CXIV. THE ISOLATION OF 17-KETOSTEROIDS FROM THE URINE OF NORMAL WOMEN

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IN a recent paper [Callow, 1939] a chromatographic method of separating the ketones in the neutral fraction of an extract of men's urine was described. By this method androsterone, aetiocholan-3(α)-ol-17-one and *transdehydroandrosterone* could readily be isolated in greater yields than had previously been possible, and actually in amounts probably not very different from those originally present in the crude extract. The present paper describes the application of the same process to an extract of normal women's urine with the object of making a roughly quantitative comparison.

EXPERIMENTAL

Collection and extraction of urine. Combined extracts from a large number of experimental extractions of bulked normal women's urine, and from the urines of a few normal patients, were used. Most of the bulk collections were made in the Nurses' Home of the Middlesex Hospital. Only extracts of fresh urine, with pH from 5.5 to 7 before hydrolysis, were included. The urine was either hydrolysed by heating with HCl and then extracted with benzene, or a combined hydrolysis and extraction was carried out, as in the method of Dingemans *et al.* [1937; cf. Callow *et al.* 1939]. The extracts were separated into neutral, acidic and phenolic fractions. After colorimetric and capon assays had been carried out, alcoholic solutions of the neutral fractions were combined and the alcohol was removed. The residue from 114 l. of urine was taken up in 200 ml. of methanol, left overnight at 0° and filtered. Evaporation of the methanol gave 3.6 g. of residue. Treatment with the Girard-Sandulesco reagent P gave 1.14 g. of ketonic fraction, i.e. 10 mg./l. of original urine, and 0.91 g. of non-ketonic fraction. Colorimetry indicated 55% of 17-ketosteroids in the ketonic fraction [cf. Callow *et al.* 1938] and a capon assay, for which we are indebted to Mr C. W. Emmens, an androgenic activity of 1.45 i.u./mg., or 14.5 i.u./l. of original urine. The non-ketonic fraction contained 0.25 i.u./l. Half of this brown, gummy ketonic fraction, equivalent to 57 l. of original urine, was taken up in 25 ml. of carbon tetrachloride and put through a 27 × 1.5 cm. column of alumina as previously described. After development with about 800 ml. of carbon tetrachloride, it showed the following coloured bands, reading from top to bottom—brown, red, white, faint pink, faint yellow, white, yellow. Successive portions of 650–800 ml. of the eluate were evaporated, yielding fractions I, II and III, wt. 26, 16 and 6.5 mg. respectively. Fraction I was a gum (72% ketone); Fraction II crystallized partially on standing, but the crystalline material has not yet been identified; Fraction III included the lowest yellow band. Evaporation of a further portion of about 700 ml. of carbon tetrachloride eluate gave only a trace of gummy residue, which was discarded. A total of 2900 ml. of carbon tetrachloride had been used at this stage. The column was then eluted with a total of 1900 ml. of carbon tetrachloride containing 0.1% of alcohol. Evaporation of successive

portions of eluate gave 7 fractions (IV–X), details of which are given in Table I. As with the extract of men's urine, this treatment caused a sharply defined yellow band to appear and to be washed rapidly down the column (Fraction V). Fractions VII, VIII, IX and X were crystalline.

Table I

Fraction	Vol. of eluate ml.	Solvent	Wt. of fraction mg.	M.P. (crude fractions) ° C.	$[\alpha]_D(\text{EtOH})$	$[\alpha]_{5461}(\text{EtOH})$	Remarks
—	230	0.1 % EtOH in CCl_4	Trace	—	—	—	Discarded
IV	200	"	10	—	—	—	Gum
V	90	"	16	—	—	—	Yellow band
VI	220	"	29	—	+ 45.7°	+ 58.7°	Gum: 8 mg. <i>transdehydroandrosterone</i> isolated
VII	200	"	14	126–161	+ 85°	+ 110°	Chiefly <i>androsterone</i>
VIII	280	"	35	166–178	+ 96°	+ 120°	<i>Androsterone</i>
IX	300	"	24	156–180	+ 92°	+ 108°	"
X	380	"	5.7	140–178	—	—	"
XI	300	0.2 % EtOH in CCl_4	Trace	—	—	—	"
XII	300	"	29	—	+ 80°	+ 100°	Gum
XIII	250	"	24.5	125–144 Tr. 140	+ 101°	+ 122°	Aetiocholan-3(α)-ol-17-one
XIV	180	"	15	130–150 Tr. 135	+ 100°	+ 120°	"
XV	600	"	36	110–146	+ 90°	+ 110°	Aetiocholan-3(α)-ol-17-one + red colouring matter
XVI	430	"	5.7	—	—	—	Gum

