CONVERSION OF PROGESTERONE TO ANDROGENS BY NON-FLAGELLATE GERMINAL CELLS ISOLATED FROM SEMINIFEROUS TUBULES OF RAT TESTIS

H. J. GALENA AND C. TERNER

Department of Biology, Biological Science Center, Boston University, Boston, Massachusetts 02215, U.S.A.

(Received 7 May 1973)

SUMMARY

A method is described for the isolation of non-flagellate germinal cells of the testis. The interstitial cells were removed by submersion of teased seminiferous tubules in distilled water. The interstitial cells exposed to water burst while the germinal cells inside the tubules remained intact. The tubules were then homogenized in isotonic saline and the non-flagellate germinal cells (spermatocytes and young spermatids) were isolated by centrifugation and filtration through a layer of Sephadex G-25 gel. On incubation with progesterone these cells produced 17α-hydroxyprogesterone, androstenedione, and testosterone. The rate of conversion of progesterone to testosterone in vitro was 0.20 μg/h/10^9 germinal cells. These results suggest that the non-flagellate germinal cells of the testis may make a significant contribution to the production of androgens.

INTRODUCTION

It has been accepted for a long time that the Leydig cells are the sole sites of steroidogenesis in the mammalian testis. However, recent investigations suggest that other cells in the testis may also possess the ability to synthesize androgens. Christensen & Mason (1965) incubated a preparation of seminiferous tubules, stripped of Leydig cells by microdissection, with progesterone and showed that the tubules synthesized androgens. This study was confirmed by Hall, Irby & de Kretser (1969), who used a similar technique. The possibility still remains that residual Leydig cells contributed to the observed biosynthesis. Also, both groups made no attempt to separate the germ cells from the tubules.

The present report presents an attempt to test the ability of isolated germinal cells, free from Leydig cells, to synthesize androgens. A method was designed to remove the interstitial tissue outside the tubules by lysis in a hypotonic solution. The germ cells, essentially spermatocytes and spermatids, were subsequently isolated by a filtration procedure. Suspensions of these germ cells were found to be capable of converting progesterone to 17α-hydroxyprogesterone, androst-4-ene-3,17-dione (androstenedione) and testosterone.
These findings were presented to the 56th Annual Meeting of the Federation of American Societies for Experimental Biology (Galena & Terner, 1972).

MATERIALS AND METHODS

Preparation of cells

Mature rats (10–20) purchased from a local breeder were killed by decapitation. The testes were weighed, decapsulated and placed into ice-cold Krebs–phosphate–saline (Krebs & Eggleston, 1940). The tufts of tubules were passed through five Petri dishes containing ice-cold Krebs–phosphate–saline. By gently pipetting fresh Krebs–phosphate–saline over the tubules with a Pasteur pipette, sliding the tubules along the side of the Petri dish and pulling them apart with forceps, the adhering connective tissue was removed. The tufts had separated into single strands by the end of the third wash. The tubules were then drained of saline, placed in ice-cold distilled water and agitated for 2 min. They were then returned to ice-cold Krebs–phosphate–saline and were cut up with fine scissors. The mine was homogenized gently with a hand homogenizer and passed twice through cheese cloth to remove debris and unbroken tubules. The homogenate was centrifuged in an International clinical centrifuge at 200 g for 2 min and the supernatant was discarded. The residue was resuspended in fresh saline and centrifuged again at 200 g for 2 min. This procedure was repeated three times in order to remove fine debris by flotation. The washed cell suspension was then passed through a thin layer of Sephadex G-25 gel supported by a sheet of glass wool in a Buchner funnel. The Sephadex had been allowed to swell in cold Krebs–phosphate–saline for 1 h before use. The spermatozoa and strands of tissue were retained by the gel, whereas the round germ cells passed through. The cells were resuspended and centrifuged again at 200 g and were suspended finally in 20 ml Krebs–phosphate–saline. The cell count was determined in a haemocytometer. The yield of germ cells obtained by this method was about 10⁸ non-flagellate cells per rat.

Radioactive materials

The radioactive steroids were purchased from the New England Nuclear Corporation, Boston, Massachusetts, U.S.A. Each was purified by thin-layer chromatography in the solvent system benzene:acetone (4:1, v/v). The labelled substrate, [1,2-³H]progesterone (50 Ci/mmol), was used either undiluted or after dilution with unlabelled progesterone. The ¹⁴C-labelled carriers, [4-¹⁴C]testosterone, [4-¹⁴C]17α-hydroxyprogesterone and [4-¹⁴C]androstenedione, were adjusted to a specific activity of 0.01–0.02 mCi/mmol.

