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BIOSYNTHESIS OF TESTICULAR STEROIDS IN THE IMMATURE, ADULT AND SENESCENT GUINEA-PIG

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Summary—The potential biosynthetic capacity of testicular hormones was studied in immature, pubertal and aging guinea-pig. In their sexual development towards puberty, changes in the relationship of the steroids involved in the steroidogenic pathways were observed. The testosterone/androstenedione ratio changes markedly, showing an important increase with pubertal proximity. The testosterone⇌androstenedione sequence, reversibly catalyzed by 17β-hydroxysteroid oxidoreductase (17β-oxido-reductase), clearly shifted towards androstenedione in immature animals irrespective of the precursor utilized. Post-pubertal animals showed a greater enzymatic activity in the 5-ene and 4-ene testicular synthesis pathways, testosterone production being greatest. In the aging animal, hormonal biosynthetic capacity falls. Reversion of the 17β-oxido-reductase activity could be one of the mechanisms responsible for the decrease in testosterone, as in immature guinea-pigs.

In order to investigate the *in vitro* steroidogenic capacity of glands at different ages, minces of testicular tissue were incubated with labelled precursors. The studies were conducted in triplicate at 35°C. For equal quantities of incubated tissue the non-metabolized amount of [^{3}H]pregnenolone and [^{14}C]progesterone, utilized as precursors, was different in post-pubertal and senescent animals: 55.7 ± 3 vs $59.3 \pm 2.3\%$ ($P < 0.01$) for pregnenolone, and 50.1 ± 3.3 vs $56.3 \pm 2.9\%$ ($P < 0.01$) for progesterone, respectively. Testosterone production was $12 \pm 2\%$ in adult and $6.7 \pm 2.7\%$ in senescent animals ($P < 0.01$). The testosterone/androstenedione ratio was not significantly different in post-pubertal and senescent animals: 2.8 ± 0.5 vs 2.4 ± 0.4, but consistently higher than found in immature animals: 0.3 ± 0.1. The lesser potential capacity of the aging tissue to synthesize testosterone could be explained by a decline in the glands capacity to metabolize the hormonal precursors.

INTRODUCTION

During the sexual development of the guinea-pig there are changes in the biosynthesis of its testicular hormones [1]. The amount of testosterone produced increases consistently from immaturity until puberty and reaches its maximum level in the adult animal. Pathways utilized in the steroidal-androgenic synthesis follow the classic 5-ene and 4-ene sequences [2]. In this way, the 17-hydroxypregnenolone, dehydroepiandrosterone and 5-androstenediol, and 17-hydroxyprogesterone and androstenedione, have been identified as intermediate metabolites in either pathway [3]. During testicular maturation, there are relationship changes between the different steroids which are part of the formation of androgens. The testosterone/androstenedione relationship is the one undergoing more significant changes as there is a noticeable testosterone increase with puberty [4, 5]. Therefore, this sequence, catalyzed by the 17β-oxido-reductase, could play a major role in normal testicular development [6]. Reversion of such enzymatic activity towards testosterone could explain the higher hormone levels found in adult animals compared with those in immature animals. In addition, there is a higher enzymatic activity of the different biosynthetic pathways which can be clearly seen in the animal that has reached puberty [7], even when the comparison involves an equal amount of glandular tissue. Testosterone levels may decrease towards senescence. It is likely that the amount of bioavailable testosterone for tissues also drops at that time [8, 9]. The final result means a decrease in the tissue androgenization [10].

To investigate androgen biosynthesis during the guinea-pig sexual development, at different

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age-levels up to senescence, the potential capacity of steroid synthesis was studied *in vitro* in testicular mince with different radioactive steroid precursors.

EXPERIMENTAL

Animals

Four groups of 10 guinea-pigs of the albino Hartley strain each were utilized:

Group 1: Immature animals, mean body wt 200–300 g, testis wt 0.1–0.3 g, 1–2 months old.

