IN VITRO PRODUCTION OF TESTOSTERONE AND ANDROSTENEDIONE IN NORMAL AND STEIN-LEVENTHAL OVARIES

By
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ABSTRACT

When compared with similar events in the normal ovary, results of incubation of progesterone-7-3H and 17α-OH-progesterone-4-14C with homogenates of typical polycystic ovaries demonstrate increased conversion of these precursors to the androgens testosterone and androst-4-ene-3,17-dione. This study provides further in vitro confirmation of increased ovarian androgen production in the Stein-Leventhal syndrome.

The role certain androgens play as intermediates in the biosynthesis of oestrogen (Baggett et al. 1956) by the ovary has been substantiated by the isolation (Anliker et al. 1957; Zander 1958) and in vitro production (Kase et al. 1961) of androst-4-ene-3,17-dione and testosterone in human ovaries. Available evidence is consistent with a partial biogenetic pathway in which the major secretory products of the ovary, progesterone and oestrogens, are biosynthetically interrelated in the following manner:

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A similar biosynthetic pathway has been demonstrated in the human testis (Slaunwhite & Samuels 1956). Since this steroidogenic pattern is shared by both testis and ovary, it has been postulated (Samuels 1955) that following differentiation from a common embryologic origin, the endocrine specificity of a gonad is achieved by modifications of the relative activities of the biosynthetic enzymes which govern the sequence of steroid conversions. Thus, masculinization could result from the exaggeration of the latent potential for elaboration of androgens inherent in the conversion of progesterone to oestrogens, without invoking a totally new biochemical capability. Evidence for the quantitative alteration of ovarian androgen biosynthesis in cases of the polycystic ovary syndrome has been supplied by Axelrod & Goldzieher (1962) and Mahesh & Greenblatt (1962). In support of this thesis, this communication reports the results of studies of in vitro androgen production by homogenates of normal and Stein-Leventhal ovaries from precursor progesterone and 17α-OH-progesterone.

CLINICAL MATERIAL

Control Case: A 39 year old white female gravida 2, para 2 underwent bilateral oophorectomy for metastatic breast carcinoma. Menses had always been regular and no signs of virilization had been noted. No hormonal therapy had been utilized prior to surgery. The ovaries were grossly and histologically normal with evidence of recent Corpus Luteum formation.

Case I: A 19 year old white female para 0000, married for 1 and ½ years, was first seen for menstrual irregularities and infertility. Since menarche at age 12, menses were persistently irregular with cycles varying from 2 weeks to 6 months. No molimina was ever noted. Physical examination was normal in all respects. No ovarian enlargement was detected. Basal body temperatures were monophasic. X-rays of the skull were negative. An endometrial biopsy on the first menstrual day revealed proliferative endometrium. 24 hour urinary 17-ketosteroid excretion was 13.5 mg. The patient was then given prednisone, 15 mg daily for 5 months without change in the irregular menses or monophasic basal body temperatures. A gynaecogram revealed bilaterally enlarged ovaries. Bilateral wedge resection was performed. The ovaries were grossly and histologically compatible with the Stein-Leventhal syndrome. Following surgery, 6 months of regular ovulatory cycles have ensued.

Case II: a 24 year old white female para 0000 complained of sterility and amenorrhoea of 3 years duration. Menses had always been irregular since the menarche at age 14 with cycles characterized by progressively increasing intervals and scanty menstrual flow. No virilizing signs were present. Physical examination revealed an
essentially normal female. An otherwise normal pelvic examination showed mild clitoral hypertrophy and right ovarian enlargement. Urinary 17-ketosteroid excretion was 9.2 mg per 24 hours. A curettage was performed and fragments of endometrium composed of sparse nonsecretory glands was obtained. A gynacogram demonstrated bilateral ovarian enlargement. Bilateral wedge resection was performed and the gross and histologic appearance of the ovaries was consistent with the Stein-Leventhal syndrome. Ovulatory cycles have followed this procedure.

