SUMMARY

1. The major pigment in the red coronas of certain cultivated varieties of *Narcissus* was separated from other pigments chromatographically on alumina.

2. The unknown pigment showed a chromatographic behaviour like that of β-carotene, and it could not be separated from β-carotene on alumina or magnesia. The spectral-absorption curve resembled that of β-carotene. In colour and shape the crystals resembled those of all-*trans* β-carotene. The pigment supported growth in rats deficient in vitamin A, and led to storage in their livers of a substance that gave a blue colour with antimony trichloride reagent. It is concluded that the pigment is β-carotene.

3. The carotene was found in the coronas in concentrations up to 3 mg./g. based on the fresh weight, or 2% of the dry weight. In red fringes only from the coronas of *N. poeticus recurvus* the carotene comprised about 16·5% of the dry weight.

4. Other pigments, probably free and esterified carotenols, were present in amounts approximately equivalent to half the β-carotene contents.

5. Pigments from trumpets of yellow forms ('daffodil'), although presumably carotenoids, included only small proportions of carotene.

6. Tests with hydrochloric acid suggested that the trumpets of yellow forms contained epoxides but that the coronas and perianths of the red-corona forms did not.

I am grateful to Dr K. R. Sporne for advice on the microscopic preparations; and to Mr H. G. W. Fogg for specimens of *Narcissus*.

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Steroids of Pregnant Mare's Urine

5. IDENTIFICATION OF TWO ANDROSTANE-3:16-DIOLS. THE STRUCTURE OF 'URANOLONE'*

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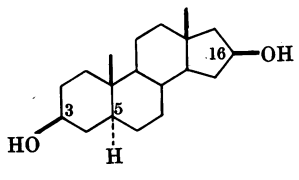
Brooks, Klyne, Miller & Paterson (1952) described the fractionation of the neutral non-ketonic lipids of pregnant mare's urine and the isolation of a number of compounds, some of which were not identified. The main purpose of the present paper is to report the identification of two of these unknown compounds (*MN* and *MM*) as the 5α-androstane-3β:16α- and -3β:16β-diols. A preliminary account of this work has already been given (Brooks & Klyne, 1956).

The structure of 'uranolone' (Marker, Lawson, Wittle & Crooks, 1938*b*) has been confirmed.

Identification of 5α-*androstane*-3β:16α- *and* -3β:16β-*diols*

The compounds *MM* and *MN* were obtained as their benzoates by the methods described by Brooks *et al.* (1952). Comparison of the properties of *MM* and its derivatives with those quoted by Fajkoš & Šorm (1955) for 5α-androstane-3β:16β-diol (I; cf. also Huffman & Lott, 1955) suggested that the two substances might be identical. Comparison of our materials with authentic samples supplied by Professor Šorm proved the identity conclusively (see Table 1, p. 665).

In the structural formulae of the steroids in this paper the convention followed by the Chemical Society has been used, i.e. interrupted lines represent α-configuration and bold continuous lines β-configuration.



(I)

5α-Androstane-3β:16β-diol

* Part 4. Brooks, Klyne, Miller & Paterson (1952).

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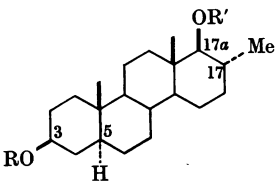
The establishment of the identity of *MM* as the 3β:16β-diol led us to consider whether one of our other unknown compounds might be the 3β:16α-epimer. Two benzoates, those of compounds *MN* and *MS*, had melting points close to that of 5α-androstane-3β:16α-diol dibenzoate. Comparison of the infrared spectra of these materials and mixed m.p. determinations showed clearly that *MN*, and not *MS*, was identical with the 3β:16α-diol, again supplied by Professor Šorm (Table 1).

This identity was confirmed by hydrolysis of *MN* dibenzoate to the diol and subsequent preparation of the diacetate; elementary analyses and determination of optical rotations were not carried out in this series, because only 4 mg. of dibenzoate was available for the hydrolysis and acetylation.

'*Uranolone*'

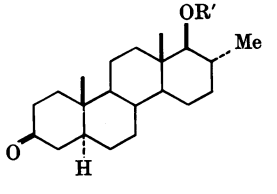
Marker *et al.* (1938*b*) isolated from pregnant mare's urine a hydroxy ketone which they called 'uran-11-ol-3-one', and to which they allotted the structure of 11-hydroxy-5α:9β-pregnan-3-one. Subsequent work has shown that the 'urane' derivatives of Marker are *D*-homosteroids (Klyne, 1950; Klyne & Shoppee, 1952) and this uranolone would therefore be 17aβ-hydroxy-17α-methyl-5α-*D*-homoandrostan-3-one (III; R′ = H). Thanks to the kindness of Dr E. J. Lawson, who placed his original sample of uranolone at our disposal, we have been able to confirm the structure indicated for this compound. This is of interest since 3-oxo steroids are not of common occurrence in urine.



