ISOLATION OF STEROIDS FROM A FEMINIZING ADRENAL CARCINOMA*

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The feminizing tumor of the adrenal gland occurs rarely in the male and is characterized clinically by gynecomastia, impotence, atrophy of the testes, and an increased excretion of estrogenic substances. In a recent review of the literature, Wallach et al. (1) found that only twenty-two cases had been reported. From the urine of several of these, a number of substances related to dehydroepiandrosterone were isolated, but until the recent work of Landau et al. (2), and Diczfalusy and Luft (3), the estrogenic steroids had not been identified. These workers found increased quantities of estradiol, estrone, and estriol, as well as pregnanediol, in the urine of their subjects.

While the urinary steroids might be assumed to reflect the secretion of the adrenal tumor, the degradative action of the liver and other tissues must be considered. The tissue analysis, therefore, was undertaken to study directly the precursors of the urinary products. This investigation resulted in the isolation of several steroids new to the human and contributed a possible method of the diagnosis of this tumor.

Materials and Methods

Tumor—The tumor weighing 920 gm. was removed from the area of the left adrenal of a 22 year-old male patient of the Salt Lake City Veterans Hospital. A detailed discussion of clinical and other laboratory findings is published by Wallach et al. (1). Pathological examination by Dr. Oscar Rambo, Salt Lake City Veterans Hospital, and confirmed by the United States Armed Forces Institute of Pathology, revealed an adrenal carcinoma of the estrogen-secreting type, characterized by anaplasia, capsular vein invasion, and cords of cells strikingly “similar to those seen in granulosa cell tumors of the ovary.”

Laboratory Methods—All solvents were purified by standard procedures.

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and by distillation prior to use. All evaporations involving more than 10 ml. were carried out in vacuo. When quantities less than 10 ml. were to be evaporated, the solvent was blown off by a fine jet of nitrogen. Acetylation was carried out in pyridine and acetic anhydride (1:3) at room temperature. The Zimmermann reaction for the estimation of 17-ketosteroids was the routine clinical procedure in which m-dinitrobenzene in 2.5 N KOH in ethanol was employed.

**Extraction of Tumor**—The portion of the tumor which was extracted chemically weighed 523 gm. The frozen tissue was homogenized in a Waring blendor and extracted three times with a total of 4 liters of acetone at 50°. Two fractions were obtained: the aqueous acetone fraction which

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Solvent</th>
<th>Volume</th>
<th>Weight of material</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane</td>
<td>100</td>
<td>Discarded</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Hexane-benzene, 3:1</td>
<td>100</td>
<td>1</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>100</td>
<td>19</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>Benzene</td>
<td>100</td>
<td>25</td>
<td>Progesterone</td>
</tr>
<tr>
<td>5</td>
<td>Benzene-CHCl₃, 3:1</td>
<td>100</td>
<td>11</td>
<td>Nothing isolated</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>100</td>
<td>15</td>
<td>&quot;</td>
</tr>
<tr>
<td>7</td>
<td>CHCl₃</td>
<td>200</td>
<td>1</td>
<td>Estrogens, other steroids</td>
</tr>
<tr>
<td>8</td>
<td>CHCl₃-CH₃OH, 1:1</td>
<td>200</td>
<td>1</td>
<td>Nothing isolated</td>
</tr>
<tr>
<td>9</td>
<td>CH₃OH</td>
<td>250</td>
<td></td>
<td>&quot;</td>
</tr>
</tbody>
</table>

was evaporated in vacuo and the protein residue which was hydrolyzed with 1 liter of 5 per cent NaOH for 2 days at room temperature.

**Acetone Fraction**—The aqueous residue from the acetone fraction was extracted quantitatively with chloroform. After the chloroform was removed in vacuo, the fatty residue was partitioned between hexane, which was discarded, and 75 per cent methanol, which was concentrated in vacuo. The aqueous residue from the methanol was extracted with chloroform and dried for column chromatography. The final weight of the yellow oil was 670 mg.

