Correlation between ultrastructure and biochemical changes in the testis of the American grey squirrel, *Sciurus carolinensis*, during the reproductive cycle

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**Summary.** Distinct differences in the ability of isolated seminiferous tubules and interstitium to utilize steroid precursors for androgen production *in vitro* were observed during the reproductive cycle of the American grey squirrel, *Sciurus carolinensis*. In spermatogenically active testes, the seminiferous tubules and the interstitium readily synthesized androgens from labelled C21 steroid precursor; seminiferous tubules also produced significant amounts of 17α,20α-dihydroxyprogesterone. However, during sexual regression androgen synthesis was drastically reduced in both testicular components, while the production of 20α-reduced metabolites of progesterone and 17α-hydroxyprogesterone was increased. Many of the fine-structural changes occurring in Leydig and Sertoli cells during the reproductive cycle could be correlated with the capacity of isolated seminiferous tubules and interstitium to formulate androgens from labelled precursors.

**Introduction**

Extensive studies on the fine structure of steroid-producing cells have demonstrated that various morphological characteristics can be ascribed to cells whose putative function is the synthesis and secretion of steroids (see review by Christensen & Gillim, 1969). However, as yet there are no absolute morphological criteria for establishing whether or not certain cells are steroidogenic.

The significance of morphological characteristics can be established only if they are typical of all cells carrying out the same function and can be correlated precisely with physiological or biochemical data. It has proved necessary, therefore, to examine the fine structure of steroid-producing cells under various experimental conditions to determine the relationship between structure and function in these cells. There have been few studies carried out with cells which have been examined morphologically and biochemically during different physiological states. Seasonally breeding mammals, however, provide a unique opportunity to examine normal morphological and biochemical changes in the testis in relation to changing endocrine states. This paper is a report of studies on the synthesis of androgens by isolated interstitium and seminiferous tubules and correlation of this activity with the fine structure of Leydig and Sertoli cells during the reproductive cycle of the seasonally breeding American grey squirrel, *Sciurus carolinensis*.

**Materials and Methods**

**Animals**

Squirrels were trapped alive and transported to the laboratory where they were killed with chloroform. Of the 12 animals used, 9 were sexually active and 3 were sexually quiescent.

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Materials

[7α-3H]Pregnenolone (3β-hydroxy-5-pregnen-20-one) (sp. act. 1·58 mCi/mg) and [4-14C]preg-
gesterone (4-pregnen-3,20-dione) (sp. act. 185 μCi/mg) were purchased from the Radiochemical Centre, Amersham, Bucks, England.

Pregnenolone, progesterone, androstenedione (4-androstene-3,17-dione), testosterone (17β-
hydroxy-4-androst-ene-3-one), 20α-dihydroprogesterone (20α-hydroxy-4-pregnen-3-one) were obtained from Steraloids Ltd, Croydon, Surrey, England. Testosterone acetate, and 17α-hydroxy-
progesterone (17α-hydroxy-4-pregnen-3,20-dione) were obtained from BDH Ltd, Poole, Dorset, England. Samples of 17α,20β-dihydroxyprogesterone (17α,20β-dihydroxy-4-pregnen-3-one) and 17α,20α-dihydroxyprogesterone (17α,20α-dihydroxy-4-pregnen-3-one) were kindly supplied by the M.R.C. Steroid Reference Collection, Westfield College, London, England.

All solvents used were of analytical grade.

Incubation conditions

Testes were removed, weighed and decapsulated. Small pieces of tissue were removed for morpho-
logical studies. The remainder was placed in ice-cold Krebs–Ringer bicarbonate buffer, pH 7·4,
containing glucose (2 g/litre), dissected into tubule and interstitial components (Christensen & Mason, 1965) and placed in separate tubes containing 5 ml buffer. Pieces of tissue were frequently processed for electron microscopy as a check on the purity of the preparation.

Amounts of tissue used for interstitial incubations varied between 65 and 385 mg wet weight and for tubule incubations between 210 and 932 mg wet weight. The buffer, plus separated interstitial and tubule fractions, was transferred to flasks containing equimolar concentrations of [7-3H]-
pregnenolone and [4-14C]progesterone as precursors. The isolated components were incubated at
33°C under an atmosphere of 95% O₂ + 5% CO₂ with continuous shaking. At timed intervals, 0·5 ml samples of incubation medium were withdrawn and replaced with 0·5 ml fresh buffer without radio-
active precursors. No carrier steroids were added for estimation of extraction losses.

