STEROID EXCRETION AND BIOSYNTHESIS,
WITH SPECIAL REFERENCE TO ANDROST-16-ENES,
IN A WOMAN WITH A VIRILISING ADRENO-
CORTICAL CARCINOMA

By

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Although the excretion and biosynthesis of a large number of steroids in adrenal cancer have been intensively studied (Lipsett & Wilson 1962; Villee et al. 1967), the C_{19}-\Delta^{18}\text{-steroids have received little attention. In some cases of adrenal tumour with virilism (Mason & Schneider 1950; Burstein & Dorfman 1962) the amount of urinary 3\alpha\text{-hydroxy-5\alpha\text{-androst-16-ene (}\Delta^{18}\text{-androstenedol was raised. Burstein & Dorfman (1962) also showed that radioactive cholesterol and pregnenolone administered in vivo were converted to }\Delta^{18}\text{-androstenedol, which was detected in the urine.}

The purpose of this paper, a preliminary report of which has been published (Gower & Stern 1967), was to determine the pre- and post-operative urinary excretion of androst-16-enes by a patient with virilising adrenal carcinoma and to see if this group of compounds was present in the peripheral blood. The levels of other steroids in urine and plasma were also measured to confirm the original diagnosis, made on clinical grounds. The formation of androst-16-enes by the neoplastic tissue from labelled precursors has been studied in vitro.

**CASE REPORT**

The patient was a 33-year-old married woman who was admitted to hospital with a history of right buttock and groin pain, amenorrhoea for six months, hirsutism of the face and deepening of the voice for one year. The breasts were atrophied and the clitoris hypertrophied. Initial laboratory investigations revealed that urinary excretion of total 17-oxosteroids and 17-hydroxycorticosteroids was above normal (Table 1) and showed little decrease after administration of dexamethasone. The plasma DHA (as sulphate) was elevated (350 \mu g/100 ml) and the plasma 11-hydroxycorticosteroid level (Mattingley 1962) was raised (30.2 \mu g/100 ml), showing only slight increase (32.9 \mu g/100 ml) after the Synacthen test (Wood et al. 1965).

Pre-sacral air insufflation revealed a large suprarenal mass on the right side and at operation a right adrenocortical tumour was found. A small portion of the adrenal tissue was sent for histological examination and approximately 5 g was obtained for incubation studies. This latter portion was immediately cooled in ice and sent to the laboratory.

The tumour weighed 1800 g and was partially encapsulated. It was yellow and showed haemorrhagic and necrotic areas. Microscopic examination revealed that the tumour, which had infiltrated the capsule, was composed of large cells of uniform size with pale faintly eosinophilic granular cytoplasm and large central nuclei which showed slight pleomorphism.

**MATERIALS AND METHODS**

Solvents, alumina and Kieselgel G used for chromatography have been described earlier (Gower & Ahmad 1967). Isotopically labelled steroids were purchased from the Radiochemical Centre, Amersham, Bucks. Purity was checked prior to use by TLC in systems described by Ahmad (1967), followed by radioautography. TLC and radioautography were performed as described earlier (Gower & Ahmad 1967). GLC was
performed as in the papers of Gower (1966), Gower & Ahmad (1967) and Gower & Thomas (1968).

Extraction of urine and plasma

50 ml aliquots of 24-hour urine specimens were hydrolysed with 5 ml Ketodase (William R. Warner & Co. Ltd., Eastleigh, Hants., England) for 66 h and extracted with ether. Steroid sulphates remaining in the aqueous layer were hydrolysed by continuous extraction with ether at pH 0.8. Plasma was extracted and steroid sulphates hydrolysed according to the method of Sündberg et al. (1965).

