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The Estimation of Androst-16-en-3α-ol in Human Urine

PARTIAL SYNTHESIS OF ANDROSTENOL AND OF ITS β-GLUCOSIDURONIC ACID

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Androst-16-en-3α-ol (II) was originally isolated, together with the 3β-epimer, from pig's testes by Prelog & Ruzicka (1944); the structure of the stenols was elucidated by Prelog, Ruzicka & Wieland (1944), who prepared other Δ^{16}-C_{19} steroids and drew attention to their remarkable odours. Other Δ^{16}-compounds in the C_{19} and C_{18} series have been synthesized, but only the androst-16-en-3-ols have been detected in biological extracts; indeed the 3β-ol has so far been found only in pig's testes. The 3α-ol was obtained from the urine of normal men and women by Brooksbank & Haslewood (1949, 1950, 1952), after treatment of a glucosiduronate extract with β-glucuronidase, in amounts which suggested that it was excreted at a rate of about 1 mg./day: the method of isolation seemed to exclude the possibility of its being a chemical artifact. Androstenol (about 15 mg./day) has also been isolated from the urine of a female patient with an adrenal tumour (Mason & Schneider, 1950), of a patient with a luteoma of the ovary (Engel, Dorfman & Abarbanel, 1953) and of a patient with adrenal hyperplasia (Miller, Rosenkrantz & Dorfman, 1953).

Burstein & Dorfman (1960) have recently shown by isotope studies in a woman with a virilizing adrenal tumour that (II) can be formed from cholesterol via 3β-hydroxypregn-5-en-20-one. All this work suggests that (II) is metabolically related to the well-known androgens, as of course its chemical structure implies. However, in some ways (II) is of unusual interest. Its occurrence in normal urine in much more than trace quantities points to its being more than an insignificant, chancy, metabolic by-product. If the biochemical precursor of (II) is a C-17-hydroxy compound, as at first sight seems likely, its formation would involve a dehydration reaction, hitherto regarded as rare and very little studied in the steroid series in mammalian systems: if the precursor is not a C-17-hydroxy compound, its origin presents an even more intriguing biochemical problem. Finally, the powerful smell of (II), described by many as 'musk-like', and its formal structural resemblance to civetone might conceivably have far-reaching physiological implications.

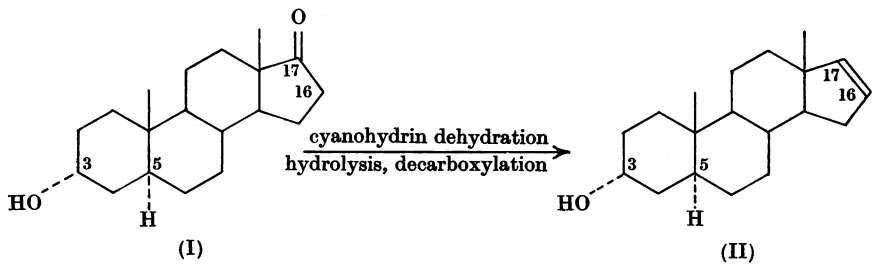
It therefore seemed worth while to begin an investigation of the metabolism of (II) by a study of its urinary excretion in health and disease. This paper describes, first, a partial synthesis of the free steroid and of its β-glucosiduronic acid, and, secondly, the details and trials of a method of estimation, including preliminary quantitative data on urinary excretion in normal men and women.

GENERAL METHODS

The partial synthesis of androst-16-en-3α-ol (II) from androsterone (I) was carried out as described in a preliminary communication (Brooksbank, Haslewood, Pollock & Hewett, 1958). In a small-scale pilot synthesis, the overall yield was about 34%; on a larger scale, it was 15–20% without recycling of intermediates.

Synthesis of methyl (androst-16-en-3α-yl tri-*O*-acetyl-β-glucosid)uronate was carried out after Bollenback, Long, Benjamin & Lindquist (1955) and Meystre & Miescher (1944), and the ester converted into androst-16-en-3α-yl β-glucosiduronic acid as described by Pelzer (1959) and Wotiz, Smakula, Lichtin & Leftin (1959).

The quantitative method, which has been already described in outline (Brooksbank & Haslewood, 1960), is



(I) (II)

designed to be simple enough for routine application, consistent with adequate specificity for androst-16-en-3α-ol. The opportunity has been taken to establish accuracy by using the β-glucosiduronic acid of androstenol for recovery experiments from urine. This refinement, which obviates uncertainties about the completeness of hydrolysis of conjugates, has not often been applied to analysis of urinary steroids conjugated with glucuronic acid.

EXPERIMENTAL

General. Elementary analysis were by Weiler and Strauss, Oxford. Melting points are uncorrected. The symbols E_{520} and E_{575} refer to extinctions measured with (green) and (yellow) light filters whose peak transmissions are stated to be at 530 and 575 mμ and transmission ranges 440–630 and 550–630 mμ. respectively. The filter transmitting maximally at 575 mμ was suitable for measurement of the colour given by androstenol in the conditions described below. For correction of E_{575} (see below) it was advantageous to use a green filter of wide transmission range.

