Steroids and Other Lipids of Pregnant Goat's Urine

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This work was undertaken in association with Dr S. J. Folley, F.R.S., and Dr A. T. Cowie at the National Institute for Research in Dairying, Shinfield, to study the steroids of pregnant goat's urine with particular attention to oestrogens and pregnanediol. It was intended to provide a basis for studies on the metabolism of administered steroids and on pathological conditions. It has also served as a preliminary to similar work on pregnant cow's urine. Preliminary accounts of this work have been given already (Wright & Klyne, 1955; Klyne & Wright, 1956*a*).

Previous work

Little work on the constituents of goat's urine has been described in the literature. Anderson (1916) studied the volatile constituents and isolated *p*-cresol and a non-phenolic ketone, $C_{10}H_{16}O$, which was identical with a substance from cow's urine. Küst & Vogt (1934) showed by biological assay that oestrogen was present in the urine of pregnant goats after the second or third month of pregnancy and then increased steadily in amount until parturition. They could not detect oestrogen in the urine of non-pregnant or male goats. Grant (1948) isolated *p*-ethylphenylsulphuric acid from normal goat's urine and from the urine of ovariectomized goats receiving hexoestrol (3:4-di-*p*-hydroxyphenylhexane) and progesterone. He concluded that this conjugate was quantitatively the most important arylsulphuric acid present. Boscott (1950) was unable to isolate any progesterone metabolites from the urine of female goats receiving 100 mg. of progesterone daily. Goto, Oshima & Ugami (1955) used a diphenylamine reagent to estimate 'corticoid' substances in goat's urine.

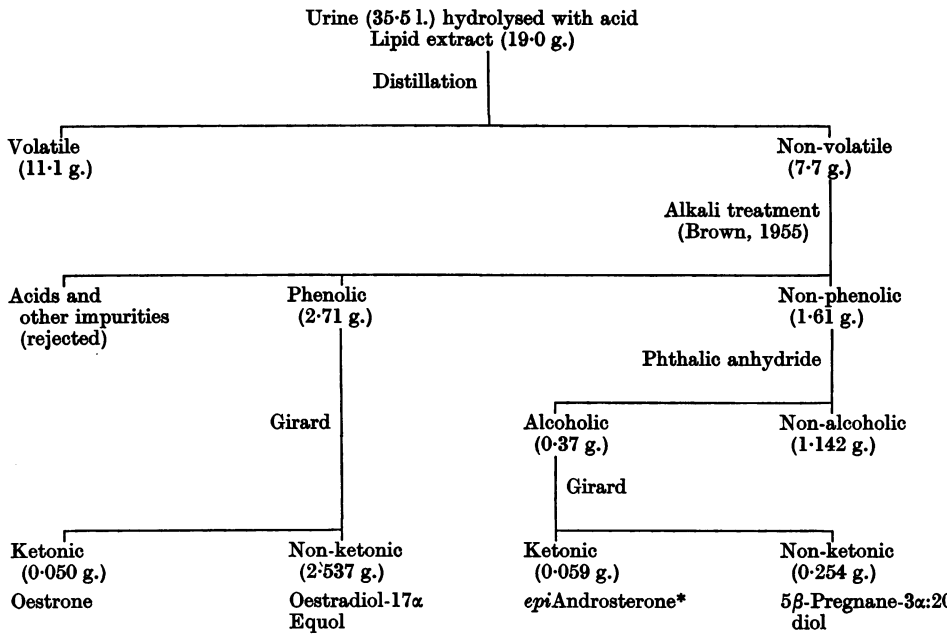
RESULTS

Separation of the major lipid fractions

The urine, which was preserved under toluene, was submitted to acid hydrolysis and extracted with toluene. The extract was distilled *in vacuo* to

Table 1. *Fractionation of lipid extract of pregnant goat's urine*

The compounds isolated are noted under the fraction in which they were found.



* Impure: three other 17-oxo steroids were partially characterized.

remove the volatile constituents, and the non-volatile material was then purified and separated into phenolic and neutral fractions according to the methods of Brown (1955).

The neutral fraction was separated into alcohols and non-alcohols with phthalic anhydride (Dobriner, Lieberman & Rhoads, 1948; cf. Chibnall, 1931). The alcohols were further fractionated by the Girard procedure and digitonin precipitation (Marker, Rohrmann & Wittle, 1938*a*) as used by Brooks, Klyne, Miller & Paterson (1952).

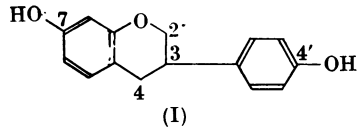
The phenols were also submitted to the Girard procedure. The non-ketonic phenols were then partitioned between water and benzene–light petroleum (Brown, 1955); this separates oestriol, (hydrophilic) from oestradiol (lipophilic). The procedure is summarized in Table 1.

The various major fractions were then submitted to chromatography on alumina, and some fractions were further investigated by paper chromatography and infrared spectrometry.

Phenolic fractions

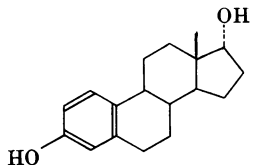
Ketonic phenols. Oestrone was isolated (approx. 0·3 mg./l.) and fully identified. No indication was obtained of the presence of other phenolic ketosteroids.

Non-ketonic phenols. The hydrophilic fraction yielded much equol (*iso*flavan-7:4′-diol, I; Marrian & Haslewood, 1932), but no oestriol. The lipophilic fraction also contained much equol, and a smaller concentration of a Kober chromogen, presumably oestradiol.