Extraction and characterization of steroid products

Steroids were extracted from incubation samples with 2 vols ethyl acetate. These crude extracts
were then subjected to initial fractionation on paper chromatography in system T/70 (1 h) and L/75
(2 h) without elution (Vinson, 1966). The chromatograms were scanned in a Packard Radiochro-
matogram scanner (Model 7200) and areas containing radioactivity were eluted. The eluates obtained from each incubation were routinely purified by reaction with the acetylation mixture of Zaffaroni
& Burton (1951) or the tert-butyl chromate oxidizing reagent of Menini & Norymberski (1962). After treatment, all samples were rechromatographed in L/75, to give adequate separation of steroid
products.

It was inevitable that some samples removed from incubations during kinetic studies contained
low yields of steroid products. To ensure adequate identification by repeated chromatography and
recrystallization procedures, the medium remaining at the end of each incubation was used for the
positive identification of steroid products. The procedures used for the characterization of these
compounds and the chromatographic systems used are shown in Table 1.

Recrystallization of products

Steroids isolated from the incubation medium were mixed with 50 mg authentic material and
recrystallized from light petroleum ether or methanol (Axelrod, Matthijssen, Goldzieher & Pulliam, 1965). Table 2 represents recrystallization data for products obtained from incubations of interstitial and seminiferous tubule tissue.

17α,20α-Dihydroxyprogesterone was subjected to oxidation with sodium bismuthate (Appleby
& Norymberski, 1955) and recrystallized as androstenedione, due to the difficulty of obtaining suffi-
cient quantities of the authentic compound. Testosterone was separated from 17α-hydroxyprogesterone by acetylation and recrystallized as testosterone acetate.

Table 1. Procedures for characterization of the compounds formed during incubation of grey squirrel testicular tissue

<table>
<thead>
<tr>
<th>Product investigated</th>
<th>Treatment</th>
<th>Authentic material with identical chromatographic behaviour</th>
<th>Chromatographic system</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H:14C]Progesterone</td>
<td>Extraction</td>
<td>Progesterone</td>
<td>T/70, L/75, TLC 4*</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Progesterone</td>
<td>L/75</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>Progesterone</td>
<td>T/70, L/75, TLC 9</td>
</tr>
<tr>
<td>[3H:14C]17α-Hydroxyprogesterone</td>
<td>Extraction</td>
<td>17α-Hydroxyprogesterone</td>
<td>T/70, L/75, TLC 8</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>17α-Hydroxyprogesterone</td>
<td>L/75, TLC 7</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>17α-Hydroxyprogesterone</td>
<td>L/75</td>
</tr>
<tr>
<td></td>
<td>Monochloroacetic anhydride</td>
<td>17α-Hydroxyprogesterone</td>
<td>L/75</td>
</tr>
<tr>
<td></td>
<td>Heptafluorobutyric anhydride</td>
<td>17α-Hydroxyprogesterone</td>
<td>L/75, TLC 3</td>
</tr>
<tr>
<td>[3H:14C]Androstenedione</td>
<td>Extraction</td>
<td>Androstenedione</td>
<td>T/70, L/75, TLC 4 and 8</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Androstenedione</td>
<td>L/75</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>Androstenedione</td>
<td>T/70/L/75, TLC 9</td>
</tr>
<tr>
<td>[3H:14C]Testosterone</td>
<td>Extraction</td>
<td>Testosterone</td>
<td>T/70, L/75, TLC 5 and 8</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Testosterone acetate</td>
<td>L/75, TLC 1, 6 and 7</td>
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<tr>
<td></td>
<td>Oxidation</td>
<td>Androstenedione</td>
<td>TLC 1</td>
</tr>
<tr>
<td></td>
<td>Monochloroacetic anhydride</td>
<td>Testosterone</td>
<td>L/75</td>
</tr>
<tr>
<td></td>
<td>Heptafluorobutyric anhydride</td>
<td>Testosterone heptafluorobutyrate</td>
<td>L/75, TLC 3</td>
</tr>
<tr>
<td>[3H:14C]17α,20α-Dihydroxyprogesterone</td>
<td>Extraction</td>
<td>17α,20α-Dihydroxyprogesterone</td>
<td>T/70, L/75</td>
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<tr>
<td></td>
<td>Acetylation</td>
<td>17α,20α-Dihydroxyprogesterone</td>
<td>TLC 2, 5, 8 and 10</td>
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<td>tert-Butyl chromatate</td>
<td>17α-Dihydroxyprogesterone</td>
<td>L/75, TLC 7</td>
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<td></td>
<td>Sodium bismuthate Reduction</td>
<td>Androstenedione</td>
<td>L/75, TLC 7</td>
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<td></td>
<td>4-Pregnene-3β, 17α,20α-triol</td>
<td>17α-Hydroxyprogesterone androstenedione</td>
<td>T/70, L/75</td>
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<td>[3H:14C]20α-Dihydroprogesterone</td>
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<td>20α-Dihydroprogesterone</td>
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<td>20α-Dihydroprogesterone acetate</td>
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<td>Oxidation</td>
<td>Progesterone</td>
<td>L/75, TLC 9</td>
</tr>
<tr>
<td>[3H]Pregnenolone</td>
<td>Extraction</td>
<td>Pregnenolone</td>
<td>T/70, L/75, TLC 4</td>
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<tr>
<td></td>
<td>Acetylation</td>
<td>Pregnenolone acetate</td>
<td>L/75</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>4-Pregnene-3,6,20-trione</td>
<td>T/70, L/75, TLC 9</td>
</tr>
</tbody>
</table>