Partial synthesis of androst-16-en-3α-ol

3α-*Acetoxy-5α-androstan-17-one cyanohydrin.* The cynaohydrin mixture was prepared from androsterone acetate as by Ruzicka, Plattner, Heusser & Pataki (1946). The amorphous white solid obtained was dried *in vacuo* over $CaCl_2$. No attempt was made to obtain a pure cyanohydrin.

3α-*Acetoxy-17β-cyanoandrost-16-ene.* The dehydration of the cyanohydrin with $POCl_3$ was carried out exactly as described by Ruzicka *et al.* (1946), but by using dry Carius tubes heated in a furnace. The dried cyanohydrin was processed in this way with batches of 1–2 g. each per Carius tube. After recrystallization of the 3α-*acetoxy-17β-cyanoandrost-16-ene* from aqueous acetone, the final (constant) m.p. was 201–204·5° (Found: C, 77·8; H, 9·1; N, 4·1. $C_{22}H_{31}O_2N$ requires C, 77·4; H, 9·2; N, 4·1%). A typical yield from 5·15 g. of androsterone acetate was (after one recrystallization) 3·10 g. of the 17-cyano compound, i.e. 58·5%.

3α-*Hydroxyaeti-16-enic acid.* The hydrolysis of the 17-cyano compound was done in small metal bombs. For example, to 3α-acetoxy-17β-cyanoandrost-16-ene (326 mg.) in a bomb were added ethanol (8·8 ml.) and N-NaOH (4·4 ml.). The sealed bomb was heated at 180–185° for 5 hr. The pooled product from seven batches (total wt., 2·58 g.) was diluted with water to about 340 ml. and the mixture extracted with ether. (Evaporation of the washed and dried ether left a neutral material, which could be subjected to a second hydrolysis, with or without further treatment with HCN.) The alkaline aqueous phase was acidified with HCl, and, after an interval, the crude aetienic acid was collected by filtration. The filtrate (after addition of excess of NaCl) and precipitate were extracted with ethyl acetate, and the extract was washed with water, dried (Na_2SO_4) and evaporated to give the crude aetienic acid. Yield (after desiccation), 1·90 g. (79·6%).

3α-*Hydroxyandrost-16-ene* (see also Sondheimer, Mancera, Urquiza & Rosenkrantz, 1958). The above crude 3α-hydroxyaeti-16-enic acid (2·15 g.) was boiled for 4 hr. in quinoline (39 ml., freshly distilled from KOH) with the addition (in two portions) of activated copper chromite catalyst (389 mg. in all, as described by Adkins & Connor, 1931). After the addition of water (1660 ml.), the mixture was extracted repeatedly with ether. The ethereal extract was washed with aqueous HCl, NaOH and water, dried (Na_2SO_4) and evaporated. The yield of neutral material (crude androstenol) was 1·48 g. (79·9%). Unchanged aetienic acid was recovered from the alkaline phase. Pure 3α-hydroxyandrost-16-ene could be obtained in good yield by sublimation of the crude neutral material at 125–135°/0·3 mm. Hg. The crystalline sublimate was recrystallized from aqueous acetone to (constant) m.p. 144·5–146°, not depressed by authentic material. As many different batches of crude androstenol were worked up together, it was not possible to calculate yields of pure androstenol in the larger-scale experiments. But in a typical sublimation, 186 mg. of crystalline sublimate were obtained from 216 mg. of crude material (yield 86%); in another experiment, 136 mg. of crude material yielded 65 mg. (m.p. 140–145·5°) after sublimation and recrystallization (yield 48%). The overall yield of pure androstenol from androsterone was usually about 15–20%. As the amount of side products formed at any stage in the synthesis seemed to be small, this yield could probably be substantially improved by recycling material through the process: this was in fact done to some extent.

Preparation of androst-16-en-3α-yl β-glucosiduronic acid

Methyl (androst-16-en-3α-yl 2:3:4-tri-O-acetyl-β-D-glucopyranosid)uronate (cf. Meystre & Miescher, 1944). Androst-16-en-3α-ol (200 mg.) in anhydrous benzene (25 ml.) containing Ag_2CO_3 (750 mg.) was treated dropwise during 1·5 hr. with a solution of methyl α-bromotri-*O*-acetylglucuronate [750 mg.; m.p. 103–105°; $[\alpha]_D^{23} + 189 \pm 1°$ in $CHCl_3$ (*c*, 2·7); prepared by slight modification of the procedure of Bollenback *et al.* (1955)] in anhydrous benzene (40 ml.). Continuous distillation of benzene (in all, about 40 ml.) was carried out for a total time of 2 hr. After all the bromo compound had been added the mixture was refluxed for a further 30 min. The benzene solution was filtered and evaporated. The dried residue was treated with ethanol–water (70:30, v/v). Crystalline material was obtained, which after recrystallization (twice) from aqueous ethanol had m.p. 171–172° (Found: C, 65·2; H, 7·9. $C_{32}H_{46}O_{10}$ requires C, 65·1; H, 7·8%). Yield, 117 mg. of material of m.p. > 164° (27%).