**Column Chromatography**—The silica gel (100 to 130 mesh) was washed extensively with acetone and ether and activated by heating overnight at 100°. A column 2 × 12 cm. in dimension was prepared with 17 gm. of silica gel in a hexane slurry. The oil was placed at the surface by dissolving it in a small amount of chloroform-hexane solution, and the flask was rinsed many times with subsequent eluent solutions. The details of the solvent system and residues are shown in Table I. Only
the benzene fraction (Fraction 4) and the chloroform fraction (Fraction 7) yielded identifiable steroids.

Reextraction of Tumor Residue after Alkali Hydrolysis—It is often difficult to extract steroids from crude tissue, and whether this is a reflection of "protein binding" or inadequate mechanical procedures is difficult to demonstrate. However, it is known that alkali digestion renders the material readily extractable with ether (5). It is often not desirable for a first step because of the degradative action of the alkali on the steroids. Progesterone and many estrogenic compounds, however, are stable to alkali, and for this reason the protein residue was hydrolyzed in 1 liter of 5 per cent NaOH for 48 hours at room temperature, extracted with ether at pH 10, and again extracted after acidification to pH 3. The dried residues of the individual extractions were not large; therefore, they were pooled, dissolved in ethyl acetate, and washed with saturated Na₂CO₃ at pH 9 according to the method of Brown (6). The extract was further purified by partition between hexane and 75 per cent methanol. The final pale yellow oil weighed 260 mg.

Paper Chromatography—Isolation of the individual steroids was accomplished by paper chromatography by the methods of Zaffaroni (7). Preliminary chromatography in the heptane-formamide system resolved the oil from the alkali digest into two gross components which were visible under an ultraviolet light scanner. The area at the origin was the source of the more polar steroids, and the area near the front yielded progesterone.

Criteria for Identification—The criteria for satisfactory identification of steroid structure are subject to controversy and are discussed in detail elsewhere (8). An RF by itself carries little weight regardless of the number of times the chromatogram has been run. A single maximum in the ultraviolet absorption spectrum may often verify the presence of a grouping, but, when several peaks are obtained, the evidence for total structure is better. It is even more reliable if it can be shown that the coefficients of absorption (E_max) of the various maxima agree with the corresponding values of the authentic compound. In our experience, the sulfuric acid spectrum (9) is extremely reliable if several maxima are obtained, and the extinction coefficients of the maxima (E_max) are compared with those of the known compound. The infrared spectrum has unique value, but requires purity and quantities greater than were available from these extracts. In most cases we have attempted to make at least one derivative and preferably two.

Results

Progesterone—Progesterone was isolated and characterized both from the acetone extract and from the alkali digest. Fraction 4 from the silica
gel column was chromatographed on paper in the hexane-formamide system as was the progesterone area of the paper chromatogram of the protein digest residue. Both gave $R_F$ values identical to those of a simultaneously run control on three separate occasions. By spectrophotometric analysis both had maxima at 240 m$\mu$, and the yields were 75 $\gamma$ from the acetone extract and 400 $\gamma$ from the hydrolysate. They were combined, and final identification was established by spectrophotometric analysis in fuming sulfuric acid and by infrared analysis. In both procedures the spectra were compatible with those of authentic progesterone.

*Equilenin*—Fraction 7 from the silica gel column (Table I) and the ori-
gin of the paper chromatogram of the alkaline extract were chromato-
graphed in the benzene-formamide system for 20 hours. The mobility of
this compound was identical to that of authentic equilenin. Spectroscopic
absorption was maximal at 235, 280, and 340 m\(\mu\) (Fig. 1, A). Both the
absorption maxima and the ratios of the extinction coefficients \((E_{\text{max}})\) at
the maxima were equivalent to those of authentic equilenin. The test
for the 3,5-unsubstituted hydroxyphenol group with Millon’s reagent was
positive (10). The Zimmermann reaction for the 17-ketone was positive,
and the Zaffaroni chromogen (9) (Fig. 2, A) gave maxima at 230, 300, 320,
400, and 472 m\(\mu\), identical both in maxima and \(E_{\text{max}}\) with those of authentic
equilenin. In concentrated sulfuric acid a green fluorescence was ob-
erved under long wave ultraviolet light similar to that obtained with au-
thentic equilenin. From these data it was inferred that the compound was
equilenin (Fig. 1, A). The total amount isolated was 327 \(\gamma\).

