Journal of Steroid Biochemistry Vol. 10, pp. 173 to 177

0022-4731/79/0201-0173$02.00/0

LACK OF METABOLISM OF PROGESTERONE TO 5α-PRODUCTS IN DOG AND GUINEA PIG TESTES COMPARED WITH IMMATURE RAT TESTES

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(*Received* 18 *May* 1978)

SUMMARY

Testicular homogenates from immature and adult dogs and guinea pigs and those from immature rats were incubated with [^{3}H]-progesterone or [^{14}C]-3β-hydroxy-5-pregnen-20-one in the presence of cofactors. After incubation, radioactive products were separated and identified by column and paper chromatography with derivative formation and recrystallization to constant specific activity.

All testes from dogs and guinea pigs of different ages converted significant amounts (up to 80%) of 3β-hydroxy-5-pregnen-20-one and progesterone to 17-hydroxy-4-pregnene-3,20-dione, 4-androstene-3,17-dione and testosterone, but these testes converted very small amounts (less than 1% in dogs and less than 5% in guinea pigs) of these precursors to 5α-reduced metabolites of 4-ene-3-oxosteroids. However, in immature rat testes under the same incubation conditions, conversion of progesterone to 5α-reduced steroids reached 70%. In contrast to rats and mice, immature testes of dogs and guinea pigs do not form large amounts of 5α-reduced C_{19}-steroids.

INTRODUCTION

Previous studies of androgen biosynthesis *in vitro* have demonstrated that rat and mouse testes are capable of a high rate of progesterone conversion to testosterone during fetal development [1], up to 15 days after birth [1–4] and when adult [2–6]. On the other hand, testicular homogenates from 20 to 40-day-old rats and mice yield 5α-reduced C_{19}-steroids such as androsterone and 5α-androstane-3α,17β-diol as major products from progesterone [2–4, 6]. We found that progesterone was converted to these 5α-reduced C_{19}-steroids primarily by a pathway through 5α-reduced 17α-OH-C_{21}-steroids in testes of immature rats and mice [7–9]. The 5α-reduction pathway was not present in testes of adult rats and mice [7–9].

Although the formation of large amounts of 5α-reduced C_{19}-steroids from progesterone was found in immature rabbit testes [10], these 5α-products were also formed as major C_{19}-steroids by adult rabbit testes [10–12]. In contrast to rats and mice, immature and adult testes of rhesus monkeys and humans, which formed testosterone and 4-androstene-3,17-dione as major C_{19}-steroids, did not form significant amounts of 5α-products including 5α-reduced C_{19}-steroids [13, 14]. These findings have suggested that the age-dependent pattern of testicular androgen biosynthesis is variable in different species of animals.

In testes of adult dogs [15, 16] and adult guinea pigs [17], synthesis of testosterone from 3β-hydroxy-5-pregnen-20-one and progesterone has been established. However, no data on age-dependent pattern of conversion of these precursors to 5α-products, including 5α-reduced C_{19}-steroids, have been reported. In the present paper, we are reporting on the *in vitro* metabolism of progesterone and/or 3β-hydroxy-5-pregnen-20-one by testes of dogs and guinea pigs at different stages of development. Although rat, mouse and guinea pig are classified in Order Rodentia, rat and mouse come under Suborder Myomorpha but guinea pig falls under Suborder Hystricomortha.

MATERIALS AND METHODS

Animals. Beagles of different ages, 1, 2, 4, 6 and 8 months, were born and raised in Fujisawa research laboratory, Osaka, Japan. Testes were removed under Nembutal anesthesia, immediately frozen and stored at −75°C for 2–3 weeks until used for incubations. Weights of testes were approximately 0.1, 0.6, 2.4, 4.2 and 14.6 g, respectively. Testes were also removed from 33-day-old rats of the Wistar strain, immediately frozen and stored at −75°C for 3 weeks until used for incubations. Guinea pigs, 10, 30, 50, 60 and 90 days old were also used. Testes were removed from 3 guinea pigs in each age group, weighed and immediately used for incubations. Weights of testes of different ages were approximately 150, 350, 550, 950 and 2300 mg, respectively.