(I)

Equol (*iso*flavan-7:4′-diol)



(II)

Oestradiol-17α

Methylation of equol (a dihydric phenol) yields a dimethyl ether, whereas oestradiol yields only a monomethyl ether, which still retains a free alcoholic hydroxyl group. These two methyl ethers could be readily separated by chromatography on alumina, as expected. It was then found that the oestradiol present in pregnant goat's urine was the 17α isomer (II); the concentration of this was about 0·1 mg./l.; none of the 17β epimer could be detected. A modification of the colour reaction of Kägi & Miescher (1939) was used to estimate oestradiol-17α; this modified reaction is also of value for other 17α-hydroxy steroids.

Neutral fractions

Neutral ketones. The only 17-oxo steroid isolated from pregnant goat's urine was *epi*androsterone (3β-hydroxy-5α-androstan-17-one) in an impure condition. Evidence from paper chromatography and infrared spectra indicated the presence of androsterone and aetiocholanolone [3α-hydroxy-5α-(and -5β-)androstan-17-ones] and of 11-oxoaetiocholanolone (3α-hydroxy-5β-androstane-11:17-dione). The total yield of non-volatile, neutral ketones was approx. 1 mg./l.

Neutral non-ketones. Chromatography of the α fraction (not precipitable by digitonin) as free alcohols and as acetates yielded 5β-pregnane-3α:20α-diol (the common 'pregnanediol' of human pregnancy urine) as the sole recognizable compound (approx. 2 mg./l.). Nothing could be isolated from the small β fraction (precipitable by digitonin).

EXPERIMENTAL

Material

The urine (98 l., worked up in two batches of 62·5 and 35·5 l. respectively) was collected from goats at a late stage of pregnancy and stored under toluene. It represented about thirty-five complete 24 hr. collections. The animals were stall-fed on hay and a concentrate of dredge corn, bran, linseed cake, maize, oats, cod-liver oil and minerals. The collections were made while the goats were confined in metabolism crates.

General methods

Methods for melting points (which are corrected), specific rotations, microanalyses, chromatograms on alumina 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.

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

Chromatography on alumina. The adsorbant was Al_2O_3 for chromatography (Savory and Moore), activity II (Brockmann & Schodder, 1941). The proportions used were lipid (1 g.), Al_2O_3 (30 g.) and eluent (each run, 100 ml.).

Paper chromatography. This was carried out with Savard's (1953) system of light petroleum–propylene glycol at a temperature of 20–25°.

Zimmermann dinitrobenzene reaction for ketones. This was carried out following the method recommended by the Medical Research Council Committee on Clinical Endocrinology (1951).

Methyl ethers. These were prepared with dimethyl sulphate in borate buffer, following the method of

Brown (1955). A third addition of dimethyl sulphate and 20% (w/v) NaOH was sometimes made.

Kober reaction for oestrogens. This was carried out as recommended by Bauld (1954). The quinol–H_2SO_4 reagent for estimating oestradiol-17β was used on fractions containing oestradiol-17α. Standards were always measured with each series of estimations.

The absorption maxima ($\lambda_{max.}$) of the oestradiols and their methyl ethers differed slightly, as follows: oestradiol-17β, 515 mμ; oestradiol-17α, 520 mμ; oestradiol-17β methyl ether, 520 mμ; oestradiol-17α methyl ether, 525 mμ. [Brown (1955) reported 514 and 518 mμ for oestradiol-17β and its methyl ether respectively.] The values of $10^{-3}\epsilon_{max.}$ varied somewhat from one run to another; approximate values were as follows: oestradiol-17β, 35; oestradiol-17α, 40; oestradiol-17β methyl ether, 38; oestradiol-17α methyl ether, 28. [The value of $10^{-3}\epsilon_{max.}$ calculated from the data of Bauld (1954) for oestradiol-17β is 38.] If the Allen (1950) formula was applied to each compound, using the extinctions at 35 mμ either side of the peak in the correction factor, more constant values of $10^{-3}\epsilon$ were obtained, as follows: oestradiol-17β, 22; oestradiol-17β methyl ether, 23; oestradiol-17α, 26·5; oestradiol-17α methyl ether, 22.

When oestradiol-17α was estimated by using oestradiol-17β as standard (the Allen correction being applied as for the 17β isomer) it produced about 10% more colour than an equal weight of the 17β compound. This procedure was followed in the first few determinations before the oestradiol was identified as the 17α isomer. It was continued, for the sake of convenience, in the determination of all fractions which contained any oestradiol.

Equol gave a yellow–brown colour in the Kober reaction, the corrected extinction value at 520 mμ being less than 1% of that for the same weight of oestradiol-17α. (It should be noted that equol may be present in 10- to 20-fold excess in crude fractions obtained after chromatography of free phenols.) Determinations carried out on mixtures of equol and oestradiol showed that the extinctions produced were approximately additive.

Acetic–sulphuric acid reaction

This procedure was based on the reaction used by Kägi & Miescher (1939) to distinguish between 17α- and 17β-hydroxy steroids.

Oestradiol-17α. The acid reagent was prepared by mixing 1 vol. of conc. H_2SO_4 with 20 vol. of acetic acid. This reagent (4 ml.) was added to the dry residue containing 10–40 μg. of oestradiol-17α in a glass-stoppered tube. The mixture was heated by plunging the tube into an oil bath at 130–140° for 2 min. After cooling to room temperature in a bath of cold water, the pink colour ($\lambda_{max.}$, 525 mμ; $10^{-3}\epsilon_{max.}$, 17) was read in a spectrophotometer at this wavelength against a reagent blank. The addition of bromine to the cooled reaction mixture, as recommended by Kägi & Miescher (1939), was found to cause rapid fading of its colour and was therefore omitted.

