CCXXVIII. THE ISOLATION OF ANDROSTERONE AND *TRANS*DEHYDROANDROSTERONE FROM THE URINE OF NORMAL WOMEN

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It was first reported by Womack & Koch [1932] that the urine of normal women yielded quantities of androgenic material which, as measured by the capon test, were of the same order as that yielded by the urine of normal men. This fact has been confirmed repeatedly [cf. Siebke, 1934; Dingemanse *et al.* 1935; 1937; Simpson *et al.* 1936; Callow, 1936; Koch, 1936; Gallagher *et al.* 1937; Callow, 1938; Callow *et al.* 1938] and, with the improved methods of extraction which have been used during the last two years, it is generally recognized that, although the average amount of urinary androgen is somewhat lower for women than for men, the limits of normal variation for the two sexes overlap. Nevertheless, there has been no report of attempts to identify chemically the androgenic compounds in the urine of normal, non-pregnant women, parallel with the work by Butenandt and his collaborators [1934; 1935; 1937], which established that androsterone and *trans*dehydroandrosterone occurred in the urine of men, and indicated the presence of *iso*androsterone and of *epi*aetiocholanediol.

Work carried out in this laboratory [Callow *et al.* 1938] has shown that in respect of the ratio of methyleneketone, or "sterone" content (estimated colorimetrically) to comb-growth activity, no distinction can be made between urine extracts from men or women, but this rather vague evidence required amplification by more precise chemical analysis. With this end in view we undertook the more detailed fractionation of extracts of urine from both males and females. The concentration of androgenic activity in the neutral ketonic fraction of men's urine obtained by the use of Girard's reagent has been described in a previous paper [Callow *et al.* 1938]. When the ketonic resin was distilled at low pressure, volatile fractions were obtained, from the first of which the oxime of androsterone could be separated in the pure state in a yield of 0·2 mg. per l. of original urine. Parallel with these experiments, a concentrate from women's urine was put through the same process. There was a striking similarity in the behaviour of the two types of extract in the whole course of the fractionation, and this culminated in the isolation from the women's urine concentrate of androsterone oxime in a yield of 0·44 mg. per l. of original urine. The identity of the compound was confirmed by hydrolysis to androsterone and conversion of the latter into the benzoate. From a second fraction, distilling at a higher temperature, *trans*dehydroandrosterone was isolated in small amount as the characteristic benzoate.

Experimental

Collection and extraction of urine. Urine was collected from seven individual women patients in Middlesex Hospital. Their ages ranged from 20 to 35 years, and none had any obvious sexual dysfunction. Toluene was used as preservative,

and the collections from periods of 6–7 days, having *p*H values from 5·5 to 7, were hydrolysed and extracted as soon as they were available, using the method described by one of us [Callow, 1936]. The neutral fractions were made up in alcoholic solution and, after removal of sufficient material for colorimetric and capon assays, the solutions were combined and evaporated to dryness. The extract from 75 l. of urine or 76 days' output obtained in this way was dissolved in 150 ml. of hot methanol, kept overnight and filtered from a small amount of insoluble material. Evaporation of the filtrate yielded a residue weighing 2·59 g. A colorimetric assay by the modified Zimmermann method [Callow *et al.* 1938] gave a value of 8·2 mg. of methyleneketone or "sterone" per litre, i.e. the chromogenic equivalent of 0·615 g. of 17-ketosteroids in the whole sample.

Separation of ketonic fraction. The methanol-soluble fraction of the extract from 32 l. of urine, wt. 1·13 g., was dissolved in 20 ml. of glacial acetic acid, 2 g. of Girard's reagent T added, and the mixture heated on the water bath for 30 min. After cooling, it was poured into 400–500 ml. of ice and water containing enough Na_2CO_3 to neutralize, to phenolphthalein, 18 ml. of acetic acid. The mixture was extracted 7 times with 100 ml. lots of ether to remove the non-ketonic fraction. The combined ether extracts were washed once with water, which was added to the aqueous ketonic layer. The latter was acidified with 10 % of its volume of conc. HCl, warmed gently and kept standing for 1·5 hr. It was then extracted 5 times with 100 ml. lots of ether; the extract was washed with water, 0·5 *N* Na_2CO_3 and again with water, dried over Na_2SO_4 and evaporated. The residue weighed 423 mg. The ethereal solution of the non-ketonic fraction was washed, dried and evaporated. Wt. of residue, 487 mg. The ketonic fraction was dissolved in acetic acid and treated again with Girard's reagent. A ketonic fraction weighing 292·7 mg. was obtained. Colorimetry indicated a content of 75 % of "sterone".

Isolation of androsterone. The ketonic material, in alcoholic solution, was transferred to the vacuum still and evaporated to dryness. The still used consisted of a vertical, cylindrical tube with rounded bottom, of 4·5 cm. internal diameter, with a concentric water-cooled condenser of 3·5 cm. external diameter reaching to within 0·5 cm. of the bottom of the outer tube. Distillation was carried out at 0·0009 mm. and a bath temperature of 90–95° for 3 hr. 77·4 mg. of an almost colourless resin distilled. A colorimetric estimation gave a value of 80 % of "sterone".

72 mg. of the distillate, 40 mg. of hydroxylamine hydrochloride and 40 mg. of anhydrous sodium acetate were dissolved in about 10 ml. of alcohol and boiled under reflux for 5½ hr. The solution was filtered to remove NaCl, and evaporated to a volume of 2–3 ml. A few drops of water were added, and an oil separated, which crystallized on scratching. Next day the solid was collected, washed rapidly with a little cold 50 % aqueous alcohol and dried: yield, 21·6 mg., M.P. 149–194°.