Histological technique. Testes were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Chemicals. [1,2-^{3}H]-Progesterone (45 Ci/mmol) and [4-^{14}C]-3β-hydroxy-5-pregnen-20-one (1.8 nmol/0.1 μCi), obtained from Daiichi Pure Chemical Co., Ltd., Japan were purified by paper chromatography using the hexan-formamide system [18] just before

Table 1. Percentage formation of [^{3}H]-steroids from [^{3}H]-progesterone by dog testes

Amount of tissue (mg) / Age (months)	20				120				Rat* (33 days)
	1	2	4	6	2	4	6	8	
Progesterone (unchanged)	7.9	11.8	33.7	29.7	3.9	12.8	10.6	30.8	4.3
20α-hydroxy-4-pregnen-3-one	<0.1	<0.1	<0.1	0.1	0.4	0.1	0.6	0.2	<0.2
16α-hydroxy-4-pregnene-3,20-dione	5.3	3.2	3.3	3.4	5.2	4.4	4.9	2.6	<0.3
5α-Pregnane-3,20-dione	<0.1	0.0	<0.1	0.2	0.0	<0.2	0.4	0.3	1.0
3ξ-hydroxy-5α-pregnan-20-one	<0.1	<0.1	0.4	0.1	<0.1	0.4	0.1	0.6	3.1
17-hydroxy-4-pregnene-3,20-dione	63.5	56.1	45.8	44.6	48.7	52.0	44.0	43.6	<0.1
17-hydroxy-5α-pregnane-3,20-dione	0.0	<0.1	<0.2	<0.4	<0.1	<0.2	0.3	<0.2	<0.3
3α,17-dihydroxy-5α-pregnan-20-one	<0.2	<0.1	<0.1	<0.1	<0.1	<0.2	<0.1	<0.3	32.9
3β,17-dihydroxy-5α-pregnan-20-one	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.9
4-androstene-3,17-dione	0.6	0.6	0.2	0.3	3.4	0.9	2.0	0.6	0.2
Testosterone	<0.2	<0.1	<0.1	<0.1	<0.2	0.4	0.3	0.3	<0.1
5α-Androstane-3,17-dione	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2
17β-hydroxy-5α-androstan-3-one	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0.1
Androsterone	<0.1	<0.1	<0.2	<0.2	<0.1	<0.2	0.2	0.2	19.1
3β-hydroxy-5α-androstan-17-one	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	4.0
5α-Androstane-3α,17β-diol	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	8.1
5α-Androstane-3β,17β-diol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4

Testicular homogenates were incubated with [^{3}H]-progesterone (1 nmol:0.5 μCi per tube) and NADPH at 34°C for 30 min in 1 ml. Values were obtained after recrystallization to constant specific activity except for 16α-hydroxy-4-pregnene-3,20-dione. *Homogenate from 120 mg testicular tissue was used.

use. Nonradioactive steroids were obtained from Steraloids, Inc., Wilton, N.H., and Ikapharm, Ramat-Gan, Israel. Other reagents were of analytical grade.

Incubation procedure. Testes from dogs, rats and guinea pigs were homogenized in 0.25 M sucrose containing 1 mM EDTA. The purified [^{3}H]-progesterone (1 or 2 nmol:0.5 or 1 μCi per tube) or [^{14}C]-3β-hydroxy-5-pregnen-20-one (1.8 nmol:0.1 μCi per tube) was introduced into 2 × 10 cm tubes dissolved in 0.02 ml ethanol. To each tube, 0.5 ml of buffer–cofactor solution was added. The buffer–cofactor solution consisted of 0.3 M potassium phosphate buffer, pH 7.4, 0.06 M nicotinamide, 2 mM $MgCl_2$ and 3 mM NADPH. When [^{14}C]-3β-hydroxy-5-pregnen-20-one was used as a substrate, 12 mM of NAD was also included. One-half ml of the tissue homogenate containing 10–120 mg of tissue was then introduced to make the total volume of the incubation mixture 1 ml. The samples were incubated in a shaking water bath in air at 34°C for 30 min. At the end of incubation, the mixtures were immediately acidified with 0.1 ml 1 N HCl and mixed with ether–chloroform (4:1, v/v) to stop the reaction.