Estimation of steroids

Steroids from urinary and plasma conjugates were chromatographed on columns of alumina using the method of Gower & Ahmad (1967) modified as follows:

$\Delta^{16}$-androstenol was eluted first, using 60 to 100 ml of a mixture of light petroleum (80–100° C) – benzene (1:1, v/v), cf. Brooksbank & Haslewood (1961), followed by androsterone, aetiocholanolone and DHA using 75 ml of benzene which contained 0.5% of ethanol. $\Delta^{16}$-androstenol was estimated by GLC of the free steroid and CMDS ether (prepared by the method of Thomas & Walton 1968) and comparison with known amounts of the authentic compounds. Recovery experiments for this method, performed by adding authentic $\Delta^{16}$-androstenol to urine samples prior to hydrolysis and extraction, indicated 87% efficiency. Androsterone, aetiocholanolone and DHA were estimated by the method of Kirschner & Lipsett (1963).

Methods used for the estimation of other steroids are given in Table 1.

Incubation of adrenal tissue

The tissue was washed with Krebs bicarbonate buffer (pH 7.4) containing glucose (200 mg/100 ml) and minced by hand. Portions of the mince (1 g) were incubated with 1.5 μc of 4-14C-pregnenolone (specific activity 24.0 mc/mm); 4-14C-progesterone (specific activity 21.7 mc/mm); 4-14C-DHA (specific activity 27.5 mc/mm) and 4-14C-testosterone (specific activity 29.2 mc/mm). Incubation conditions and cofactors added were as described previously (Gower & Ahmad 1967). After homogenising, 50 μg each of the following carrier steroids were added: $\Delta^{16}$-androstenol, 3α-hydroxy-5β-androst-16-ene, 3β-hydroxy-androsta-5,16-diene, 3β-hydroxy-5α-androst-16-ene, androsta-4,16-dien-3-one, 3-hydroxy-oestra-1,3,5(10),16-tetraene, DHA, testosterone and androst-4-ene-3,17-dione. Extraction was performed with ethyl acetate (3 × 4 ml), the combined extracts evaporated, dried and chromatographed on alumina columns (Gower & Ahmad 1967).