Androst-16-en-3α-yl β-D-glucopyranosiduronic acid (cf. Pelzer, 1959; Wotiz *et al.* 1959). The above methyl (androstenyl tri-*O*-acetylglucosid)uronate (m.p. 166–171°; 87 mg.) in ethanol (1·5 ml.) was treated with 0·49 N-$Ba(OH)_2$ (5 ml.). The mixture was heated for 2–3 min. on a boiling-water bath and then kept overnight at 5°. The precipitated barium salt was filtered off and washed with ice-cold water and with acetone. It was dissolved in methanol (9 ml.) containing N-HCl (0·15 ml.), and the gently boiling solution was treated dropwise with 0·1 N-H_2SO_4 (1·5 ml.). After removal of the precipitated $BaSO_4$ by centrifuging, the supernatant was filtered, concentrated under a N_2 stream at 40° and cooled to 0–5°. The yield of white solid which separated was enhanced by addition of water; the solid was isolated by centrifuging. On slow recrystallization from a mixture of ethanol (1·5 ml.) with water (4·5 ml.) there was obtained *androst-16-en-3α-yl β-D-glucopyranosiduronic acid* of m.p. 169–174° (yield 37 mg.; 56%) [Found: androstenol,

60·7 (method described below); glucurone, 38·7 (method of Fishman & Green, 1955, slightly modified); C, 65·9, H, 8·7. $C_{25}H_{38}O_7$ requires androstenol, 60·8; glucurone, 39·2; C, 66·7; H, 8·4%].

Quantitative estimation of urinary androstenol

Apparatus. The columns for chromatography were of length, 200 mm. and diameter, 10 mm., with 0·5–0·7 mm. bore at the jet; they were fused to 150-ml. round-bottomed flasks at the top and fitted with flat-type stopcocks to facilitate adjustment of running rates. All glassware was of standard-joint and kept scrupulously clean; particular care was taken to avoid contamination by bench-wax and similar materials.

Reagents. These were as follows. Ether: anaesthetic ether, B.P., was redistilled from fresh bottles, or recovered by distillation from spent ether after washing with $FeSO_4$ and water. The ether was tested for peroxide with vanadic acid before use. Ethanol: for standard solutions, etc., was purified by shaking with *p*-phenylenediamine and redistilled. Methanol: for rinsing glassware, was redistilled. Benzene: was dried with Na and redistilled. Light petroleum: dried with Na and the fraction of b.p. 83–97° was used. Acetic acid: A.R., was treated with acetic anhydride and CrO_3 (Weissberger & Proskauer, 1935), and redistilled. Sulphuric acid: A.R., was N-free. Resorcylaldehyde: 2:4-dihydroxybenzaldehyde, was recrystallized from ether–light petroleum (b.p. 80–100°) and from water under N_2. Alumina: type H (Peter Spence and Co. Ltd., Widnes, Lancs.) was equilibrated in 1 kg. batches with 3–4% (v/w) of water by mechanical shaking for 6 hr. The partially deactivated alumina was stored in a calibrated dispenser to minimize entry of atmospheric water vapour during sampling. β-Gluronidase: was prepared from limpets after Dodgson & Spencer (1953) to powder *B* stage. To improve the yield, powder *A* (120 g.) was extracted twice with water (400 ml.), which was then treated with acetone (1200 ml.) Assay figures for the β-glucuronidase activity of powder *B* were obtained by the method of Fishman, Springer & Brunetti (1948), scaled down to conserve substrate, at final concentrations of powder of 10–20 μg./1·5 ml. of incubation mixture.

Method

Hydrolysis. Urine (24 hr. specimens) is collected in glass (not plastic) bottles and sent to the laboratory as soon as possible. The volume is noted, and, if from an adult and less than 1 l., made up to 1 l. with water. No preservative is used until the urine is received in the laboratory, when 1 ml. of a solution of $Hg(CN)_2$ (0·2 g./100 ml. in water)/100 ml. of urine is added. Duplicate samples taken for analysis are 1/20 of the 24 hr. vol. for urines of young adult men and 1/10 of the 24 hr. vol. for others. The samples are adjusted with 5N-HCl (a few drops) to approximately pH 4·5 and to each 100 ml. are added 4M-sodium acetate buffer, pH 4·5 (6·25 ml.) and 100 000–150 000 Fishman units of limpet β-glucuronidase [40–60 mg. of Dodgson & Spencer's (1953) powder *B*]. The samples are then incubated at 37–40° for 65–66 hr.

Extraction. The incubated urine is extracted with 1·0, 0·5 and 0·5 vol. of ether, and the combined ethereal extract washed with N-NaOH (0·1 vol.) and water (3 × 0·1 vol.). Care is taken to rinse precipitated material thoroughly in the incubation flask with ether, and to rinse alkali from the stopper of the separating funnel. A few drops of Bradosol (Ciba Ltd.; 5%, w/v) may be added, if necessary, to prevent emulsions. The washed ether extract is dried with Na_2SO_4 (6–7 g., freshly dried) for 1–2 hr. and filtered into a long-necked round-bottomed flask, containing three glass-beads; the Na_2SO_4 and filter paper are rinsed carefully with dry ether (2 × 25 ml.) without delay. The ether is evaporated rapidly at atmospheric pressure, not quite to dryness, on a boiling-water bath, with the flask set at an angle of about 45° and carrying a splash-head. The last drops of solvent are evaporated under reduced pressure in a vacuum desiccator (containing $CaCl_2$), in which the extract is stored at atmospheric pressure. The details of this evaporation procedure and the subsequent evaporation described below must be carefully followed, in view of the volatility of androstenol.

