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ACIDIC STEROID METABOLITES: PREDOMINANT EXCRETION OF 20-OXO-PREGNANOIC ACID METABOLITES OF PROGESTERONE IN RABBIT URINE

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SUMMARY

The major acidic metabolite of progesterone excreted in rabbit urine has been isolated and identified as 3α, 6ξ-dihydroxy-5α-pregnane-20-one-21-oic acid. Several related steroidal acids have been synthesised for the first time. The rabbit excretes mainly 20-oxo-21-oic acids. A 20-hydroxylated-21-oic acid and 17-carboxylic acid metabolites were also detected, but were of less quantitative significance. The trivial name, pregnanoic acid, is proposed to describe the ring A saturated-21-oic acids of the 17-deoxy pregnane series.

INTRODUCTION

The excretion of steroidal-21-oic acids by the rabbit has been demonstrated by the isolation of 3,6,20-trioxo-5α-pregnan-21-oic acid after oxidation of the acidic metabolites of progesterone excreted in rabbit urine [1]. This compound was also identified by gas–liquid chromatography (GLC) after oxidation of rabbit pregnancy urine, but could not be detected in pregnancy urines from the rat, guinea pig, pig or human [2].

The complex mixture of steroidal acids excreted in rabbit urine following the administration of progesterone has now been partially resolved by Celite partition and gas–liquid chromatography and related steroidal acids have been synthesised. Evidence is presented for the predominant excretion of 20-oxo-steroidal-21-oic acids.

MATERIALS AND SUPPLIES

Solvents and chemicals were of similar purity and obtained from the same sources as previously reported [1]. GLC columns were purchased pre-packed with either 3% SE-30 or 3% XE-60 on 100/120 GCQ (Applied Science Lab. Inc., U.S.A.). Lead tetracetate was obtained from Aldrich Chemical Co. Inc., U.S.A.; boron fluoride ethyl ether from Eastman Kodak, U.S.A., and boron trifluoride in methanol (14%, v/v) from sigma Chemical Co., U.S.A. Other chemicals and the Celite 545 and Florisil (60–100 mesh) column packings were obtained from Fisher Scientific Co., U.S.A. Celite columns were packed in glass Konte Chromatoflex columns (1 × 60 cm) (Kontc, New Jersey, U.S.A.) with Teflon plunger assemblies. [4-^{14}C]Progesterone (52.8 mCi/mmol) was purchased from New England Nuclear (Canada Ltd.) and non-labelled steroids from Ikapharm, Israel or Steraloids, U.S.A.

METHODS

Injection of progesterone. Female New Zealand white rabbits (3.5 kg) housed in metal metabolic cages were injected i.m. with [4-^{14}C]progesterone (20 μCi) dissolved in 2 ml 50% ethanolic saline. Urine was collected every 24 h for 48 h and stored at −17°C until processed. Four days later the same rabbit was injected i.m. with non-labelled progesterone (50 mg) plus [4-^{14}C]progesterone (2.5 μCi) dissolved in 5 ml sterile peanut oil. Further injections were given daily for 9 consecutive days, for a total dosage of 500 mg/25 μCi progesterone, and 24 h urines were collected for 14 consecutive days.

Preliminary fractionation of urine. Crude urine from the carrier and carrier-free experiments were individually percolated through Amberlite XAD-2 columns as previously described [1]. The methanol eluates were evaporated and the residues partitioned between 8% $NaHCO_3$ and ethyl acetate to remove the free neutral steroids. The alkali soluble fraction was acidified to pH 1 with HCl, saturated with NaCl and extracted with ethyl acetate. After evaporation of the extract the residue was fractionated on a Florisil column and the acidic fractions methylated with boron trifluoride in methanol (14%, w/v) as previously described [1]. The methyl esters were further fractionated on Celite partition columns.

Celite partition chromatography. Celite 545 was purified as described by Kelly *et al.* [3] and 12 g was treated with the stationary phase of the first developing solvent at the rate of 0.5 ml/g Celite. A 1 × 45 cm column bed was packed with a glass packer and the columns developed consecutively with 2 solvents: toluene–*iso*-octane–methanol–water in the proportions of 40:60:85:15 (by vol.) (400 ml) and 90:10:84:15 (by vol.) (200 ml). Ten ml fractions were collected and 0.5 ml aliquots counted.