Separation and identification of radioactive metabolites

Each alumina column fraction was submitted to TLC, followed by radioautography. Fractions which might contain androst-16-enes were run in toluene-ethyl acetate (9:1, v/v) or benzene – ether (9:1, v/v) (Gower 1964). More polar metabolites were run in benzene – acetone (4:1, v/v) first, followed by other systems known to separate compounds of interest (Gower 1966; Gower & Ahmad 1967). Whenever possible, tentative identifications were confirmed by the preparation of derivatives and re-running in appropriate TLC systems until the specific activity, determined after each run, was constant (Gower 1966). The weight of steroid eluted was determined by GLC and radioactivity measured.
Table 1.
Urinary steroid levels (mg/24 h) in a woman with virilising adrenal carcinoma before and after unilateral adrenalectomy.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Pre-operative</th>
<th>Post-operative 1 month</th>
<th>Post-operative 6 weeks</th>
<th>Post-operative 6 months</th>
<th>Mean normal values for females</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total 17-OS</td>
<td>200; 160°; 176**</td>
<td>6.1</td>
<td>5.5</td>
<td>8.8</td>
<td>5–15</td>
<td>Medical Research Council Committee (1951)</td>
</tr>
<tr>
<td>Total 17-OHCS</td>
<td>70; 65°; 46**</td>
<td>39.2</td>
<td>17.7</td>
<td>10.8</td>
<td>5–17</td>
<td>*Appleby et al. (1955)</td>
</tr>
<tr>
<td>Androsterone</td>
<td>19 (G 13; S 6)</td>
<td>0.11</td>
<td>2.6</td>
<td></td>
<td>1.4</td>
<td>**Kirschner &amp; Lipsett (1964)</td>
</tr>
<tr>
<td>Aetiocholanolone</td>
<td>42.5 (G 23.5; S 19)</td>
<td>4.0</td>
<td>N. D.</td>
<td></td>
<td>1.8</td>
<td>(1963), modified</td>
</tr>
<tr>
<td>DHA</td>
<td>123.5 (G 46.3; S 77.2)</td>
<td>0.13</td>
<td>N. D.</td>
<td></td>
<td>0.56</td>
<td>†Brooks (1964)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.19</td>
<td>0.005</td>
<td>0.004</td>
<td></td>
<td>&lt; 0.02</td>
<td>modified</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0.20</td>
<td>0.0015</td>
<td>&lt; 0.001</td>
<td></td>
<td>&lt; 0.02</td>
<td>See text</td>
</tr>
<tr>
<td>Δ18-androstenol</td>
<td>2.06 (G 1.46; S 0.6)</td>
<td>0.115</td>
<td>0.04</td>
<td></td>
<td>0.35 (Gower, unpubl.)</td>
<td>Kopper et al. (1955)</td>
</tr>
<tr>
<td>Pregnanediol</td>
<td>32.6; 18.8°</td>
<td>0.3</td>
<td>0.7</td>
<td></td>
<td>0.5–2.0†</td>
<td>Stern (1957)</td>
</tr>
<tr>
<td>Pregnanetriol</td>
<td>1.2; 1.6°</td>
<td>–</td>
<td>–</td>
<td></td>
<td>&lt; 2.2</td>
<td>By TLC with authentic compounds</td>
</tr>
<tr>
<td>Pregnanetriolone</td>
<td>N. D.</td>
<td>–</td>
<td>–</td>
<td></td>
<td>N. D.</td>
<td>Stern &amp; Barwell (1963)</td>
</tr>
<tr>
<td>Pregnanetetrol</td>
<td>N. D.</td>
<td>–</td>
<td>–</td>
<td></td>
<td>N. D.</td>
<td>‡Brown (1955), modified by Brown et al. (1957)</td>
</tr>
<tr>
<td>Δ9-Pregnenetriol</td>
<td>32.0</td>
<td>0.3</td>
<td>0.1</td>
<td></td>
<td>&lt; 0.2</td>
<td></td>
</tr>
<tr>
<td>17β-Oestradiol</td>
<td>0.024; 0.025°</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td></td>
<td>0–0.003 0.004–0.014</td>
<td></td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.093; 0.074°</td>
<td>0.0025</td>
<td>0.002</td>
<td></td>
<td>0.004–0.007 0.010–0.031</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.187; 0.162°</td>
<td>0.0168</td>
<td>0.0176</td>
<td></td>
<td>0–0.015 0.008–0.072</td>
<td></td>
</tr>
</tbody>
</table>

N. D. = not detected  * = after administration of 2 mg dexamethasone
G = glucuronide      ** = after administration of 8 mg dexamethasone (McHardy-Young et al. 1967)
S = sulphate         † = proliferative phase   ‡ = progestational phase
RESULTS

Table 1 summarizes results of some urinary steroid analyses before, and at intervals after operation. Pre-operatively, the ratio of DHA glucuronide to DHA sulphate was high. This was also found by Burstein & Dorfman (1962) in a patient with a virilising adrenal adenoma. As with other cases of adrenal carcinoma, there was little change in the 17-oxosteroids after administration of dexamethasone.

Excretion of androst-16-enes

The amount of $\Delta^{16}$-androstenol excreted as glucuronide before operation was found to be 1.46 mg/d. This value was approximately four times the mean for a normal woman of the same age (Gower, unpublished). In addition to $\Delta^{16}$-androstenol, GLC of this fraction revealed the presence of another compound, as yet unidentified, which had a retention time similar to, though not identical with, that of androsta-4,16-dien-3-one (Fig. 1).