* TLC 1 = light petroleum ether/benzene/ethyl acetate (1:1:4 by vol.); TLC 2 = methylene chloride/acetone (9:1 v/v); TLC 3 = benzene/ethyl acetate (9:1 v/v); TLC 4 = benzene/ethyl acetate (2:1 v/v); TLC 5 = toluene/ethyl acetate (8:2 v/v), followed by benzene/ethyl acetate (2:1 v/v), followed by toluene/methanol (2:1 v/v); TLC 6 = benzene/ethyl acetate (2:1 v/v); TLC 7 = toluene/ethyl acetate (8:2 v/v); TLC 8 = benzene/methanol (9:1 v/v); TLC 9 = benzene/ethyl acetate (8:2 v/v); TLC 10 = benzene/methanol (9:1 v/v) followed by benzene/acetone (8:2 v/v); TLC 11 = ethyl acetate/cyclohexane (1:1 v/v).
Table 2. Recrystallization data of products isolated from incubations of testicular interstitial and seminiferous tubule tissue of grey squirrels

<table>
<thead>
<tr>
<th>Substance (recrystallized as)</th>
<th>Solvent</th>
<th>Recrystallization No.</th>
<th>Crystals</th>
<th>Mother Liquor</th>
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<tbody>
<tr>
<td>Interstitial tissue</td>
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<td></td>
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<tr>
<td>Progesterone</td>
<td>Methanol</td>
<td>1</td>
<td>10.05</td>
<td>10.91</td>
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<td></td>
<td></td>
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<td>9.89</td>
<td>10.43</td>
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<td></td>
<td>3</td>
<td>10.59</td>
<td>9.32</td>
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<tr>
<td>17α-Hydroxyprogesterone</td>
<td>Light petroleum</td>
<td>1</td>
<td>19.74</td>
<td>9.61</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td>3</td>
<td>9.73</td>
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<td>9.57</td>
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<td>3</td>
<td>9.30</td>
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<td>Testosterone (recrystallized as testosterone acetate)</td>
<td>Light petroleum</td>
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<td>9.99</td>
<td>10.49</td>
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<td></td>
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<td>2</td>
<td>8.74</td>
<td>9.61</td>
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<td></td>
<td></td>
<td>3</td>
<td>9.91</td>
<td>9.73</td>
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<tr>
<td>Seminiferous tubule tissue</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>Methanol</td>
<td>1</td>
<td>2.89</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2.05</td>
<td>2.92</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>2.75</td>
<td>2.97</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>Light petroleum</td>
<td>1</td>
<td>5.59</td>
<td>10.26</td>
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<tr>
<td></td>
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<td>2</td>
<td>6.45</td>
<td>12.13</td>
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<td>4</td>
<td>10.45</td>
<td>10.24</td>
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<td>1</td>
<td>9.41</td>
<td>10.98</td>
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<td></td>
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<td>3</td>
<td>10.26</td>
<td>10.85</td>
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<td>17α,20α-Dihydroxyprogesterone</td>
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<td>8.18</td>
<td>10.72</td>
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<td>3</td>
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<td>4</td>
<td>8.65</td>
<td>10.71</td>
</tr>
</tbody>
</table>

**Determination of radioactivity**

After identification, steroids were evaporated into vials and 10 ml toluene containing 4 g 2,5 diphenyl oxazole and 50 mg 1,4-di-[2-(5-phenyl oxazolyl)] benzene per litre were added. The radioactive content of these steroids was estimated by liquid scintillation spectroscopy in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3380, adjusted for double isotope counting. This machine gave efficiencies of 60-09% and 24-28% for 14C and 3H respectively, with 6-98% efficiency for 14C in the 3H channel and 0-2% efficiency for 3H in the 14C channel. After correcting for dilution factors incurred during the incubations, the radioactive content of isolated steroids was expressed as a percentage of the added precursor and plotted against time.