Case III: A 26 year old white female para 0000, married 6 and 1/2 years, complained of infertility. Menarche at age 13 was followed by persistently irregular cycles varying from 3 to 8 weeks in duration. No abnormal menstrual flow or accompanying molimina had been noted. Physical examination revealed an essentially normal female with mild facial hirsutism. On pelvic examination, an enlarged right ovary was palpable. Basal body temperatures were monophasic. Vaginal cytology demonstrated decreased oestrogen effect. Protein-bound iodine was 3.6 µg/100 ml and urinary 17-ketosteroid excretion per 24 hours was 9.5 mg. Proliferative endometrium was obtained on a first menstrual day biopsy. A gynacogram revealed bilaterally enlarged ovaries of smooth contour. A trial of Meticorten, 10 mg daily for two months, failed to effect menses or basal body temperature curve. A previous trial of therapy with thyroid extract had also been unsuccessful. A curettage and bilateral wedge resection of the ovaries were performed and ovarian tissue grossly and microscopically consistent with the diagnosis of the Stein-Leventhal syndrome was found. Twenty-four days following surgery, menses occurred and thereafter three regular ovulatory cycles ensued. Four months after wedge resection the patient conceived and consequently delivered a normal female child.

Case IV: A 29 year old Puerto Rican female, gravida 1 para 1 noted progressive oligomenorrrhea following uncomplicated delivery of her child 5 years ago. Prior to this pregnancy, her menstrual cycle had been normal. In addition to altered cycles, the patient also had noted increasing facial hirsutism for 3 years. Physical examination was normal except for facial hirsutism and acne. Pelvic examination revealed bilaterally enlarged rotund ovaries of smooth contour. Basal metabolic rate and protein-bound iodine were normal. Urinary 17-ketosteroids were 11 mg per 24 hours. A bilateral wedge resection was performed. The appearance of the ovaries grossly and histologically confirmed the clinical diagnosis of Stein-Leventhal syndrome. Following surgery 9 monthly ovulatory menstrual cycles have occurred.

MATERIAL AND METHODS

Tissue: In all instances the tissues from wedge resections were immediately placed in dry ice and stored at -18° C. These were subsequently thawed and investigated simultaneously, thereby achieving the same incubation conditions for all ovaries. Two grams of each specimen were utilized for study.

Incubation: The tissues was finely minced and suspended in the cofactor solution based on the tissue weight of two grams (Table 1). Following homogenization in a Potter-Elvehjem homogenizer at 4° C, each homogenate was transferred to individual incubation flasks containing 35.5 × 10⁶ dpm. (16.1 microcuries) progesterone-7-3H and 2.7 × 10⁶ dpm. (1.23 microcuries) 17α-hydroxyprogesterone-4-14C. An equal volume of KCl was utilized to facilitate this transfer and guard against evaporation losses during incubation. Incubations were performed in open flasks at 37° C in air with constant mixing for three hours.

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Table 1.
Composition of cofactor solution used for homogenizing ovarian tissue.*

<table>
<thead>
<tr>
<th>Glucose-6-phosphate</th>
<th>32.3 micromoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triphosphopyridine nucleotide</td>
<td>3.85 &quot;</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>10.00 &quot;</td>
</tr>
<tr>
<td>Phosphate buffer, pH 7.2</td>
<td>30.00 &quot;</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>1.00 K-unit</td>
</tr>
<tr>
<td>Potassium chloride 0.154 M q.s. ad.</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

* Each gram of tissue was homogenized with 1.0 ml of the cofactor solution.

Extraction: Each incubate was covered with five volumes of acetone and allowed to stand overnight at 4°C. The debris was filtered and extracted three times with additional solvent. The combined extract was taken to dryness under reduced pressure. The dried extract was taken up in 70% aqueous methanol and partitioned against 1/2 volume petroleum ether two times for fat removal. Following back extraction of the petroleum ether with fresh 70% methanol (1/2 volume) the aqueous methanol solutions were combined and concentrated to an aqueous sludge and extracted with equal volumes of redistilled methylene chloride three times. These extracts were partitioned with one third volume 1.0 N NaOH two times to remove phenolic compounds. The resulting methylene chloride extracts were washed with small volumes of water to neutrality, dried over sodium sulfate, concentrated to dryness in vacuo, and the dried residue was taken up in 10 ml of benzene.