GLC of the »androstenol« fractions obtained after alumina chromatography of urine and plasma sulphates revealed a peak in each case having a retention time relative to cholestane (used as internal standard) of authentic $\Delta^{16}$-androstenol. The tentative identification of this steroid was made as follows: One half of each extract was re-chromatographed on the same GLC column after chloromethylation, when a peak was obtained in each case which had a relative retention time (RRT) equal to that of the chloromethylidimethylsilyl ether of $\Delta^{16}$-androstenol (Fig. 2). The rest of each extract was submitted to TLC (see Methods Section). After elution of the zones corresponding to authentic $\Delta^{16}$-androstenol used as marker, the extracts were submitted to GLC. Again, a peak was obtained in each case with an RRT of $\Delta^{16}$-androstenol. After oxidation (Chamberlain et al. 1965), the products showed a peak in each case which had an RRT of 5$\alpha$-androst-16-en-3-one, the expected oxidation product. These results are summarized in Table 2. The amounts of $\Delta^{16}$-androstenol as sulphate in pre-operative urine and plasma were approximately 0.6 mg/d and 2.8 $\mu$g/100 ml respectively. After operation the amount of urinary $\Delta^{16}$-androstenol excreted as glucuronide decreased to 0.1 mg/d or less. None was detected in the urinary sulphate fraction nor in the peripheral plasma.

Formation of radioactive steroids in vitro

The yields of metabolites obtained are summarised in Table 3. Radioactive testosterone was formed from pregnenolone, progesterone and DHA, although the yields were fairly small. Androst-4-ene-3,17-dione and 11$\beta$-hydroxy-androst-4-ene-3,17-dione were formed from the three above-mentioned precursors as well as from labelled testosterone.
GLC of $\Delta^{18}$-androstenol. (a) Mixture of $\Delta^{18}$-androstenol (peak 1), androsta-4,16-dien-3-one (peak 2) and cholestane (peak 3); (b) »$\Delta^{18}$-androstenol« fraction (eluted from alumina) obtained after hydrolysis of urinary glucuronides of a woman with virilising adrenal carcinoma. Cholestane (peak 3) was added as internal standard. The 'unknown' peak has a retention time similar to, though not identical with, that of androsta-4,16-dien-3-one. Column conditions are given in Table 2.

**Fig. 1.**

*Formation of radioactive androst-16-enes from $4-^{14}C$-pregnenolone*

TLC and radioautography of alumina column fractions which might contain radioactive androst-16-enes revealed a zone with the mobility of $3\beta$-hydroxy-androsta-5,16-diene and/or $3\beta$-hydroxy-5α-androst-16-ene. These compounds were not resolved in toluene – ethyl acetate (9:1, v/v). After elution and acetylation (Brooksbank & Gower 1964), the steroid was run in toluene – ethyl acetate (19:1, v/v) which just separated the acetates of these alcohols. The spot seen after radioautography coincided exactly with that formed by the authentic acetate of the $\Delta^{5,16}$-3-alcohol. The relevant zone was eluted, hydrolyzed and the product run in toluene – ethyl acetate (9:1, v/v) when the radioactivity corresponded to $3\beta$-hydroxy-androsta-5,16-diene. After oxidation (Chamberlain et al. 1965) and TLC in the same system, the radioactive zone moved with the mobility of authentic androsta-4,16-dien-3-one, the expected oxidation pro-
Fig. 2.
GLC of the chloromethyldimethyl silyl ether of $\Delta^{16}$-androstenol. (a) Standard (0.025 $\mu$g); (b) $\Delta^{16}$-androstenol fraction eluted from alumina and chlorasilanised (see text) obtained from plasma of a woman with virilising adrenal carcinoma. Column conditions are given in Table 2. Peak 3, cholestane.

The yield of this metabolite from pregnenolone (0.2 $\%$) was too small to permit any further identification studies.