**Electron microscopy studies**

The tissue removed from each testis before incubation was immediately fixed in ice-cold Millonig's phosphate-buffered glutaraldehyde for 2-3 h. This tissue was washed overnight in a phosphate-buffered rinse and then post-fixed for 1 h in Millonig's phosphate-buffered OsO4. After rapid dehydration the tissue was embedded in Araldite (Glauert & Glauert, 1958), sections were stained with uranyl acetate (Glauert, 1965) and lead citrate (Reynolds, 1963) and examined in a Siemens Elmiskop 1a or a JEOL JEM 7a electron microscope.
Results

Interstitium isolated from sexually functional testes

Product–yield/time graphs for 14C and 3H compounds were constructed from incubations of 6 separate kinetic experiments, using preparations collected from 6 different animals. A typical example for 3H steroids is shown in Text-fig. 1(a).

All metabolites isolated were double-labelled and the results were similar for both isotopes. [14C]Progesterone was readily converted into 14C-labelled 17α-hydroxyprogesterone, androstenedione and testosterone. These metabolites, together with [3H]progesterone, were also effectively formed from [3H]pregnenolone. A distinctive feature of these incubations was the extremely high yields of androstenedione formed from both substrates: it was the major steroid synthesized and within the first 30 min of most incubations over 20% of both added precursors had been metabolized to this compound. Maximal yields of androstenedione varied between 27 and 49% of the added activity. Compared with the yields of androstenedione the amounts of testosterone formed from both substrates were low and varied between 0.55 and 13% of the added activity.

Interstitium isolated from regressing testes

The results from incubations of interstitium taken from testes during early regression (i.e. with many tubules containing degenerating germ cells but with some still spermatogenically active) were similar to those obtained for the interstitium of sexually functional testes.

Text-figure 1(b) shows a product–yield/time graph for 3H products isolated from one of two separate incubations of interstitium from testes during sexual quiescence (i.e. no tubules producing spermatozoa and with the lumen occluded with degenerating spermatids). The experiments were carried out using interstitial preparations from testes of different animals which were at the same stage of regression. All metabolites produced were double-labelled and the yield/time graphs were similar for both isotopes.

The production of [3H]progesterone from [3H]pregnenolone was extremely efficient, with yields of up to 30% of the added substrate being converted after 1 h of incubation. However, once this step had been accomplished, the further metabolism of progesterone was not as effective. This resulted in the accumulation of progesterone during the incubations with the formation of 17α-hydroxyprogesterone, androstenedione and testosterone occurring at very low rates. Although [3H]- and [14C]progesterone remained as the major steroid throughout these incubations, significant yields of androstenedione (up to 12%) and 17α,20α-dihydroxyprogesterone (up to 8%) were formed from both substrates. In contrast, the amounts of testosterone synthesized from both precursors were extremely low and never exceeded 2% of the added activity.

Spermatogenically active seminiferous tubules

Product–yield/time graphs were constructed from the results of incubations of seminiferous tubules obtained from the testes of animals used for interstitial incubations and a characteristic graph is illustrated in Text-fig. 1(c). Six incubations of seminiferous tubule preparations from 6 different animals were carried out. The yield/time graphs exhibited a degree of variability in the amounts of steroid intermediates and end-products synthesized. Despite this, most graphs were comparable with each other.

All metabolites isolated from these incubations were double-labelled and the results were consistently similar for both isotopes. The production of [3H]progesterone from [3H]pregnenolone proceeded efficiently with maximal yields of up to 17% being obtained within the 1st hr of incubation. Both [14C]progesterone and [3H]progesterone were equally efficiently transformed to 17α-hydroxyprogesterone, androstenedione and testosterone. An interesting feature was the synthesis of 17α, 20α-dihydroxyprogesterone, which was often the major steroid formed from both precursors with yields of up to 22% of the added activity being converted. Smaller amounts of 20α-dihydroprogesterone (up to 2% yield) were also isolated from some incubations.
Text-fig. 1. Product-yield/time graphs for radioactive compounds from [7α-³H]pregnenolone during incubations of (a) interstitium isolated from sexually functional testis; (b) interstitium isolated from regressed testis; (c) spermatogenically active seminiferous tubules; (d) seminiferous tubules from regressed testis of grey squirrels. ●, Pregnenolone; ■, progesterone; △, 17α-hydroxyprogesterone; ▲, androstenedione; ○, testosterone; □, 17α,20α-dihydroxyprogesterone; ×, 20α-dihydroprogesterone.
The maximal yields of 17α,20α-dihydroxyprogesterone and testosterone varied during the different incubations. The synthesis of these products usually reached a maximum, but sometimes the production of the two steroids continued to increase throughout a particular incubation.