Incubation

Carrier-diluted [³H]progesterone (100 µg 0.3 mCi/mmol; 10⁶ c.p.m./mg) in benzene solution and three drops of 5% propylene glycol in ethanol were added to the incubation flasks. When products of higher specific activity were required for carrier crystallization, 2 µCi undiluted [³H]progesterone of negligible mass were added. The volatile solvents were evaporated under a stream of nitrogen. The suspension of germinal cells in Krebs–phosphate–saline was adjusted to a count of 1–2 x 10⁸ cells/ml. Portions of the suspension (20 ml) were pipetted into each of the incubation
and zero-time control flasks; glucose (0·003 mol/l) was also added. The reaction was stopped by adding 20 ml ethyl acetate to the controls at time zero and to the experimental flasks after 3 h shaking in a water-bath at 37 °C; ¹⁴C-labelled carrier steroid (50–100 µg; 0·01–0·02 mCi/mmol) was then measured into the control and incubated flasks. For the final identification of products, extracts from three incubations were pooled.

**Extraction and isolation of products**

The tissue and incubation medium were extracted five times with ethyl acetate and the combined extracts were washed three times with distilled water. The solvent extract was dried by filtering through anhydrous sodium sulphate and the solvent was removed in a rotary evaporator. The extract was redisolved in benzene and a sample (5% of the total amount) pipetted into a vial with 10 ml scintillation fluid (1000 ml toluene and 42 ml Liquifluor, New England Nuclear Corporation) and counted in a Packard Tri-Carb scintillation counter with counting efficiencies for ¹⁴C and ³H of 70 and 21% respectively. After each further step of purification a small sample (5%) was counted; at least 10000 ¹⁴C counts were collected.

The extract was chromatographed on paper in the system described by Bush (1961), light petroleum (boiling range 37–53 °C): methanol:water (100:75:25, by vol.). The zones located by their absorption of u.v. light were eluted with methylene chloride:methanol (85:15, v/v) and the solvents were evaporated.

The zone corresponding to androstenedione recovered by paper chromatography was run in t.l.c. system I, toluene: methanol (99:1, v/v). The zone resembling androstenedione was then dissolved in 10 ml methanol, reduced with NaBH₄ (10 mg/ml) at 0 °C (Bush, 1961), and run in solvent system II, benzene: light petroleum (boiling range 30–60 °C): ethyl acetate (1:1:2, by vol.). The zone from the paper chromatogram corresponding to testosterone was eluted, acetylated overnight and run in solvent system III, benzene:acetone (4:1, v/v).

The zone isolated from paper containing a mixture of testosterone and 17α-hydroxyprogesterone was rechromatographed in t.l.c. solvent system I. The material was recovered, acetylated overnight, applied to t.l.c. plates and developed in system II. The resulting zones were treated as follows: (i) the zone corresponding to 17α-hydroxyprogesterone recovered from system II was re-run in solvent system III and then oxidized to androstenedione with 0·5% CrO₃ in glacial acetic acid for 2 h at room temperature (Bush, 1961). The steroid was extracted with methylene chloride and chromatographed in solvent system II. (ii) The testosterone acetate zone recovered from the t.l.c. plate was run in system III. Then it was hydrolysed by standing in 2% K₂CO₃ in methanol overnight at room temperature (Ward & Grant, 1963). The steroid was extracted and applied to a t.l.c. plate and developed in system II.

To each isolated product 10 mg of the corresponding authentic crystalline compound were added; they were crystallized to constant ³H:¹⁴C ratio using the solvent systems, acetone:water and methanol:water.
RESULTS

Isolation of non-flagellate germ cells

To remove the interstitial cells the tubules had been teased carefully and immersed in distilled water for 2 min before homogenization. To test the effectiveness of this technique a drop of a suspension of testicular cells in isotonic saline was mixed with water on a slide under the microscope. All cells could be seen to burst within 20 s. However, when intact seminiferous tubules were placed in distilled water the cells within the tubules were protected by the tubular wall, and when returned to Krebs–phosphate–saline the tubules were found to contain undamaged cells. Plate, figs 1 and 2 show sections of tubules before and after exposure to water.

On microscopic examination of smears of crude homogenates only a few Sertoli cells were seen among the large number of flagellate and round cells. The Sertoli cells can be recognized by their vase-like shape, often with tufts of sperm tails protruding from the neck (Plate, fig. 3). Because of the scarcity of Sertoli cells in the whole homogenates it may be assumed that most of these cells and also the spermatogonia which were attached to the basement membrane had been torn during homogenization. The few remaining Sertoli cells and practically all spermatozoa and spermatids with fully developed flagellae were removed during the subsequent procedure of filtration through Sephadex. The homogenates and the filtrates also contained a small number of multinucleate cells; these may have resulted at the time of homogenization from the confluence of spermatocytes which are connected by intercellular bridges (Dym & Fawcett, 1971). A differential count of the cell suspension (Plate, fig. 4) showed: primary (mainly pachytene) spermatocytes, 27%; spermatids, 30%; residual bodies (originating from stage 18–19 spermatids), 12%; unidentified cells, 10% and non-nucleated cytoplasmic fragments, 21%. No Sertoli cells or spermatogonia were seen.

