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SUBSTRATE SPECIFICITY FOR ANDROGEN BIOSYNTHESIS IN THE PRIMATE TESTIS

JAMES P. PRESLOCK and EMIL STEINBERGER
Department of Reproductive Medicine and Biology, The University of Texas Medical School at Houston and The Graduate School of Biomedical Sciences, P.O. Box 20708, Houston, TX 77025, U.S.A.

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SUMMARY

The metabolism of selected substrates of the 4-ene and 5-ene pathways was investigated in the testis of the marmoset *Sanguinus oedipus*. Testicular fragments (50 mg) were incubated in duplicate with 10 μCi of the following radiolabeled substrates: pregnenolone, progesterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, and testosterone. Incubations were for 3 h in Krebs–Ringer bicarbonate buffer, pH 7.4, at 37°C. Pregnenolone was converted to 17-hydroxyprogesterone (44.6)%, with lower conversion to testosterone (21.3%), androstenedione (10.8%), and progesterone (9.5%). Progesterone was metabolized to 17α-hydroxyprogesterone (47.3%) with testosterone (22.8%) and androstenedione (11.1%) identified in the incubates. 17-Hydroxypregnenolone was converted to testosterone (57.0%), dehydroepiandrosterone (22.1%) and androstenedione (13.6%). However, 17-hydroxyprogesterone was not well metabolized as 59.8% of the substrate remained, while testosterone and androstenedione were 21.2% and 10.7% of the respective radioactivities. Dehydroepiandrosterone was converted to testosterone (61.0%), and androstenedione (16.2%). Androstenedione was metabolized to testosterone (83.0%), while testosterone substrate was primarily nonmetabolized (85.4%), with some conversion to androstenedione (6.6%). These results suggest a predominant 4-ene pathway in marmosets due primarily to a lack of pregnenolone 17α-hydroxylase activity, while C_{17}-C_{20} lyase may be the rate-limiting step for testosterone synthesis in the testis of this subhuman primate species.

INTRODUCTION

Reports which have been published regarding the biosynthesis of androgens in the subhuman primate testis have described the primary pathways for conversion of pregnenolone to testosterone as either the 4-ene or the 5-ene pathways [1, 2]. We recently demonstrated in the marmoset testis [3, 4] that the 4-ene pathway, with formation of progesterone, 17α-hydroxyprogesterone and androstenedione as intermediates, was predominant. However, there have been no reports published with marmosets which describes the metabolism of intermediates of the 4-ene or the 5-ene pathways as an index of the specificity of testicular steroidogenic enzymes for selected substrates. The following studies were therefore undertaken to investigate the specificity of selected steroidogenic enzymes in the marmoset testis, and to further substantiate the 4-ene pathway as predominate for the synthesis of testosterone from pregnenolone in this subhuman primate specie.

MATERIALS AND METHODS

Materials. Nanograde solvents and silica gel (Silicar TLC-7Gf) were obtained from Mallinckrodt Chemical Works, St. Louis, MO. Cofactors for incubations were from Sigma Chemical Company, St. Louis, MO. Chromatography paper (Whatmann No. 1) was purchased from Whatmann Paper Company and was washed with methanol prior to use. Non-radioactive carriers were obtained from Steraloids, Inc., Rawling, NY, and radiolabeled substrates and tracers were from Amersham Searle, Inc., Arlington Heights, IL. Steroids were checked for purity by thin-layer chromatography prior to use.

Experimental animals. Adult male *Sanguinus oedipus* were housed at constant temperature and humidity with lighting maintained at 14L:10D. They were fed a commercial diet with water provided *ad libitum*. The testes were surgically removed and placed into ice cold 0.25 M sucrose–Tris buffer, pH 7.4, whereupon they were weighed and cut into approximately equal 50 mg fragments.