Testosterone synthesis proceeded efficiently in most tubule incubations, with yields of up to 37% being obtained from both substrates. However, in one incubation the formation of testosterone was extremely low with yields of not more than 3% being obtained.

Seminiferous tubules isolated from regressing testes

Incubations of tubules from testes undergoing early regression did not differ from those of previous incubations using seminiferous tubules from sexually functional testes.

Text-figure 1(d) demonstrates a product–yield/time graph for 3H compounds from results of incubations of seminiferous tubules from fully regressed testes; these tubule preparations were obtained from the same testes as used for the interstitial tissue experiments.

All metabolites formed were double-labelled and the results were similar for both isotopes. [3H]Pregnenolone was efficiently transformed to [3H]progesterone with yields of up to 21% being produced in the 1st hr of incubation. After this, however, progesterone was further metabolized at a very slow rate. This resulted in large amounts of [3H]- and [14C]progesterone accumulating during the incubations. The major steroid synthesized from both substrates was 17α,20α-dihydroxyprogesterone with yields of up to 6% being obtained. Other steroids produced in significant amounts were 20α-dihydroprogesterone (up to 2%) and 17α-hydroxyprogesterone (up to 7%). The synthesis of androstenedione and testosterone from both precursors was extremely low with yields never exceeding 0.5% of the added activity.

Fine structure

Leydig cells. Low power micrographs demonstrated that the Leydig tissue in spermatogenically active testis is composed of large cells, associated in compact islands or chains between seminiferous tubules. These Leydig cells appeared fully differentiated with the cytoplasm containing numerous cellular organelles (Pl. 1, Fig. 1). Mitochondria are numerous, often large and polymorphic with tubular cristae. Lipid droplets are often surrounded by elements of the agranular reticulum. The granular reticulum is only poorly represented and consisted of short, isolated tubules. In contrast, there is extensive development of the agranular reticulum which pervades most of the cytoplasm as a system of anastomosing tubules.

The interstitial tissue of squirrels during testicular regression is composed of a loose assemblage of severely involuted Leydig cells. These cells possess small amounts of cytoplasm and nuclei which are extremely irregular in outline, with the chromatin condensed around the nuclear membrane (Pl. 1, Fig. 2). There is a reduction in the amount of cellular inclusions, especially in the amount of agranular reticulum which is now composed of a small number of tubules and vacuoles. A distinctive feature of regressed Leydig cells is the presence of many large electron-dense bodies consisting of a granular matrix containing small droplets, possibly lipoidal in nature.

Sertoli cells. In spermatogenically active testes, Sertoli cells are represented by areas of cytoplasm, observed as strips of tissue running between and around developing germ cells. The Sertoli cell cytoplasm is distinct because it appears more dense than germ cell cytoplasm. In certain regions, usually at the base of the germinal epithelium, expanded areas of Sertoli cell cytoplasm were observed. These regions contain numerous small mitochondria, lipid droplets and elements of the agranular reticulum (Pl. 2, Fig. 3). A common feature previously described (Pudney, 1968) was an extreme development of the agranular reticulum by Sertoli cytoplasm surrounding maturing spermatids. These large areas of agranular reticulum are composed of parallel and fenestrated cisternae. Also present in the basal region of the seminiferous tubule are specialized junctions between adjacent Sertoli cells (Flickinger & Fawcett, 1968).
During sexual regression there is an apparent increase in volume of Sertoli cell cytoplasm. This is due in part to the degeneration of many germ cells resulting in an increase in area occupied by Sertoli cells. Also, since the Sertoli cells engulf these germ cells an increase in volume of Sertoli cytoplasm due to the phagocytosis of this material would be expected. The large areas of Sertoli cytoplasm contain numerous cellular organelles as well as many structures derived from the degeneration of germ cells. A noticeable feature is an increase in the amount of agranular reticulum which occurs in many different configurations (Pl. 2, Fig. 4). Areas of parallel cisternae, fenestrated cisternae, anastomosing tubules and diffuse regions of tubules and vacuoles are found in Sertoli cytoplasm. These areas of agranular reticulum differ from those present in Sertoli cells during spermatogenesis in that they are not normally associated with a particular germ cell type. However, numerous vacuoles surrounding degenerating spermatids were consistently observed (see Pl. 2, Fig. 4). It is possible that these vacuoles result from the dissolution of the ordered arrays of cisternal agranular reticulum which had previously surrounded developing spermatids in the spermatogenically active testis. The numerous empty vacuoles, dense inclusions and vacuoles containing cellular structures or a flocculent material present in Sertoli cells at this time were presumed to be involved with, or products derived from, the phagocytosis of degenerating germ cells.