Group 2: Pre-pubertal animals, mean body wt 400–600 g, testis wt 0.9–1.5 g, 3–4 months old.

Group 3: Post-pubertal animals, mean body wt 700–900 g, testis wt 2.1–2.5 g, 6–9 months old.

Group 4: Senescent animals, mean body wt 1000–1600 g, testis wt 1.8–2.3 g, over 18 months old.

Incubations

After tunica removal, the other gland piece was minced on ice and incubated in a Krebs–Ringer phosphate buffer, pH 7.4. Testes from three immature animals were minced together and the group was treated as one animal in the result calculations. In some experiments, immature animals were processed with pre-pubertal animals. Testicular tissue were studied in triplicate. They were preincubated for 15 min in the Krebs–Ringer phosphate medium. Steroid precursors were then added to the incubation flasks: 2 μCi of [^{3}H]pregnenolone plus 0.5 μCi of [^{14}C]progesterone, plus non-labelled precursors in order to reach a final amount of 5 μg of each precursor per flask. Several other experiments were performed with: 1.5 μCi of ^{3}H-dehydroepiandrosterone; 0.5 μCi of [^{14}C]androstenedione; and 1 μCi of [^{3}H]androstenediol. There were also 1 mg of NADP and 5 mg of glucose-6-phosphate per flask. Incubation flasks contained approx. 40 mg wet wt testicular tissue per ml of buffer. In most incubations 5 ml was utilized per flask. Incubation was performed in air at 35°C for different time-periods as indicated under Results. The radioactive steroids were obtained from New England Nuclear Corporation (Boston, Mass).

Steroid studies

Reaction was stopped by adding methylene chloride to the flasks and the reaction products were quickly extracted. Chromatographic separation, identification and purification of the synthesized steroids were performed as previously described [2]. The isotope dilution method was used to identify most of the radioactive metabolites. The constancy of the specific activities was considered sufficient proof of purity. The chromatograms were scanned for radioactivity using a Packard Radiochromatogram scanner. Carbon-14 and tritium in fractions were determined in a Nuclear Chicago liquid scintillation spectrometer. In some cases, radioimmunoassays were used in order to measure steroid concentrations, using reagents obtained from Diagnostic Products Corporation (Los Angeles, Calif.). Analysis of variance was used to assess differences between the groups at each incubation time. Student's t-test was used for other comparisons, as noted in Results.

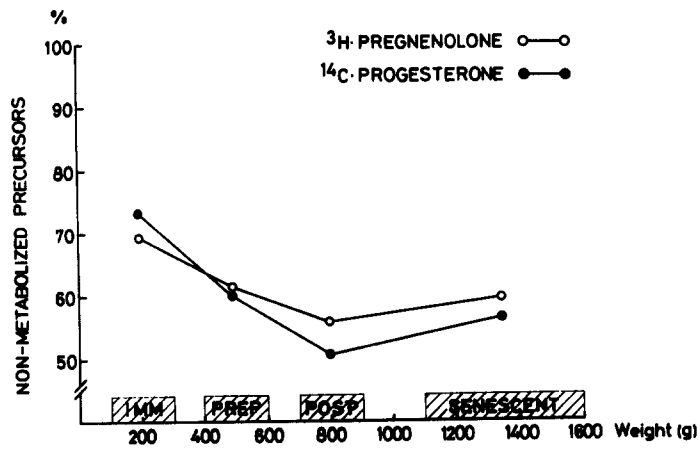

Fig. 1. Non-metabolized [^{3}H]pregnenolone and [^{14}C]progesterone after a 30 min incubation of minced immature (IMM), pre-pubertal (PREP), post-pubertal (POSP) and senescent (SENESCENT) guinea-pig testes (mean from triplicates).