Incubations. Quadruplicate determinations with 10 μCi of the following radiolabeled precursors were utilized in these studies: [7-^{3}H]-pregnenolone, [7-^{3}H]-progesterone, [7-^{3}H]-17-hydroxypregnenolone, [7-^{3}H]-17-hydroxyprogesterone, [7-^{3}H]-dehydroepiandrosterone, [7-^{3}H]-androstenedione and [7-^{3}H]-testosterone. Incubations were for 3 h in Krebs–Ringer bicarbonate buffer, pH 7.4, at 37°C in a 95% O_2/5%CO_2 atmosphere. The buffer contained NADH and an NADPH generating system. Upon completion of the incubations the reactions were terminated with 1N HCl and the incubates rapidly frozen.

Extraction, separation and purification of metabolites. The incubates were thawed at room temperature and the fragments homogenized in Krebs–Ringer bicarbonate buffer, pH 7.4. The homogenates were pooled with their respective incubation media, with unlabeled carriers and ^{14}C-labeled tracers added to

each pool. The pools were extracted ten times with cold diethyl ether–chloroform, (4:1, v/v) and the extract evaporated with nitrogen. Aliquots for estimation of recoveries were taken prior to each chromatographic procedure, and prior to recrystallization of metabolites.

Initial separation of incubates was by paper chromatography (Whatmann No. 1, 2.5 cm × 50 cm) in hexane–formamide, with a second separation in benzene–hexane–formamide. Peaks were located with a Packard Model 385 Recording Ratemeter and eluted with 80 ml methanol. Further isolation of metabolites was by thin-layer chromatography in selected solvent systems.

Identity of metabolites. Upon separation, identity of metabolites was established by recrystallization to constant specific activities and $^3H/^{14}C$ ratios through three successive solvent combinations. Percent conversion of substrates to metabolites was determined by calculating the total ^3H-d.p.m. for each metabolite from recrystallization data, correcting the total ^3H-d.p.m. for procedural losses, and dividing the corrected ^3H-d.p.m. for each metabolite by the ^3H-d.p.m. of the total incubate.

RESULTS

Radiolabeled pregnenolone was converted primarily to 17-hydroxyprogesterone (44.6%), with testosterone (21.3%), androstenedione (10.8%) and progesterone (9.5%) also identified as metabolites of pregnenolone. Progesterone was also converted primarily to 17-hydroxyprogesterone (47.3%), with significant conversion to testosterone (22.8%), and to androstenedione (11.1%). Figure 1 demonstrates percent conversion of these substrates into their respective metabolites, while Table 1 lists recrystallization data for the metabolites.

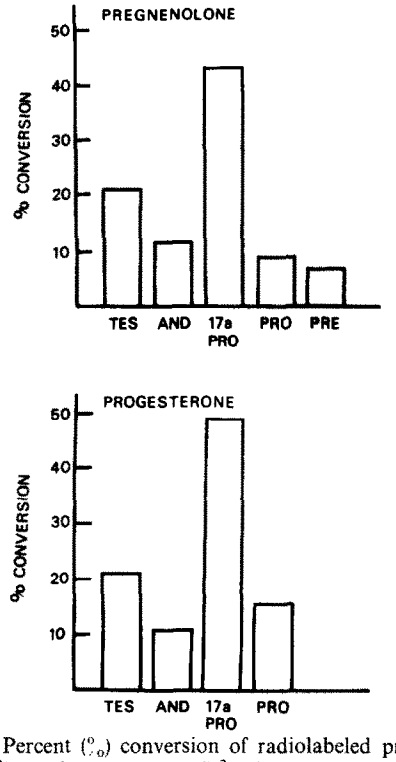

Fig. 1. Percent (%) conversion of radiolabeled pregnenolone-7-^3H and progesterone-7-^3H by marmoset testicular fragments at 3 h of incubation. The following abbreviations are used in the figures: TES, testosterone as testosterone acetate; AND, androstenedione, 17-PRO, 17-hydroxyprogesterone; PRO, progesterone; PRE, pregnenolone.