Gas–liquid chromatography. Steroid methyl esters and their trimethyl silyl ether (TMSE) and methoxime (MO) derivatives was chromatographed on 3% SE-30 or 3% XE-60 columns at 220–240°C with a Hewlett–Packard high efficiency gas chromatograph (Model 402) equipped with a hydrogen flame detector. Compounds were injected in tetrahydrofuran solution and the relative retention time of each peak (r) was calculated relative to cholesterol TMSE or 5α-cholestane run as the internal standards. TMSE and MO derivatives were prepared as previously described [1].

Hydrolyses. Florisil fraction C was hydrolysed with β-glucuronidase (Ketodase), as previously described [4].

Reduction of steroid methyl esters. Reduction with 20β-hydroxysteroid dehydrogenase, (E.C.1.1.1.53; Sigma Chemical Co.) was carried out overnight as described by Martin and Monder [5] except that the steroids were dissolved in 0.1 ml of propylene glycol and the reduction was carried out in sodium phosphate buffer (0.2 M; pH 7.6).

Synthesis of reference steroidal acids

The synthetic pathways are outlined in Fig. 1. Melting points were determined with a Fisher Johns heated block and are reported as read. Infra red spectra were obtained with potassium bromide discs with a Beckman Acculab 4 spectrometer. Peaks are designed as weak (w), medium (m) or strong (s). Thin layer chromatography was carried out on glass plates coated with silica gel GF_{254} (0.25 mm thickness). Elemental analyses were obtained from the Galbraith Laboratories, Tennessee, U.S.A. and Mass spectra with a Perkin–Elmer–Hitachi (RMu-6E) mass spectrometer with an ionising voltage of 70 V.

3α,6α-Dihydroxy-5β-pregnan-20-on-21-acetate **(II).** 3α,6α-Dihydroxy-5β-pregnan-20-one (I) (8 g; 0.024 mol) in benzene (400 ml) and methanol (20 ml) was oxidised with lead tetraacetate (15 g; 0.034 mol) and boron trifluoride–ethyl ether complex (50 ml) [6]. The mixture was shaken mechanically for 4 h at room temperature, then filtered and the filtrate washed with 0.1 N NaOH and water. The organic phase was dried over Na_2SO_4, filtered and decolorised by heating with activated charcoal. After filtration the solvent was evaporated *in vacuo* and the residue crystallised from benzene to give a white solid. Yield 91.5%.

t.l.c. In benzene–ethanol (8:2, v/v), R_F 0.66 (**I** 0.73); methyl cyclohexane–acetone (1:1, v/v) R_F 0.31 (**I** 0.37). Mp. 84–86°C. Elemental analysis: Calc. for $C_{22}H_{36}O_5$ C 70.4; H 9.2, Found; 70.6, 9.38. I.R. (KBr): 3400(*s*), 2950(*s*), 2890(*s*), 1751(*s*), 1451(*m*), 1420(*w*), 1380(*m*), 1235(*m*), 1051(*s*), 1090(*s*).

3α,6α,21-Trihydroxy-5β-pregnan-20-one **(III).** **II** (1 g; 2.55 mmol) dissolved in 70% methanolic NaOH (30 ml) was gassed with nitrogen and incubated at room temperature for 4 h [7]. Water was added and the mixture extracted three times with ethyl acetate. The organic phase was washed twice with water, dried over Na_2SO_4 and evaporated *in vacuo*. The oily product was dried in a vacuum oven and crystallised as a white solid from methyl cyclohexane–acetone (1:1, v/v). Yield 72%.

t.l.c. In benzene–ethanol (8:2, v/v), R_F 0.33, brown color with H_2SO_4–ethanol (1:1) spray; chloroform–methanol (9:1, v/v) R_F 0.34 (**II**, R_F 0.44). Mp. 99–102°C. Elemental analysis; Calc. for $C_{21}H_{34}O_2$ C 72.0, H 9.7, Found; 71.4, 9.82. I.R. (KBr): 3420(*s*), 2980(*s*), 2900(*s*), 1735(*s*), 1475(*m*), 1420(*w*), 1100(*m*), 1060(*s*).