RESULTS

The testicular tissue of the four groups of animals under study led to the formation of different radioactive metabolites which participated in the hormonal biosynthesis. Testes of immature and pre-pubertal guinea-pigs, as well as those of adult and senescent animals, are able to convert the different isotopic precursors utilized into testosterone. Starting from [^{3}H]-pregnenolone, synthesis can occur via the 5-ene route, through 17-hydroxypregnenolone, dehydroepiandrosterone and 5-androstenediol, or continue towards the corresponding 3-oxo-4-ene steroids due to the action of the 3β-ol-dehydrogenase-5-ene-4-ene isomerase. When incubation was carried out with [^{3}H]pregnenolone and [^{14}C]progesterone, the different [^{3}H]/[^{14}C]ratio found in testosterone with reference to 17-hydroxyprogesterone and androstenedione, supports the presence of alternative synthesis pathways with 5-ene steroid participation. Table 1 shows the contribution of [^{3}H] and [^{14}C] in the studies dealing with testes of post-pubertal animals (group 3). The percentage of steroids formed after a 30 min incubation period and [^{3}H] and [^{14}C] contribution to such synthesis is shown in Table 2.

After 30 min incubation, maximum conversion was achieved in those flasks with glands of post-pubertal animals and then, in a decreasing order, in those of senescent, pre-pubertal and immature animals (Fig. 1). Calculations were made on an equal-glandular-weight basis. It was observed that the non-metabolized amount of [^{3}H]pregnenolone and [^{14}C]progesterone, utilized as precursors, was different in post-pubertal and senescent animals after 30 min incubation: 55.7 ± 3 vs $59.3 \pm 2.3\%$ ($P < 0.01$) for pregnenolone; and 50.1 ± 3.3 vs 56.3 ± 2.9 ($P < 0.01$) for progesterone, respectively. In order to study the kinetics of this lower metabolism by testes of aging animals, incubation was continued to a total of 90 min. As incubation time increased, the percentage of remaining pregnenolone not only remained constant but even increased.

Table 1. [^{3}H]/[^{14}C] relationship present in the 17-hydroxyprogesterone (17-OH-PROG), androstenedione (4-A) and testosterone (TESTO) synthesized after [^{3}H]pregnenolone and [^{14}C]progesterone incubation with minced post-pubertal guinea-pig testicular tissue for 30, 60 and 90 min.

Synthesized steroids	[^{3}H]/[^{14}C] 30 min	60 min	90 min
17-OH-PROG	0.3	0.4	0.3
4-A	0.6	0.7	0.7
TESTO	2.0	1.6	1.5

Table 2. Synthesis of testicular steroids from [^{3}H]pregnenolone and [^{14}C]progesterone after 30 min incubation with minced testes of immature, pre-pubertal, post-pubertal and senescent guinea-pigs. Mean from triplicates

	% Yield (per 100 mg tissue)															
	PREGN		PROG		17-OH-PREGN		17-OH-PROG		DHEA		4-A		5-A		TESTO	
Animals	[^{3}H] NM	[^{14}C]	[^{3}H]	[^{14}C] NM	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
Immature	69.6	—	7.6	73.3	3.1	—	3.0	12.4	3.6	—	2.6	4.9	0.4	—	0.5	1.2
Pre-pubertal	62.9	—	10.8	61.7	4.4	—	4.7	18.2	4.1	—	2.8	5.6	0.7	—	1.3	2.3
Post-pubertal	55.7	—	15.0	50.1	5.3	—	7.3	23.3	3.1	—	1.9	2.7	1.0	—	5.6	6.4
Senescent	59.3	—	11.4	56.3	4.9	—	6.6	23.0	2.3	—	1.3	1.7	0.9	—	3.0	3.8

PREGN = pregnenolone; PROG = progesterone; 17-OH-PREGN = 17-hydroxy-pregnenolone; DHEA = dehydroepiandrosterone; 5-A = 5-androstenediol; 4-A = androstenedione; TESTO = testosterone; NM = non-metabolized.