Analysis and identification of steroids. To the incubation mixtures, 2–50 μg quantities of 17 steroids shown in Table 1 were added as nonradioactive carriers. The extraction of steroids, analysis of these steroids and 5-ene-steroids by paper [18] and elution [19] chromatography with acetylation of steroids and calculation of metabolite found in each steroid fraction were the same as previously described [7, 20]. Finally, all the separated radioactive steroids or their acetates except for 3ξ-hydroxy-5α-pregnan-20-one, 17-hydroxy-5α-pregnane-3,20-dione, 3α,17-dihydroxy-5α-pregnan-20-one and 16α-hydroxy-4-pregnene-3,20-dione were recrystallized with 10–15 mg nonradioactive standard steroids to constant specific activity in order to identify the steroids formed. 3ξ-hydroxy-5α-pregnan-20-one, 17-hydroxy-5α-pregnane-3,20-dione and 3α,17-dihydroxy-5α-pregnan-20-one were tentatively identified by derivative formation and recrystallization to constant specific activity of the derivative formed with chromium trioxide, as previously described [20]. 16α-Hydroxy-4-pregnene-3,20-dione and its acetylated derivative were tentatively identified by 4 different chromatographic systems [13]. The amount of each steroid on the final chromatogram was corrected for the decrease of specific radioactivity on 4 repeated crystallizations. The present procedure for calculating the rate of formation of metabolites can permit an approximate estimation of the percentage conversion of radioactive precursors, which seems to be satisfactory for the present purpose.

RESULTS

1. *Histological findings of dog and guinea pig testes*

At 1 and 2 months in dogs and 10 days in guinea pigs, the seminiferous tubules were small and contained spermatogonia and Sertoli cells. At 30 days in guinea pigs, a few primary spermatocytes appeared. At 4 months in dogs and 50 days in guinea pigs, primary spermatocytes increased. At 6 months in dogs and 60 days in guinea pigs, immature spermatids appeared indicating that at least in part reduction division had already been completed. At 8 months in dogs and 90 days in guinea pigs, full spermatogenesis was noted. The histological findings found in 6-month-old dogs and 60-day-old guinea pigs are similar to those found in 33-day-old rats.

Table 2. Percentage formation of [^{14}C]-steroids from [^{14}C]-3β-hydroxy-5-pregnen-20-one by 120 mg of dog testes

Age (months)	4	6	8	No tissue
3β-hydroxy-5-pregnen-20-one	1.1	3.1	4.0	94.2
Progesterone	1.1	<0.1	2.1	<0.5
5α-Pregnane-3,20-dione	<0.3	<0.4	<0.3	<0.4
3ξ-hydroxy-5α-pregnan-20-one	<0.3	<0.4	<0.1	<0.2
3β,17-dihydroxy-5-pregnen-20-one	1.5	<0.4	<0.4	<0.1
17-hydroxy-4-pregnene-3,20-dione	6.5	1.9	5.1	0.0
17-hydroxy-5α-pregnane-3,20-dione	<0.4	<0.2	<0.3	<0.1
3α,17-dihydroxy-5α-pregnan-20-one	<0.3	<0.5	<0.5	0.0
3β,17-dihydroxy-5α-pregnan-20-one	<0.3	<0.2	<0.2	<0.1
Dehydroepiandrosterone	25.5	46.5	37.9	<0.5
5-androstene-3β,17β-diol	<0.5	<0.3	<0.3	<0.1
4-androstene-3,17-dione	38.0	18.9	21.7	<0.4
Testosterone	4.5	3.3	4.5	0.0
5α-Androstane-3,17-dione	<0.3	<0.1	<0.2	<0.4
17β-hydroxy-5α-androstan-3-one	<0.4	<0.6	<0.6	<0.5
Androsterone	0.6	0.5	0.8	<0.3
5α-Androstane-3α,17β-diol	<0.1	<0.2	<0.2	0.0
5α-Androstane-3β,17β-diol	<0.6	<0.1	<0.2	<0.1

Testicular homogenates were incubated with [^{14}C]-3β-hydroxy-5-pregnen-20-one (1.8 nmol:0.1 μCi per tube), NAD and NADPH at 34°C for 30 min in 1 ml. Values were obtained after recrystallization to constant specific activity.

