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BINDING AND METABOLISM OF TESTOSTERONE IN THE RAT BRAIN DURING SEXUAL MATURATION—II. TESTOSTERONE METABOLISM

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SUMMARY

Slices of cerebral cortex, hypothalamus, hypophysis and prostate of three- to nine-week-old male rats castrated for 24–48 h, were incubated with tritiated testosterone. Steroids were identified in the incubation medium, and in the total protein fraction from the cytosol and nuclear fluid of the incubated tissues. With the four tissues, the metabolites found in the incubation medium presented no appreciable differences in relation to age. In the hypothalamus, more 17β-hydroxy-5α-androstane-3-one bound to the cytosol proteins was found in the immature than in the adult animals, but a small quantity of this compound bound to the nuclear proteins was always present regardless of age. In the hypophysis, less testosterone and more 17β-hydroxy-5α-androstane-3-one bound to the cytosolic and nuclear proteins were found during puberty, with more nuclear than cytosolic bound 17β-hydroxy-5α-androstane-3-one. These two series of results suggest a role of this compound as the active form of testosterone in the hypophysis.

INTRODUCTION

The *in vitro* metabolism of testosterone in nervous tissue was studied almost exclusively in adult animals: rat [1–5], dog [6] and cattle [7]. Jouan *et al.* [8, 9] were the only workers concerned with the nature of the androgens bound to the total cytosol and nuclear proteins of cerebral tissues of adult male rats. The same workers [10] found an increase of 5α steroid reductase and 3α hydroxysteroid dehydrogenase induced by castration in the hypophysis, but not hypothalamus, of both adult and young (22 days) rats. Massa *et al.* [11] measured 17β-hydroxy-5α-androstane-3-one (DHT‡) in the incubation media of nervous and hypophyseal tissues of rats aged 7–60 days.

In the preceding paper, we have shown that there was an increase in the quantity of androgens bound to the total protein fraction of the hypophyseal cytosol in puberty [12]. In the present study, an attempt was made to identify the specific steroids involved. The results from the hypophysis and hypothalamus were compared with those from the cerebral cortex which was used as a reference tissue, and from the prostate gland used as a classical target tissue for androgens. We have demonstrated that if the metabolites present in the incubation medium showed no major difference with age, the proportion of DHT to testosterone bound to the cytosol and nuclear proteins of the rat hypophysis and hypothalamus showed a direct relationship to the animal's maturity.

‡ Testosterone: 17β-hydroxy-4-androsten-3-one. DHT: 17β-hydroxy-5α-androstan-3-one. Δ^4-androstenedione: 4-androstene-3,17-dione. Isoandrosterone: 3β-hydroxy-5α-androstan-17-one. β DHT: 17β-hydroxy-5β-androstan-3-one. Etiocholanolone: 3α-hydroxy-5β-androstan-17-one. Androsterone: 3α-hydroxy-5α-androstan-17-one. Androstanedione: 5α-androstan-3,17-dione. Etiocholanedione: 5β-androstan-3,17-dione. Epitestosterone: 17α-hydroxy-4-androsten-3-one.

MATERIAL AND METHODS

Male Sprague–Dawley rats castrated 24 or 48 h before the incubation were used. The animals were divided into groups according to age, from 3, 4–5, 6 and 9 weeks, which correspond to immature, prepubertal, pubertal, and adult animals respectively. The confirmation of puberty was made by testicular smears, as described previously [12]. The hypophysis, hypothalamus, fragments of the cerebral cortex and the prostate gland from 10, 15 or 20 rats, depending upon the number of glands necessary to obtain the same average weight in all instances, were used. This weight was 80–100 mg for hypophysis tissue, 170–210 mg for hypothalamus and cortex tissues, 300–400 mg for prostate tissue. One ml of incubation medium per

25 mg tissue was used. Each tissue was incubated for 120 min with radioactive testosterone, using either [1,2-^{3}H] (42·6 Ci/mmol) or [1,2-6,7-^{3}H] (91 Ci/mmol), in amounts corresponding to 20 ng/ml of incubation medium. Thereafter, each tissue was homogenized and fractionated by centrifugation in a Beckman centrifuge (L 3-40) (12). Two of the subcellular fractions were used in the present study: "cytosol", i.e. 100,000 *g* supernatant and "nuclear fluid", i.e. a soluble nuclear fraction containing soluble proteins and low mol. wt. nucleic acids. These fractions were filtered through a Sephadex G 25 column saturated with cold testosterone, in an attempt to remove any non-specific binding which may occur. The protein bound radioactivity was thus separated from the free radioactivity.