Chromatography. The column of alumina is prepared by filling the glass column with eluent mixture and running in alumina (5 g.) from the dispenser on to a small plug of fat-free cotton-wool set in the constriction of the column; the liquid is at the same time allowed to run out and the column is tapped to drive out air bubbles and to level the surface. A layer (5 mm. thick) of acid-washed sand is placed above the alumina.

Only one eluent mixture, light petroleum (b.p. 83–97°)–benzene (1:1, v/v), is used throughout. Androst-16-en-3α-ol (about 300 μg.) is dissolved in this mixture and the solution is used to determine the volume of eluent required for each batch of deactivated alumina, as follows. The androstenol in eluent mixture (4 + 2 + 2 ml.) is run into a column of alumina prepared as described above and then eluted. The eluate, running at 30–40 ml./hr., is collected automatically as 2-ml. fractions in tubes (0·5 in. diam.). Solvent is evaporated in an oven and the colour reaction applied to the residues. The apparent activity of the alumina, in terms of the elution volume for androstenol, is not quite constant during the first 4 weeks or so after the preparation of the alumina, and, apart from testing each new batch, checks should be made from time to time on the same batch especially when a new lot of eluent mixture is made up. However, with a 'safety margin' of 10–15 ml. of eluent on each side of the determined elution range of androstenol, and adherence to the procedure described, no losses of androstenol on the alumina columns need be expected.

The dry urinary extract, prepared as described under Extraction, above, is leached with 4, 2, and 2 ml. of eluent mixture, and each rinse carefully and separately applied and run into a fresh alumina column. Two fractions are then eluted as above, the second, containing the androstenol, being collected in a 150 ml. long-necked round-bottomed flask. [The volumes of eluent used for these two fractions will depend on the elution volume of androstenol; the volumes should be in the ranges 25–50 ml. for the first fraction (excluding the 8 ml. used for application of the extract) and 75–85 ml. for the second fraction.] The 'androstenol fraction' is evaporated, if necessary the following day, on a water bath at 80–90° under reduced pressure (water-pump). To prevent losses, the long-necked flask is fitted with a splash-head, and set at an angle of about 45° only just dipping into the hot water; three glass beads are added and the flask is tapped gently until boiling is quite steady. As soon as evaporation is complete, the flask is immediately removed from the bath and evacuated in a

vacuum desiccator at about 22° until the last traces of eluent are removed (about 5 min.). The flask is then kept in the desiccator at atmospheric pressure.

Colorimetry. The dry 'androstenol fraction' is dissolved in the 150 ml. flask in 3·0 ml. of purified acetic acid containing resorcylaldehyde (0·5 g./100 ml.), the neck of the flask being carefully rinsed down. To allow for complete and homogeneous solution, the mixture is kept for 10 min. in the stoppered flask with occasional mixing. Two 1·0 ml. samples are then pipetted into colour reaction tubes (10 cm. × 1 cm.) and 1·0 ml. of acetic acid–H_2SO_4 mixture (95:5, v/v) is added to each tube, with careful mixing. 'Standards' of pure androstenol are conveniently prepared by evaporating measured samples of a stock solution (500 μg./ml. in ethanol) in colour reaction tubes over $CaCl_2$ in a vacuum desiccator, and dissolving the residues in 1·0 ml. of acetic acid–resorcylaldehyde reagent. 'Blanks' consist of 1·0 ml. samples of acetic acid–resorcylaldehyde reagent in colour reaction tubes. 'Standards' and 'blanks' are each treated as above with 1·0 ml. of acetic acid–H_2SO_4 mixture. In view of the reproducibility and linearity of the 'standard' calibration curves, a pair of identical 'standards', containing 20–30 μg. of androstenol, will usually suffice for each series of estimations.

The tubes are placed without delay in a water bath at 100° for 8 min. The size of the tubes is not critical, and stoppering is unnecessary, but the bath should be such that the liquid in the tubes is fully immersed in the water and out of direct illumination, while the mouths of the tubes are protected from condensing steam. A convenient arrangement for 10 cm. × 1 cm. tubes consists of two perforated metal plates, 7 cm. apart in the water, the lower supporting the tubes, the upper resting on top of the bath and having holes 1·2 cm. in diameter through which the tubes pass. After heating, the tubes are cooled in ice–water and kept in the dark. After about 20 min., the coloured solutions are diluted with 2·0 ml. of acetic acid and measured spectrophotometrically at about 575 and 530 mμ. (With an EEL photoelectric colorimeter, suitable filters are Ilford Spectrum Yellow, no. 626 and EEL Green, no. OGR1.) The corrected extinctions at about 575 mμ [E_{575} (corr.)] may be found from the observed extinctions less 'blank' [E_{575} (obs.)] by using the expression: E_{575} (corr.) = [$2E_{575}$ (obs.) − E_{530}]/0·95, where E_{530} is the extinction, less 'blank', at about 530 mμ (for explanation, see Results). If any other colour filter combination is used a different expression, empirically determined, is of course applied. As slight spectral changes occur on standing, it is advisable to measure the colours after very approximately the same interval. The 'blank' should have an extinction of not more than 0·01 in 1 cm. tubes. Smaller colorimeter tubes can be used, as it is not necessary to dilute the colour mixture after heating. When colours giving E_{575} (corr.) > 0·4 are obtained, the androstenol content should be calculated by reference to a calibration curve: E_{575} (corr.) *versus* μg. of androstenol (see Fig. 2 and Results).