After three recrystallizations from acetone, a product was obtained crystallizing in well-formed rhombic plates, M.P. 208–213·5° after softening at 204° (Kofler apparatus; see note below). The M.P. was unchanged by admixture with an authentic specimen of androsterone oxime (M.P. 210–211°).

14 mg. of once recrystallized oxime, M.P. 203–206°, were dissolved in 2 ml. alcohol, 1 ml. of 3 *N* H_2SO_4 added and the mixture boiled under reflux for 3 hr. The crude ketone, precipitated by water, weighed 3 mg.; M.P. 168·5–175°. It crystallized from methanol in thin plates, M.P. 178–181°, after subliming to needles. A mixture with an authentic specimen of androsterone (M.P. 184–185°) melted at 178–181·5°.

About 2·5 mg. of crude androsterone (recrystallized product and material obtained by evaporation of the mother liquors above) were dissolved in 0·5 ml. of pyridine, and 2 drops of benzoyl chloride added. The mixture was warmed on the water bath for 5 min., cooled and treated with water drop by drop. An oil separated, which was extracted with ether. The extract was washed with water, dilute HCl, $NaHCO_3$ solution and again with water, dried over Na_2SO_4 and evaporated. The residue was crystallized from 2 drops of methanol. The product, M.P. 166·5–171° (soft at 166°), was again recrystallized from methanol. It then had M.P. 172–175° (soft at 169°), and a mixture with an authentic specimen of androsterone benzoate (M.P. 170–174°) melted at 170–174·5° (soft at 168°).

Separation of trans*dehydroandrosterone benzoate.* 192 mg. of ketonic fraction (from 20 l. of urine) were distilled as described above at 90–95°/0·002 mm. for 2 hr. After removal of the distillate, the still was evacuated to 0·0017 mm., the oil bath heated slowly to 145° and then kept at 145–150° for 1½ hr. 84 mg. of distillate were obtained. Colorimetric assay indicated a content of 87 % of "sterone". A comparative experiment showed that *trans*dehydroandrosterone slowly sublimed between 120 and 150° under the above conditions.

The distillate was dissolved in about 2 ml. of dry pyridine, treated with 3 drops of benzoyl chloride and the mixture warmed for 5 min. It was then cooled and treated with water. An oil separated which was well washed with water and dissolved in about 2 ml. of methanol. After leaving overnight in the refrigerator, 1–2 mg. of a crystalline solid separated, M.P. 223·5–243·5°. After recrystallization once from methanol and once from ethyl acetate, the M.P. was 246–248° (softening 245°). A mixture with an authentic specimen of *trans*-dehydroandrosterone benzoate (M.P. 245·5–250°) melted at 245–248·5°. A further crop of crystals separated from the original methanol mother liquors which after recrystallization melted at 238–248° (mixture with *trans*dehydroandrosterone benzoate, M.P. 237–252°).

All the melting points recorded were observed on a microscope slide on an electrically heated stage, using Kofler's micro-melting point apparatus. The temperatures recorded are those of the first appearance of liquid and of complete fusion.

Discussion

It is abundantly clear that complete chemical analysis of extracts of normal urine into their steroid components is a difficult matter. The processes of partition between solvents, distillation and crystallization, not ideal for quantitative work at the best of times, are not well adapted to the separation of closely related steroids. Our yields of androsterone amounted to about the equivalent of 0·2 and 0·4 mg. per litre from urines of men and women, respectively. The higher yield in the latter case is to be attributed to practice in the manipulation, and there are clear indications that extracts of urine from normal men or women contain similar amounts. We do not feel, however, that it is possible to estimate the amounts originally present more accurately than Butenandt and his collaborators [1935] did when they concluded that the amounts of androsterone and *trans*dehydroandrosterone actually present in extracts of men's urine were at least twice those separated in the pure state. With the available methods of separation we are, in fact, very far from the stage of being able to account, by isolation of recognizable derivatives, either for the whole androgenic activity, or for the colorimetrically determined methylene-ketone content of normal urine extracts.

The importance of the present work lies in its demonstration that androsterone and *trans*dehydroandrosterone are not specific products of the male

organism. It is possible, however, that the excretion of identical androstane derivatives by the two sexes is simply due to these products being common degradation products, particularly adapted for excretion, of original secretions which, although different, are closely related in chemical structure. The alternative assumption of actual identity of the original secretions in the two sexes is more speculative, and could only apply to a part of the endocrine steroids, since the nature of only a fraction of the excretory transformation products is established. It has, however, some plausibility as regards products from the suprarenal glands, for evidence from suprarenal tumour cases leads to the belief that *trans*dehydroandrosterone at least is metabolically derived from the family of steroid compounds present in the suprarenal glands, and that its excretion is not directly connected with gonadal function (cf. the discussion by Callow [1938]). The degree of direct responsibility of the gonads for any part of the urinary excretion of androstane derivatives remains to be investigated; at present there is no clue to products which can be recognized as derived from the testis or the ovary, and any characteristic difference in the androstane derivatives excreted by the two sexes still eludes detection. It is hoped that more light on these problems will be obtained as chemical and physical methods are developed and applied to the more detailed analysis of urines from abnormal clinical types of patient. Work along these lines is now in progress.

SUMMARY

Androsterone and *trans*dehydroandrosterone have been isolated from extracts of the urine of normal women, in a yield comparable with that from normal men's urine.

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