The absorption spectrum of the pink colour produced by oestradiol-17α resembled that given by the product of the Kober reaction. In applying the method to urinary residues, many of which were brown gums, the colour correction of Allen (1950) was used in order to correct for non-specific absorption. The colour was then read at 490, 525 and 560 mμ.

Concentrations of 2–5 μg. of oestradiol-17α/ml. of the final reaction mixture gave convenient extinction values (0·1–0·3; 1 cm. cell) which were proportional to concentration; typical values for the corrected extinction at 525 mμ were 0·103 and 0·197 for concentrations of 2·25 and 4·5 μg. of oestradiol 17α/ml. respectively.

Other 17α-hydroxy steroids. The absorption spectra of the colours given by some other 17α-hydroxy steroids in this reaction are shown in Fig. 1. 5α-Androstan-17α-ol and 5α-androstane-3β:17α-diol both gave a pink colour resembling that given by oestradiol-17α. The products obtained from the two 5β-androstane-3:17α-diols (aetiocholanediols) were green, but that of androst-5-ene-3β:17α-diol was blue (cf. Kägi & Miescher, 1939). The last-named compound was the only one in this series which gave a colour ($\lambda_{max.}$ 550 mμ) of intensity ($10^{-3}\epsilon_{max.}$ 10·4) comparable with that given by oestradiol-17α.

Oestradiol-17β, 5α-androstane-3β:17β-diol and androst-5-ene-3β:17β-diol gave negligible colours in this reaction.

Equol. Equol was tested at concentrations, 25–50 μg./ml., similar to those present in some of the urinary fractions that were estimated for oestradiol-17α. It gave a green colour, $\lambda_{max.}$ 340, 400 and 597 mμ with $10^{-3}\epsilon_{max.}$ of 1·08, 1·31 and 0·47 respectively. At the wavelengths at which the absorption of oestradiol-17α was normally measured (490, 525, 560 mμ) the spectrum of equol showed a minimum ($10^{-3}\epsilon$ 0·50, 0·39, 0·45 respectively). Determinations of oestradiol-17α in the presence of a 10- to 20-fold excess of equol nevertheless showed a slight increase (5–10%) in the corrected extinction values at 525 mμ as compared with those for pure oestradiol-17α.

Hydrolysis and extraction of urine

The urine was received with some toluene added as a preservative. It was hydrolysed by adding 0·1 vol. of 10 N-HCl and heating in a boiling-water bath for 1·5 hr. in the presence of toluene. The hydrolysate was brought to pH 6 with solid Na_2CO_3 and filtered. More toluene was added to a total of 0·2 vol. and the mixture shaken. The aqueous phase was further extracted with toluene (2 × 0·2 vol., 1 × 0·1 vol.). Precipitates, filtered off after hydrolysis or

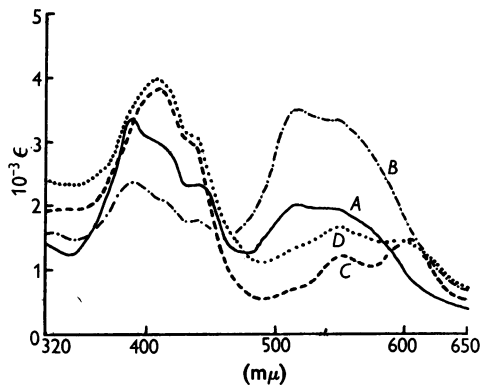


Fig. 1. Absorption spectra of products obtained in acetic–sulphuric acid reaction with some 17α-hydroxy steroids. *A*, 5α-Androstan-17α-ol; *B*, 5α-androstane-3β:17α-diol; *C*, 5β-androstane-3α:17α-diol; *D*, 5β-androstane-3β:17α-diol.

from the emulsions formed during extraction, were also extracted with toluene, which was then added to the bulk. The toluene extract was washed with water (4 × 0·05 vol.) and evaporated *in vacuo*.

Separation of the major fractions

The lipid material obtained (22·25 g. from 62·5 l. of urine) was distilled at 120°/10–15 mm. for 1 hr., and then for a further 1 hr. at 120°/1 mm., giving two volatile fractions (V_1, 4·27 g., and V_2, 5·4 g., respectively).

The non-volatile residue, 12·36 g., was dissolved in ether (4 l.) and acidic or oxidizable constituents were removed by the method of Brown (1955) as follows. (*A*) The ethereal solution was first washed with 2 × 800 ml., 2 × 400 ml. of conc. carbonate solution [prepared by adding NaOH (20%, w/v; 150 ml.) to $NaHCO_3$ (8%, w/v, 1 l.)]; these washings were rejected. (*B*) The ethereal layer was next shaken with 2N-NaOH (200 ml.); $NaHCO_3$ (8%, w/v; 800 ml.) was then added and the mixture shaken again before the layers were separated; the washing was rejected. (*C*) Stage *B* was repeated with half the volumes of NaOH and $NaHCO_3$. (*D*) and (*E*) The ethereal solution was finally washed with $NaHCO_3$ (8%, w/v; 400 ml.) and water (4 × 100 ml.), all washings being rejected. The residue after evaporation of the ether (6·68 g.) was dissolved in benzene (1 l.) and the phenols were extracted by washing with 2N-NaOH (1 × 100 ml., 7 × 50 ml.). The benzene was then washed with water (3 × 25 ml.), dried over Na_2SO_4 and evaporated, giving a neutral fraction (2·647 g.). The NaOH solutions were brought to pH 2·0 with 10N-HCl and saturated with NaCl. They were then extracted with ether (3 × 100, 3 × 50 ml.). After drying over Na_2SO_4 the ether was evaporated to give the phenolic fraction (2·435 g.).

