Uniformly Gram-positive in young culture tending to variability in older culture with Gram-negative cytoplasm beaded with Gram-positive granules. Not acid-fast.

*Cultural characteristics.* Colonies on peptone agar 1–2 mm. in diameter, high convex, circular with entire edges, white, smooth non-glistening surface. Growth in peptone broth: granular sediment with little general turbidity. Aerobic. Good growth at 25 and 37°.

*Physiological characteristics.* Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk alkaline, casein not digested. Nitrates slowly reduced to nitrites. $H_2S$ not produced. Indole not formed. Cellulose not digested. Starch not hydrolysed. Acid from levulose. No acid from dextrose, galactose, arabinose, xylose, rhamnose, maltose, lactose, saccharose, raffinose, trehalose, mannitol, dulcitol, sorbitol, inositol, glycerol or inulin. Urea not hydrolysed.

Unless otherwise indicated, *Achromobacter* sp. (1) has been used for all the manometric studies.

In order to determine whether or not species of bacteria other than those isolated from the perfused soil might attack pyruvic-oxime with the production of nitrite, a series of laboratory stock cultures which had been maintained on peptone agar were transferred to pyruvic-oxime agar. The results are outlined in Table 2. It is seen that none of a variety of species held in stock possessed, initially at least, the ability to produce nitrite from pyruvic-oxime. Indeed many of the species tested failed to grow on this medium. Growth where it occurred was in all instances rather sparse requiring from 3 days to 1 week of incubation, depending upon the species. The finding that certain heterotrophic organisms may oxidize pyruvic-oxime to nitrite has been confirmed by the recent work of Jensen (1951).

It was felt that adaptation might be a factor in determining the ability of an organism to attack pyruvic-oxime. In order to test this, four selected species, *Achromobacter hartlebii*, *Achromobacter perolens*, *Agrobacterium radiobacter* and *Bacterium globiforme*, were carried through six successive transfers on pyruvic-oxime agar. On the sixth transfer the two species of *Achromobacter* produced very small amounts of nitrite. *Agrobacterium radiobacter* and *Bacterium globiforme* failed to produce nitrite. It is of interest to note that the two species which gained, presumably by adaptation, the ability to attack the substrate with the liberation of free nitrite were both members of the same genus as the three active species isolated from perfused soil and faeces.

Table 2. *The ability of various stock cultures of bacteria to grow on pyruvic-oxime agar and to produce nitrite from pyruvic-oxime*

| Organism | Growth | Nitrite |
|---|---|---|
| *Achromobacter hartlebii* | + | – |
| *Achromobacter perolens* | + | – |
| *Achromobacter* sp. (isolated from fish) | + | – |
| *Alcaligenes faecalis* | + | – |
| *Agrobacterium radiobacter* | + | – |
| *Chromobacterium violaceum* | – | – |
| *Pseudomonas aeruginosa* (2 strains) | + | – |
| *Escherichia coli* | – | – |
| *Aerobacter aerogenes* | + | – |
| *Klebsiella pneumoniae* | + | – |
| *Serratia marcescens* | + | – |
| *Proteus vulgaris* | – | – |
| *Micrococcus ureae* | – | – |
| *Micrococcus pyogenes* var. *aureus* | – | – |
| *Micrococcus epidermidus* | – | – |
| *Micrococcus agilis* | – | – |
| *Sarcina lutea* | – | – |
| *Corynebacterium segmentosum* | – | – |
| *Bacterium globiforme* | + | – |
| *Bacillus mesentericus* | – | – |
| *Bacillus cereus* | – | – |
| *Bacillus megatherium* | – | – |

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# The Isolation of Androst-16-en-3α-ol from Women's Urine

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The musk-smelling androst-16-en-3α-ol, first isolated from swine testes by Prelog & Ruzicka (1944), was obtained from a glucuronide fraction from men's urine by Brooksbank & Haslewood (1949, 1950) and from the urine of women with adrenal cortical tumours by Mason & Schneider (1950). When Brooksbank & Haslewood (1950) tried to obtain this steroid from the urine of women not showing any endocrine abnormality, they were able to isolate from the musk-smelling fraction only a small amount of a substance melting at about 160°, and therefore different from the androstenol. It has seemed of interest to us to repeat this work in order to settle, if possible, the question of the nature of the musk-smelling constituent of (hydrolysed) urine from normal women.