**Formation of androst-16-enes from $4^{-14}C$-progesterone**

TLC and radioautography of alumina column fractions which might contain androst-16-enes revealed a small amount ($< 0.03 \%$) of a compound which had the mobility of androsta-4,16-dien-3-one. The amount formed, however, was too small for purification to be carried out.
Table 2.
Gas-liquid chromatography of $\Delta^{18}$-androstenol obtained from the urine and plasma of a woman with virilising adrenal carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>Urine Glucuronide</th>
<th>urine Sulphate</th>
<th>Plasma</th>
<th>Authentic steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free steroid*</td>
<td>0.290*§</td>
<td>0.285*</td>
<td>0.295</td>
<td>0.291 ± 0.005§</td>
</tr>
<tr>
<td>Chloromethyl-</td>
<td>0.427</td>
<td>0.428</td>
<td>0.425</td>
<td>0.480</td>
</tr>
<tr>
<td>dimethyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>silyl ether*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After thin-layer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free steroid†</td>
<td>0.248</td>
<td>0.245</td>
<td>0.248</td>
<td>0.246</td>
</tr>
<tr>
<td>Oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>product†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5α-androst-16-en-3-one)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.255</td>
<td>0.251</td>
<td>0.252</td>
<td>0.256</td>
</tr>
</tbody>
</table>

Column used: cyclohexanediethanol succinate / JXR silicone gum (0.6% / 0.75%) with carrier gas flow rate 50 ml/min.
* Run at 205° C; cholestane time 23.6 min.
† Run at 193° C; cholestane time 31.9 min.
‡ Run at 188° C; cholestane time 35.8 min.
§ Mean of two estimations
§§ S. D. with no. of estimations in parenthesis

The formation of androst-16-enes from labelled DHA or testosterone could not be demonstrated.

**DISCUSSION**

This patient with a virilizing adrenal carcinoma excreted larger than normal quantities of many steroids as shown in Table 1. Similar findings have been reported by other workers (Keller et al. 1958; Lipsett & Wilson 1962). In general, the in vitro metabolism of steroids by the tumour confirmed previously reported results (Roversi et al. 1963; Villee et al. 1967) but the extent of 11β-hydroxylation was relatively great in the present case, as shown by the yields of 11β-hydroxyandrostenedione formed from pregnenolone, progesterone, DHA and testosterone (Table 3). This is in accord with the raised 11-hydroxycortico-
Table 3.
Yields (as percentage radioactivity incorporated/g wet wt. of tissue) of steroid metabolites formed in minces of adrenal carcinoma.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>4-14C Pregnenolone</th>
<th>4-14C Progesterone</th>
<th>4-14C DHA</th>
<th>4-14C Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>&lt; 0.1</td>
<td>1.8</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>7.9</td>
<td>4.2</td>
<td>13.2</td>
<td>35.6</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.33</td>
<td>0.25</td>
<td>0.34</td>
<td>5.9</td>
</tr>
<tr>
<td>11β-Hydroxy-androstenedione</td>
<td>1.69</td>
<td>2.88</td>
<td>2.56</td>
<td>6.8</td>
</tr>
<tr>
<td>DHA</td>
<td>2.5</td>
<td>N. D.</td>
<td>6.1</td>
<td>N. D.</td>
</tr>
<tr>
<td>3β-Hydroxy-androsta-5,16-diene</td>
<td>0.2</td>
<td>N. D.</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Androsta-4,16-dien-3-one</td>
<td>N. D.</td>
<td>&lt; 0.03</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
</tbody>
</table>

* All yields, except for those of 11β-hydroxyandrostenedione, have been fully corrected for analytical losses.
N. D. = not detected

steroid levels of plasma and the total 17-hydroxycorticosteroids in the urine (Table 1).

Progesterone synthesis from pregnenolone was found to be very small; indeed, it was possible to obtain only presumptive evidence for its presence. This was also noticed by Villee et al. (1967) and may be due to the inhibition of the 3β-hydroxy-C21-steroid dehydrogenase by excessive quantities of DHA (Kowal et al. 1964).