(II)

'Uranediol'

17α-Methyl-5α-*D*-homoandrostane-3β:17aβ-diol



(III)

'Uranolone'

17aβ-Hydroxy-17α-methyl-5α-*D*-homoandrostan-3-one

The preparation of the hydroxy ketone (III; as its benzoate, R′ = Bz) was facilitated by a chance finding, that treatment of the dibenzoate of uranediol (17α-methyl-5α-*D*-homoandrostane-3β:17aβ-diol (II; R = R′ = Bz) with potassium carbonate in aqueous methanol hydrolysed only the 3β-benzoyloxy group, yielding the 17aβ-monobenzoate (II; R = H; R′ = Bz). Oxidation of the monobenzoate with chromium trioxide in acetic acid yielded the benzoyloxyketone (III; R′ = Bz), which was identical with the benzoate of Lawson's compound (Table 2, p. 665).

The presence of the keto group at C-3 in (III) was demonstrated by the dinitrobenzene reaction (Zimmermann, 1935, 1936; Callow, Callow & Emmens, 1938; cf. also Marker *et al.* 1938*b*); the absorption spectra of the colours obtained by reaction for 5 and 60 min. were almost identical with those given by 5α-cholestan-3-one (Broadbent & Klyne, 1954). The other hydroxy ketone derived from uranediol, i.e. 3β-hydroxy-17α-methyl-5α-*D*-homoandrostan-17*a*-one, gives no colour in the dinitrobenzene reaction, because it has no free methylene group adjacent to the carbonyl at C-17*a*. (This compound has recently been prepared by reduction of uranedione with sodium borohydride; W. Klyne, unpublished observations.)

The partial hydrolysis of the dibenzoate (II; R = R′ = Bz), in which the 17aβ-benzoyloxy group is resistant to alkali, provides an interesting example of steric hindrance by adjacent methyl groups on both sides of the benzoyloxy group, although this is most probably equatorial (cf. Brooks *et al.* 1952). The hindered benzoate group of the compound (III; R′ = Bz) was hydrolysed to a considerable extent by refluxing with 8% (w/v) potassium hydroxide in aqueous methanol for 9 hr.

EXPERIMENTAL

General methods

Methods for melting points (which are corrected), specific rotations, microanalyses, chromatograms on alumina, 'usual working up' and preparation of acetates and benzoates were as described by Brooks *et al.* (1952). Ultraviolet absorption spectra were determined in ethanol with either a Beckman (DU) or a Unicam (SP. 500) spectrophotometer. Alumina for chromatography was of Activity II (Brockmann & Schodder, 1941).

Infrared absorption spectra were measured in a Grubb–Parsons S3A single-beam instrument between 1200 and 900 cm.$^{-1}$ (the 'finger-print' region) in CS_2 or in $CHCl_3$.

Identification of compounds MN *and* MM *as* 5α-*androstane*-3β:16α- (*and* -3β:16β-*diols*

The properties of the two urinary compounds (Brooks *et al.* 1952) are compared with those of the authentic 3:16-diols (Fajkoš & Šorm, 1955) in Table 1.

Urane derivatives

17α-*Methyl*-5α-D-*homoandrostane*-3β:17aβ-*diol* 17a-*monobenzoate* (*II;* R = H, R′ = Bz; *uranediol* 17a-*monobenzoate*). Uranediol dibenzoate (32 mg., m.p. 212–213°) in methanol (10 ml.) was treated with potassium carbonate (140 mg.) in water (1·4 ml.) and refluxed for 5 hr. The product (29 mg.) was obtained by the usual working up (extraction with ether; washing with dilute alkali, dilute acid and water; drying with Na_2SO_4); it was chromatographed on Al_2O_3 (1·8 g.). Light petroleum–benzene mixture (1:3, v/v) and benzene eluted 23 mg. of material, m.p. 213–217°. Recrystallization of this from methylene chloride–methanol yielded the 17a-*monobenzoate* as plates, m.p. 219–220°; this

Table 1. *Identification of compounds* MM *and* MN *as 5α-androstane-3β:16-diols*