With glands of immature and pre-pubertal guinea-pigs, apart from a lower precursor metabolism, androstenedione accumulation was observed, thus explaining the uneven testosterone/androstenedione ratio found: under 1 in the studies of immature and pre-pubertal testes and over 2 in those of post-pubertal and senescent animals (Table 3). Such a difference remained stable through incubations up to 90 min.

Figure 2 shows the contribution of [^{3}H]-pregnenolone and [^{14}C]progesterone towards the synthesis of androstenedione and testosterone in the different groups of animals studied, after 30 min of incubation. In the aging animal, testosterone synthesis is lower than in the post-pubertal state but higher in animals which did not reach full sexual development: testosterone yield was $12 \pm 2\%$ in adults and $6.7 \pm 2.7\%$ in senescent animals ($P < 0.01$). Although the testosterone/androstenedione ratio was slightly lower than that present in the flasks of adult animals (2.4 ± 0.4 vs 2.8 ± 0.5), it was always much higher than that in the immature and pre-pubertal tissues (0.3 ± 0.1 and 0.5 ± 0.2), respectively. When using [^{3}H]dehydroepiandrosterone as a precursor, hormonal synthesis in post-pubertal animals followed two different pathways: it continues towards 5-androstenediol or to androstenedione. Both steroids are then efficiently turned into testosterone. However, in immature animals or those that have not yet reached puberty, 5-androstenediol production is very small and androstenedione accumulates in the incubation medium. The 5-androstenediol/dehydroepiandrosterone ratio remains under 1 after 90 min incubation while it is always over 2 in adults. Aging guinea-pigs have a sequence similar to that of post-pubertal animals but with a lower testosterone yield after 90 min incubations: 38.1 ± 2.8 vs 49.0 ± 3.1 ($P < 0.001$), respectively (Fig. 3). Testosterone/androstenedione ratio is shown in Table 4.

When using [^{3}H]androstenediol and performing incubation for 10, 20 and 30 min, the initial amount of testosterone produced in the groups of animals under study decreases after 20 min with immature and pre-pubertal testes and there is a parallel increase in androstenedione which continues after 30 min incubation. On the other hand, post-pubertal and aging animals always showed low androstenedione percentages but post-pubertal animals had a

Table 3. Testosterone/androstenedione ratio after incubation of minced guinea-pig testes for different time-periods with [^{3}H]pregnenolone and [^{14}C]progesterone

	TESTO/4-A		
Animals	30 min	60 min	90 min
Immature	0.3	0.4	0.3
Pre-pubertal	0.5	0.6	0.6
Post-pubertal	2.8	3.2	3.4
Senescent	2.4	2.6	2.7

Table 4. Testosterone/androstenedione ratio after incubation of minced guinea-pig testes of immature + pre-pubertal, post-pubertal and senescent animals with [^{3}H]dehydroepiandrosterone

	TESTO/4-A		
Animals	30 min	60 min	90 min
Immature + pre-pubertal	1.9	1.4	0.9
Post-pubertal	6.8	10.7	14.8
Senescent	5.7	7.8	9.0

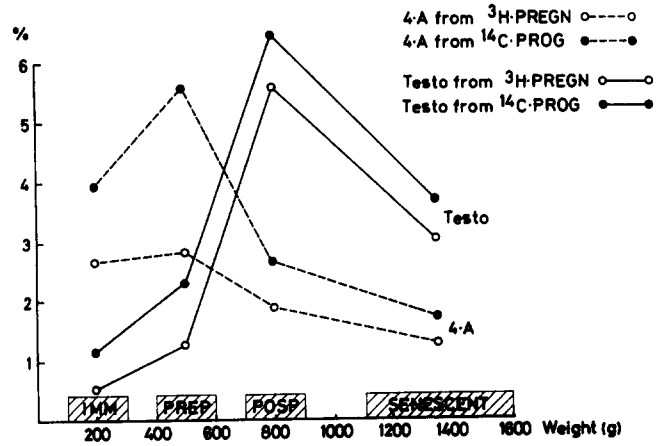

Fig. 2. Synthesis of androstenedione (----) (4-A) and testosterone (——) (TESTO) by minced testes of immature (IMM), pre-pubertal (PREP), post-pubertal (POSP) and senescent (SENESCENT) guinea-pigs after 30 min incubation with [^{3}H]pregnenolone (^{3}H-PREGN) (○) and [^{14}C]progesterone (^{14}C-PROG) (●).