Phenols. These were separated into ketonic and non-ketonic fractions by the Girard procedure (cf. Brooks *et al.* 1952).

Non-phenolic fraction. This was treated with phthalic anhydride (Dobriner *et al.* 1948) to obtain alcoholic and non-alcoholic fractions. The alcohols were further fractionated to obtain ketonic and non-ketonic alcohols (Table 1), each of which gave digitonin-precipitable and -non-precipitable fractions (Brooks *et al.* 1952). The digitonin precipitations were carried out in 95% ethanol as described by Marker *et al.* (1938*a*). As only a slight cloudiness was obtained after the ethanol solutions had stood for 3 hr., the solutions were concentrated and precipitated with ether. The β fractions were then contained wholly in the ether-precipitated material.

Phenolic fractions

Ketonic phenols

After the Girard separation, 85 mg. of ketonic material was obtained from 62·5 l. of urine. It was chromatographed on alumina (2·7 g.) and the following fractions were obtained: chromatogram I *A* (benzene), 4 mg.; *B* (runs 4–8; benzene–ether, 90:10), 27 mg.; *C* (benzene–ether, 80:20) 6 mg.; *D* (benzene–ether, 50:50, ether), traces; *E* (ether–methanol, 50:50), 22 mg. Fraction *E* was methylated and chromatographed again, but no compounds were isolated.

Isolation of oestrone. The first part of fraction I *B* (run 4) was crystalline (16 mg.), and was therefore chromatographed again (chromatogram II). Benzene eluted only traces of gum, but benzene containing 1% of ether-eluted material which crystallized spontaneously. Higher concentrations of ether gave fractions containing only small amounts of gum. The solid fractions were combined and recrystallized from ethyl acetate. The product had m.p. 268–269°, not depressed on admixture with authentic oestrone. A comparison of the properties of this compound and its derivatives with those of authentic material is given in Table 2.

Non-ketonic phenols

The non-ketonic fraction (2·159 g. from 62·5 l. of urine) was dissolved in ethanol (40 ml.) and transferred to a separating funnel with benzene (1 l.). Light petroleum (1 l.) was added and the organic phase extracted with water (2 × 1 l.) (cf. Brown, 1955).

Hydrophilic fraction. The aqueous solutions were allowed to stand two days at 4°, when pale-buff crystals were deposited. These were collected (fraction *A*). One-tenth of the supernatant was methylated (fraction *B*) and the remainder acidified and extracted with ether (fraction *C*).

Equol. The crystals (*A*, 0·357 g.) had m.p. 150–155°. They were apparently insoluble in saturated $NaHCO_3$ and 2N-Na_2CO_3, but dissolved readily in 2N-NaOH and methanol. After recrystallization from water they had m.p. 187–190°, and showed no depression on mixing with authentic equol (Marrian & Haslewood, 1932). Table 3 shows a comparison between the equol isolated in this work and authentic material. The product of the methylation (*B*, 0·057 g.) was chromatographed on alumina (chromatogram III). Light petroleum–benzene (95:5) eluted 43 mg. of material which was identified as equol dimethyl ether (cf. Table 3). Further elution with light petroleum–benzene, benzene and benzene–ether gave no identifiable material. More impure equol was obtained by chromatography of fraction *C*. This fraction contained only a small amount of Kober chromogen, and nothing else was obtained from it.

Table 2. *Identification of oestrone*

Phenol	Urinary compound		Authentic oestrone
M.p.	268–269°		266–267°
Mixed m.p.		270–271°	
Ultraviolet absorption ($\lambda_{max.}$)	280 mμ		280 mμ
Infrared spectrum (KBr disk)		Identical	
Kober reaction ($\lambda_{max.}$)	515 mμ		515 mμ
Acetate			
Infrared spectrum (CS_2)		Identical	

Lipophilic non-ketonic phenols

The phenols remaining in the organic phase at the partition (1·284 g.) were chromatographed on alumina (chromatogram IV; Table 4). Kober determinations indicated that the oestrogen was mainly in fraction *C* and the adjacent eluates in fractions *B* and *D*; a smaller amount was present in fraction *G*.

Oestradiol-17α. The oestrogen was only a small proportion of each fraction. The predominant material appeared from its behaviour on chromatography and its solubility in various solvents to be equol. After methylation of the crude product, equol dimethyl ether and oestradiol monomethyl ether could be separated readily by chromatography (Table 4).