Steroids were identified: (1) in the incubation medium, (2) in the total protein fraction of the cytosol, obtained by filtration through Sephadex G 25, and (3) in the total protein fraction of the nuclear fluid, obtained by the same method. The isolation of the steroids was made by reverse isotope dilution. [4-^{14}C]-testosterone, DHT, Δ^4-androstenedione, and cold carriers for the steroids to be analysed were added to the biological sample. These ^{14}C or cold carriers were used to calculate recovery, and to measure a ^{3}H/^{14}C or ^{3}H/μg cold carrier ratio. In the course of purification, this ratio remained constant when no other radioactive metabolites could be separated from the metabolite being assayed. After adding these carriers, the sample was extracted with chloroform. In a few instances, this was followed by an ethyl acetate extraction, since this is a better solvent for highly polar steroids. Only the conjugated (esterified) steroids, and the very tightly protein bound steroids remained in the aqueous phase, after the organic solvents extraction. Steroids were separated by paper chromatography (Whatman no. 3

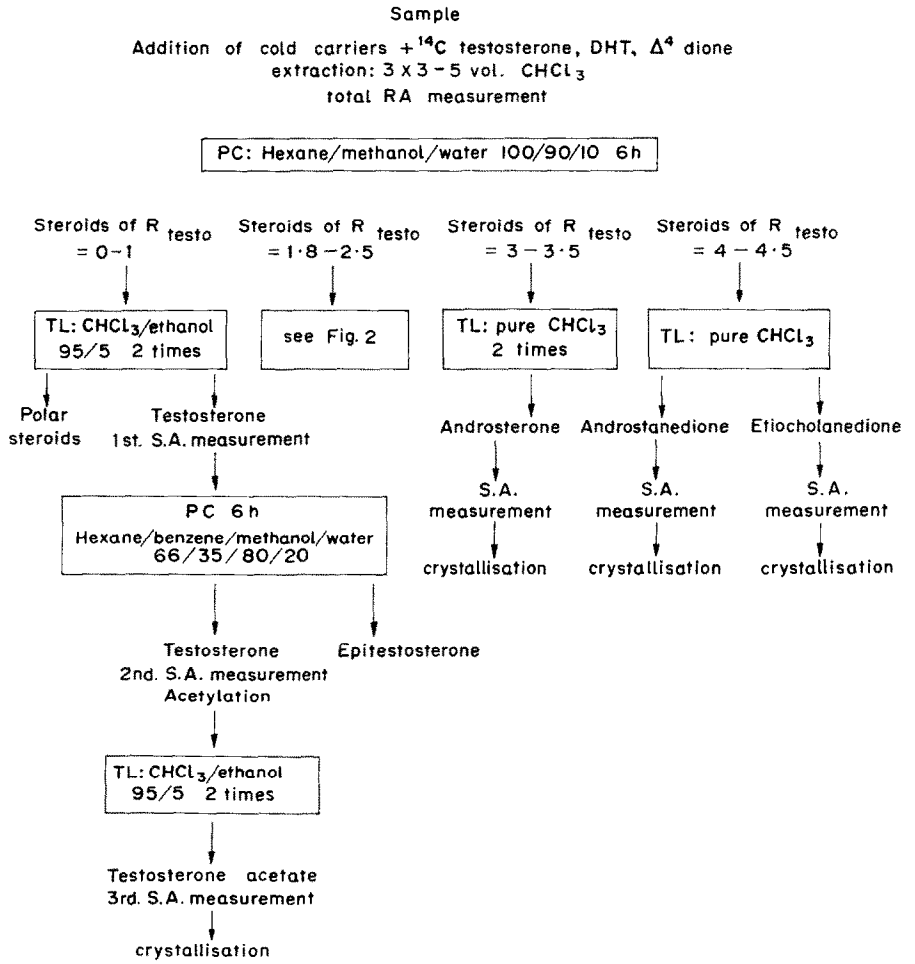

Fig. 1. Steroid isolation. PC: paper chromatography; TL: thin layer chromatography (silica gel); S.A.: "specific activity" ^{3}H/^{14}C ratio of ^{3}H/μg of steroid carrier ratio.