**Table II**

<table>
<thead>
<tr>
<th>Substances Isolated from 523 Gm. of Feminizing Adrenal Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Products isolated</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Progesterone</td>
</tr>
<tr>
<td>Equilenin</td>
</tr>
<tr>
<td>AAE-I-B1</td>
</tr>
<tr>
<td>AAE-I-A1</td>
</tr>
<tr>
<td>AAE-I-A2</td>
</tr>
<tr>
<td>AAE-I-C1</td>
</tr>
<tr>
<td>AAE-I-C2</td>
</tr>
<tr>
<td>AAE-I-D</td>
</tr>
</tbody>
</table>

Compound AAE-I-B1 (Structure Not Identified)—Isolated from both
acetone and alkaline extracts, this compound was slightly more polar than
equilenin and at first appeared to be similar to 6-dehydroestradiol. It
was overrun for 6 hours in benzene-formamide and traveled 9.5 cm, identi-
cally with authentic 6-dehydroestradiol. The Millon test was positive.
An acetate was formed, and this derivative had an \(R_f\) of 0.15, whereas the
acetate of 6-dehydroestradiol did not move from the origin in this amount
of time. The absorption maxima in methanol were seen at 220, 255, and
310 m\(\mu\) as compared to those of 6-dehydroestradiol which occurred at
225, 260, and 305 m\(\mu\) (Fig. 1, B). The \(E_{\text{max}}\) ratios at those wave lengths
were not comparable. The sulfuric acid chromogen yielded a sharp ab-
sorption maximum at 300 and a shoulder at 355 m\(\mu\) (Fig. 2, B). This
confirmed the difference from 6-dehydroestradiol which gave sharp peaks
at 295, 370, 430, and 460 \(\text{m} \mu\) (Fig. 2, B). (The precariousness of deducing structure by ultraviolet absorption and \(R_F\) is reemphasized by the study of this compound.) Temporarily we postulate from Woodward’s rule (11) an additional conjugated double bond either endocyclic or exocyclic to the B ring, but we cannot define the remainder of the structure. The total amount isolated was 389 \(\gamma\).

Other Substances—No substance isolated reacted positively with triphenyltetrazolium chloride. Consequently, there was no evidence for the presence of the \(\alpha\)-ketol side chain characteristic of the normal adrenal hormones. A search for the expected phenolic estrogens failed to reveal the presence of estradiol, estrone, or estriol. Other substances which were found but not identified are presented in Tables II and III in order of decreasing polarity. Thus, AAE-I-A1 and AAE-I-A2 were slightly less polar than estriol and presumably were phenolic substances. Substance AAE-I-D was slightly less polar than testosterone, but more polar than 4-androstene-3,17-dione.

### DISCUSSION

The previously published advantages of hydrolysis with sodium hydroxide prior to the extraction of steroids are reaffirmed in this work (5). While it would be difficult to postulate the cohesiveness of the steroid-protein “binding,” certainly, the hydrolysis of the protein rendered the steroid more extractable. Thus, the alkaline digestion of the acetone residue yielded 5 to 10 times more of each of the steroids than was obtained by acetone extraction, even though the acetone mixture was heated to about 50\(^\circ\) (Table II).