Discussion

Steroid products isolated from incubations indicated that the interstitium and the seminiferous tubules from spermatogenically active testes possess the enzyme systems $\Delta^2$-3$\beta$-hydroxysteroid dehydrogenase–isomerase, 17a-hydroxylase, $C_{17}$–$C_{20}$ lyase and 17$\beta$-hydroyxysteroid dehydrogenase, capable of converting pregnenolone to testosterone (see Text-fig. 2).

(a) **Interstitial tissue**

\[
\text{Pregnenolone} \rightarrow \text{progesterone} \rightarrow 17a-\text{hydroxyprogesterone} \rightarrow \text{androstenedione} \rightarrow \text{testosterone}
\]

**Seminiferous tubules**

\[
\begin{align*}
20a-\text{dihydroprogesterone} & \quad 17a,20a-\text{dihydroxyprogesterone} \\
\text{Pregnenolone} & \rightarrow \text{progesterone} \rightarrow 17a-\text{hydroxyprogesterone} \rightarrow \text{androstenedione} \rightarrow \text{testosterone}
\end{align*}
\]

(b) **Interstitial tissue**

\[
17a,20a-\text{dihydroxyprogesterone}
\]

\[
\text{Pregnenolone} \rightarrow \text{progesterone} \rightarrow 17a-\text{hydroxyprogesterone} \rightarrow \text{androstenedione} \rightarrow \text{testosterone}
\]

**Seminiferous tubules**

\[
\begin{align*}
20a-\text{dihydroprogesterone} & \quad 17a-20a-\text{dihydroxyprogesterone} \\
\text{Pregnenolone} & \rightarrow \text{progesterone} \rightarrow 17a-\text{hydroxyprogesterone} \rightarrow \text{androstenedione} \rightarrow \text{testosterone}
\end{align*}
\]

Text-fig. 2. Pathways for androgen production by the testis of the grey squirrel during (a) active spermatogenesis and (b) sexual regression. $\rightarrow$, minor pathway; $\rightarrow$, dominant pathway.

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**EXPLANATION OF PLATE 1**

Fig. 1. Electron micrograph of Leydig cells during the sexually active season of the grey squirrel. The cytoplasm contains large areas of agranular reticulum (arrows) in the form of anastomosing tubules, and numerous mitochondria (M). Also shown is the Golgi complex (GC). $\times 13,000$.

Fig. 2. The involuted Leydig cells of the grey squirrel do not possess a well developed agranular reticulum. The cytoplasm contains many large mitochondria (M) and dense bodies (D). Note the irregular shape of the nucleus (N). $\times 11,000$. 
The similarity between yield/time curves for $^3$H and $^{14}$C products suggests that the two precursors are metabolized similarly by the two tissues and is consistent with the hypothesis that progesterone is an early and obligatory intermediate in the formation of steroids from pregnenolone (Vinson, 1966; Whitehouse & Vinson, 1967). Product yield/time curves may be further interpreted on the basis that sequential maxima in isotope content of different compounds reflect the order in which they occur in the biosynthetic pathway (see Frost & Pearson, 1961). Therefore, in the present work, the early maximum in isotope content of 17α-hydroxyprogesterone and subsequently in androstenedione indicated that the biosynthetic pathway for androgen production in both interstitium and seminiferous tubule incubations proceeded from progesterone, through 17α-hydroxyprogesterone and androstenedione to testosterone. This is in accord with the $\Delta^4$ pathway for androgen formation as described by Slaunwhite & Samuels (1956). Other evidence (Slaunwhite & Burgett, 1965) has suggested an alternative pathway for androgen synthesis which involves various $\Delta^5$ intermediates. However, with the absence of intermediates bearing the $^3$H label alone, it would appear that the $\Delta^4$ pathway is the major route for androgen synthesis in the interstitial and seminiferous tubule tissue of grey squirrel testis.