Fraction V*A* on recrystallization from methanol yielded crystals, m.p. 88·5–90°, not depressed on admixture with authentic equol dimethyl ether. Fraction V*D* was recrystallized from pentane–ether, yielding crystals, m.p. 111–112·5°, not depressed by admixture with an authentic sample of oestradiol-17α methyl ether. This material was further investigated by means of the Kober and acetic–

Table 3. *Identification of equol*

Phenol	Urinary compound		Authentic equol
M.p.	187–190°		190–192°
Mixed m.p.		190–192°	
Infrared spectrum ($CHCl_3$)		Identical	
$[\alpha]_D$ (EtOH)	−17°		−22°*
Methyl ether			
M.p.	86·5–87·5°		88·5–89·5°
Mixed m.p.		88–89·5°	
Infrared spectrum (CS_2)		Identical	
Diacetate			
M.p.	123–125°		122–124°
Mixed m.p.		123–125°	
Infrared spectrum (CS_2)		Identical	
Dibenzoate			
M.p.	187–190°; 219–220·5°		189·5–190·5°; 209–212°
Mixed m.p.		188–190°; 216–219·5°	
Infrared spectrum (CS_2)		Identical	

* Marrian & Haslewood (1932) found $[\alpha]_{5461}$ −21·5° (EtOH).

Table 4. *Chromatography of lipophilic phenols as free phenols and as methyl ethers*

Tube no.	Eluent	Eluate: Fraction	Wt. (mg.)	Appearance	Kober chromogen
	Chromatogram IV: free phenols. 40 g. of Al_2O_3; each run 120 ml. of eluent				
1–4	Benzene	IV*A*	44	Gum	−
5–6	Benzene–ether (90:10)	IV*B* (1)	2	Gum	−
7–9	Benzene–ether (90:10)	IV*B* (2)	37	Gum	+ +
10–13	Benzene–ether (80:20)	IV*C*	56	Solid	+ + +
14–15	Benzene–ether (60:40)	IV*D* (1)	42	Solid	+ +
16–21	Benzene–ether (60:40)	IV*D* (2)	59	Solid	tr.*
22–23	Benzene–ether (40:60)	IV*E*	20	Solid	tr.
24–26	Ether	IV*F*	18	Solid	tr.
27–30	Ether–methanol (90:10)	IV*G*	329	Gum	+ +†
31–37	Ether–methanol (90:10; 50:50)	IV*H*	—	Gum	tr.
	Chromatogram V: methyl ethers. Fractions IV *B* (2), *C*, *D* (1) after methylation. 3 g. of Al_2O_3; each run 10 ml. of eluent				
1–6	Light petroleum–benzene (95:5)	V*A*	49	Solid	−
7–12	Light petroleum–benzene (90:10; 80:20)	V*B*	.	Gum	−
13–16	Light petroleum–benzene (50:50)	V*C*	.	Gum	+ +
17	Benzene	V*D*	7	Solid	+ + +
18–20	Benzene	V*E*	.	Gum	+ +
21–22	Benzene–ether (90:10)	V*F*	.	Gum	tr.
23–26	Benzene–ether (80:20); ether	V*G*	.	Gum	−

* tr. = trace. † This fraction contained much equol; see text, chromatogram VI.

Table 5. *Identification of oestradiol-17α*

	Urinary compound		Authentic oestradiol-17α
3-Methyl ether			
M.p.	111–112·5°		113–114°
Mixed m.p.		112–114°	
Infrared spectrum (CS_2)		Identical	
Kober reaction, $\lambda_{max.}$	526 mμ		525 mμ
Acetic–sulphuric acid reaction, $\lambda_{max.}$	523 mμ		524 mμ
17α-Acetate 3-methyl ether			
M.p.	126·5–129°		129–130°
Mixed m.p.		128·5–130·5°	
Infrared spectrum (CS_2)		Identical	

Oestradiol-17α-acetate 3-methyl ether, authentic (Found: C, 76·7; H, 8·5. $C_{21}H_{28}O_3$ requires C, 76·8; H, 8·6%).

Table 6. *Chromatography of the α-ketonic alcohols*

Chromatogram VII: α-ketonic alcohols, 87 mg.; Al_2O_3, 2·7 g.; each run 9 ml. of solvent. 'Ketosteroids' mean substances giving Zimmermann-positive spots with R_F of the same value as the known compounds indicated. An, androsterone; Ae, aetiocholanolone; K, 11-oxoaetiocholanolone. Figures, thus (+2), show the number of additional Zimmermann-positive spots of unknown origin. tr. = trace.

Tube no.	Eluent	Eluate: Fraction	Eluate: Weight (mg.)	Eluate: 'Ketosteroids' present
1	Light petroleum–benzene (25:75)	VII*A*	2	—
2	Light petroleum–benzene (25:75)	VII*B*	12	An
3	Light petroleum–benzene (25:75)	VII*C*	7	An, Ae, +2
4–6	Benzene	VII*D*	10	An (faint), Ae, +4
7–10	Benzene	VII*E*	6	+2
11–14	Benzene–ether (90:10)	VII*F*	7	Ae, K, +2
15–17	Benzene–ether (80:20)	VII*G*	tr.	K, +1
18–20	Benzene–ether (50:50)	VII*H*	tr.	K, +1
21–23	Ether	VII*J*	tr.	K (faint), +1
24–26	Ether–methanol (90:10)	VII*K*	tr.	K, +1
27–29	Ether-methanol (50:50)	VII*L*	—	—

sulphuric acid reactions. To provide an additional comparison, samples of oestradiol-17α 17-acetate 3-methyl ether were prepared. Comparisons of the compound from goat's urine with authentic material are shown in Table 5.

Fraction IV*G* was also methylated and chromatographed on alumina (chromatogram VI); fraction VI*A* (light petroleum–benzene, 90:10 and 80:20) contained 229 mg. of material which proved to be impure equol dimethyl ether. No other crystalline material was obtained. Fractions VI*D*, *E* and *F* (eluted with various benzene–ether mixtures) contained small amounts of Kober chromogens (see next paragraph).