RESULTS

Evaluation of the method of analysis

Colour reaction. The colorimetry is a quantitative adaptation of one of the reactions described by Miescher (1946) for 17-hydroxy and Δ^{16}-C_{19} steroids. The final concentrations chosen for the resorcylaldehyde and for the H_2SO_4 (2·5 vol. %) represent the best compromise found between maximum specific colour and minimum 'blank' colour. The purple colour obtained shows maximal absorption at 580–585 mμ and an inflexion at 520–540 mμ (Fig. 1): Fig. 1 also gives the absorption spectra of the 'blank', of a chromatographic eluate from a urinary extract of average apparent androstenol content, and of one from an extract giving only a trace of the androstenol purple colour.

Heating for 8 min. was found to be optimum. Provided the coloured reaction mixture was then kept in the dark, the extinction at about 580 mμ remained constant for at least 3 hr. whereas the extinction at about 530 mμ fell by about 3–5 % in this time. Fading of the colour occurred rather more rapidly after dilution with acetic acid. In daylight, the colours faded much more rapidly before than after dilution, whereas exposure to bright sunlight (through glass) caused a complete change in under 20 min. to a pinkish colour with absorption maximum at 555 mμ.

The relationship between intensity of colour and concentration of androstenol is shown in Fig. 2. The molecular extinction coefficient, ϵ_{585}, is 11 850. As Fig. 2 shows, the extinctions at about 575 mμ are linear over a wider range of concentration than are those at about 530 mμ and the ratio E_{530}/E_{575} rises at extinctions greater than 0·4.

The data of Miescher (1946) on the specificity of the colour reaction have been confirmed and

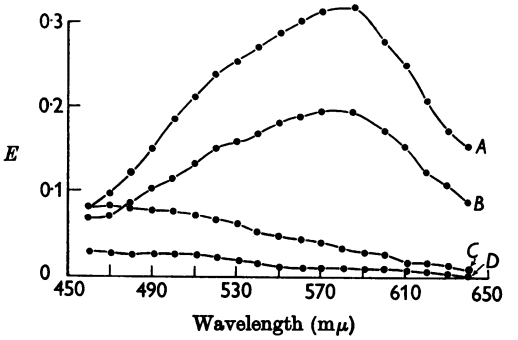


Fig. 1. Absorption spectra of colours obtained in the resorcylaldehyde–H_2SO_4–acetic acid reaction. Curve *A*, 29 μg. of pure androst-16-en-3α-ol; curve *B*, a typical urinary androstenol fraction; curve *C*, a urinary androstenol fraction containing almost entirely non-specific chromogens; curve *D*, the reagent 'blank'. Reaction mixture contained: resorcylaldehyde (0·5 %, w/v) in acetic acid (1 ml.) and H_2SO_4 (5 %, v/v) in acetic acid (1 ml.); acetic acid (2 ml.) was added after heating; extinctions were measured in 1 cm. cuvettes on a Unicam SP. 600 spectrophotometer against water; figures for curve *D* are subtracted from those for curves *A–C*.

extended. The following steroids occurring in urine extracts gave no visible colour at the concentration of 10–20 μg. in 2 ml. of reaction mixture: androsterone, dehydro*epi*androsterone (as acetate), 6β-hydroxy-3:5-*cyclo*androstan-17-one, androsta-3:5-dien-17-one, androst-2-(or-3-)-en-17-one, 3β-chloroandrost-5-en-17-one, progesterone, 3β-hydroxypregn-5-en-20-one (as acetate) and tetrahydrocortisone. A larger amount (50 μg.) of free dehydro*epi*androsterone still gave no colour. About 100 μg. of androstane-3:17-dione and pregnane-3:20-dione gave a barely perceptible yellow, but 100 μg. of cholesterol (purified) gave a pink colour (ϵ_{540}, 978); 25 μg. of androstane-3β:17α-diol and of aetiocholane-3α:17α-diol respectively gave colours of intensities of 1·33 and 1·12 relative to that given by the same weight of androst-16-en-3α-ol; androstane-3β:17β-diol and aetiocholane-3α:17β-diol gave no colour at this concentration. It seems likely that no appreciable interference can be expected in the colour reaction by known urinary steroids of polarity similar to that of androstenol, with the possible exception of cholesterol.

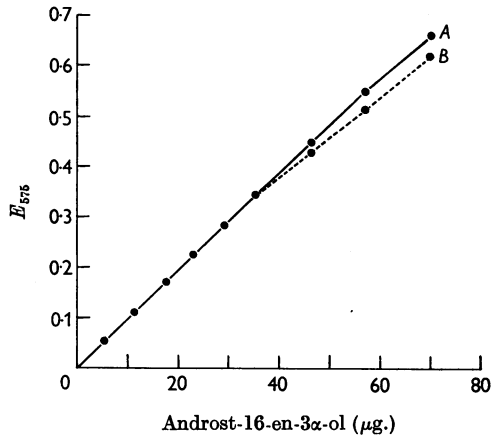


Fig. 2. Relation between μg. of androst-16-en-3α-ol and extinction (E_{575}, see text) without (*A*) and with (*B*) empirical correction as described in the text. Reaction mixture contained: resorcylaldehyde (0·5%, w/v) in acetic acid (1 ml.) and H_2SO_4 (5%, v/v) in acetic acid (1 ml.); acetic acid (2 ml.) was added after heating; extinctions were measured in 1 cm. diameter tubes in an EEL colorimeter.