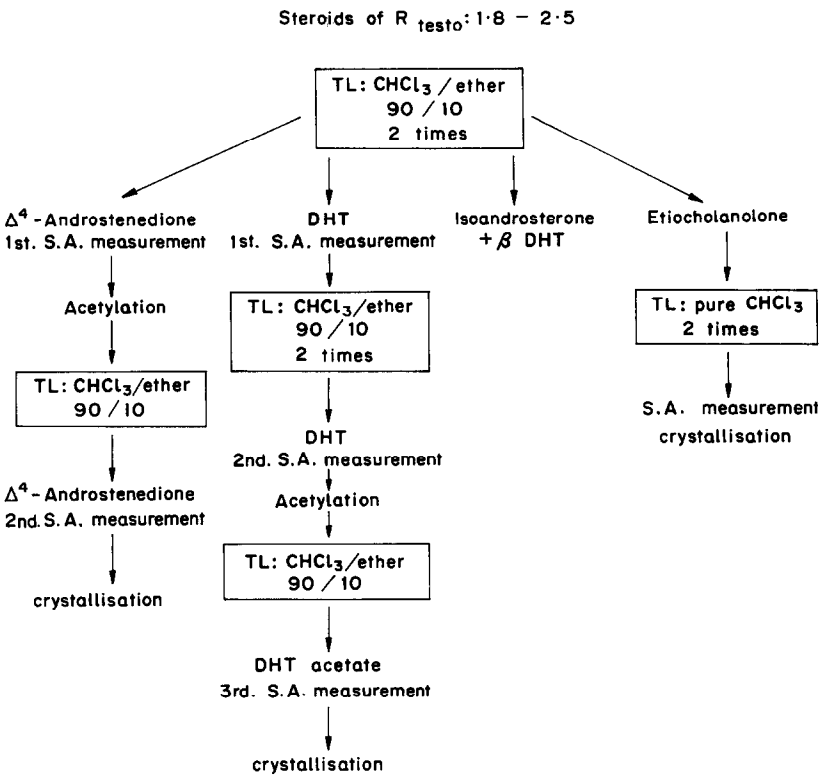

Fig. 2. Steroid isolation (continued). Abbreviations as in Fig. 1.

MM for the first chromatogram, and no. 2 for the second), and silica gel thin layer chromatography, as shown in Figs. 1 and 2. The purity of the isolated steroids, as already suggested by the constancy of the $^3H/^{14}C$ or $^3H/\mu g$ cold carrier ratio, was then confirmed by crystallization.

RESULTS

(A) *Incubation media*

(1) *Distribution of the radioactivity between organic solvent and water phases.* After chloroform extraction, 96–100% of the radioactivity was found in the organic phase, with no appreciable difference when ethyl acetate was used. It can be said consequently that very little radioactivity is present as conjugated steroids in the medium.

(2) *Testosterone metabolites.* The results are shown in Tables 1–4. Each table shows the results obtained with one tissue for each age group.

The difference between the four tissues at the same age was examined. The prostate gland had the greatest metabolic effect on the testosterone and the neural tissue the least. The hypophysis had an activity that was between these other two tissues. Polar compounds constituted the major part of testosterone metabolites isolated in the cephalic tissues. DHT was present in the incubation media of the four tissues, although the quantity in the cortex samples was very small. Δ^4-androstenedione appeared to be a minor metabolite in our incubations, with a slightly higher percentage found with the hypophysis. In the prostate, acetylation separated Δ^4-androstenedione from an unknown compound, accounting for 0·1–0·2% of the total radioactivity. Another unknown compound was separated from androsterone by thin layer chromatography. This metabolite was proportionally more abundant in the incubation media of the nervous tissues than of the prostate, although a minor metabolite in all instances: 0·1–3% of the total radioactivity. This compound could well be the "X compound" bound to the cytosol proteins of rat nervous tissue [8] or the "non polar compound" found in the neural tissues of dog [6].

The effect of age on each of the four tissues was examined: only small differences were observed between the various age groups, although tissues of prepubertal and pubertal animals (4–5 and 6-weeks) used slightly more testosterone than the other age

Table 1. Steroids isolated from the incubation medium of male rat hypophysis incubated with tritiated testosterone. Results are expressed in percentage of radioactivity extracted from the medium by organic solvents