Elution was continued with carbon tetrachloride containing 0.2 % of alcohol. The first 1630 ml. of eluate were divided into five fractions (XI–XV, Table I), the last of which included the red colouring matter. Further elution (XVI and subsequent fractions) gave small quantities of gummy material which were not examined further.

Isolation of steroids from crude fractions. (a) *transDehydroandrosterone*. Fractions IV and V were not further examined. From the behaviour of the extract of men's urine it was suspected that *transdehydroandrosterone* would be found in Fraction VI, which was a yellow gum, weighing 29 mg. Its specific rotation was comparatively low, and colorimetry indicated 67 % of 17-ketosteroids. The fraction was treated with 150 mg. of digitonin in 14 ml. of 60 % aqueous alcohol. The precipitate which formed overnight was collected and washed with a little ether. It weighed 49 mg. It was then decomposed with pyridine, the digitonin precipitated by the addition of ether, and the solution filtered, washed with dilute HCl and water, dried and the ether removed. The crystalline residue weighed 8.5 mg., and had M.P. 127–140° and $[\alpha]_D + 18^\circ$ (EtOH). The identity of the compound with *transdehydroandrosterone* was established by preparation of the benzoate. This was very sparingly soluble in methanol; recrystallized from ethyl acetate, it had M.P. 243–252°, and a mixture with an authentic specimen of *transdehydroandrosterone benzoate* had M.P. 250–254.5° (soft at 243.5°).

The melting point and specific rotation suggested that Fraction VII might be a mixture of *androsterone* and *transdehydroandrosterone*. An approximate absorption spectrum of the colour produced with Zimmermann's reaction showed that it was about 90 % 17-ketosteroid. It was taken up in 1.5 ml. of 50 % aqueous alcohol and treated with a hot solution of 80 mg. of digitonin in 8.5 ml. of 50 % alcohol. After standing overnight the precipitate was collected and weighed. Yield: 7.5 mg. This was decomposed in the usual way and the product benzoylated, when the characteristic benzoate of *transdehydroandrosterone* was obtained. The total yield of *transdehydroandrosterone* from Fractions VI and VII was about 10 mg.

(b) *Androsterone*. The filtrate from the above digitonin precipitate was extracted with ether, the extract washed with water, dried and the ether removed. The slightly gummy crystalline residue weighed 12 mg. Recrystallized from methanol it melted at 178–181.5°, after subliming on to the cover slip. It was combined with later androsterone fractions for further recrystallization. The specific rotations and melting points of the crude Fractions VIII and IX suggested that they were androsterone. An approximate absorption spectrum of the colour reaction was that of a 17-ketosteroid. Fraction VIII recrystallized from methanol gave plates, m.p. 181–183°, after subliming to needles, and, mixed with an authentic specimen of androsterone, melted at 179–183°. The identity of the compound as androsterone was confirmed by preparation of the oxime. This, recrystallized from aqueous alcohol, had m.p. 208–211°, which was unchanged by admixture with an authentic specimen. Fraction IX recrystallized from methanol gave 10 mg. of plates m.p. 182–184° after subliming to needles. Mixed with an authentic specimen of androsterone, the m.p. was 183–185°. The total amount of androsterone isolated in a moderately pure state was 76 mg. It may be noted that this quantity accounts for 90% of the biological activity of the crude ketonic fraction as measured by the comb-growth method.