The specificity of the method might be improved if the correction formula of Allen (1950) for non-specific chromogens were applicable. This possibility was carefully studied, but was rejected after consideration of the spectral characteristics of colours given by androstenol and interfering substances.

The fraction eluted from alumina columns as described above, and which should contain the androstenol of urine extracts, gave a colour which had not precisely the spectral characteristics of that generated from pure androstenol (Fig. 1): it was pinker and absorbed more at lower wavelengths, whereas the ratio (E_{530}/E_{575}) of the extinctions (as defined under Experimental and corrected for the 'blank') was greater than that for pure androstenol. Under conditions given above, the ratio E_{530}/E_{575} for pure androstenol was 1·05; and the highest ratio observed for a urinary fraction was 2·0. If the latter figure is taken as the limiting value for an extract containing representative interfering chromogens and no androstenol, and if the assumption is made that the colour due to the interfering chromogens obeys Beer's Law, then E_{575} (less 'blank') can be corrected by the formula (cf. Gibson & Evans, 1937):

$$E_{575}\text{ (corr.)} = \frac{K_I E_{575}\text{ (obs.)} - E_{530}\text{ (obs.)}}{K_I - K_A}$$

where $K_I = E_{530}/E_{575}$ (= 2·0, in our case, for interfering chromogen) and $K_A = E_{530}/E_{575}$ (= 1·05, for androstenol).

Hence:

$$E_{575}\text{ (corr.)} = [2E_{575}\text{ (obs.)} - E_{530}\text{ (obs.)}]/0{\cdot}95.$$

Table 1 shows the extent of this correction with typical urines. Obviously, the ideal of absolute

Table 1. *Application of colour correction formula to urinary androstenol fractions*

Colorimetric extinctions with resorcylaldehyde –H_2SO_4–acetic acid reaction on urinary androstenol fractions with and without empirical correction for non-specific colour. For details, see text. Extinctions are less 'blank' as defined in text.

Type of extract	E_{575} (obs.)	E_{530}	E_{530}/E_{575}	Apparent androstenol (μg./24 hr.)*	E_{575} (corr.)	Corrected androstenol (μg./24 hr.)†
Low androstenol titre, much pink chromogen	0·058	0·085	1·47	186	0·033	99
	0·022	0·038	1·73	112	0·006	31
	0·062	0·114	1·84	109	0·011	37
Medium titre, moderate pink chromogen	0·157	0·180	1·15	546	0·141	495
	0·146	0·178	1·22	483	0·108	360
	0·078	0·090	1·16	489	0·070	438
High titre	0·163	0·179	1·10	1120	0·155	1040
	0·257	0·286	1·11	1760	0·240	1640
	0·423	0·457	1·08	1330	0·410	1340

* Calculated from E_{575} (obs.). † Calculated from E_{575} (corr.).

Table 2. *Recoveries of androstenol in later stages of the method described in the text*

	Amount added (μg.)	Amount recovered (%)	No. of tests
After chromatography on alumina, in pure solution	29·3	101, 104	2
	58·6	101, 101	2
	65·2	99, 99	2
			(no control)
Added before evaporation of eluent mixture	29·0	100–103	9
Added to urine extract fraction (equivalent to 0·0375 of a 24 hr. specimen and containing the colour equivalent of 4·2 μg. of androstenol after chromatography and evaporation of eluent)	62·8	100–103	4

Table 3. *Effect of various amounts of β-glucuronidase and time of incubation on the yield of androstenol from pooled male urine*

Figures are for 100 ml. of urine.

Wt. of limpet powder *B* added (mg.)	β-Glucuronidase added (Fishman units)	Duration of incubation (hr.)	Androstenol found (duplicate results) (μg.)
37·5	100 000	66	77·9, 81·0
75	200 000	66	84·5, 83·6
75	200 000	44	79·4, 81·0
150	400 000	44	82·4, 83·1
150	400 000	22	69·3, 67·6

specificity is not achieved by this manoeuvre, but the corrected values are likely to be substantially nearer the true values than are those calculated from E_{575} alone. The occurrence of contaminants in urinary androstenol fractions seems to be capricious, depending upon the simultaneous presence of material extracted from the urine and impurities in reagents. We have found that the contaminating colour is usually not more than the equivalent of 50 μg. of androstenol/24 hr. (uncorrected E_{575}); it has rarely amounted to the equivalent of about 100 μg. No manoeuvre has yet been devised to eliminate this non-specific chromogenic material.