	Compound *MM*		5α-Androstane-3β:16β-diol*
	MM		
Diol			
M.p.	180–182°		182–183°†
Mixed m.p.		180–182°	
$[\alpha]_D$ (EtOH)	−6°		−6°
Analysis	C, 78·3; H, 10·9%		C, 78·1; H, 10·9%‡
Infrared spectra ($CHCl_3$)		Identical	
Diacetate			
M.p.	108–109°		105–107°
Mixed m.p.		105–107°	
$[\alpha]_D$ ($CHCl_3$)	−14°		−8°
Infrared spectra (CS_2)		Identical	
Dibenzoate			
M.p.	188–190°		188–190°
Mixed m.p.		188–190°	
$[\alpha]_D$ ($CHCl_3$)	−15°		−14°
Analysis	C, 78·8; H, 8·2%		C, 79·2; H, 8·1%
Infrared spectra (CS_2)		Identical	
	Compound *MN*		
	MN		5α-Androstane-3β:16α-diol*
Diol			
M.p.	186–187°		186–187°
Mixed m.p.		186–188°	
Diacetate			
M.p.	172–173°		172–173°
Mixed m.p.		173–175°	
Infrared spectra (CS_2)		Identical	
Dibenzoate			
M.p.	183–184°		184–185°
Mixed m.p.		183–184°	
Infrared spectra (CS_2)		Identical	

* Samples from Professor F. Šorm; m.p.'s determined in this Laboratory; values for $[\alpha]_D$ are those given by Fajkoš & Šorm (1955).

† A sample of this compound from Dr M. N. Huffman had m.p. 182–184°; mixed m.p. with compound *MM*, 182–183°.

‡ Analysis values in this column are calculated for $C_{19}H_{32}O_2$ and $C_{33}H_{40}O_4$ respectively.

compound does not sublime before melting—contrast uranediol itself. $[\alpha]_D^{20}$ +21 in $CHCl_3$ (c = 0·8), molecular rotation (M_D), +91. Ultraviolet absorption (in ethanol) $\lambda_{max.}$ 230 and 273 mμ.; log $\epsilon_{max.}$ 4·16 and 2·98 respectively. (Found: C, 79·0; H, 9·5; $C_{33}H_{40}O_4$ requires C, 79·2; H, 9·5%.)

17aβ-Benzoyloxy-17α-methyl-5α-D-homoandrostan-3-one (III; R′ = Bz; uranolone benzoate). Uranediol monobenzoate (II; R = H, R′ = Bz; impure, containing some free uranediol: 84 mg.) was dissolved in acetic acid (20 ml.); chromium trioxide (0·2 M in acetic acid; 2 ml.) was added, and the mixture was allowed to stand overnight at room temperature. The neutral product (71 mg.) was isolated by extraction with ether, washing with dilute alkali, dilute acid and water, and drying (Na_2SO_4); it was chromatographed on Al_2O_3 (5 g.). Light petroleum–benzene mixtures (1:1 and 1:3, v/v) eluted 43 mg. of the benzoyloxyketone. Benzene and benzene–ether mixtures eluted small quantities of material which had infrared spectra very similar to that of uranedione.

The *benzoyloxyketone*, after recrystallization from methanol containing a little $CHCl_3$, formed leaflets, m.p. 239–241°. $[\alpha]_D^{20}$ +60 in $CHCl_3$ (c = 0·7), $[M]_D$ +254. Ultraviolet absorption (in ethanol) $\lambda_{max.}$ 230 and 272 mμ.; log $\epsilon_{max.}$ 4·10 and 2·76 respectively. (Found: C, 79·5; H, 9·2. $C_{28}H_{38}O_3$ requires C, 79·0; H, 9·3%.) For comparison with the benzoate of Lawson's uranolone see Table 2.

The dinitrobenzene reaction (Callow *et al.* 1938), carried out according to Broadbent & Klyne (1954) for periods of 5 min. and 60 min. on the benzoyloxyketone, gave absorption spectra (Fig. 1) which were almost identical with those given by 5α-cholestan-3-one.