Steroids / Animals age	Polar	Testosterone	DHT	Δ^4 androstenedione	Isoandrosterone (+β DHT)	Etiocholanolone	Androsterone	Androstanedione	Etiocholanedione
3 weeks (20 rats)	17·8	70·5*	6·9*	2·2*	0·2	0·1	0·6*	0·3	<0·1
4–5 weeks (10 rats 4 weeks + 5 rats 5 weeks)	17·0	69·0*	5·3*	1·9*	0·3	0·1	0·5		
6 weeks (15 rats)	—	62·7*	2·3*	—	—	0·3	—	0·2	<0·1
(15 rats)	18·7	66·3*	10·2*	1·3*	—	0·1*	0·1*	0·2*	<0·1*
(15 rats)	32·2	53·6*	7·2*	0·8*	—	<0·1*	0·4*	0·4*	<0·1*
9 weeks (10 rats)	14·6	79·1*	1·9*	2·7*	0·2	0·2	0·2*	<0·1	<0·1
(10 rats)	12·0	80·0*	3·8*	1·1*	—	0·1*	<0·1*	<0·1*	<0·1

* Crystallised steroid.

Table 2. Steroids isolated from the incubation medium of male rat hypothalamus incubated with tritiated testosterone. Results are expressed in percentage of radioactivity extracted from the medium by organic solvents

Steroids / Animals age	Polar	Testosterone	DHT	Δ^4 androstenedione	Isoandrosterone (+β DHT)	Etiocholanolone	Androsterone	Androstanedione	Etiocholanedione
3 weeks (20 rats)	5·9	90·0*	1·8*	0·8*	—	<0·1	<0·1	<0·1	<0·1
4–5 weeks (10 rats 4 weeks + 5 rats 5 weeks)	15·3	71·5*	2·2*	0·8*	0·4	0·1	0·1	0·4	
6 weeks (15 rats)	—	84·1*	1·3*	—	—	0·2		<0·1	<0·1
(15 rats)	—	—	1·0*	0·7*	—	<0·1	<0·1*	<0·1	<0·1
(15 rats)	21·9	72·1*	1·5*	0·5*	—	<0·1*	<0·1*	<0·1	<0·1
9 weeks (10 rats)	11·2	85·2*	0·9*	1·2*	—	<0·1	<0·1*	<0·1	<0·1
(10 rats)	15·0	80·4*	1·6*	0·3*	—	0·1*	<0·1*	<0·1	<0·1

* Crystallised steroid.

Table 3. Steroids isolated from the incubation medium of male rat frontal cortex incubated with tritiated testosterone. Results are expressed in percentage of radioactivity extracted from the medium by organic solvents

Steroids / Animals age	Polar	Testosterone	DHT	Δ^4 androstenedione	Isoandrosterone (+β DHT)	Etiocholanolone	Androsterone	Androstanedione	Etiocholanedione
3 weeks (20 rats)	8·7	88·7*	0·4*	0·6*	—	0·1	<0·1*	<0·1	<0·1
(20 rats)		85·2*	0·3*						
4–5 weeks (10 rats 4 weeks + 5 rats 5 weeks)	13·6	81·2*	0·6*	0·8*	0·1	0·4	0·2	—	—
6 weeks (15 rats)	—	79·5*	0·6*	—	—	0·2	—	<0·1	<0·1
(15 rats)	20·1	75·9*	0·4*	0·4*	—	<0·1*	<0·1*	<0·1	<0·1
(15 rats)	22·6	70·1*	0·2*	0·3*	—	0·2*	<0·1*	<0·1	<0·1
9 weeks (10 rats)	4·7	92·9*	0·3*	0·7*	<0·1	<0·1	<0·1	<0·1	<0·1
(10 rats)	4·2	92·3*	0·6*	0·3*	—	0·2	<0·1*	<0·1*	<0·1*

* Crystallised steroid.

Table 4. Steroids isolated from the incubation medium of male rate prostrate incubated with tritiated testosterone. Results are expressed in percentage of radioactivity extracted from the medium by organic solvents

Steroids / Animals age	Polar	Testosterone	DHT	Δ^4 androstenedione	Isoandrosterone (+β DHT)	Etiocholanolone	Androsterone	Androstanedione	Etiocholanedione
3 weeks (20 rats)	19·8	36·8*	30·8*	0·5*	—	0·1	2·8*	7·2*	0·1
6 weeks (15 rats)	42·4	40·2*	11·8*	0·3*	—	0·4*	1·2*	1·2*	<0·1
9 weeks (10 rats)	19·3	60·6*	16·9*	0·4*	0·3	0·1	0·1*	1·0*	0·2
(10 rats)	24·1	38·3*	30·1*	0·2*	—	0·1*	0·5*	2·6*	<0·1*

* Crystallised steroid.

groups, as evidenced by the lower percentage of testosterone found in the incubation media. Prostatic androsterone and androstanedione were found in greater quantities at 3-weeks of age than at 9-weeks. The same phenomenon, albeit less pronounced, was observed in the hypophysis, but not in the hypothalamus and cerebral cortex.

