{-7} M). The values in parentheses indicate percent recovery of subcellular fraction. Procedural loss of 5α-reductase during subcellular fractionation was not corrected except for the values in 800 *g* pellet and 2·2 M sucrose pellet, which were corrected by recovery of DNA.

Table 5. Effect of testosterone propionate on 5α-reductase activity of the hypothalamus and prostate

		Hypothalamus			Prostate			
			5α-Reductase				5α-Reductase	
Group	No. of studies	mg Protein g tissue wt^{-1}	pmol g tissue wt^{-1} h^{-1}	pmol mg protein^{-1} h^{-1}	wt mg	mg Protein g tissue wt^{-1}	pmol g tissue wt^{-1} h^{-1}	pmol mg protein^{-1} h^{-1}
Normal	4	98·1 ± 5·7	1748 ± 206	18·2 ± 3·0	342 ± 36	103·1 ± 4·4	2230 ± 257	21·4 ± 2·1
Castration	4	103·5 ± 1·3	1665 ± 199	16·2 ± 2·2	45·4 ± 8·9†	67·5 ± 1·6*	960 ± 255*	28·0 ± 6·4
Castration plus testosterone treatment	4	101·0 ± 2·0	1785 ± 125	17·8 ± 1·1	202·0 ± 7·0*	86·0 ± 6·8	48480 ± 5130‡	555·0 ± 67·5†

Animal treatment and incubation methods were as described under "Materials and Methods". Each value represents the mean ± S.E. of 4 separate studies. Tissues from two animals were used for each flask.

* $P < 0{\cdot}05$. † $P < 0{\cdot}01$. ‡ $P < 0{\cdot}001$: significance from normal value.

DISCUSSION

Several authors have observed that testosterone was converted to some 5α-reduced steroids in the rat hypothalamus [2–4]. However, quantitative analysis of this testosterone metabolism seems incomplete.

Although Jaffe reported that linear increase in the formation of dihydrotestosterone was observed for 120 min during incubation with mince of the rat hypothalamus, our time course study showed that the dihydrotestosterone formed was rapidly converted to 5α-androstan-3α-17β-diol and that the rate of the formation of dihydrotestosterone was levelled off by 30 min. These different results may be caused by the difference in the tissue preparation. Our results indicate that the quantitative analysis of the 5α-reduction in the hypothalamus should be on the basis of the formation of the 5α-reduced steroids (the sum of dihydrotestosterone and 5α-androstan-3α-17β-diol) as previously reported for the prostate [16].

Subcellular distribution of the 5α-reductase in the rat hypothalamus was in accord with the data reported by Rommerts and van der Molen[3]. However, they stated that the 5α-reductase activity in the nuclear fraction was due to contamination of the microsome fraction because of the diminution of the enzyme activity relative to DNA during purification of the nuclei. However, in an enzyme distributed both in nuclear fraction and in a cytoplasmic fraction, the enzyme activity relative to DNA in the nuclear fraction, is diminished during purification of nuclei because of removal of the enzyme in the cytoplasmic components. The diminution in the 5α-reductase activity relative to DNA during purification of nuclei was also shown with prostate tissue which was considered to have most of the 5α-reductase in the nuclei. Therefore, the 5α-reductase in the nuclear fraction of the hypothalamus seems to be associated with nuclei itself.

The 5α-reductase in the hypothalamus was not significantly changed by testosterone treatment in contrast to the prostate. Massa *et al.*[2] also reported that, in the rat hypothalamus, the effect of castration and treatment with testosterone on the 5α-reductase activity was not so large and not constant at all times.

Recent findings *in vivo* on the rat [5] and the human [17] have shown that some 5α-reduced androgens have a more potent effect on the LH suppression than testosterone. These observations and studies *in vitro* suggest that 5α-reduction of testosterone in the hypothalamus may have some biological significance with regard to LH feedback. 5α-androstan-3α-17β-diol, which is considered to be an inactive metabolite in the prostate [18], has been shown to be more potent on LH suppression than the other androgens [5]. The biological significance of 5α-androstan-3α-17β-diol, however, remains to be determined since, as shown in the present paper, 5α-androstan-3α-17β-diol is convertible to dihydrotestosterone in the hypothalamus.

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