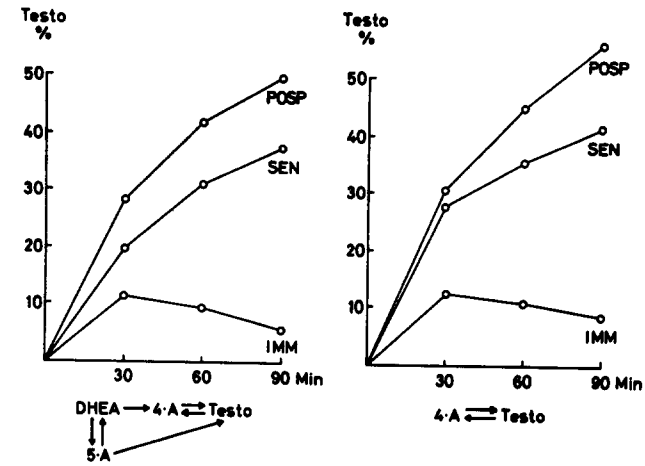

Fig. 3. Testosterone yield after incubation of minced testes of immature (IMM), post-pubertal (POSP) and senescent (SEN) guinea-pigs with [^{3}H]dehydroepiandrosterone (DHEA) and ^{14}C-androstenedione (4-A).

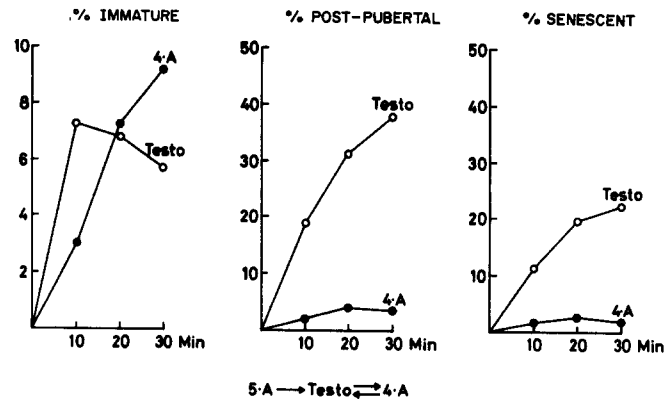

Fig. 4. Percentage contribution from [^{3}H]5-androstenediol (5-A) into androstenedione (●) and testosterone (○) formed in immature, post-pubertal and senescent guinea-pig testes, incubated for varying time intervals.

higher production of testosterone: 38 ± 3.9 vs 21.1 ± 3 ($P < 0.001$), respectively (Fig. 4).

Last but not least, when [^{14}C]androstenedione was the precursor, immature and pre-pubertal animals showed a poor initial yield of testosterone after 10 min incubation and a slight decrease after 30 min. Instead, testosterone synthesis was significantly higher in post-pubertal and senescent animals. Post-pubertal animals also showed a higher testosterone yield than senescent guinea-pigs after 90 min incubation: 54.9 ± 5.3 vs 40.8 ± 4.3 ($P < 0.001$), respectively (Fig. 3).

Figure 5 shows the enzymatic activity calculated from percentage metabolism of [^{3}H]pregnenolone after a 30 min incubation.

Fig. 5. Enzymatic activity calculated from percentage metabolism of [^{3}H]pregnenolone after 30 min incubation of minced testes of immature (IMM), pre-pubertal (PREP), post-pubertal (POSP) and senescent (SENESCENT) guinea-pigs. Enzymatic activity was obtained from addition of its products. Numbers indicate enzyme relationship.