(B) *Radioactivity bound to the total cytosol proteins*

(1) *Distribution of the radioactivity between organic solvent and aqueous phases.* Chloroform extracted 90–99% of the radioactivity from the hypophyseal and prostate total cytosol proteins fractions but the extraction was less complete in the hypothalamus and cortex: 80–90%. Consequently a maximum of 20% of the radioactivity may have been present in conjugated form and/or strongly bound to proteins.

(2) *Testosterone metabolites.* These results are given in Table 5. In all tissues, there was less testosterone than in the incubation medium. The proportion of DHT, on the other hand, was found to have increased, especially in the hypophysis and prostate, while the quantity remained low in the cortex. Δ^4-androstenedione had little quantitative importance. An appreciable quantity of steroids other than testosterone and DHT was also bound to the cytosol proteins. These metabolites were mainly polar products.

Neither the prostate nor the cortex, the two "reference" tissues, showed any difference in the percentage of testosterone and DHT bound to proteins related to age. On the contrary, in the hypophysis there was less testosterone bound to cytosol proteins of the 4–5 and 6-week-old animals than that found in the adult and immature rats. The difference between the 3 and 6 and

Table 5. Steroids isolated from the cytosol total protein fraction of various tissues of male rats castrated for 24 (×) or 48 (○) h, tissues incubated with tritiated testosterone. Results are expressed in percentage of radioactivity extracted with organic solvent

Steroids		Hypophysis				Hypothalamus				Frontal cortex				Prostate			
Animals age		T	DHT	Δ^4	NI	T	DHT	Δ^4	NI	T	DHT	Δ^4	NI	T	DHT	Δ^4	NI
	(22 rats) ×	61·5	15·1	—	23·4	54·8	16·7	—	28·5					1·7	47·7	—	50·6
3	(19 rats) ×	50·2	10·1	—	39·7	62·5	9·2	—	28·1								
weeks	(24 rats) ○	64·9	4·4	—	30·7	50·8	12·1	—	37·1	90·1	0·9	—	8·0				
	(20 rats) ○	46·8*	—	2·1	—	82·1*	—	1·3	—	94·9*	4·7*	0·5	0·0				
4–5	(10 rats																
weeks	4 wk.																
	+ 5 rats ×	43·3	21·7	—	35·0									1·9	62·5	—	35·8
	5 wk.)																
	(21 rats) ×	36·3	21·5	—	42·2	43·6	6·0	—	50·4	60·9	2·6	—	36·5				
6	(14 rats) ×	41·4	13·4	—	45·2	90·2	9·6	—	5·2	89·7	2·1	—	8·2				
weeks	(16 rats) ○	46·0	8·6	—	45·4	84·0	2·7	—	13·3	89·6	1·6	—	8·8				
	(20 rats) ○	38·7*	—	2·3	—	84·7*	10·7*	1·1	3·5	93·8*	3·0*	0·9	2·3				
	(16 rats) ×	43·7	10·0	—	46·3	51·5	3·1	—	45·4					5·9	49·9	—	44·2
9	(10 rats) ×	47·4	11·8	—	40·8	43·3	9·7	—	47·0	58·9	3·3	—	37·8				
weeks	(10 rats) ×	65·4	12·5	—	22·1	81·5	2·5	—	16·0					2·4	55·0	—	42·6
	(10 rats) ○	55·8	5·7	—	38·5	52·4	14·3	—	33·3	88·9	2·0	—	9·4				
	(10 rats) ○	54·1*	—	2·0	—	69·8*	16·9*	0·2	13·1	88·1*	3·2*	—	8·7				

T = testosterone; Δ^4 = Δ^4 androstenedione; NI = non identified steroids; * = crystallised steroid.

Table 6. Steroids isolated from the nuclear fluid total protein fraction of various male rat tissues incubated with tritiated testosterone. Results are expressed in percentage of radioactivity extracted by organic solvents

Steroids	Hypophysis			Hypothalamus			Frontal cortex			Prostate		
Animals age	T	DHT	NI	T	DHT	NI	T	DHT	NI	T	DHT	NI
3 weeks	34·4	6·6	59·0	36·7	5·1	58·2	50·9	2·4	96·7	1·3	52·7	46·0
4–5 weeks	31·3	28·2	40·5	-	-	-	-	-	-	1·7	57·7	40·6
6 weeks	32·3	24·3	43·4	33·4	4·8	61·8	63·4	<2·0	34·6	-	-	-
9 weeks	26·1	18·5	55·4	35·0	7·4	57·6	64·9	4·9	30·2	0·9	65·9	33·2

T = testosterone; NI = non identified steroids.

the 6 and 9-week-old rats is statistically significant ($0{\cdot}05 > P > 0{\cdot}02$ in both instances). It also appeared that more DHT was bound to proteins of the 4–5 and 6-week-old animals than in the two other groups, but the difference was not significant.