17aβ-Hydroxy-17α-methyl-5α-D-homoandrostan-3-one (III; R′ = H; uranolone). The benzoate (IV; R′ = Bz; 12 mg.) was dissolved in 5 ml. of boiling methanol; KOH (400 mg.) in water (0·8 ml.) was added and mixture was heated under reflux for 9 hr. The product obtained by the

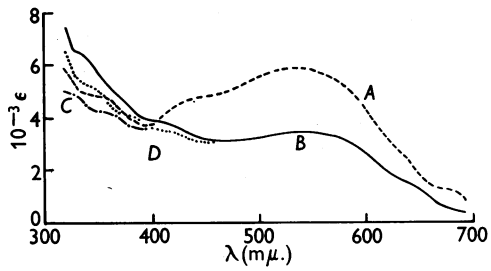


Fig. 1. Absorption spectra of colours produced in Zimmermann dinitrobenzene reactions. Uranolone benzoate, *A* (- - -), 5 min.; *B* (—), 60 min.; 5α-Cholestan-3-one, *C* (-·-·-), 5 min.; *D* (. . . .), 60 min. Curves *C* and *D* are almost superposable on *A* and *B* respectively above 400–450 mμ.

Table 2. *Comparison of uranolone and 17aβ-hydroxy-17α-methyl-5α-D-homoandrostan-3-one*

	Uranolone*		17aβ-Hydroxy-17α-methyl-5α-D-homoandrostan-3-one
Hydroxy compound (III; R′ = H)			
M.p.	162–165°		167–169°
Mixed m.p.		165–170°	
Infrared spectrum (in CS_2)		Identical	
Benzoate (III; R′ = Bz)			
M.p.	234–237°		237–239°
Mixed m.p.		236–240°	
Infrared spectrum (in CS_2)		Identical	

* Sample provided by Dr E. J. Lawson.

usual working-up (extraction with ether; washing with dilute alkali, dilute acid and water; drying with Na_2SO_4) was chromatographed on Al_2O_3 (1 g.) and the infrared spectra of selected fractions were examined for the presence of the characteristic benzoate band at about 1110 cm.$^{-1}$. The first four fractions obtained with light petroleum–benzene (1:3, v/v) contained 1 mg. of material (strong benzoate bands); the second four fractions with the same eluent contained 3 mg., m.p. 164–169° (faint benzoate bands). The last three fractions with the same eluent and five fractions with benzene (3 mg.) had m.p. 165–169° and showed no benzoate bands. Recrystallization from acetone yielded crystals, m.p. 167–169°. For comparison with Lawson's uranolone see Table 2.

DISCUSSION

The present work emphasizes once again the important species differences which may occur in the nature of the urinary steroids between mammals in pregnancy. The only two species hitherto investigated thoroughly are the human female and the mare. (For reviews see Pearlman, 1948; Hirschmann, 1955.) Some work on two other species—goat and cow—has been presented briefly (Wright & Klyne, 1955; Klyne & Wright, 1956*a*, *b*).

The differences in steroid content between human and mare's pregnancy urine may be considered under the following headings.

Phenolic steroids. Human urine contains only *A*-ring aromatic steroids; mare's urine contains considerable quantities of compounds with additional double bonds in the *B* ring (equilin) or with both *A* and *B* rings aromatic (equilenin).

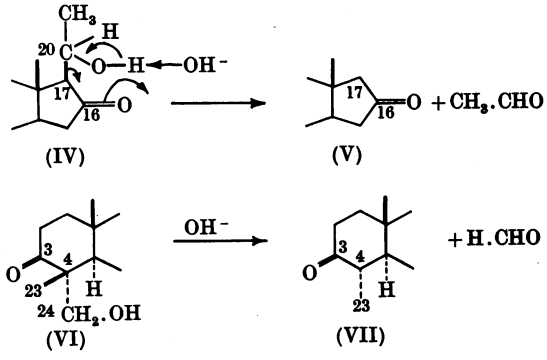
Pregnanediol isomers. Although human urine contains a great preponderance of one isomer, 5β-pregnane-3α:20α-diol, mare's urine contains considerable quantities of two isomers with the common steroid skeleton (5α-pregnane-3β:20α- and -3β:20β-diols), of which the latter seems to preponderate towards the end of pregnancy. Mare's urine contains in addition the *D*-homosteroid uranediol, and, in smaller quantity, a number of other constituents, which are probably pregnanediol or androstanediol isomers.

Urane derivatives. There is no mention in the literature of the occurrence of these compounds in any natural source but mare's urine—except for one claim by Marker, Kamm, Oakwood, Wittle & Lawson (1938*a*) to have obtained uranetrione by oxidation of a fraction from human pregnancy urine, and a statement by Dobriner (1952) that small quantities of uranediol (identified by infrared spectra) had been isolated from the same source. Although many schools have worked intensively on human pregnancy urine for years, these findings have not been confirmed. Fukushima & Gallagher (1956) have discussed the identification of some *D*-homosteroids obtained from extracts of human urine; these, as they say, are most probably artifacts.