Chromatography of androstenol. Unfractionated neutral lipid extracts from urine gave a purple colour with the resorcylaldehyde–H_2SO_4–acetic acid reagent far more intense than could be accounted for by androstenol alone. Purification by some form of chromatography was therefore necessary. Androstenol could be separated readily from cholesterol and other very non-polar steroids in the paper-chromatographic system heptane–phenylethane-1:2-diol of Neher & Wettstein (1952), and separations could be achieved with reversed-phase partition chromatography on hydrophobic Celite. However, simple alumina-column chromatography was found effective and much easier to apply quantitatively. The procedure described under Method above was rigorously tested for possible variables and disturbing factors. It separated androstenol from most chromogenic impurities in the extract with a convenient elution volume.

The accuracy of the analyses in the chromatographic stages of the method is demonstrated by the results in Table 2.

Conditions for enzymic hydrolysis of androstenyl β-glucosiduronic acid. The availability of synthetic androstenyl β-glucosiduronic acid made possible the establishment of optimum conditions for the enzymic hydrolysis of the conjugate in urine. It was assumed that limpet glucuronidase will hydrolyse androstenyl β-glucosiduronic acid in urine buffered with acetate at pH 4·5, which is approximately optimum for hydrolysis of 17-oxo steroid β-glucosiduronic acids (Stitch & Halkerston, 1956). Tests of the best conditions were confined to those of enzyme concentration and durations of incubation. With β-glucuronidase made from limpets and assayed with phenolphthalein β-glucosiduronic acid, data as regards amount of enzyme and duration of incubation were determined and are listed in Table 3. It is apparent that a shorter time of incubation cannot be offset by adding more enzyme. An initial enzyme concentration of 1000 units/ml. of urine is probably adequate, as was confirmed in recovery experiments with added androstenyl β-glucosiduronic acid (Table 4), but the slightly larger amount of 1340 units/ml. of urine was chosen, for security.

Precision of the whole method. The conditions finally chosen, as well as the accuracy of the whole method, were checked in a series of recovery experiments (Table 4). For some of these, androstenyl β-glucosiduronic acid was added to urines from subjects who apparently excreted very small amounts of androstenol, in others a pool prepared by mixing eight assorted normal male and female specimens was used. In this way, reasonable checks were made against the possibility of any of these specimens, and particularly those from subjects with low androstenol excretion, containing abnormally large amounts of β-glucuronidase inhibitors.

The occasional occurrence of marked β-glucuronidase inhibition, as in any method employing

Table 4. *Recovery of androst-16-en-3α-ol, with the whole method as described in the text, after addition of androstenyl β-glucosiduronic acid to urine*

Androstenol is expressed as μg. of free androstenol/100 ml. of urine.

No. of determinations	Initial content of androstenol	Androstenol added	Recovery (%)		Enzyme units added/ml. of urine incubated
			Mean	S.D. (±)	
		(a) On low-content urine			
2	12·0	61·7	82·5	2·3	1600
2	10·2	61·7	88·5	1·5	1500
2	5·4	30·9	92	0	1340
2	5·4	61·7	86	2·0	1340
3	8·4	15·5	94	0·8	1340
3	8·4	30·9	99	4·1	1340
4	5·9	61·7	89	3·0	1340
3	11·7	15·5	95	8·6	1340
3	11·7	30·9	90	2·6	1340
2	47·4*	71·0	90	2·0	1340
2	53·7†	71·0	88·5	1·5	1340
2	10·2	61·7	89	3·0	1000
		(b) On higher-content (pooled) urine			
2	60·3	61·7	86·5	1·5	1500
3	55·5	30·9	96	0·7	1340
3	55·5	61·7	87	1·4	1340
2	60·3	61·7	85	2·0	1000

* Rotten urine sterilized by boiling before incubation. † Rotten urine not boiled before incubation.

Table 5. *Preliminary figures for urinary androstenol in normal human subjects*

Males		Females	
Age (yr.)	Androstenol (μg./24 hr.)	Age (yr.)	Androstenol (μg./24 hr.)
11	30	4	53*, <10
12	267	6	<10
13	162	9	<10
15	78	15	549
18	495, 501, 1330	16	1100
19	126, 105, 540, 1090, 1430, 1620, 1340	18	1010*, 431*, 450, 46[illegible]
		19	698*, 509*, 72, 552
20	2040, 486, 1120, 921, 1960, 1080, 1160, 1280, 1910	20	500*, 423, 696, 642
		21	390
21	2040, 906, 846, 1610, 2630, 1870, 591	22	138
		23	819
22	1730, 547, 1480, 2330	25	411
23	1290	28	225, 417, 249
24	420	31	183
25	(947, 1044, 624)†, 544	37	66
27	1110	38	183
31	1130*	39	141*
34	(96, 19)†	69	258
35	1450	72	62
40	1450*	—	—
41	1390	—	—
45	805	—	—
53	228	—	—
71	540, 625	—	—
80	60	—	—

* Values from E_{575}, uncorrected by formula given in text.

† Values obtained on urines collected on different occasions, from the same person. All other values refer to different individuals.

this enzyme in urine, cannot of course be guaranteed, but the recovery figures show that incomplete hydrolysis of β-glucosiduronic acid conjugates, described, for example, by Gallagher *et al.* (1954), is unlikely to be serious with androstenyl β-glucosiduronic acid. For the 40 tests (Table 4), the overall mean percentage recovery and its standard deviation were 90·4 ± 5·3.