2. *Metabolism of 3β-hydroxy-5-pregnen-20-one and progesterone by dog testes of different ages*

The percentage formation of [^{3}H]-steroids from [^{3}H]-progesterone by testicular homogenates from dogs of different ages is shown in Table 1. In 20 and 120 mg tissue incubations with 1 nmol of [^{3}H]-progesterone for 30 min, testes from 1–8-month-old dogs converted approximately 50% of progesterone to 17-hydroxy-4-pregnene-3,20-dione and 0.2–3.5% to 4-androstene-3,17-dione and testosterone. When both amounts of tissue from the same testes were used, the rate of production of C_{19}-4-ene-3-oxo-steroids was roughly proportional to the weight of tissue used. However, no or very small amounts of 5α-reduced metabolites of all 4-ene-3-oxosteroids were found in all dog testes of different ages examined. On the other hand, testes from 33-day-old rats converted 70% of progesterone to 5α-reduced steroids such as 3α,17-dihydroxy-5α-pregnan-20-one and 5α-reduced C_{19}-steroids under the same incubation conditions. In immature rat testes, accumulation of 17-hydroxy-4-pregnene-3,20-dione, 4-androstene-3,17-dione and testosterone was less than 1%.

The percentage formation of [^{14}C]-steroids from 1.8 nmol of [^{14}C]-3β-hydroxy-5-pregnen-20-one for 30 min by 120 mg testicular homogenates from dogs is shown in Table 2. Although small amounts of 4-androstene-3,17-dione and testosterone were formed from progesterone in dog testes (Table 1), markedly increased amounts of these C_{19}-4-ene-3-oxosteroids were produced from 3β-hydroxy-5-pregnen-20-one in all dog testes examined under the similar conditions suggesting that dog testes were able to form testosterone mainly by the 5-ene-pathway. This finding is in agreement with those reported by van der Molen and Eik-Nes [15] and Oh and Tamaoki [16] in adult dog testes. Again, 5α-products formed from 3β-hydroxy-5-pregnen-20-one were found in very small amounts in 4-, 6- and 8-month-old dog testes examined. In the present study, control incubations without tissue were included when [^{3}H]- and [^{14}C]-substrates were used. There was no or very little transformation of radioactive substrates. One of the results is shown in Table 2.

3. *Metabolism of progesterone by guinea pig testes of different ages*

The percentage formation of [^{3}H]-steroids from 2 nmol of [^{3}H]-progesterone for 30 min by 10 and 60 mg testicular homogenates from guinea pigs is shown in Table 3. In 10 mg tissue incubations, testes from 10–90-day-old guinea pigs converted 25–50% of progesterone to 17-hydroxy-4-pregnene-3,20-dione and 15–50% to 4-androstene-3,17-dione and testosterone. In 60 mg tissue incubations, accumulation of 17-hydroxy-4-pregnene-3,20-dione decreased markedly and formation of C_{19}-4-ene-3-oxosteroids reached 70–80%. However, 5α-products formed were found in small amounts in all guinea pig testes used. Although small but significant amounts of 5α-androstane-3α,17α-diol were formed in 120 mg tissue incubations, the typical age-dependent pattern of 5α-reduced C_{19}-steroid formation demonstrated in rat and mouse testes [2–4] was not present in guinea pig testes.

DISCUSSION

The present investigation demonstrated that large amounts of 3β-hydroxy-5-pregen-20-one and/or progesterone were metabolized to 17-hydroxy-4-pregnene-3,20-dione, 4-androstene-3,17-dione and testosterone *in vitro* in testes from immature and adult

Table 3. Percentage formation of [^{3}H]-steroids from [^{3}H]-progesterone by guinea pig testes