The formation of Δ16-steroids in adrenal tumours in vitro has not previously been demonstrated. 3β-Hydroxy-androsta-5,16-diene (0.2 %) was formed from pregnenolone and it is possible that progesterone yielded trace amounts of androsta-4,16-dien-3-one although the evidence is only of a preliminary nature due to the small quantity of steroid formed. These results are in accord with recent experiments performed by Gower & Ahmad (1967) in which the Δ4,16-3-ketone was formed from pregnenolone in 2 % yield in boar testis minces but in less than 0.1 % yield in adrenal tissue from the same species. Further results using progesterone instead of pregnenolone suggest that the Δ4,16-3-ketone may be the precursor of the Δ16-3-alcohols since the ketone is formed very early in the incubation and subsequently falls to very low levels as the yield of the Δ18-alcohols increases (Ahmad 1967; Ahmad & Gower 1968). If this is the
case in the adrenal tumour tissue it is not surprising that the ketone was detected only in trace amounts after an incubation of 3 hours with progesterone. Recent results using boar testis tissue show that testosterone and DHA form little or no $\Delta^{18}$-steroids (Ahmad & Gower 1968). In accord with these results, the tumour tissue was unable to form 3$\beta$-hydroxy-androsta-5,16-diene or androsta-4,16-dien-3-one from DHA or testosterone (Table 3). Moreover, attempts to show that testosterone and DHA (Ahmad & Morse 1965; Bulbrook et al. 1963; Brooksbank 1962) and epitestosterone (Wilson & Lipsett 1966) are precursors of $\Delta^{18}$-steroids in man have not succeeded. But it is significant that in the adrenal adenoma patient of Burstein & Dorfman (1962) both cholesterol and pregnenolone gave rise to $\Delta^{18}$-androstenol in vivo. It appears that the adrenal tumour tissue studied here is able to perform the biosynthetic transformation of C$_{21}$-steroids to C$_{19}$-$\Delta^{18}$-steroids even if the latter are formed in small yields. The significance of 3$\beta$-hydroxy-androsta-5,16-diene, known to be excreted in small quantities in normal human male urine (Brooksbank & Gower 1964), is as yet unknown. The closely related androsta-4,16-dien-3-one has recently been discovered in normal male peripheral plasma (Brooksbank 1968).

$\Delta^{18}$-Androstenol was excreted by this patient in several times the normal amount for a female of comparable age. The excretion of this compound as sulphate has not previously been reported; Brooksbank & Gower (1964) could find no evidence for this in normal males. $\Delta^{18}$-Androstenol has also been found in other cases of adrenal tumour (Mason & Schneider 1950; Burstein & Dorfman 1962), in adrenal hyperplasia (Miller et al. 1953) and in luteoma of the ovary (Engel et al. 1953). Its presence in plasma in a case of virilising adrenal cancer has not previously been reported and it has not so far been detected in normal plasma, and was not found in this patient after adrenalectomy. We were unable to detect $\Delta^{18}$-androstenol as a metabolite of pregnenolone and progesterone. It is possible, however, that it was formed in the ovaries of this patient but this has not yet been demonstrated.

ACKNOWLEDGEMENTS

We are indebted to Dr. P. M. F. Bishop and to Mr. F. N. Glover for permission to study their patient and to Professor S. de Nevasquez for the report on the histology of the tumour. We are also most grateful to Dr. B. W. L. Brooksbank for helpful discussion, to Miss C. Lyne for the plasma 11-hydroxycorticosteroid estimations, to Mr. B. S. Thomas for preparing the chloromethyldimethyl silyl ether of $\Delta^{18}$-androstenol. Miss J. A. Jackson, Miss Jean Jepson and members of the Endocrine Laboratory Staff, Chelsea Hospital for Women, rendered valuable assistance. D. B. G. thanks the Medical Research Council for financial support (Grant no. G. 964/248/B) and for the purchase of the gas chromatograph. We are indebted to Mr. Lawrence Armitage for photographing the gas chromatographic tracings.

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Medical Research Council Committee on Clinical Endocrinology: Lancet 2 (1951) 585.

Received on July 9th, 1968.