(c) *Aetiocholan-3(α)-ol-17-one*. The crystalline Fractions XIII, XIV and XV were separately treated with charcoal in methanol solution. Addition of water to the filtrates gave material crystallizing in long needles which partially melted and changed to a platy crystalline form at 140–143° and finally melted at about 150°. This behaviour suggested that the material in all cases was aetiocholan-3(α)-ol-17-one, which has a transition point at 140–142°, but a sharp m.p. could not be obtained. The fractions were therefore combined and converted into the benzoate. This had m.p. 161.5–163.5° (from methanol) and the m.p. was unchanged by admixture with an authentic specimen of aetiocholan-3(α)-ol-17-one benzoate. About 76 mg. of aetiocholan-3(α)-ol-17-one were isolated in a moderately pure state.

(d) *Unidentified material*. In addition to the small amount of crystalline material separating from Fraction II already mentioned, there is an indication of an unidentified material in the non-crystalline Fraction XII. The position in the series, the specific rotation (see Table I) and the 17-ketosteroid content (72%) suggest that it might be a mixture of aetiocholan-3(α)-ol-17-one with a non-ketonic impurity, but the presence of other compounds cannot be excluded. A crystalline benzoate, m.p. 80–140°, was prepared, but the amount was not sufficient for purification. Further investigation of these materials will require larger amounts of urine extract.

Note. All melting points recorded were observed in Kofler's micro-melting point apparatus. The optical rotations were measured in a 4 dm. tube.

DISCUSSION

The origin of the 17-ketosteroid compounds in women's urine is as yet a matter only for surmise. Androsterone and aetiocholan-3(α)-ol-17-one were isolated in abnormally high yield from the urine of a man receiving testosterone [Callow, 1939]. This observation indicates that testosterone is transformed by the body into androsterone and aetiocholan-3(α)-ol-17-one. The further observations that these two compounds were major constituents of the neutral ketonic fraction of extracts of normal men's urine was consistent with the assumption that they were derived from endogenous testosterone, assumed to be secreted by the testis. The observations now reported show that normal women's urine yields 1.3 mg./l. of androsterone and 1.3 mg./l. of aetiocholan-3(α)-ol-17-one, as

compared with the yields of 1.6 mg./l. and 1.4 mg./l., respectively, now obtained from normal men's urine by the same method. The excretion of these two compounds is, therefore, not an index of the secretory activity of the testis. The simple assumption that these compounds are excretory transformation products characteristic of testosterone only, would lead to the conclusion that testosterone was secreted by the normal woman in amounts comparable with those secreted by the normal man.

There are, however, no grounds for the assumption that these compounds cannot also be derived from other steroids in the body, whether of gonadal or of adrenal cortical origin. Although ovaries may secrete material with androgenic activity [Hill & Gardner, 1936; Parkes, 1937; Hill & Strong, 1938; Deanesly, 1938], the nature of the compounds produced is unknown. The idea that normal males and females both secrete the same highly active androgen in comparable amounts is contrary to current physiological theories, although Korenchevsky [1939] considers it reasonable to suppose that male hormones present in the female should be of the same nature as the other natural male hormones. The next step we propose to take in the chemical investigation is to examine the nature of the compounds excreted by eunuchs and ovariectomized women. Presumably this will give a clue to the contribution of the adrenal cortex to the urinary 17-ketosteroids. It may be that the latter will include the compounds now regarded as derived from testosterone. Up to the present the evidence points to *transdehydroandrosterone* being a characteristic transformation product from the adrenal cortex in tumour cases [Callow, 1936; Crooke & Callow, 1939], whilst Butler & Marrian [1938, 1, 2] have isolated aetiocholan-3(α)-ol-17-one and androstan-3(β)-ol-17-one from the urine of a case of adrenal hyperplasia. It may be noted that the amounts of *transdehydroandrosterone* now isolated from urine of normal women, although higher than those obtained previously [Callow & Callow, 1938], are still small and comparable with those from normal men's urine.

SUMMARY

1. Aetiocholan-3(α)-ol-17-one (1.3 mg./l.), androsterone (1.3 mg./l.) and *transdehydroandrosterone* (0.2 mg./l.) have been isolated from extracts of the urine of normal women. These yields are comparable with those of the same compounds from normal men's urine.

2. Androsterone is the principal androgenic compound in extracts from the urine of either sex.

3. Although urinary aetiocholan-3(α)-ol-17-one and androsterone are known to be derived from testosterone when this has been given by injection, it does not follow that their presence in urine indicates the secretion of testosterone by women.

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