Amount of tissue (mg)	10					60				
Age (days)	10	30	50	60	90	10	30	50	60	90
Progesterone (unchanged)	29.0	22.1	5.8	22.0	11.7	4.1	3.2	5.8	4.2	3.3
20α-hydroxy-4-pregnen-3-one	<0.2	<0.3	<0.3	<0.3	<0.2	<0.1	<0.1	<0.1	<0.3	<0.1
5α-Pregnane-3,20-dione	<0.1	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
3ξ-hydroxy-5α-pregnan-20-one	1.2	1.2	1.1	1.2	0.7	0.7	0.6	0.4	0.6	0.2
17-hydroxy-4-pregnene-3,20-dione	36.6	39.2	26.6	41.1	45.5	0.3	0.3	0.3	0.2	0.1
17-hydroxy-5α-pregnan-3,20-dione	<0.2	<0.1	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
3α,17-dihydroxy-5α-pregnan-20-one	0.5	0.4	0.5	0.4	0.2	1.0	0.8	0.5	0.7	0.4
3β,17-dihydroxy-5α-pregnan-20-one	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
4-androstene-3,17-dione	12.3	13.2	28.9	12.0	12.6	46.4	32.1	11.7	25.2	4.9
Testosterone	3.9	4.9	20.0	6.5	13.4	32.4	47.1	61.1	51.5	73.6
5α-Androstane-3,17-dione	<0.2	<0.1	<0.1	<0.1	<0.1	<0.2	<0.1	0.0	0.0	0.0
17β-hydroxy-5α-androstan-3-one	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Androsterone	0.1	0.1	0.1	0.1	0.1	0.5	0.4	0.2	0.3	0.1
3β-hydroxy-5α-androstan-17-one	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1	<0.1	0.1	<0.1
5α-Androstane-3α,17β-diol	0.1	<0.1	0.5	0.1	0.1	2.6	3.1	3.7	2.4	2.7
5α-Androstane-3β,17β-diol	<0.1	0.0	<0.1	0.0	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2

Testicular homogenates were incubated with [^{3}H]-progesterone (2 nmol:1 μCi per tube) and NADPH at 34 C for 30 min in 1 ml. Values were obtained after recrystallizations to constant specific activity.

dogs and guinea pigs, but no or little 5α-reduced metabolites of all these 4-ene-3-oxosteroids were formed (Tables 1–3). Concentrations of endogenous progesterone in testes of 1–8-month-old dogs and 10–90-day-old guinea pigs estimated by competitive protein binding method were 0.03–0.1 nmol per 100 mg tissue. Since 1–2 nmol of [^{3}H]-progesterone or [^{14}C]-3β-hydroxy-5-pregnen-20-one was added to 10–120 mg testicular homogenates, the effect of dilution by endogenous steroids on the metabolism of radioactive substrates seems to be limited. These observations seem to indicate that efficient formation of 5α-reduced C_{19}-steroids instead of testosterone, already demonstrated in testes of immature rats and mice [2–4, 6–9] is not present in testes of immature dogs and guinea pigs.

From Tables 1–3, it is apparent that only about 80% of the added substrates was recovered. The deficient 20% was not due mainly to procedural losses, because of the method for calculation of metabolite used in the present study [7, 20]. The deficient 20% was due mainly to unidentified polar metabolites including 17,20α-dihydroxy-4-pregnen-3-one and 17,20β-dihydroxy-4-pregnen-3-one in Table 1, 3β,16α-dihydroxy-5-pregnen-20-one, 16α-hydroxy-4-pregnene-3,20-dione and 17,20α-dihydroxy-4-pregnen-3-one in Table 2 and 16α-hydroxy-4-pregnene-3,20-dione, 17,20α-dihydroxy-4-pregnen-3-one and 17,20β-dihydroxy-4-pregnen-3-one in Table 3. Since the formation of oestrogen was not examined, the possibility of some oestrogen formation was also present. In the present study, the testes of the dogs and rats were stored at −75°C for 2–3 weeks while the guinea pig testes were used immediately. Since the dog testes of different ages were generously supplied from Fijisawa research laboratory, it was difficult to obtain fresh dog testes of different ages at the same time. By preliminary experiments in dog and rat testes, the effect of storage at −75°C for 3 weeks on the enzyme activities was shown to be insignificant.

In rats and mice, significant amounts of 5α-reduced C_{19}-steroids are formed by immature testes but not by adult testes [2–4, 6–9]. However, in humans [13, 14], rhesus monkeys [13], dogs and guinea pigs (Tables 1–3), no or very small amounts of 5α-androgens are formed by immature and adult testes. In rabbits, significant amounts of 5α-androgens are formed by immature as well as adult testes [10–12]. These findings suggest that the typical age-dependent change of testicular androgen synthesis may be found only in some groups in Rodentia, though future studies are needed in order to clarify prepubertal changes of testicular androgen synthesis in many species other than human, rhesus monkey, dog, rabbit, guinea pig, mouse and rat. Our observations might reflect a different mechanism controlling testosterone accumulation in immature testes in different species of animals.

Acknowledgement—The authors are grateful to Dr. N. Watanabe, Fujisawa research laboratory, for his generous supply of dog testes.

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