The incubations of seminiferous tubule tissue were carried out at the same time as the interstitium incubations so that the steroid-producing activity of the two components under the same conditions could be directly compared. Steroid synthesis by the seminiferous tubules differed (both qualitatively and quantitatively) from that occurring in the interstitium. This, coupled with histological checks on the purity of each preparation, demonstrated that the steroid-producing activity of the tubules was not due to contaminating interstitial tissue enzymes.

Certain features of the yield/time graphs constructed from incubations of squirrel tubules were comparable with those obtained by Bell, Vinson & Lacy (1971) for rat tubule incubations. In both cases testosterone was one of the major steroids formed. Evidence for a reductive pathway, leading to various hydroxylated and/or reduced C$_{21}$ compounds was also found in rat tubule incubations. A hydroxylating pathway was also demonstrated during incubations of squirrel seminiferous tubules. The presence of the enzyme system 20α-hydroxysteroid dehydrogenase was demonstrated by the identification of 20α-dihydroprogesterone and 17α,20α-dihydroxyprogesterone. As in rat tubules, therefore, there are two pathways for the elaboration of steroids in squirrel tubules, one leading to the production of androgens, the other to the formation of hydroxylated compounds (see Text-fig. 2). In contrast, there was no evidence to suggest the existence of a hydroxylating pathway in squirrel interstitium isolated from spermatogenically active testes.

The production of 17α,20α-dihydroxyprogesterone has been found to occur in teased tubule preparations from rats (Ellis & Berliner, 1969), while the enzyme system 20α-hydroxysteroid dehydrogenase has been demonstrated in the seminiferous tubules of the English sparrow (Passer domesticus) (Fevold & Eik-Nes, 1962).

It has been suggested (Sweat et al., 1960; Axelrod & Goldzieher, 1962) that 17α,20α-dihydroxyprogesterone could be cleaved to form C$_{19}$ steroids. However, only a limited amount of direct evidence for the side-chain cleavage to C$_{19}$ steroids has been available for the testis (Berliner & Ellis, 1965). Ichii, Kobayashi & Matsuba (1965) observed that whereas C$_{19}$ steroids could be derived from 17α,20α-dihydroxyprogesterone in hog adrenal homogenates, 17α-hydroxyprogesterone was the immediate precursor of C$_{19}$ steroids in rat testis homogenates. There was little evidence from the kinetic incubation studies to suggest that in the spermatogenically active seminiferous tubules of the grey squirrel 17α,20α-dihydroxyprogesterone was cleaved to form androstenedione.

**EXPLANATION OF PLATE 2**

Fig. 3. An area of Sertoli cytoplasm (S) from spermatogenically active testis of a grey squirrel. The Sertoli cytoplasm contains numerous small mitochondria (M) and a well developed agranular reticulum (arrows). The Sertoli cell nucleus (N) is present, and a germ cell (G) is also shown. x18,000.

Fig. 4. During sexual quiescence in the grey squirrel the area occupied by Sertoli cell cytoplasm increases and contains large amounts of agranular reticulum. In this micrograph anastomosing tubules (At) and numerous vacuoles (V) are shown. A Sertoli cell nucleus (N) and a germ cell (G) are also present. x9000.
Inano, Nakano, Shikita & Tamaoki (1967) suggested that 20α-hydroxysteroid dehydrogenase plays an important role in controlling androgen synthesis by competing with C17-C21 lyase for the common substrate 17α-hydroxyprogesterone. The present work shows that competition between the enzymes, 20α-hydroxyprogesterone dehydrogenase and C17-C20 lyase, for 17α-hydroxyprogesterone did not affect the production of testosterone or 17α,20α-dihydroxyprogesterone since both steroids were the major compounds produced during the various incubations. It does not appear, therefore, that the formation of 17α,20α-dihydroxyprogesterone is significant in the regulation of androgen synthesis in squirrel seminiferous tubules. De Bruijn & van der Molen (1974) also concluded that 17α,20α-dihydroxyprogesterone was not involved in the regulation of androgen production in vivo in rabbit or rat testes.