## RESULTS

The work described below shows that the urine examined contained androst-16-en-3α-ol (approximately 0·2–0·3 mg./l.), as well as pregnane-3α:20α-diol (in a concentration of the order of 0·4 mg./l.); these substances were almost certainly present as glucuronides. The previously described substance of melting point about 160° was not again encountered.

## DISCUSSION

The urine used was collected from women in whom there was no evidence of endocrine abnormality, and it probably consisted almost entirely of 'follicular' urine, i.e. excreted in the menstrual cycle before the formation of functioning corpora lutea. It is clear, therefore, that in man, the androstenol is truly characteristic of female, as well as of male, urine. It may be of adrenal origin, as suggested by Mason & Schneider (1950) for a female case of adrenal cortical neoplasm. There is no definite evidence to suggest that the testis is involved in its formation by the male, although it was our impression that rather greater amounts were excreted by men than by women. The amount of pregnanediol isolated was about what might have been expected in 'follicular' urine.

## METHODS

In general the methods of isolation of Brooksbank & Haslewood (1950) were closely followed.

'Pregnanediol-like glucuronide' (PLG) was estimated as previously described, and the results expressed as pure sodium pregnanediolglucuronidate (NaPG). Melting points are uncorrected. Light petroleum was A.R., b.p. 40–60°. $Al_2O_3$, supplied by Hopkin and Williams Ltd., was neutralized as described by Shoppee (1949).

*Isolation and purification of PLG.* Urine (191 l.) was collected from healthy women and from patients (in surgical wards) not suffering from endocrine diseases, cancer, kidney or bladder disturbances. All subjects menstruated normally; they were instructed to collect urine only during the first 14 days after menstrual bleeding had ceased. Urine was preserved with thymol-salicylic acid or butanol. It was extracted with butanol and the PLG entrained as described by Brooksbank & Haslewood (1950) in a manner corresponding to the 'concentrated' conditions of the original small-scale estimation (Bisset, Brooksbank & Haslewood, 1948). The total yield of PLG recovered from barium phosphate precipitates was 1·629 g., which was assayed as approx. 50% pure glucuronide (as NaPG). Of the 1·629 g., 96 mg. was used in experiments, kindly carried out by Dr Mary Barber, on bacterial hydrolysis; it was found that this was unsatisfactory, as the extracts apparently contained an inhibitor of glucuronidase activity. An unsuccessful attempt to remove the glucuronidase inhibitor was made by dissolving the remaining PLG (1532 mg. =789 mg. NaPG) in *n*-butanol/methanol (2:1 v/v) and eluting it from $Al_2O_3$ (10 g.) in a swiftly moving column (2 × 3 cm.). The original solvent eluted 283 mg. (27% NaPG), methanol (300 ml.) gave 956 mg. (39% NaPG) and further methanol (approx. 1 l.) gave 147 mg. (48% NaPG). The total solid recovered thus weighed 1·386 g.; it was equivalent to 517 mg. NaPG and hence 789–517 = 272 mg. (34%) of glucuronide (as NaPG) were lost on the column.

*Enzymic hydrolysis of PLG.* The PLG (517 mg. as NaPG) recovered from $Al_2O_3$ (above) in 0·1 M-citrate buffer (770 ml., pH 5·2, Kerr, Graham & Levvy, 1948), was treated with 18 200 units of purified calf-spleen glucuronidase (prepared according to instructions kindly supplied by Dr G. T. Mills of the University of Glasgow) dissolved in water (90 ml.). The mixture, together with about 5 ml. $CHCl_3$, was incubated at 37°. Commercial deoxyribonucleic acid, to a concentration of about 0·3% (w/v), was added after 2 days as a glucuronidase accelerator (Bernfeld & Fishman, 1950) and incubation was continued for a further 2·5 days. The mixture was then diluted with water to about 1 l. and extracted continuously with ether for about 16 hr.; the aqueous portion was finally shaken with two lots of about 200 ml. each of fresh ether. The combined ether extracts were washed with water, dilute $Na_2CO_3$ solution, water, dried ($Na_2SO_4$) and evaporated. The residue weighed 247 mg.; it was partially crystalline and had a strong musk-like smell. If this residue had been pregnanediol, it would correspond to about 401 mg. of NaPG; hence about 517–401 = 116 mg. of glucuronide (as NaPG) remained unhydrolysed. Hence, the approximate volume of urine from which the above 247 mg. of hydrolysed material was obtained corresponded to

$$\frac{1532}{1629} \times \frac{401}{789} \times 191 = 91 \text{ l.}$$

*Isolation of steroids.* The above hydrolysed ether-soluble neutral material (247 mg.) was left for a few days at 0–5° with acetone (about 10 ml.). The separated solid was collected and washed with a little cold acetone. The insoluble material (58 mg.) was crystallized from ethanol/aqueous NaOH and gave pregnane-3α:20α-diol (30 mg., m.p. 228–232°, not depressed by authentic material).