Viability of isolated germ cells

The average respiratory rate, \(-Z_{O_2} (\mu l/10^8 \text{ cells/h}; 22.4 \mu l = 1 \mu mol)\) of the round cell suspension was 70 (endogenous) and 170 (in presence of glucose). The cells also incorporated \([14\text{C}]\)glucose into neutral and phospholipids (Terner & MacLaughlin, 1973).

Metabolism of progesterone

The cell suspensions incubated with \([1,2-\text{3H}]\)progesterone converted it to 17\(\alpha\)-hydroxyprogesterone, testosterone and androstenedione. This was demonstrated by addition at the end of the incubation period of \([14\text{C}]\)-labelled carriers, 17\(\alpha\)-hydroxyprogesterone, androstenedione and testosterone, followed by isolation of the products and crystallization to constant isotope ratio. The isolation procedure was also carried out on the zero-time controls which contained \([1,2-\text{3H}]\)progesterone, the \([14\text{C}]\)-labelled carriers and germinal cells. A very low \(^{3}\text{H}:^{14}\text{C}\) ratio in the steroids isolated from the control mixtures indicated that the products isolated from the incubated cells carried minimal contamination by \(^{3}\text{H}\)-labelled progesterone (Table 1). In further control experiments with heat-inactivated cells the \(^{3}\text{H}:^{14}\text{C}\) ratios of the isolated androgens were low and fell to the level of the zero-time control values after the third t.l.c. run. The rates of conversion of progesterone to its metabolites were calculated from the
Androgen biosynthesis by round germinal cells

isotope ratios observed in those experiments in which 100 µg of carrier-diluted [3H]progesterone had been added as substrate. The intermediate 17α-hydroxyprogesterone accumulated in the reaction mixture. Testosterone was the predominant androgen. The relative rates of formation of the three products shown in Table 2

Table 1. Purification of steroid products produced by non-flagellate germinal cells isolated from rat seminiferous tubules

<table>
<thead>
<tr>
<th>Product</th>
<th>Purification step</th>
<th>Solvent</th>
<th>Zero-time control</th>
<th>3-h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Hydroxyprogesterone (crystallized as androstenedione)</td>
<td>Zone from last t.l.c. plate</td>
<td>—</td>
<td>0.7</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>First crystals</td>
<td>Methanol:water</td>
<td>0.6</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>4.0</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>Second crystals</td>
<td>Acetone:water</td>
<td>0.5</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>0.3</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Third crystals</td>
<td>Methanol:water</td>
<td>0.5</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>0.2</td>
<td>8.0</td>
</tr>
<tr>
<td>Androstenedione (crystallized as testosterone acetate)</td>
<td>Zone from last t.l.c. plate</td>
<td>—</td>
<td>1.6</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>First crystals</td>
<td>Methanol:water</td>
<td>0.7</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>5.0</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>Second crystals</td>
<td>Acetone:water</td>
<td>0.3</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>0.4</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>Third crystals</td>
<td>Methanol:water</td>
<td>0.3</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>0.3</td>
<td>6.6</td>
</tr>
<tr>
<td>Testosterone (crystallized as testosterone)</td>
<td>Zone from last t.l.c. plate</td>
<td>—</td>
<td>1.3</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>First crystals</td>
<td>Methanol:water</td>
<td>0.6</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>2.0</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>Second crystals</td>
<td>Acetone:water</td>
<td>0.5</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>0.5</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Third crystals</td>
<td>Methanol:water</td>
<td>0.5</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Mother liquor</td>
<td></td>
<td>0.5</td>
<td>19.0</td>
</tr>
</tbody>
</table>

The germinal cells were incubated with [1H]progesterone. 14C-Labelled carrier steroid was added at the end of incubation. Steroids were isolated by repeated paper and thin-layer chromatography (t.l.c.) of the compounds and derivatives. The zones isolated from the last t.l.c. plate were crystallized with 10 mg authentic carrier.

Table 2. Metabolism of [1,2-3H]progesterone by round germinal cells of the rat

<table>
<thead>
<tr>
<th>Product</th>
<th>Conversion (µg/10^6 cells in 3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>1.38</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.33</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.54</td>
</tr>
</tbody>
</table>

The cells (10^4/ml in 5 ml Krebs–phosphate–saline) were incubated with 100 µg [1,2-3H]progesterone (0.3 mCi/mmol) at 37 °C. 14C-Labelled carrier steroids (100 µg, 0.01 mCi/mmol) were added and the products were isolated and purified to a constant ^3H:^14C ratio.
were calculated from one experiment in which the three products were isolated simultaneously. The data are supported by a number of other experiments in which one or two of the steroids were isolated.

