LXIX. THE ISOLATION OF TWO TRANSFORMATION PRODUCTS OF TESTOSTERONE FROM URINE

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INCREASES in the amount of androgenic material, assayed biologically, which could be extracted from human urine following administration of androgens, have been reported by Bühler [1933], by McCullagh *et al.* [1938], who treated a eunuch with urine extract, and by Kochakian [1937]. These were small and uncertain, and were produced by low doses. Dorfman & Hamilton [1939], using efficient methods of extraction, have recently reported three cases in which large increases in the amount of androgenic material occurred as a result of the administration of testosterone propionate. Prof. J. W. Cook has carried out chemical work on these extracts [Cook *et al.* 1939]. Callow *et al.* [1939, 1, 2] have investigated independently a series of cases in which large doses of hormones were administered. One of these patients, a man under the care of Dr E. P. Sharpey-Schafer, was receiving 100 mg. of testosterone propionate daily. A clinical account of this case has been published elsewhere [Schrire & Sharpey-Schafer, 1939, case 3]. During the administration of testosterone propionate the excretion of 17-ketosteroids (measured colorimetrically by the modified Zimmermann reaction described by Callow *et al.* [1938]), and of androgenic activity, increased to about four times the level of that of a subsequent control period, when this excretion was within normal limits, so that there can be little doubt that the increase was directly caused by the injections; sufficient extract was available for chemical separation of the 17-ketosteroids, with which the urine was enriched, to be attempted.

The neutral fraction of the benzene extract of acid-hydrolysed urine was separated into ketonic and non-ketonic fractions by the Girard-Sandulesco reagent P. The ketonic fraction after chromatographic adsorption followed by elution of the column yielded androsterone and a compound which was identified as aetiocholan-3(α)-ol-17-one (Fieser's nomenclature [1937]; the "epi-oxy-aetiocholanon-17" of Ruzicka & Goldberg [1935]). A total of 60 mg. of crude androsterone and 58 mg. of crude aetiocholan-3(α)-ol-17-one were obtained from 7½ l. of urine or about 6½ days' output.

As a control, the extract from 50 l. of a bulk collection of normal men's urine was put through the same process. A total of 60 mg. of androsterone and 70 mg. of crude aetiocholan-3(α)-ol-17-one were separated. The isolation of the latter compound from normal urine has not previously been reported.

Thus from the patient receiving testosterone propionate 8 mg./l. of crude androsterone and 7.7 mg./l. of crude aetiocholan-3(α)-ol-17-one were isolated, while from the normal men's urine the same two compounds were obtained in yields of 1.2 and 1.4 mg./l. respectively. The androsterone which was isolated accounts for about 74 % of the androgenic activity, assayed on capons, of the extracts of the "testosterone urine", and about 45 % of the activity of the normal urine extract.

EXPERIMENTAL

All melting points in this paper were observed under the microscope on a slide on an electrically heated stage (Kofler's micro-melting point apparatus). The optical rotations were measured in absolute alcoholic solution in a 4 dm. tube.

Collection and extraction of urine. 24-hr. collections of urine were made for 8 days while the patient was receiving 100 mg. of testosterone propionate daily by intramuscular injection, beginning on the second day of injection. Toluene was used as preservative, and the completed collections were stored at 0° until hydrolysis, which was carried out not more than 5 days after collection. Before hydrolysis the pH of all the samples was 6, indicating that little, if any, putrefaction had taken place. The 24-hr. collections were hydrolysed, extracted, and separated into neutral, acidic, and phenolic fractions by the routine method in this laboratory [Callow, 1936]. Colorimetry indicated a content of 17-ketosteroids varying from 28 to 45 mg., average 38 mg. daily. By capon assay (for which I am indebted to Mr C. W. Emmens) the average androgen content was 126 I.U. per day.

The neutral fractions, equivalent to $6\frac{1}{2}$ days' excretion or $7\frac{1}{2}$ l., were combined and taken up in about 15 ml. of methanol. Next day the solution was filtered from traces of insoluble material; evaporation of the filtrate gave 0.54 g. of neutral fraction. Treatment with the Girard-Sandulesco reagent P (carbohydrazidomethylpyridinium chloride) [cf. Callow & Callow, 1938] gave a "ketonic fraction" weighing 0.255 g. and a "non-ketonic fraction" weighing 0.24 g. Colorimetry indicated that the ketonic fraction contained about 65% of 17-ketosteroids.