Evaporation of the above acetone liquors left a strongly smelling brown gum (189 mg.) which was purified by chromatography as shown in Table 1.

Fraction 4 (75·2 mg.) from column 1 was crystallized from ethanol/aqueous NaOH and gave pregnane-3α:20α-diol (5 mg., m.p. 224–227° not depressed by an authentic sample). Hence, the total weight of purified pregnanediol isolated was about 35 mg., i.e. about 0·4 mg./l. of urine.

Fractions 5 (5·8 mg.) and 6 (2·0 mg.) from column 2 were purified by cooling with a little light petroleum in solid $CO_2$-acetone mixture, decanting and washing the crystalline residue with a little light petroleum similarly cooled. The residues were recrystallized from aqueous acetone, as also was the residue left on evaporation of the light petroleum liquors. The three samples (none of which weighed more than about 1 mg.) thus obtained had melting points as follows: from fraction 5, m.p. 140–142°; from fraction 6, m.p. 136–138°; from the combined light petroleum liquors, m.p. 142–143°. None of these melting points was depressed by mixture with authentic androst-16-en-3α-ol. The last sample (m.p. 142–143°) was sent to Dr K. Dobriner, of the Sloan-Kettering Institute for Cancer Research, New York, for an infrared spectral examination. Dr Dobriner reported that the absorption taken in the 'finger-print' region (900–1200 cm.$^{-1}$) of our sample was identical with that of

Table 1. *Chromatography of hydrolysed ether-soluble neutral material from women's 'follicular' urine*

| Fraction no. | Eluted with: Vol. (ml.) | Eluted with: Solvent | Eluate: Wt. (mg.) | Eluate: Appearance |
|---|---|---|---|---|
| | | Column 1. Hydrolysed material (189 mg.) on $Al_2O_3$ (2 g.) | | |
| 1 | 40 | 50% (v/v) Benzene/light petroleum | 73·1 | Gum |
| 2 | 40 | 50% (v/v) Benzene/light petroleum | 9·3 | Gum |
| 3 | 40 | Benzene | 7·8 | Gum |
| 4 | 40 | Ether | 78·2 | Crystalline |
| 5 | 40 | Acetone | 10·0 | Gum |
| 6 | 40 | Ethanol | 10·0 | Gum |
| | | Total | 188·4 | |
| | | Column 2. Fraction 1 (73·1 mg.) of column 1 on $Al_2O_3$ (1 g.) in a column (5 × 0·5 cm.) | | |
| 1–3 | 10 | Light petroleum | 20·4 | Colourless oil, slight smell |
| 4 | 5 | Light petroleum | 18·6 | Reddish oil, musk smell |
| 5 | 10 | Light petroleum | 5·8 | Crystalline, musk smell |
| 6 | 10 | Light petroleum | 2·0 | Crystalline, musk smell |
| 7 | 20 | Light petroleum | 4·3 | Partially crystalline, musk smell |
| 8 | 10 | 50% (v/v) Benzene/light petroleum | 7·4 | Brown gum, smell |
| 9 | 20 | Benzene | 12·6 | Gum |
| | | Total | 71·1 | |

androst-16-en-3α-ol. Less pure samples of the androstenol were obtained from fractions 7 and 8 of column 2: more of this substance might have been present in fraction 4 from this column. The previously purified substance of m.p. 160–161° (Brooksbank & Haslewood, 1950) was not detected.

## SUMMARY

1. Enzymic hydrolysis of a partially purified 'pregnanediol-like glucuronide' fraction from the urine of women in the 'follicular' phase of the menstrual cycle gave a mixture from which was isolated androst-16-en-3α-ol and pregnane-3α:20α-diol.

2. The amount of the latter substance obtained indicated an original concentration of the order of 0·4 mg./l. of the urine, whilst the androstenol might have been present in amounts of 0·2–0·3 mg./l. The musk-smelling androst-16-en-3α-ol is thus truly characteristic of the (hydrolysed) urine of men and women.

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