Experimental: Two ml of each neutral fraction were added to 10 mg of carrier androst-4-ene-3,17-dione (mp. 171°-172°C) and chromatographed in ligroin/proplylene glycol paper system. Similarly, two ml of extract and 10 mg of carrier testosterone (mp. 150°-151°C) was chromatographed on paper in cyclohexane:benzene (1:1)/propylene glycol.

In the case of testosterone, the U-V reactive zone was eluted with methanol, and acetylated in acetic anhydride and pyridine. The acetate was then chromatographed on an alumina column (Woelm Grade II) with crystalline material obtained in fractions eluted with 10% benzene-90% petroleum-ether. Several crystallizations of the acetate (mp. 136°-138°C) in ether:petroleum-ether were performed and the radioactivity of a portion of each was measured in a Packard tricarb scintillation counter. When constant specific activity of the acetate was achieved, hydrolysis to the free compound was performed by refluxing the acetate in the presence of two equivalents of NaOH for two hours. Free testosterone was recrystallized and its radioactivity measured. If the constant specific activity achieved corresponded to that of the acetate, the compound was considered radiochemically homogeneous.

Following elution from paper androst-4-ene-3,17-dione was also chromatographed on alumina (Woelm Grade II). Crystalline material was eluted with 20% benzene-80% petroleum-ether. Material recrystallized from an ether:petroleum-ether was submitted to liquid scintillation counting. Following three recrystallizations yielding material of constant specific activity, the dioxime was prepared by refluxing the free steroid for three hours in methanol containing hydroxylamine hydrochloride and sodium acetate. This derivative (decomp. 140°-143°C) was recrystallized from ether:petroleum ether to constant specific activity to further demonstrate the radiochemical homogeneity of the dione.
RESULTS

Table 2 summarizes the specific activities (dpm/mg of carrier) of testosterone and androst-4-ene-3,17-dione and their respective derivatives after purification to constant specific activity. It is clear from this data that all ovarian homogenates were capable of converting the precursor compounds to both testosterone and androst-4-ene-3,17-dione.

Table 3 shows the total number of counts produced from the precursors per gram of ovarian tissue utilized and the per cent conversion from each precursor to testosterone and androst-4-ene-3,17-dione respectively.

A normal control ovary was capable of 0.27% and 0.22% conversion of 17α-hydroxyprogesterone and progesterone to testosterone. Androst-4-ene-3,17-dione production by this homogenate from the same precursors was 2.6% for 14C and 0.72% for 3H. These values correspond to those previously reported (Kase et al. 1961) as well as those of Ryan, in which a different technique was utilized (Smith & Ryan 1961). In all the homogenates of the Stein-Leventhal ovaries, testosterone production was greater than in the control ovary. Precursor conversion to androst-4-ene-3,17-dione was similarly enhanced.