Androstenol excretion in normal subjects, preliminary results. In Table 5 are listed values obtained for the urinary output of material appearing in the same alumina chromatogram fraction as androst-16-en-3α-ol and giving the characteristic purple colour reaction. Some figures which were not corrected for non-specific colour are included, as the absolute reduction of the values incurred by the correction is small (Table 1).

DISCUSSION

Quantitative method for urinary androstenol. The recovery data show that the degree of precision is quite adequate for the purpose of obtaining information on the physiological significance of this steroid; the method is also simple to carry out. Drawbacks are the apparent volatility of the steroid and the chance development of interfering chromogens in the colour reaction.

The material which is estimated as androst-16-en-3α-ol and referred to as 'androstenol' is not of course proven to be the 3α:5α-epimer. However, other work has shown that androst-16-en-3β-ol, aetiochol-16-en-3α-ol and androsta-5:16-dien-3β-ol are not eluted from alumina in the 'androstenol fraction' of this method.

Contamination of urinary androstenol fractions by non-specific chromogens does not lead to serious inaccuracy except in urine extracts of very low androstenol content, and in any case its effect is greatly reduced by application of the colour correction formula as described above. Recoveries at the concentration of 15 μg. of androstenol/100 ml. of urine, where the actual amount of androstenol being estimated in the colorimeter tubes was about 8 μg., demonstrate that amounts of androstenol corresponding to 100 μg./24 hr. of urine can be distinguished from zero satisfactorily. Nevertheless, it is obviously advisable to repeat determinations on low-titre urines with a larger sample, to improve the precision of the analysis. In order to minimize contamination, particular attention must be paid to avoiding 'non-polar' organic material such as vaseline, silicone grease, bench wax etc.: it is also necessary to avoid the use of plastic bottles for urine collection, especially if organic solvents are added as preservatives.

As regards preservation of urine, we have found that a falling-off in the androstenol titre of a sample occurs in 1–2 weeks at about 22°, whereas at 5° a slight decrease may be apparent after about 6 weeks unless a preservative has been added. Urine samples need not, and should not, be boiled, for this also incurs some loss of androstenol.

The possible occurrence of androstenyl sulphate in urine was tested by incubation at 40° of an acidified ethyl acetate extract of sulphate conjugates (Burstein & Lieberman, 1958) from a mixture of male and female urines of varying androstenol glucuronide content. Added androstenol was recovered after this procedure, but no endogenous sulphate-conjugated androstenol was detected.

Normal values for urinary androstenol. Our data are insufficient to enable any firm conclusions to be made as to the physiological significance of this steroid. The very great variation, amounting to some 100-fold, between male subjects of the same age group is most striking. It is also clear that the actual amounts of androstenol excreted in the urine are not insignificant, being of the same order as that of the 5β-17-oxo steroids with oxygen functions at C-11 and only a little less than that of dehydro*epi*androsterone. In spite of this large variation between individuals, the mean figure so far found for women is under one-half of that for men, and only 15% of the male subjects so far investigated excreted less androstenol than the mean for women of reproductive age.

SUMMARY

1. The partial synthesis is described of androst-16-en-3α-ol from androsterone, through the dehydration of androsterone acetate cyanohydrin, saponification, and decarboxylation of the derived aeti-16-enic acid.

2. The synthesis of methyl (androst-16-en-3α-yl tri-*O*-acetyl-β-D-glucopyranosid)uronate and the preparation from it of androst-16-en-3α-yl β-D-glucosiduronic acid are described.

3. A method for the estimation of glucuronide-conjugated androst-16-en-3α-ol in human urine is described in detail. The procedure includes hydrolysis of the conjugate with β-glucuronidase, separation of the androstenol by chromatography on alumina, and quantitative application of a colour reaction employing resorcylaldehyde and sulphuric acid in acetic acid.

4. The specificity and limitations of the colour reaction have been studied.

5. The precision of the whole method has been evaluated. Recoveries of androstenol, added to urine as the pure β-glucosiduronic acid, exceeded 82% in the range 15–71 μg. of androstenol/100 ml. of urine and averaged 90·4 (S.D. ± 5·3)%.

6. Preliminary figures for androstenol excretion in urine are given. In the age group 18–45 years they ranged from 19 to 2630 μg./24 hr. in men and from 66 to 1010 μg./24 hr. in women.

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The Reaction of Mono- and Di-functional Alkylating Agents with Nucleic Acids

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In attempts to relate the chemical reactions of the alkylating agents with their biological effects two general principles have emerged. First, a general positive correlation has been established between chemical reactivity, as measured by rate of hydrolysis, and cytotoxicity (Haddow, Kon & Ross, 1948; cf. review by Ross, 1953). Secondly, the difunctional agents, i.e. those possessing two alkylating groups in the molecule, generally exert a markedly more powerful cytotoxic action than the corresponding monofunctional agents (Loveless & Ross, 1950). This has been ascribed to their ability to cross-link fibrous macromolecules, in particular those involved in duplication of the chromosomes (Goldacre, Loveless & Ross, 1949), reaction of this type between di-(2-chloroethyl) sulphide (mustard gas) and deoxyribonucleic acid having been proposed by Elmore, Gulland, Jordan & Taylor (1948). The importance of deoxyribonucleic acid as a site of biological alkylation was also suggested by observation of the mutagenic activity of mustard gas (Auerbach & Robson,