Separation by fractional adsorption. The ketonic resin was dissolved in 10 ml. of carbon tetrachloride (A.R.), left overnight and filtered. The filtrate, diluted to about 25 ml., was put through a 26×1.5 cm. column of alumina (Merck's "Aluminium oxide standardized according to Brockmann") in carbon tetrachloride. It was developed in the usual way with about 1.5 l. of carbon tetrachloride, and then with carbon tetrachloride containing successively 0.1, 0.2 and 0.3% of absolute alcohol. Successive portions of 200–500 ml. of the eluate were evaporated. The pure carbon tetrachloride eluate gave only a trace of coloured gummy material. The 0.1% alcohol solution caused a narrow yellow band to move rapidly down the column, and evaporation of 300 ml. of eluate gave 11.7 mg. of gummy residue. Elution with the 0.2 and 0.3% alcohol solutions gave crystalline residues. The weights, volumes of eluate, and the melting points and specific rotations of the residues are shown in Table I. After these fractions had been removed, no more solid was obtained by further elution with 0.3% of alcohol in carbon tetrachloride, and subsequent treatment with 600 ml. of 0.5% and 500 ml. of 1.0% alcohol solutions yielded only 6 mg. of gummy material.

Table I

Frac- tion	Vol. and nature of solvent	Wt. crude crystals mg.	M.P. ° C.	Optical rotation		Main product subsequently identified as
				$[\alpha]_D$	$[\alpha]_{5461}$	
I	350 ml. 0.2% EtOH in CCl ₄	42	175–181	+92°	+104°	Androsterone
II	225 ml. " "	18	170–184 (recryst.)	+85°	+89°	"
III	250 ml. 0.3% EtOH in CCl ₄	21.5	130–146	+89°	+124°	Aetiocholan- 3(α)-ol-17-one
IV	225 ml. " "	15.6	115–151	+91°	+98°	"
V	225 ml. " "	13	136–149	+102°	+122.5°	"
VI	240 ml. " "	5.7	—	—	—	"

Characterization of androsterone

The specific rotation, and the characteristic habit of subliming on to the coverslip during the determination of the M.P. on the micro-melting point apparatus suggested that both fractions I and II were androsterone. After two recrystallizations from methanol, fraction I yielded 24 mg. of crystals, M.P. 183–184° (soft at 179°). A mixed melting point with an authentic specimen of androsterone was 181–184·5°. The identity of the compound as androsterone was confirmed by preparation of the oxime. 10 mg. of the ketone, 6 mg. of hydroxylamine hydrochloride and 6 mg. of anhydrous sodium acetate were boiled under reflux in alcoholic solution for 2½ hr. The solvent was removed under reduced pressure, and the crystalline residue taken up in about 3 ml. of acetone, the solution filtered and evaporated to about 0·5 ml. On cooling 8 mg. of the oxime, M.P. 203–206 were obtained. After recrystallizing from acetone it had M.P. 206–212·5° and mixed M.P. with an authentic specimen of androsterone oxime 208–212·5°.

Fraction II after recrystallization from methanol had M.P. 182–184, which was not depressed by admixture with an authentic specimen of androsterone.

Identification of aetiocholan-3(α)-ol-17-one

The colour reaction of material from fraction III showed the absorptiometric spectrum characteristic of a 17-ketosteroid [cf. Callow *et al.* 1938]. 21 mg. of the crude fraction III were treated with a hot solution of 100 mg. of digitonin in 10 ml. of 50 % aqueous alcohol. Next day the slight precipitate was removed by centrifuging and the supernatant liquid was diluted with water and extracted with ether; the ether extract, washed with water, and dried over sodium sulphate, yielded on evaporation a residue, 19 mg., which was recrystallized from aqueous methanol. The crystals showed a characteristic transition from a needle-shaped to a platy crystalline form at 137–139°, with partial fusion and resolidification. The final M.P. was 150°. Further recrystallization gave material with M.P. 147–151° and $[\alpha]_D + 100^\circ$: $[\alpha]_{5461} + 130^\circ$ (EtOH); and finally with M.P. 151–152° (transition point 140–142°). This M.P. was not depressed by admixture with an authentic specimen of aetiocholan-3(α)-ol-17-one, M.P. 152–153° (transition point 140°). Found: (Weiler) C, 78·2; H, 10·0 %; calc. for $C_{19}H_{30}O_2$: C, 78·6; H, 10·4 %.