A substantial portion of radiolabeled 17-hydroxyprogesterone substrate remained unmetabolized throughout the incubations, representing 59.8% of the

Table 1. Recrystallization data for metabolism of pregnenolone-7-^3H (10 μCi) and progesterone-7-^3H (10 μCi) by marmoset testicular fragments (*Sanguinus oedipus*) at 3 h of incubation

Metabolite*†	^3H d.p.m. (I)§	^3H d.p.m./mg‖¶	^{14}C d.p.m./mg‖¶	$^3H/^{14}C$	^3H d.p.m. (T)**	% Purity††
Pregnenolone substrate						
Testost.	2,507.6	114.5	1.2	89.0	2,347.2	93.6
Andros.	1,657.6	75.1	0.9	76.0	1,510.1	91.1
17-OH Prog.	6,267.5	279.7	—	—	5,622.0	89.7
Prog.	1,424.1	64.0	1.3	48.4	1,287.4	90.4
Preg.‡	937.0	40.2	1.1	35.4	808.7	86.3
Progesterone substrate						
Testost.	2,838.0	130.2	1.4	92.3	2,616.7	92.2
Andros.	1,595.6	69.8	1.0	69.5	1,418.5	88.9
17-OH Prog.	6,141.4	288.3	—	—	5,785.2	94.2
Prog.‡	2,097.9	92.5	1.2	71.8	1,923.8	91.7

* The following abbreviations are used in the table: Testost., Testosterone as testosterone acetate; Andros., androstenedione; 17-OH Prog., 17-hydroxyprogesterone; Prog., progesterone, Preg., pregnenolone.

† Approximately 20 mg of crystalline steroid was added to each sample prior to recrystallization.

‡ Non-metabolized substrate.

§ Initial ^3H-d.p.m. ($\times 10^3$) before recrystallization.

‖ Data as mean d.p.m. ($\times 10^3$) of three successive recrystallization of duplicate samples.

¶ Solvent systems for recrystallizations were: I. acetone–hexane; II. acetone–cyclohexane; III. acetone–hexane.

** Total ^3H d.p.m. ($\times 10^3$) following recrystallization.

†† % Purity = ^3H d.p.m. (T)/^3H d.p.m. (I).

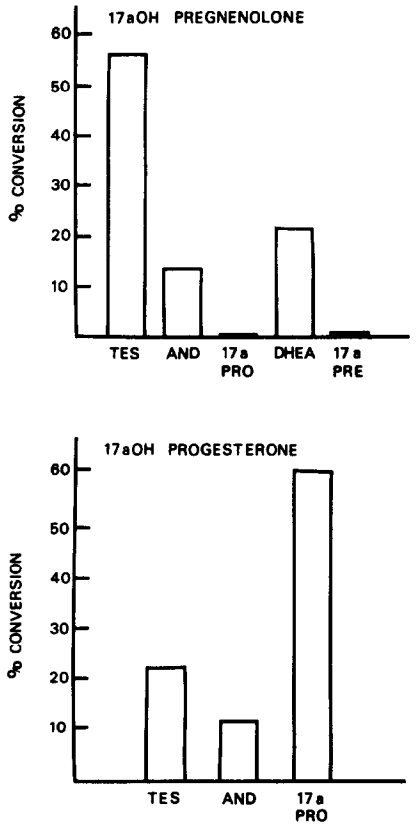

Fig. 2. Percent (%) conversion of radiolabeled 17-hydroxyprogesterone and 17-hydroxypregnenolone by marmoset testicular fragments at 3 h of incubation. The following abbreviations are used in the figure: TES, testosterone as testosterone acetate; AND, androstenedione; 17-PRO, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone as dehydroepiandrosterone acetate: 17-PRE, 17-hydroxypregnenolone.

total radioactivity at 3 h of incubation. Testosterone and androstenedione were 22.4% and 11.0% of the respective radioactivity from 17-hydroxyprogesterone (Fig. 2; Table 2). Conversely, 17-hydroxypregnenolone substrate was efficiently converted to testosterone, which represented 57.0% of the total radioactivity in the 3 h incubate. A significant portion of the 17-hydroxypregenenolone was also converted to dehydroepiandrosterone (22.1%), and to androstenedione (13.6%), with only a trace of 17-hydroxyprogesterone (< 1%) identified in the incubates (Fig. 2; Table 2).