Possible occurrence of oestradiol-17β. Kober and acetic–sulphuric acid estimations were carried out on all fractions of the above chromatograms that might contain oestradiol. Values for total oestradiol (Kober) and oestradiol-17α (acetic–sulphuric) agreed to within 10% for all fractions except VI*D*, *E* and *F*. Interfering substances prevented accurate evaluation of the proportions of 17α and 17β isomers in these three fractions. Calculation from the differences between the two series of results as a whole indicated that oestradiol-17β could not have represented more than 10% of the total oestradiol present; in fact there was no positive evidence for the presence of the 17β isomer.

Non-phenolic fractions

α-Ketonic alcohols

The ketonic alcohols not precipitated by digitonin (87 mg.) were chromatographed on alumina (chromatogram VII). The eluates were examined by paper chromatography with the light petroleum–propylene glycol system of Savard (1953). The results are shown in Table 6. Spots were obtained with the same R_F values as androsterone, aetiocholanolone and 11-oxoaetiocholanolone. Five other spots were obtained with the Zimmermann reagent, the colours ranging from blue to brown.

Some selected fractions of this main chromatogram were combined and rechromatographed on alumina (chromatogram VIII). Examination by paper chromatography (Savard, 1953) and infrared spectrometry of the fractions so obtained indicated that they were still complex mixtures. The infrared spectra indicated the presence of aetiocholanolone and 11-oxoaetiocholanolone by reason of similarities with authentic spectra in the regions near 1036 $cm.^{-1}$ and 1000–1200 $cm.^{-1}$ respectively. Many fractions also showed a peak at or about 1670 $cm.^{-1}$ typical of a 4-en-3-one grouping. We are indebted to Dr A. E. Kellie of the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, for measuring and interpreting these infrared spectra.

Fraction VII*C* was submitted to gradient-elution chromatography (Kellie & Wade, 1956) by Dr A. E. Kellie. He reported that the 17-oxo steroids were separated from one another (as in samples from human urine) but that they could not be separated from the other substances present, and no quantitative estimation could therefore be carried out. Paper chromatography with the system *A* of Bush (1950) confirmed our finding of aetiocholanolone and androsterone; many other substances were also found.

β-Ketonic alcohols

The ketonic alcohols precipitated by digitonin (21 mg.) were also chromatographed on alumina (chromatogram IX) and the fractions were investigated by paper chromatography. When the Zimmermann reagent was used for identification, only two spots could be obtained. One of these, eluted from the alumina with ether–methanol mixtures, was faint. The other substance was eluted from alumina with benzene–ether (90:10) and appeared from its R_F value to be *epi*androsterone. The relevant fractions were combined and chromatographed again on alumina (chromatogram X). Material eluted with benzene and benzene–ether mixtures (95:5) partly solidified on treatment with ether and methanol. The most solid fraction was recrystallized from ethyl acetate–pentane. Crystals were obtained which melted at 173–176° after considerable previous softening; there was insufficient material for a mixed-melting-point determination with authentic material (m.p. 174–178°). The infrared spectra of the crystals showed a clear pattern of *epi*androsterone, but indicated the presence of impurities, including some 20-oxo steroid as shown by the typical peak (1705 cm.$^{-1}$). Dr Kellie estimated that the material contained approx. 70% of *epi*androsterone. No dehydro*epi*androsterone could be detected on the paper chromatograms, but the colour reaction of Allen, Hayward & Pinto (1950) indicated that a trace might be present.

α-Non-ketonic alcohols

This fraction was chromatographed on alumina (12 g.; chromatogram XI); the following fractions (all gums) were eluted with the solvents indicated: *A*, benzene, 37 mg.; *B*, *C* and *D*, benzene–ether mixtures, 33, 42 and 54 mg.; *E*, ether, 17 mg.; *F*, ether–methanol (90:10), 176 mg.; *G*, ether–methanol (80:20 and 50:50), 19 mg. Fraction XI*F* was immediately chromatographed again (chromatogram XII, Table 7). Fractions XI*E* and XII*B*, which showed signs of solidification, were combined and chromatographed (chromatogram XIII). Finally, fractions XII*C* and XII*D* were also chromatographed again (chromatograms XIV, XV respectively). Many fractions eluted with ether and with ether–methanol (99:1, 98:2) solidified. Attempts were made to recrystallize these from ether and then methanol, but the melting points of the fractions varied from 200° to 230°. The best fractions were therefore acetylated separately, and the products recrystallized from methanol. All of these had m.p. 164–168°, and on admixture with authentic 5β-pregnane-3α:20α-diol diacetate the m.p. showed no depression (Table 8).

Table 7. *Chromatography of the α-non-ketonic alcohols*

Chromatogram XII: fraction XI*F*. Al_2O_3, 10 g.; each run 20 ml. of solvent.

		Eluate			
Tube no.	Eluent	Fraction	Wt. (mg.)	Appearance	M.p.
1–6	Benzene–ether (50:50; 20:80)	XII*A*	8	Gum	—
7–13	Ether	XII*B*	12	Gum and solid	—
14	Ether–methanol (98:2)	XII*C*	47	Gum and solid	—
15–16	Ether–methanol (98:2)	XII*D*	57	Gum	—
17	Ether–methanol (98:2)	XII*E*	3	Gum and solid	230–231·5°*
18	Ether–methanol (98:2)	XII*F*	5	Solid	200–210°
19	Ether–methanol (98:2)	XII*G*	3	Gum and solid	215–218°*
20	Ether–methanol (95:5)	XII*H*	5	Solid	224–227°*
21–32	Ether–methanol (95:5; 90:10; 80:20; 50:50)	XII*J*	15	Gum	—

* M.p. after crystallization.