Preparation of aetiocholan-3(α)-ol-17-one benzoate

Material, M.P. 147–151°, obtained by evaporation of the mother liquors from the last recrystallization of the above aetiocholan-3(α)-ol-17-one was dissolved in 0·25 ml. of dry pyridine and two drops of benzoyl chloride added. After heating on the water bath for 5 min., the mixture was cooled, and diluted with water drop by drop. The sticky crystals which separated were well washed with water, and recrystallized from aqueous methanol, from which characteristic bundles of needles, M.P. 140–160°, separated. After four recrystallizations from aqueous methanol, it had M.P. 153–161°. There was insufficient for further purification.

For comparison a specimen of aetiocholan-3(α)-ol-17-one benzoate, which has not been described before, was prepared from the authentic hydroxyketone as described above. After recrystallization from aqueous methanol, and from absolute methanol, it had a constant M.P. 161·5–163·5°, and crystallized well in long needles similar to those already described. The melting point of the sample prepared from the urine extract was not depressed by admixture with this authentic specimen.

Fractions IV, V and VI were combined, after preliminary measurements of their melting points and specific rotations, and recrystallized from benzene. The top fraction partially melted and recrystallized at 137° and finally melted at 152°. The last fraction, obtained by evaporation of the mother liquors, m.p. 142–148°, yielded a benzoate, m.p. 151–160°. There was insufficient for further purification, but it was similar in crystalline form and behaviour to the substance identified above as aetiocholan-3(α)-ol-17-one benzoate. The top and intermediate fractions from the benzene recrystallizations were united (15 mg.) and treated with a hot solution of 80 mg. of digitonin in 10 ml. of hot 50 % aqueous alcohol. After standing overnight there was only a slight precipitate, and 11 mg. of material were recovered from the filtrate. This was treated with acetic anhydride in presence of pyridine on the water bath for 30 min. The mixture was cooled, water was added and the oil which separated was extracted with ether; the extract was washed with aqueous Na₂CO₃ and with water, dried and evaporated. The residue, twice recrystallized from aqueous methanol, had m.p. 91–94.5°. The m.p. of a slightly impure authentic specimen of aetiocholan-3(α)-ol-17-one acetate (m.p. 89.5–94°) was not depressed by admixture with this specimen.

The normal urine extract used as a control was part of a bulk collection of 250 l. of normal men's urine worked up by Messrs Boots Pure Drug Co. This opportunity is taken to acknowledge their generosity in undertaking this work. The urine was collected with Brilliant Green as preservative, hydrolysed with HCl and extracted with chloroform. The chloroform was evaporated, and the extract sent to this laboratory, where it was taken up in benzene, and separated into neutral, acidic and phenolic fractions in the usual way. Colorimetry of the neutral fraction indicated 8 mg./l. of 17-ketosteroids, and capon assay 26 i.u./l. of androgenic activity. The neutral fraction of the extract from 50 l. of urine was separated into "ketonic" and "non-ketonic" fractions. These weighed 0.48 and 1.12 g. respectively. The ketonic fraction was taken up in carbon tetrachloride, and put through a column of alumina. The column was developed with carbon tetrachloride, and carbon tetrachloride containing 0.1, 0.2 and 0.3 % of absolute alcohol, as described for the "testosterone urine". Development with any one solvent was continued until evaporation of about 250 ml. of eluate gave a residue weighing less than 10 mg. Evaporation of 310 ml. of the 0.1 % alcohol eluate gave 49 mg. of a semicrystalline residue. After treatment with charcoal and repeated recrystallization from aqueous methanol, 6 mg. of a product, m.p. 142–150°, were obtained. This was subsequently identified as *transdehydroandrosterone*. 15 mg. of material from the mother liquors were treated with 60 mg. of digitonin in 70 % aqueous alcohol. The precipitated digitonide was decomposed in the usual way with pyridine, and yielded 2.5 mg. of a crystalline substance, m.p. 136–145°. This was combined with the material obtained by fractional crystallization and converted into the benzoate. This, after recrystallization from ethyl acetate, was recognized as *transdehydroandrosterone benzoate*. It then had m.p. 242–248°, not depressed by admixture with an authentic specimen. Elution with a total of 810 ml. of 0.2 % alcohol in carbon tetrachloride yielded 60 mg. in all of crystalline residue. This was identified as *androsterone* by its m.p., mixed m.p. and property of subliming. The next fraction, obtained by elution with 370 ml. of 0.3 % alcohol in carbon tetrachloride, yielded 70 mg. of crystalline material. After repeated recrystallization from aqueous methanol, the m.p. became constant at 144–147°. A mixed m.p. with an authentic specimen of aetiocholan-3(α)-ol-17-one was 145–150°. The identity of the compound was confirmed by preparation of the benzoate. This had m.p. 159–162°, and a mixed m.p. 162–163.5° with an authentic specimen of aetiocholan-3(α)-ol-17-one benzoate.