When utilized as a substrate dehydroepiandrosterone was also efficiently converted to testosterone, which was 62.3% of the total radioactivity of the incubate (Fig. 3; Table 3). The remainder of the radioactivity was identified as androstenedione (16.2%) with only a trace of the dehydroepiandrosterone substrate remaining in the incubates (0.9%).

Androstenedione substrate was converted primarily to testosterone (83.5%), while only a small portion (7.8%) of the androstenedione was not metabolized (Fig. 3; Table 3). However, testosterone substrate was poorly metabolized by marmoset testicular fragments as 85.4% of this substrate was still present at 3 h of incubation (Fig. 3), while only 6.6% of the radioactivity in the testosterone incubates was identified as androstenedione.

Neither estrogens nor 5α-reduced metabolites were identified in any of these incubations with marmoset testicular fragments.

DISCUSSION

It has been demonstrated within the mammalian testis that androgens are formed from pregnenolone

Table 2. Recrystallization data for metabolism of 17-hydroxyprogesterone-7-^{3}H (10 μCi) and 17-hydroxypregnenolone (10 μCi) by marmoset testicular fragments (*Sanguinus oedipus*) at 3 h of incubation

Metabolite*†	^{3}H d.p.m. (I)§	^{3}H d.p.m./mg‖¶	^{14}C d.p.m./mg‖¶	^{3}H/^{14}C	^{3}H d.p.m. (T)**	%Purity††
17-hydroxyprogesterone substrate						
Testost.	2,435.5	109.2	1.1	92.9	2,201.7	90.4
Andros.	1,786.3	76.8	1.1	66.5	1,568.4	87.8
17-OH Prog.‡	5,780.4	299.2	—	—	5,352.7	92.6
17-Hydroxypregnenolone substrate						
Testost.	8,038.1	357.2	—	—	7,162.0	89.1
Andros.	2.526.1	116.8	1.3	86.7	2,349.3	93.0
17-OH Prog.	1.3	0.05	0.9	0.06	1.2	88.7
DHEA	3,082.1	139.1	1.2	111.0	2,810.9	91.2

* The following abbreviations are used in the table: Testost., testosterone as testosterone acetate; Andros., androstenedione; 17-OH Prog., 17-hydroxyprogesterone; 17-OH Preg., 17-hydroxypregnenolone; DHEA, dehydroepiandrosterone as dehydroepiandrosterone acetate.

† Approximately 20 mg of crystalline steroid was added to each sample prior to recrystallization.

‡ Non-metabolized substrate.

§ Initial ^{3}H-d.p.m. ($\times 10^3$) before recrystallization.

‖ Data as mean d.p.m. ($\times 10^3$) of three successive recrystallizations of duplicate samples.

¶ Solvent systems for recrystallization were: I. Acetone–Hexane; II, Acetone–Cyclohexane; III. Acetone–Hexane.

** Total ^{3}H d.p.m. ($\times 10^3$) following recrystallization.

†† % Purity = ^{3}H d.p.m. (T)/^{3}H d.p.m. (I).