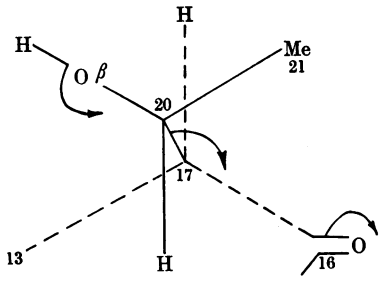
The suggestion has been made (Klyne, 1953) that urane derivatives might arise in the mare by rearrangement *in vivo* of 17α:20β-diols to 17-methyl-*D*-homoandrostan-17*a*-ones, followed by reduction; this hypothesis was based on theoretical considerations regarding the preferred conformation of the side chain. Some support is lent to this idea: first, by the finding in this Laboratory (Brooks, unpublished work) that the ratio of 3β:20β to 3β:20α isomers is much higher in late-pregnancy than in mid-pregnancy urine (uranediol has been isolated by us only from late-pregnancy urine); secondly, by the work of Ramirez & Stafiej (1955*a*, *b*, 1956), who have shown that the rearrangement of 20ξ-amino-17ξ-hydroxy compounds to *D*-homo ketones *in vitro* is strictly stereospecific in the way suggested for 17:20-diols by Klyne.

16-*Substituted androstanes.* The existence of a compound now identified as 3β-hydroxy-5α-androstan-16-one in pregnant mare's urine has been known for some years (Heard & McKay, 1939; Oppenauer, 1941), although its structure was not proved until recently (Huffman & Lott, 1951). The two 3β:16-diols reported in this paper are clearly related to Heard's hydroxy ketone. Such 3:16-disubstituted androstanes have not been reported from any other natural sources, although 5α-pregnane-3β:16α:20β-triol (Haslewood, Marrian & Smith, 1934; Hirschmann, Hirschmann & Daus, 1949) is one of the major constituents of the triol fraction from mare's urine, and 3:16:17-triols (including oestriol) are well-known constituents of human urine (for references see Hirschmann, 1955; Marrian & Bauld, 1955).

The 5α-androst-16-en-3α-ol of Brooksbank & Haslewood (1950) may of course be considered as an *in vivo* dehydration product of either a 16-hydroxy or a 17-hydroxy compound.

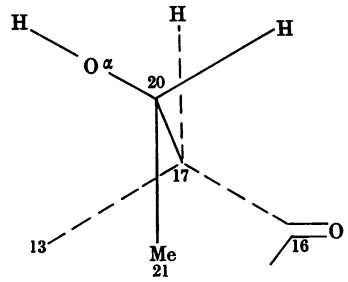


A plausible mechanism for the formation of the 3:16-disubstituted androstanes without a C-17 substituent *in vivo* is the following. Any process involving side-chain oxidation must necessarily give a 17-hydroxy or 17-oxo compound; a hydrolytic



(VIII)

20β-Hydroxy-16-one



(IX)

20α-Hydroxy-16-one

(Perspective views looking down C-20—C-17 bond.)

process of some kind must therefore be considered. A 20-hydroxypregnan-16-one (as IV) is a β-hydroxy ketone and might undergo a reverse aldol condensation to give a 16-oxoandrostane (V); an analogy *in vitro* is provided by the ready formation of formaldehyde by the action of alkali on the triterpene icterogenin (VI), yielding a 24-nortriterpene (VII) (Barton & de Mayo, 1954).

If the 16-one (V) does arise by a process like that suggested above, the 20β-hydroxy-16-one should react more readily than its 20α-epimer for the following reason. Only the 20β-compound can easily take up the side-chain conformation (VIII) required for a concerted reaction, in which C-16, C-17, C-20 and O-20β are coplanar and the bonds 16–17 and 20–O are antiparallel [cf. formula (XX) in Klyne, 1953]; here the hydrogen atom at C-20 is close to the angle methyl group at C-13.

For the 20α-hydroxy isomer, the similar conformation (IX) is unfavourable, because here the methyl group at C-20 (C-21) would be close to the angle methyl group and subject to steric strain.

SUMMARY

1. Two neutral non-ketonic steroids of pregnant mare's urine (compounds *MN* and *MM* of Brooks *et al.* 1952) have been identified as the 5α-androstane-3β:16α- (and -3β:16β-)diols.