Table 8. *Identification of 5β-pregnane-3α:20α-diol*

	Urinary compound		Authentic 5β-pregnane-3α:20α-diol
Diol			
M.p.	238·5–239°		236·5–239°
Mixed m.p.		241–243°*	
Diacetate			
M.p.	167–168°		168–169°
Mixed m.p.		167–168·5°	
Infrared spectrum (CS_2)		Identical	
Dibenzoate			
Infrared spectrum (CS_2)		Identical	

* A similar rise was found when the mixed melting point was repeated. No explanation can be given.

5β-Pregnane-3α:20α-diol. The acetates prepared as described above were combined and hydrolysed with KOH (5%, w/v) in aqueous methanol under reflux for 2 hr. The reaction product was dissolved in ether (25 ml.) and washed with water (1 × 4 ml., 4 × 2 ml.). The ether was evaporated and the residue recrystallized from methanol. The product was compared with authentic 5β-pregnane-3α:20α-diol (see Table 8). Finally the benzoate was prepared for a comparison of the infrared spectrum with that of authentic material.

β-Non-ketonic alcohols

This fraction was small (18 mg.). After chromatography on alumina, the fraction eluted with ether–methanol (95:5 and 90:10) was the largest; this was chromatographed again. Nothing was identified, even after acetylation and chromatography of the acetates on alumina.

DISCUSSION

The results of this work emphasize two points which are also obvious from previous work on species differences in urinary steroids (cf., for example, Brooks & Klyne, 1957), but which have unfortunately been disregarded in some other studies: first, that a thorough qualitative investigation of the urinary steroids in any species should be made before their estimation is undertaken; secondly, that care must be taken in applying to animal urines, which may contain unknown interfering constituents, methods designed for human urine. The pregnant goat excretes only small quantities of steroids, whose estimation may be considerably hampered by other materials present.

Pregnanediol

We have isolated 5β-pregnane-3α:20α-diol from pregnant goat's urine; the presence of other isomers could not be demonstrated. 5β-Pregnane-3α:20α-diol is the common 'pregnanediol' of human pregnancy urine, which contains up to 50 mg./day or more in late pregnancy; the goat excretes a much smaller quantity (approx. 5 mg./day). Meites, Webster, Young, Thorp & Hatch (1951) found that pregnant goats from which corpora lutea had been removed required about 10 mg. of progesterone/day to maintain pregnancy. This has been confirmed by Amoroso (unpublished observations).

The pregnant mare excretes a mixture of diols (Brooks *et al.* 1952; Brooks & Klyne, 1957). 5β-Pregnane-3α:20α-diol has also been demonstrated in the pregnancy urine of the chimpanzee (Fish, Dorfman & Young, 1942) and the rabbit (Verly, Sommerville & Marrian, 1950). In the latter animal it was identified by Heard, Bauld & Hoffman (1941) as the principal metabolite of progesterone. Marker (1938) and Marker, Wittle & Lawson (1938*b*) claimed that 5β-pregnane-3α:20α-diol was the main diol component of the neutral steroids of bull's and cow's urine; Klyne & Wright (1956*b*) were, however, unable to find any pregnanediol in pregnant cow's urine.

Oestrogens

The chief C_{18} steroid in pregnant goat's urine is oestrone (about 0·3 mg./l. or 0·8 mg./24 hr.); a smaller quantity (about 0·1 mg./l.) of oestradiol-17α has been isolated. The application of the Kober reaction and the acetic–sulphuric acid reaction to all the urinary fractions containing oestradiol indicated that little or none of the 17β-isomer was present.

Oestrone is the major phenolic steroid excreted by the mare at mid-pregnancy [up to 200 mg./l. (Beall & Grant, personal communication); 30 mg. of oestrogen (as oestrone)/l. (Kober, 1938)], but in late pregnancy the *B*-ring unsaturated ketones (equilin and equilenin) predominate. Some confusion exists about the proportions of the non-ketonic phenols of mare's urine. The work of Grant and his colleagues (Beall & Grant, personal communication; Glen, Barber, McConkey & Grant, 1956) indicates that 3:17α-diols [oestradiol-17α, dihydroequilin-17α and dihydroequilenin-17α (previously called 'β'-oestradiol, 'β'-dihydroequilin and 'β'-dihydroequilenin respectively)] are the major constituents of the non-ketonic phenolic fraction; the proportions of the compounds differ at different stages of pregnancy. Earlier workers (Hirschmann & Wintersteiner, 1938; Stolk & Lenchère, 1937) had found that oestradiol-17β could be isolated in larger amounts than the 17α-isomer.

The rabbit excretes oestradiol-17α after injection with oestrone (Stroud, 1939; Pearlman & Pearlman, 1944) or with oestradiol-17β (Heard *et al.* 1941; Fish & Dorfman, 1941); oestrone, the only other product, is excreted in smaller amounts. Pearlman & Pearlman (1944) found no evidence for the presence of oestradiol-17α in human pregnancy urine.

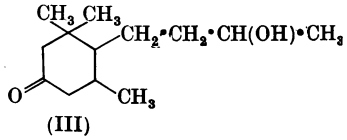
Ketosteroids

The ketosteroids of goat's urine have not been completely characterized in this work, mainly because the quantities are very small. The concentrations of the individual 17-oxosteroids have not been estimated, but the total non-volatile ketonic-alcohol fraction amounts to little more than 1 mg./l. This is similar to the value estimated by Holtz (1956) in cattle urines (0·3 mg. of 17-oxo steroids/l.).