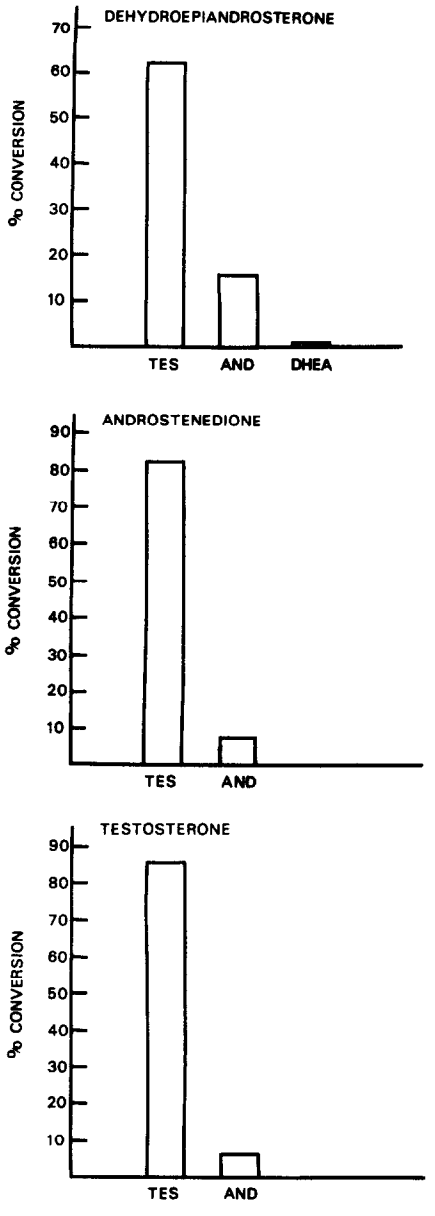

Fig. 3. Percent (%) conversion of radiolabeled dehydroepiandrosterone-7-^{3}H, androstenedione-7-^{3}H, and testosterone-7-^{3}H by marmoset testicular fragments at 3 h of incubation. The following abbreviations are used in the figure. TES, testosterone as testosterone acetate; AND, androstenedione; DHEA, dehydroepiandrosterone as dehydroepiandrosterone acetate.

through the 4-ene or the 5-ene pathway. Metabolism via the 4-ene pathway involves conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase and isomerase [5, 6], with conversion of progesterone to 17-hydroxyprogesterone by steroid 17α-hydroxylase [7, 8]. 17-Hydroxyprogesterone is then converted to testosterone through androstenedione [5, 9]. Metabolism of pregnenolone via the 5-ene pathway involves an initial conversion of pregnenolone to 17-hydroxypregnenolone by steroid 17α-hydroxylase, with a subsequent conversion of 17-hydroxypregnenolone to dehydroepiandrosterone, and then to testosterone [10, 11].

The preferred substrate of steroid 17α-hydroxylase has not been conclusively determined [12]. However, Tamaoki[6] postulated that progesterone is the preferred substrate, while other investigators [10, 11] have reported prenenolone as the preferred substrate of steroid 17α-hydroxylase. More recently, Kremers[12] reported the affinity of microsomal steroid 17α-hydroxylase as 4–6-fold greater for mitochondrial pregnenolone than for progesterone, suggesting that 17-hydroxypregnenolone is an important intermediate in the biosynthesis of androgens.

The present studies with marmosets suggests that progesterone is the preferred substrate of testicular 17α-hydroxylase as progesterone was efficiently converted to 17-hydroxyprogesterone within the incubates, while pregnenolone was not converted into 17-hydroxypregnenolone in the present, or in previous [3, 4], studies. This finding in marmosets differs from that reported by other investigators [10–12] in which pregnenolone was the preferred substrate. However, the results in the present studies with marmosets are somewhat difficult to correlate with studies utilizing rat testicular tissue or microsomes as species variations, enzymic sources, and methodological approaches differ among the studies.

This substrate specificity demonstrated by steroid 17α-hydroxylase in the present study with the marmoset testis is of particular interest. Kremers[12] recently speculated that testicular 17α-hydroxylase in the rat is a single enzymatic complex which is preferential for pregnenolone, resulting in the 5-ene pathway as the normal pathway for androgen biosynthesis. However, the present studies with marmosets suggest this may not be valid in the subhuman primate testis. The fact that marmoset testicular fragments converts progesterone to 17-hydroxyprogesterone, but does not convert any detectable pregnenolone to 17-hydroxypregnenolone, suggests separate isozymic forms of steroid 17α-hydroxylase, namely progesterone 17α-hydroxylase and pregnenolone 17α-hydroxylase, in the marmoset testis. The evidence further suggests that progesterone 17α-hydroxylase efficiently converts progesterone to its 17α-hydroxylated derivative, while pregnenolone 17α-hydroxylase is either inactive or lacking within the testis of this subhuman primate species.

The results of these studies demonstrate that the intermediates of the 5-ene pathway are more efficiently converted to testosterone by the marmoset testis than are the intermediates of the 4-ene pathway. There was nearly complete conversion of 17-hydroxypregnenolone and dehydroepiandrosterone to testosterone, while progesterone and 17-hydroxyprogesterone were less efficiently converted. These results are particularly significant in light of our previous data which demonstrated a predominant 4-ene pathway in marmosets for conversion of pregnenolone to testosterone [3, 4].