DISCUSSION

The results of this study confirm the in vitro capability of normal and abnormal ovarian tissue to convert progesterone and 17α-hydroxyprogesterone to testosterone as well as androst-4-ene-3,17-dione. Testosterone production by Stein-Leventhal ovaries has been noted by O'Donnell & McCaig (1960) and Axelrod & Goldzieher (1962). Biosynthesis of this highly active androgen has also been shown in abnormal ovarian tissue from a hirsute patient by Mills & Brooks (1959) and by Savard et al. (1961) in studies of arrhenoblastoma tissue. The existence of the same biosynthetic pathways in the ovary, the testis, and in part of the adrenal cortex (Kase & Kowal 1962) suggests that organ specific steroid elaboration may be the result of modifications in the activity of certain biosynthetic enzymes possessed by all steroid producing cells. It is well documented that altered activity of adrenal hydroxylating enzymes can produce significant clinical disease. Similarly, unknown factors may alter ovarian steroid biosynthetic enzyme capability. As a result, steroid production and cellular function are no longer parallel, and evidence of pathologic histology and physiology may appear. On this basis, assuming in vitro data qualitatively reflect in vivo events (Mahesh & Gleenblatt 1962), the results of this study appear to confirm the ovarian origin of a potentially masculinizing syndrome in human females caused by excessive ovarian elaboration of testosterone and androst-4-ene-3,17-dione. The exceptional activity
### Table 2.
Final specific activities (dpm/mg carrier) of testosterone and androst-4-ene-3,17-dione and their respective derivatives obtained from incubation of 7α-3H-progesterone and 17α-hydroxyprogesterone-4-14C with homogenates of five different human ovarian samples.

<table>
<thead>
<tr>
<th>Case</th>
<th>Isotope</th>
<th>Testosterone</th>
<th>Testosterone acetate</th>
<th>Androst-4-ene-3,17-dione</th>
<th>Androst-4-ene-3,17-dioxime</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14C</td>
<td>3H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>68</td>
<td>56</td>
<td>321</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>860</td>
<td>655</td>
<td>13006</td>
<td>9763</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>202</td>
<td>159</td>
<td>2562</td>
<td>2123</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>345</td>
<td>224</td>
<td>2410</td>
<td>1757</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>769</td>
<td>818</td>
<td>15641</td>
<td>11440</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.
Conversion of 17α-hydroxyprogesterone-4-14C and progesterone-7-3H to testosterone and androst-4-ene-3,17-dione in five ovarian samples.

<table>
<thead>
<tr>
<th>Case</th>
<th>Isotope</th>
<th>dpm/g of tissue</th>
<th>per cent conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Testosterone</td>
<td>Androst-4-ene-3,17-dione</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14C</td>
<td>3H</td>
</tr>
<tr>
<td>Normal</td>
<td>3381</td>
<td>24170</td>
<td>0.27 %&lt;sup&gt;o&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>20292</td>
<td>325150</td>
<td>1.76 %&lt;sup&gt;o&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>4778</td>
<td>61488</td>
<td>0.4 %&lt;sup&gt;o&lt;/sup&gt;</td>
</tr>
<tr>
<td>III</td>
<td>8159</td>
<td>60250</td>
<td>0.93 %&lt;sup&gt;o&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>18918</td>
<td>387115</td>
<td>2.72 %&lt;sup&gt;o&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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of testosterone can be presumed to account for the fact that increased production is sufficient to exert clinical effects without significant elevations of 17-ketosteroid excretion.

This study does not establish the locus or type of enzyme defect involved in the initiation of excess androgen production by Stein-Leventhal ovaries. Short & London (1961) and others (Axelrod & Goldzieher 1962) have suggested that the impetus for the increased efficiency of C₂₁ to C₁₉ conversions in this type of ovary is a diminution in the activity of enzymes governing C₁₈ elaboration with consequent «back-up» excess of C₁₉ precursors. In addition, Mahesh & Greenblatt (1961) have demonstrated a relative deficiency in 3β-ol-dehydrogenase function in the Stein-Leventhal ovary with excess production of the androgen dehydroepiandrosterone and C₂₁,Δ⁴-3β-ol compounds. Since progesterone and its 17α-hydroxy derivative were utilized as precursors in the present study, no statement on the implicit contradiction of the existence of this enzyme defect and an excessive testosterone and androst-4-ene-3,17-dione production in Stein-Leventhal ovaries can be made. This alternate route to ovarian androgen and oestrogen biosynthesis (via pregnenolone → 17α-OH-pregnenolone → dehydroepiandrosterone) is currently under investigation in our laboratory.

ACKNOWLEDGEMENT

The authors wish to express their appreciation to Dr. Normal Hertzig and Dr. David Zakin for the case material utilized in this study.

REFERENCES


Received on January 2nd, 1963.