It is impossible to interpret our results on the basis of other workers’ findings. Only about 2 dozen cases of “feminizing tumors” have been reported, and there is so much variation clinically, pathologically, and in

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Millon test</th>
<th>Zimmermann reaction</th>
<th>Ultraviolet (MeOH) maximum (m\mu)</th>
<th>Chromogen (H(_2)SO(_4)) maximum (m\mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAE-I-B1</td>
<td>+</td>
<td>-</td>
<td>220, 255, 310</td>
<td>300, 355</td>
</tr>
<tr>
<td>AAE-I-A1</td>
<td>+</td>
<td>+</td>
<td>242, 260, 295</td>
<td>240, 265, 330</td>
</tr>
<tr>
<td>AAE-I-A2</td>
<td>-</td>
<td>+</td>
<td>240, 300-325*</td>
<td>385</td>
</tr>
<tr>
<td>AAE-I-C1</td>
<td>-</td>
<td>-</td>
<td>240</td>
<td>280, 370, 435</td>
</tr>
<tr>
<td>AAE-I-C2</td>
<td>-</td>
<td>-</td>
<td>240</td>
<td>280, 370, 435</td>
</tr>
<tr>
<td>AAE-I-D</td>
<td>-</td>
<td>-</td>
<td>240</td>
<td>280, 370, 435</td>
</tr>
</tbody>
</table>

* Plateau.
the urinary steroid analyses that comparison is not profitable. However, to our knowledge, this is the only feminizing tumor tissue from which steroids have been characterized and the only isolation of equilenin from a human source (12).

Equilenin was not the only unusual phenolic substance isolated, for analysis of compound AAE-1-B1 would certainly indicate a substance with a phenolic A ring and additional conjugated unsaturation. It could not be determined whether this unsaturation were endocyelic in the B ring or exocyelic, but the $R_F$ would tend to support the former concept. This substance was neither 6-dehydroestradiol nor dihydroequilenin. The implication of finding equilenin and this substance in the human is that they are at least quantitatively unique to this malignancy and reflect either an aberrant aromatizing enzyme system or an overly active one. On the other hand, at least three different groups have found one or all three of the usual phenolic estrogens in the urine of their patients with feminizing tumors (2, 3), and Mason and Kepler (13) found estrone in the urine of a patient with adrenocortical hyperplasia.

Progesterone was the substance isolated in greatest quantity from the tumor. The amount isolated was as much as was found in term placenta (1 mg. per kilo), but less than was found in sow ovaries (15 mg. per kilo) (14). The normal ox adrenal, on the other hand, has been reported to contain 0.5 mg. per kilo (15). Whether progesterone is present in the role of a precursor to the estrogens and other steroids (16–21) or whether the estrogens and progesterone are synthesized from common or different precursors is a consideration beyond the scope of this report. However, it is of interest that one is rarely found without the other.

On a clinical level, the isolation of progesterone is interesting because its presence in so many secretory tumors is reflected by increased levels of pregnane-3α,20α-diol in the urine. Since this tumor occurs most often in males (1), who normally have low urinary levels of pregnanediol, the presence of increased amounts of the latter substance should certainly arouse clinical suspicion of endocrine malignancy. Thus, to cite a few examples, Landau et al. (2) found elevated levels of pregnanediol in the urine of their patient, and Twombly (22) emphasizes the excretion of pregnanediol in cases of secretory ovarian tumors. If progesterone is a precursor to other steroids, its diagnostic role in cases of secretory tumors of the gonads and adrenals and their functional metastases deserves further investigation.

**SUMMARY**

Steroids were extracted and identified from a feminizing adrenal carcinoma. Preliminary hydrolysis of the tissue with sodium hydroxide was found to be 5 to 10 times more effective than extraction with warm acetone.
Progesterone and equilenin were isolated and identified; six other steroids were studied, but their structures could not be elucidated. There is evidence, however, for the presence of phenolic groups and other conjugated unsaturation in the molecule.

It is suggested that urinary study of pregnanediol and equilenin-like steroids would be of diagnostic value in patients suspected to have secretory tumors of this type. The significance of the findings in relation to endocrine tumors and their function is briefly discussed:

The authors wish to express their appreciation to Dr. Harold Brown and Dr. Stanley Wallach for the tumor and clinical information associated with this case, to Dr. Oscar N. Rambo for permission to quote his pathological report, to Dr. George Fujimoto for his cooperation with the infrared analyses, and to Dr. E. G. Holmstrom for his support and interest in this investigation.

BIBLIOGRAPHY