DISCUSSION

The isolation of androsterone and the stereoisomeric compound aetiocholan-3(α)-ol-17-one from the urine of a man receiving testosterone propionate, and, in much smaller quantities, from normal men's urine, is particularly interesting from the point of view of determining the parent substances of the steroid compounds excreted in urine, whether the former have their origin in the gonads or the adrenals. Testosterone is the only androgen which has been isolated from gonadal tissue, but there is no evidence that it is ever excreted in urine. This work owed its inception to the hope that some degradation product might be recognized as an index of testosterone production, just as pregnanediol is recognized as an index of progesterone production [cf. Venning & Browne, 1936; 1937; Venning, 1937]. Since androsterone has been isolated from normal women's urine [Callow & Callow, 1938] and aetiocholan-3(α)-ol-17-one from the urine of a woman with adrenal hyperplasia [Butler & Marrian, 1938], the meaning of the excretion of these two compounds must remain a matter of speculation until further work has been done. The possible occurrence of aetiocholan-3(α)-ol-17-one in the urine of normal women is now being investigated.

Hartmann & Locher [1935] suggested that pregnanediol and *allo*pregnanediol, which can be isolated from the urine of pregnant women, were derived from progesterone by reduction. A completely analogous process in ring I can be postulated for the degradation of testosterone (I) to androsterone (II) and aetiocholan-3(α)-ol-17-one (III), involving the reduction of the 3-keto-group to give a 3(α)-hydroxy compound, and of the 4:5 double bond to give both the two possible configurations at position 5, with oxidation of the 17-hydroxyl group.

Butler & Marrian [1938] suggested that the aetiocholan-3(α)-ol-17-one which they isolated from the urine of a woman with adrenal hyperplasia was derived by partial oxidation from pregnane-3(α):17:20-triol, which they isolated from the same urine. The finding of the former compound as a degradation product of testosterone does not, of course, disprove this hypothesis, but it shows that another mechanism is possible.

The degradations of testosterone to androsterone and aetiocholan-3(α)-ol-17-one now experimentally demonstrated may be compared with the speculative scheme of degradation of male hormones in the body recently put forward by Marker [1938]. He suggested that androsterone and aetiocholan-3(α)-ol-17-one were stages in the reduction of Δ^4 -androstene-3:17-dione. This scheme would be in accordance with the facts, provided that Δ^4 -androstene-3:17-dione were derived from testosterone, and not the reverse, as assumed by Marker. He also suggested that *trans*dehydroandrosterone (Δ^5 -androstene-3(β)-ol-17-one) is derived from Δ^4 -androstene-3:17-dione. In the case investigated there was actually no large increase in the excretion of *trans*dehydroandrosterone during the administration of testosterone propionate. From the behaviour of the extract from normal urine, it is known that this compound is eluted from the adsorption column with carbon tetrachloride containing 0.1% of alcohol. The

corresponding fraction from the "testosterone urine" was a gum which weighed only 11.7 mg., and was obviously a mixture. The yield of this fraction from seven times the volume of normal men's urine was 49 mg. of semicrystalline material, from which 8 mg. of *trans*dehydroandrosterone were isolated. *trans*Dehydroandrosterone is more probably derived from the adrenal cortical secretions [cf. Callow, 1938].

When this work was nearing completion it was learnt from Prof. J. W. Cook that he had isolated androsterone as a degradation product of administered testosterone, and accounts of both his and our investigations were given to the meeting of the Biochemical Society on 10 February 1939 [Cook *et al.* 1939; Callow *et al.* 1939, 1].

SUMMARY

Androsterone and aetiocholan-3(α)-ol-17-one have been isolated from the urine of a man receiving 100 mg. of testosterone propionate daily in yields of 8 and 7.7 mg./l., respectively. The same two compounds have been obtained from normal men's urine in yields of 1.2 and 1.4 mg./l.

I have pleasure in thanking Dr E. P. Sharpey-Schafer of the British Post-Graduate Medical School for putting the urine from his case at my disposal, and Dr D. Beall for arranging its collection and transport. I am greatly indebted to Prof. Ruzicka for a specimen of aetiocholan-3(α)-ol-17-one, which he worked up from the semicarbazone so that a comparison with the natural product might be made, and to Prof. G. F. Marrian for a specimen of the acetate. Finally I have to acknowledge much encouragement and helpful criticism from my husband, Dr R. K. Callow.

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