DISCUSSION

Other investigators who claimed to have observed steroidogenic activity in seminiferous tubules (Christensen & Mason, 1965; Bell, Vinson, Hopkin & Lacy, 1968; Ellis & Berliner, 1969; Hall et al. 1969; Dufau, de Kretser & Hudson, 1971) separated the tubules from the interstitium by microdissection or teasing the tubules apart. This technique leaves open the possibility that part or all of the observed steroidogenic activity was due to residual Leydig cells. Also, since intact tubules were incubated, the reported steroidogenic capacity could not be attributed to any specific cell type. Histochemical evidence, notably the presence of smooth endoplasmic reticulum (Fawcett & Ito, 1958) and the detection of \( \Delta^5 \)-3\( \beta \)-hydroxysteroid dehydrogenase (Woods & Domm, 1966), suggested that all components of the seminiferous tubules might be capable of steroidogenesis. However, some workers favour the Sertoli cells as the site of steroidogenic activity (Huggins & Moulder, 1945; Teilum, 1950; Collins & Lacy, 1969).

The results of the present study suggest that suspensions of germinal cells possess the ability to convert progesterone to androgens. These cells were isolated from the seminiferous tubules after a treatment which included lysis of the interstitial cells and homogenization which disrupted the tubular wall and structures attached to it, leaving intact only the free non-flagellate and flagellate cells from the interior of the tubules. The round cells were then isolated by passing the suspension through Sephadex which retained the spermatozoa and the Sertoli cells. Although 70\% of the cells could be classified as spermatocytes and spermatids, the presence of unidentified cells and fragments may seem to preclude the total exclusion of the Leydig cells, at least by cytological criteria. However, it is most improbable that functioning interstitial tissue could have survived the washing in distilled water to which the tubules, teased out to single strands, had been subjected. This treatment was so drastic that it caused some damage to the walls of the tubules and the cells adjacent to the basement membrane. Only the cells in the interior of the lumen were protected and remained intact (Plate, fig. 2). Furthermore, cells attached to the basement membrane, such as Sertoli cells and spermatagonia, are vulnerable to disruption by homogenization. Since, in the seminiferous tubules of the rat, spermatagonia constitute less than 10\% of the total non-flagellate cells (Clermont & Morgenthaler, 1955), their relative number may have been further reduced by homogenization. Any contribution of spermatagonia to the overall metabolism of the final germ cell suspension could have been only minimal. Despite extensive scanning of stained slides, Sertoli cells with or without attached spermatozoa were not found in the final suspensions. The irregular shape of the Sertoli cells and the protrusion of sperm tails from most of these seem to facilitate the retention in the gel of Sephadex of those cells which had escaped disruption.

Whereas the present study does not provide evidence confirming or denying the possibility that other non-germinal cells within the seminiferous tubules may be
steroidogenic, it shows that a population of non-flagellate germ cells, consisting mainly of spermatocytes and spermatids and free from Leydig cells, Sertoli cells and spermatogonia, possesses the ability to metabolize progesterone to androgens.

In a further study we have shown that the non-flagellate germ cells contain cytoplasmic binding proteins for progesterone and for androgens (Galena, Pillai & Terner, 1973). The effects of sex hormones on the metabolism of the germinal cells have been described and utilized in developing a treatment for the control of male fertility (Terner & MacLaughlin, 1973).

This work was supported by the National Institutes of Health, U.S. Public Health Service and the Population Council, New York, N.Y. We wish to thank Dr Y. Clermont, McGill University, Montreal, Canada, for the analysis of the germ cells by the criteria of Meistrich, Bruce & Clermont (1973).

REFERENCES


DESCRIPTION OF PLATE

Fig. 1. Section of seminiferous tubules passed through Krebs-phosphate-saline. Arrow indicates a blood vessel surrounded by interstitial tissue. Stain: Bouin's haematoxylin-eosin. (x 160.)

Fig. 2. Section of seminiferous tubules after exposure to water for 2 min. Stain: Bouin's haematoxylin-eosin. (x 160.)

Fig. 3. Crude homogenate of rat testis tubules. Arrows indicate Sertoli cells with tufts of sperm tails. Stain: Harris' haematoxylin-eosin. (x 410.)

Fig. 4. Non-flagellate germ cells. A few nuclei can be seen separating from cytoplasm of spermatids. Stain: formalin, Harris' haematoxylin-eosin. (x 600.)