Ionone derivatives

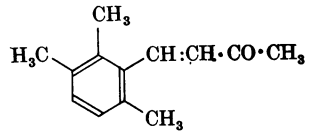
The estimation of 17-oxo steroids in pregnant goat's urine by the routine Zimmermann procedure (M.R.C. Committee on Clinical Endocrinology, 1951) gives values very much higher than the quantities isolated. 'Apparent 17-ketosteroid'

values for individual 24 hr. specimens were 7–10 mg./l., or 10–23 mg./24 hr. Holtz (1954, 1956) has investigated the application of this method to cattle urines, and has produced evidence for the presence of a number of non-steroid substances which interfere. These compounds, related to the ionones (e.g. III), have been isolated by Prelog and his coworkers from mare's urine (Prelog, Führer, Hagenbach & Schneider, 1948). One of the volatile ketonic fractions obtained by us from pregnant goat's urine gave a Zimmermann product, the absorption curve of which was similar to those given by a 17-oxo steroid and by the ketone *J* of Prelog *et al.* (1948) (2:3:6-trimethylbenzylideneacetone, IV).



(III)

1-(3-Hydroxybutyl)-2:2:6-trimethyl*cyclo*hexan-4-one
'Oxyketone *E*'



(IV)

2:3:6-Trimethylbenzylideneacetone
'Ketone *J*'

The origin of these compounds is unknown, but it has been suggested that they might be breakdown products of carotenoids (Prelog *et al.* 1948; Holtz, 1954, 1956). It would be interesting to compare the output of 'apparent ketosteroids' by animals on diets of different carotenoid content.

A recent paper by Simon, Eisengart, Sundheim & Milhorat (1956) describes two C_{16} metabolites of α-tocopherol, which must have arisen by fission of a long polyisoprene side chain in a manner similar to that by which the C_{13} ionone derivatives might be derived from carotenoids.

Equol

Many fodder plants contain oestrogenic substances, some of which are *iso*flavones, e.g. formononetin and genistein. This subject has been reviewed by Bradbury & White (1954). Bassett, Sewell & White (1954), using a biological assay, found that sheep grazing on oestrogen-potent red clover excreted, in the urine, appreciably more oestrogen than grass-fed animals. Equol (I), which has been isolated from goat's urine (10–15 mg./l.) in this work, is *iso*flavan-7:4′-diol (Marrian & Haslewood, 1932; Marrian & Beall, 1935; Anderson & Marrian, 1939), and could arise by reduction of the 2:3 double bond and the 4-oxo group of an *iso*flavone. Equol itself is inactive oestrogenically (Marrian & Haslewood, 1932) and would not interfere with biological assay. It might, nevertheless, interfere with chemical estimation; pilot experiments reported on p. 94 indicate that in the Kober reaction the errors introduced by the equol present in goat's urine would probably not exceed about 10%. The effect of equol on the chromatographic separations used in recent chemical estimations of oestrogen (Brown, 1955; Bauld, 1956) has not yet been studied.

SUMMARY

1. Pregnant goat's urine was hydrolysed with acid, and the lipid material obtained was submitted to the fractionation procedures customary in the study of urinary steroids.

2. Oestrone (0·3 mg./l.) and oestradiol-17α (0·1 mg./l.) were isolated from the phenolic fraction. Oestradiol-17β could not be detected.

3. The heterocyclic phenol equol (*iso*flavan-7:4′-diol) was also isolated (10–15 mg./l.).

4. 5β-Pregnane-3α:20α-diol (2 mg./l.), the common 'pregnanediol' of human pregnancy urine, was isolated from the neutral non-ketonic fraction. There was no evidence for the presence of any isomeric pregnanediols.

5. The neutral non-volatile ketonic fraction was small (1 mg./l.). *epi*Androsterone (3β-hydroxy-5α-androstan-17-one) was isolated from it in impure form. Three other α-17-oxo steroids were partially characterized.

6. Much other volatile material giving a purple colour in the Zimmermann dinitrobenzene reaction was present. This 'apparent 17-ketosteroid' material probably includes ionone derivatives (cf. Holtz, 1954).

7. Species differences in urinary steroids are discussed.

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Protein Synthesis in Ripening Pea Seeds

1. ANALYSIS OF WHOLE SEEDS

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Under conditions conducive to protein synthesis, the amount of protein nitrogen increases at the expense of soluble nitrogen; this has been shown by a great number of workers to hold true for developing seeds (cf. Chibnall, 1939; Schulze, 1911; Schulze & Winterstein, 1910; Emmerling, 1880, 1887, 1900; Zaleski, 1905; Nedokutschajew, 1902, 1904; Boswell, 1924; Bisson & Jones, 1932), for leaves (Wood & Petrie, 1942) and for actively metabolizing potato disks (Steward & Preston, 1940, 1941). This fact, however, does not provide any decisive information about the path of protein synthesis, and can be interpreted in the light of several current theories. Wood & Petrie (1942), for example, considered the correlation between amino acid and protein nitrogen as strong evidence in favour of the hypothesis of direct condensation of amino acids, whereas Steward & Preston (1940, 1941), from their studies with metabolizing potato disks, thought that the amino acids were not utilized directly in protein synthesis, but were first deaminated.

It is not possible, at present, to decide between the two hypotheses of protein synthesis mentioned

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