The accumulation of progesterone during incubations of seminiferous tubules and interstitium isolated from regressed testis is of interest because progesterone and its metabolites have been shown to possess antiandrogenic activity (Huseby, Dominguez & Samuels, 1961; Ellis & Berliner, 1969) and to interfere with, and suppress, spermatogenesis (Heller, Laidlaw, Harvey & Nelson, 1958; Heller, Moore, Paulsen, Nelson & Laidlaw, 1959). Ellis & Berliner (1969) have suggested that progesterone and 17α-hydroxyprogesterone might accumulate in Sertoli cells while androgen synthesis is diminished. It was further suggested that these antiandrogens inhibited the normal functioning of the seminiferous tubules after irradiation by suppressing spermatogenesis (Ellis, 1970).

In interstitial and seminiferous tubule preparations isolated from spermatogenically active testes, the results demonstrated that the dominant pathway proceeded via C17-C20 lyase with the production of a high yield of androgens. When the testis underwent regression, the formation of androgens became reduced but there was an increase in activity of 20α-hydroxyprogesteroid dehydrogenase. The significance of the increased activity of this enzyme at this time could be to inactivate intermediates for androgen synthesis by means of a 'shunt pathway' with the production of androgenically inert steroids such as 17α,20α-dihydroxyprogesterone.

It has been established (Christensen & Mason, 1965; Hall, Irby & de Kretser, 1969; Bell et al., 1971) that isolated rat seminiferous tubules are capable of producing androgens in vitro. The present work has shown that, in a different species, seminiferous tubules undergoing spermatogenesis also possess the capacity to formulate androgens under in-vitro conditions. The production of androgens by the seminiferous tubules was commensurate with normal germ cell development; however, natural regression of the germinal epithelium was accompanied by modified steroid metabolism and a reduction in the formation of androgens.

Seasonal changes in the capacity of testicular tissue of the masked civet cat to produce testosterone, in vitro, from labelled pregnenolone has been studied by Tsui, Tam, Lofts & Phillips (1974). From the limited number of animals studied it was suggested that a close correlation existed between gross histological changes, such as nuclear size and presence of lipid, in the interstitial cells and the ability of testicular tissue to produce testosterone from a labelled precursor. However, studies on the pipistrelle bat demonstrated no apparent change in either morphology of Leydig cells or presence of steroidogenic enzymes in the interstitium during the reproductive cycle (Racey & Tam, 1974). High levels of androgen were synchronous with spermatogenesis, but were still produced by the testis during the sexually quiescent phase. However, the bat is unusual in that spermatozoa are stored in the cauda epididymidis during the sexually inactive period, and the survival of these spermatozoa is presumably dependent upon a continuous secretion of androgen by the testis.

It is possible to correlate many of the changes in ability of the isolated interstitial and tubular tissue to metabolize steroid precursors during the periods of sexual activity and sexual regression with changes in the fine structure of Leydig and Sertoli cells. Leydig cells in spermatogenically active testes possessed all the necessary cytological criteria which have been established for steroid-producing cells. This was confirmed by demonstration that the interstitial tissue possessed the capacity to efficiently metabolize steroid precursors to androgens in vitro. In contrast, Leydig cells during sexual quiescence were atrophied and did not possess an abundant agranular reticulum. Morphologically these Leydig cells appeared less capable of formulating steroid hormones than those of spermatogenically active testes, and the incubation results showed that the amounts of androgen synthesized were insignificant. In fact, the major steroid obtained was progesterone (plus small
quantities of hydroxylated pregnanes). Since these hydroxylated pregnanes were not produced by active Leydig cells and the synthesis of androgens was drastically reduced, it was apparent that the difference in fine structure of Leydig cells was associated, not only with their ability to utilize androgen precursors, but also with the pathways involved in the metabolism of these precursors. The enzyme system, Δ5-3β-hydroxysteroid dehydrogenase isomerase, which converts pregnenolone to progesterone, appeared to be equally as active in the interstitial tissue of regressed testes as that isolated from spermatogenically active testes. The interstitium tenaciously retained this enzyme complex even when other steroid biosynthetic enzyme activities were diminished, and also at a time when the amount of agranular reticulum in the cells had become drastically reduced. However, there were some features which were not compatible with the normally accepted criteria established for steroid-producing cells. For example, the Sertoli cells of regressed testes possessed enormous amounts of agranular reticulum. An abundance of agranular reticulum is, by morphological dogma, indicative of active steroid metabolism. However, the seminiferous tubules of the grey squirrel, during sexual regression, only poorly metabolized steroid precursors during incubation. Such observations demonstrate the danger of extrapolating morphological data from one cell type to another without reservations or physiological facts to support the extrapolation.

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References


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