DISCUSSION

Guinea-pig testes are capable of converting pregnenolone to testosterone by two principal

pathways: one involving progesterone, 17-hydroxyprogesterone and androstenedione as metabolic precursors and the other via 17-hydroxypregnenolone. Contribution of the 5-ene pathway to the testosterone synthesis keeps increasing from immaturity to adulthood and somewhat declines in senescence (Table 2). On the other hand, the hormonal synthesis capacity of the testis greatly varies with age. Work carried out with glands of newly born animals shows a testosterone synthesis potential as important as that found in adults. However, this is short-lived and hormonal production drops to its lowest levels a short time after birth [11]. As the animal grows and develops sexually, there are changes in the androgenic biosynthesis which clearly mark the difference between immature and post-pubertal animals. The lower yield of testosterone in animals that did not reach sexual maturity could be caused by a lesser concentration and/or different cytologic quality of the interstitial elements capable of androgenic production [12, 13]. This different testosterone yield between immature and adult animals remains the same even if results are calculated from equal amounts of testicular tissue. Lower hormonal synthesis is consistent with the difference in the activity of some of the enzymes participating in the various synthesis sequences. A good examle of this are the results found herein; they point to a displacement of the androstenedione$\rightleftharpoons$testosterone reaction towards androstenedione in immature animals with the ensuing drop in testosterone levels, no matter the precursor selected. In puberty, the enzyme activity reverses. The reducing activity predominates thus leading to a higher synthesis of testosterone which reaches its maximum peak during adulthood. Steroid precursors added to the incubation medium are more efficiently utilized and the activity of enzymes which are part of the synthesis of the 5-ene and 4-ene pathways reaches its maximum level. The testosterone/androstenedione ratio increases steadily as androstenedione drops. Afterwards, with the onset of the early signs of senescence, hormonal yield declines. Although in senescent animals there is a slight decrease in the testosterone/androstenedione ratio with reference to that found in post-pubertal animals, the lower testosterone synthesis is not due to the reducing enzyme reversion, as is the case with immature animals. The lower potential could be because of a decrease in the total amount of androgen-producing cells or on account of a drop in its synthesis potential. In the latter case, there may be different possibilities, namely: (a) drop in the enzymatic activity of one or more synthesis sequence; (b) alteration in the availability of precursors and cofactors necessary to achieve an adequate stereoidogenesis; (c) decrease of the stimulating central activity bearing on testicular function; (d) presence of inhibitors or blocking substances, whether local or circulating; (e) general metabolic disfunction leading to an abnormal local glandular metabolism; (f) drop in the blood concentration of compounds which help to a correct biosynthesis; and (g) inadequate oxygen contribution due to a circulatory deficit, etc. In this regard, a relative steroidogenic insufficiency also was observed in the elderly males compared to that of younger adult testes [14]. The results of these experiments indicate that the lower hormonal synthesis in senescent animals could be caused by a decreased capacity to utilize the radioactive steroid precursors in the incubation medium, probably because of a lower amount of androgen-producing cells. Also, there would be a slight drop in the activity of the 17,20-desmolase responsible for the cleavage of the C_{21} steroid side chain (Fig. 7). This is supported by Axelrod's [15] report of reduced C_{17-20} lyase activity in an aged testis. The hypothesis stating that lower testosterone concentration could be due to reversion of the 17β-oxido-reductase with androstenedione accumulation, as happens in immature animals, was discarded after analysis of all the experiments performed by using different precursors of hormonal biogenesis. In such experiments, it was verified that the lower testosterone synthesis found in the aging animals did not correlate with a parallel androstenedione accumulation. Our findings do not discard modifications prior to pregnenolone formation, specially in the mitochondrial enzymes that catalyze the cleavage of the chain linked to C_{17} of cholesterol, as has been suggested for human males as a consequence of an abnormal hypothalamic-pituitary–gonadal axis activity [16–18] or of the study of intratesticular steroid concentration [19], facts that would lead to an even lower precursors contribution in hormonal synthesis.

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