In the hypothalamus, an inverse distribution to that of the hypophysis might exist, that is to say more testosterone and less DHT during puberty in contrast to adult and immature animals, but the difference between the three groups was not significant.

(C) *Radioactivity bound to total proteins of the nuclear fluid*

The results are found in Table 6. Minute quantities of radioactivity were found in the nucleus within the nervous tissue and even less in the hypophysis. Therefore, for each age group, the protein fractions of the nuclear fluid were combined, though cytosol fractions were analysed separately.

In the nucleus, less testosterone was found than in the corresponding cytosol fraction: practically absent in the prostate, it accounted for 25–35% of the radioactivity bound to proteins in the nuclear fluid of the hypophysis, 33–37% in the hypothalamus and 50–65% in the cerebral cortex. Only in the hypophysis was more DHT bound to the nuclear fluid proteins than to the cytosol proteins. Between 30 and 60% of the radioactivity of the nuclear fraction represented other testosterone metabolites. The hypophyseal DHT reached a peak at puberty while there was no evident modifications with age in all other cases.

DISCUSSION

Our earlier studies [12] have shown that there is an increase in the total amount of steroids bound to the cytosol proteins of the hypophysis, and to a lesser extent, of the hypothalamus at puberty. The aim of the present research was to see if the metabolism of testosterone by these tissues in sexually immature animals was different in adult animals. In particular, we wished to identify those steroids that were involved in this change in their binding to the tissue proteins.

In the cerebral cortex, as might have been expected for a presumed non-target organ, there was no difference between the four age groups studied. Similarly, in the hypothalamus there was no age related change in the metabolites appearing in the incubation medium. However, there seemed to be more DHT bound to the cytosol proteins in the immature than in the pubertal rats. This observation, if it is confirmed, is difficult to interpret since the proportion of testosterone and DHT bound to the nuclear proteins was remarkably constant in the analysis of all age groups.

There was a possible age related decline in DHT production by the hypophysis *in vitro*, as has been described by Massa *et al.* [11]. On the other hand, less testosterone and more DHT were found bound to the cytosol proteins at puberty than before or after this time. Furthermore, there was more DHT bound to the nuclear proteins in prepubertal and pubertal animals than in the youngest group or the adults. This was accompanied by the increase in the macromolecules binding the proteins. In all, this leads to an increase in the absolute amount of DHT available in the hypophyseal cells of adult animals, and an even greater rise during puberty. This is probably true for other steroids. These changes are probably related to variations in gonadotrophin secretion which typify the onset of puberty.

The hypophysis contained also more Δ^4-androstenedione than the other tissues studied, as previously found by Jaffe [2]. This steroid has some androgenic potency [13] but it is our belief that its presence has no significant importance here. The hypophysis is a tissue difficult to free of all blood prior to incubation and thus it is likely that 17β oxydoreductase activity of some red blood cells [14] along with that of the hypophysis are measured.

In theory, the incubation medium, cytosol proteins and nuclear protein sequentially should contain an increasing amount of active hormone and decreasing amount of inactivated end products. DHT is contained in increasing amount from medium to nucleus in the hypophysis. By contrast, testosterone itself remains the main androgen in the tissues of the central nervous sys-

tem and this appears to be true whatever the age group studied. This metabolic approach is in agreement with the present concept of the role of androgens in the central nervous system: DHT plays no part in hypothalamic sex differentiation nor in sexual behaviour [15–17], but participates in gonadotrophin regulation [15, 17, 18].

Finally, in the hypophysis and perhaps in the hypothalamus and cerebral cortex, the percentage of testosterone in the incubation medium is lower when tissue from 4–5 and 6-week-old animals was studied than from younger and older animals.

An increase in the metabolism of testosterone in the testicles of rats as they grow from 20–50 days old has been demonstrated by Fisher *et al.* [19] and Coffey *et al.* [20]. In view of their results as well as our own observations, this increase of metabolism of testosterone could be a generalized phenomenon throughout the body as it prepares for puberty.

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