Table 3. Recrystallization data for metabolism of dehydroepiandrosterone-7-^{3}H (10 μCi), androstenedione-7-^{3}H (10 μCi) and testosterone-7-^{3}H (10 μCi) by marmoset testicular fragments (*Sanguinus oedipus*) at 3 h incubation

Metabolite*†	^{3}H d.p.m. (I)§	^{3}H d.p.m./mg‖¶	^{14}C d.p.m./mg‖¶	^{3}H/^{14}C	^{3}H d.p.m. (T)**	% Purity
Dehydroepiandrosterone substrate						
Testost.	7,437.2	330.9	1.1	—	6,604.3	88.8
Andros.	2,611.9	121.5	1.1	105.2	2,431.7	93.1
DHEA‡	137.6	6.2	1.2	4.8	124.3	90.3
Androstenedione substrate						
Testost.	7,852.3	316.9	0.95	—	7,271.3	92.6
Andros.‡	1,265.0	55.3	1.0	51.8	1,128.4	89.2
Testosterone substrate						
Testost.‡	7,423.8	324.4	0.82	—	6,807.7	91.7
Andros.	998.0	48.0	1.0	47.1	941.1	94.3

* The following abbreviations are used in the table: Testost., testosterone as testosterone acetate; Andros., androstenedione; DHEA, dehydroepiandrosterone as dehydroepiandrosterone acetate.

† Approximately 20 mg of crystalline steroid was added to each sample prior to recrystallization.

‡ Non-metabolized substrate.

§ Initial ^{3}H-d.p.m. ($\times 10^3$) before recrystallization.

‖ Data as mean d.p.m. ($\times 10^3$) of three successive recrystallizations of duplicate samples.

¶ Solvent systems for recrystallization were: I. Acetone–Hexane; II. Acetone–Cyclohexane; III. Acetone–Hexane.

** Total ^{3}H d.p.m. ($\times 10^3$) following recrystallization.

†† % Purity = ^{3}H d.p.m. (T)/^{3}H d.p.m. (I).

The only other subhuman primate in which the testicular biosynthesis of androgens has been investigated is the rhesus monkey. In this primate, Hoschoian and Brownie[1] incubated testicular homogenates and minces with radiolabeled progesterone and pregnenolone, with 17-hydroxyprogesterone and 17-hydroxypregnenolone, or with androstenedione and dehydroepiandrosterone Pregnenolone and 17-hydroxypregnenolone were more efficiently converted to testosterone than were progesterone and 17-hydroxyprogesterone, suggesting a predominant 5-ene pathway. However, Sharma *et al.*[2] subsequently reported that 17-hydroxyprogesterone was a better substrate for testosterone than was dehydropiandrosterone, postulating testosterone formation through the 4-ene pathway.

The accumulation of 17-hydroxyprogesterone from pregnenolone and progesterone substrates in the present studies is similar to that which we earlier reported [3, 4]. This accumulation of 17-hydroxyprogesterone may reflect low C_{17}-C_{20} lyase activity in the marmoset testis, suggesting that lyase may be the rate-limiting step for testosterone synthesis in this primate species in which the 4-ene pathway is predominant.

In conclusion, the data obtained from the present studies suggest that the predominant 4-ene pathway in marmosets may be a consequence of suppressed pregnenolone 17α-hydroxylase activity, resulting in conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase and isomerase, with progesterone subsequently metabolized to testosterone through the less efficient 4-ene pathway. However, it is also possible that the localization of these steroidogenic enzymes within the smooth endoplasmic reticulum may enable pregnenolone to be more rapidly metabolized by 3β-hydroxysteroid dehydrogenase than by 17α-hydroxylase. This "spatial specificity" of the enzymes may determine their accessibility to pregnenolone, and thereby establish the predominant pathway for conversion of pregnenolone to testosterone within the testis of this subhuman primate species.

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