2. The constitution of 'uranolone' (Marker *et al.* 1938*b*) has been confirmed as 17aβ-hydroxy-17α-methyl-5α-*D*-homoandrostan-3-one.

3. Species differences in urinary steroids between the human female and the mare are discussed.

We are indebted to the Agricultural Research Council for grants for expenses which have largely defrayed the costs of this work, and for a personal grant to one of us (R.V.B.). We are most grateful to Ayerst, McKenna and Harrison (Montreal), British Drug Houses Ltd. (London), N. V. Organon (Oss, Netherlands) and Ovaltine Laboratories (King's Langley, Herts) for their generous help in the provision of mare's urine; Professor F. Šorm (Czechoslovak Academy of Science, Prague) and Professor M. N. Huffman (Oklahoma Medical Research Foundation) for reference compounds; Dr E. J. Lawson (Sterling–Winthrop Research Institute, Rensselaer, N.Y.; formerly of Pennsylvania State College) for putting at our disposal his original sample of uranolone; Dr I. D. P. Wootton, Mrs P. Bane and Miss Sylvia Saunders for the infrared spectra; and Miss Miriam Mattock for much technical assistance in the preparation of urane derivatives.

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Studies on Sulphatases

15. THE ARYLSULPHATASES OF HUMAN SERUM AND URINE*

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Conflicting reports concerning the arylsulphatase activity of serum and urine have appeared in the literature. Thus Pantlischko & Kaiser (1952), using phenolphthalein disulphate as substrate, could detect no arylsulphatase activity in urine, whereas Russo (1947), using phenyl sulphate, stated that the arylsulphatase activity of urine towards this substrate was quantitatively comparable with that of animal tissues excluding liver. Huggins & Smith (1947) found activity equivalent to the liberation of 0·3–15·0 and 0·9–19·7 μg. of *p*-nitrophenol/hr./ml. from *p*-nitrophenyl sulphate (NPS) by serum and urine respectively, and Abbott & East (1949), using the same substrate, observed increased serum arylsulphatase activity after induced liver damage in the rat. On the other hand, Abbott (1947) could detect no hydrolysis of phenyl sulphate by human serum over a period of 18 hr. Dodgson & Spencer (1954) were unable to confirm the observations of Huggins & Smith (1947), the activities recorded corresponding to the release of only 0–0·2 μg. of *p*-nitrophenol/hr./ml. by either urine or serum. More recently Boyland, Wallace & Williams (1955) used potassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) as the assay substrate, and reported that the arylsulphatase activities of normal urine and serum were respectively 0–0·82 and 1·2–3·3 μg. of nitrocatechol liberated/hr./ml. These authors observed increased activity in several infected urines and in urines from patients with carcinoma of the bladder and other sites and from patients with tuberculosis. There was no parallel rise in serum arylsulphatase activity.

Recently, the arylsulphatase activity of mammalian tissues has been shown to be due to three separable enzymes which can be distinguished by their relative substrate affinities, behaviour towards inhibitors and ease of solubilization (Dodgson, Spencer & Thomas, 1955; Dodgson, Spencer & Wynn, 1956). Two of the enzymes (arylsulphatases *A* and *B*) are easily obtained in solution from mammalian livers after rupture of the mitochondria of the liver cells (cf. Viala & Gianetto, 1955) and have been called 'soluble' or mitochondrial arylsulphatases (Dodgson *et al.* 1955, 1956). They have a high affinity and activity towards NCS but have little affinity or activity towards NPS or potassium *p*-acetylphenyl sulphate (APS). The substrate specificity of the other arylsulphatase (arylsulphatase *C*) is the converse of that shown by the 'soluble' enzymes. This enzyme is found exclusively in the microsomes of the liver cell (Dodgson, Spencer & Thomas, 1954, 1955) and has been obtained in a soluble form only after treatment with crude pancreatic extracts in the presence of a non-ionic surface-active agent (Dodgson, Rose & Spencer, unpublished results).

A re-assessment of the urinary and serum arylsulphatase patterns has now been made in the light of these findings.

* Part 14: Dodgson, Lloyd & Spencer (1957).

EXPERIMENTAL

Serum and urine samples. Blood samples from humans, dogs and rabbits were collected intravenously and from rats by cardiac puncture. Ox and sheep bloods were fresh slaughter-house specimens. Whenever possible sera were separated and assayed within 1 hr. of collection of the blood. Urines were assayed immediately after voiding.

Assay of arylsulphatase activity. The substrates APS, NPS and NCS were prepared as previously described (Dodgson *et al.* 1955). No attempt was made to purify the NCS