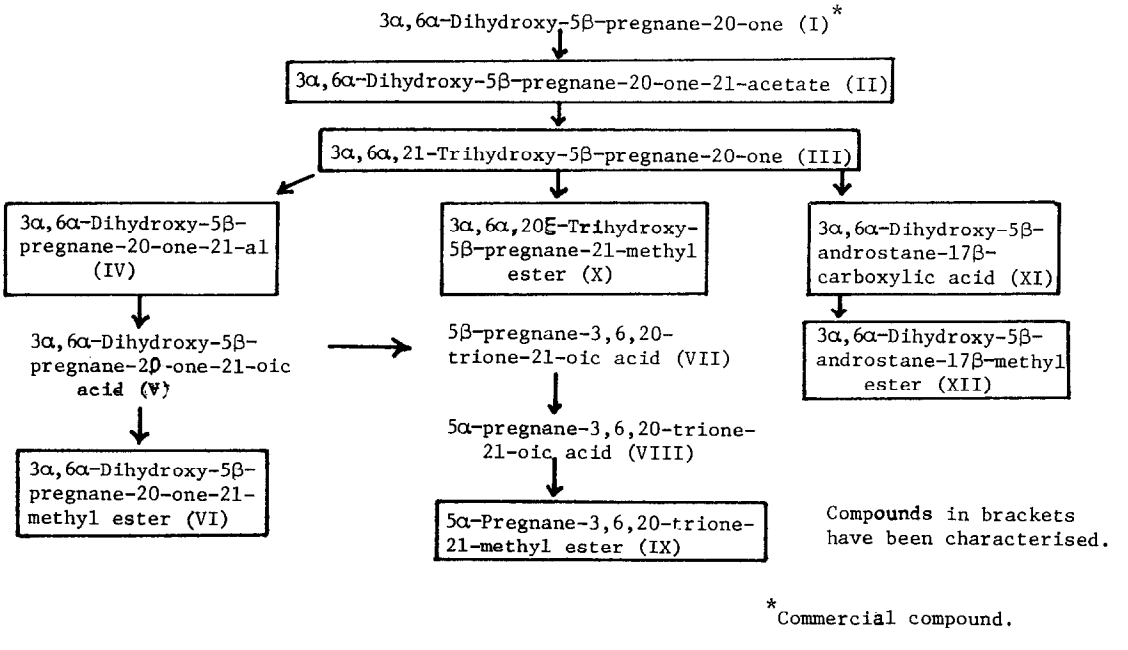

Fig. 1. Outline of chemical syntheses.

3α,6α-Dihydroxy-5β-pregnan-3-one-21-al (**IV**). **III** (596 mg; 1.70 mmol) dissolved in methanol (20 ml) was oxidised with cupric acetate H_2O (0.15 g; 0.75 mmol) dissolved in methanol (30 ml) [8]. After standing 10 min the mixture was aerated for 30 min, and the reaction stopped by the addition of EDTA (0.3 g in 0.1 M sodium carbonate solution (20 ml). After a further 10 min the methanol was evaporated to low vol. *in vacuo*, water was added and the mixture extracted with ethyl acetate three times. The extract was washed twice with 8% $NaHCO_3$ solution and water and dried over Na_2SO_4. Evaporation gave a yellow oil that was crystallised by dissolving in acetone (10 ml) and carefully adding 40 ml sodium phosphate buffer (0.2 M; pH 7.5) under the surface. The crystals deposited overnight at 4°C were filtered, washed with water and dried in a vacuum oven. Yield 63.1%.

t.l.c. in benzene–ethanol (8:2, v/v) gave a major spot R_F 0.53 (minor spot at R_F 0.41) with H_2SO_4–ethanol (1:1) spray. Mp. 171–173°C. Elemental analysis: calc. for $C_{21}H_{32}O_4$ C 72.4, H 9.19, Found; 71.4, 10.02. I.B. (KBr): 3200(*s*), 2940(*s*), 2880(*m*), 1710(*m*), 1460(*w*), 1380(*m*), 1035(*s*).

3α,6α-Dihydroxy-5β-pregnan-20-one-21-methyl ester (**VI**). **IV** (300 mg; 0.86 mmol) dissolved in acetone (30 ml) and diluted with 0.2 M phosphate buffer (15 ml; pH 6.8) was oxidised by the dropwise addition of 1% aqueous methylene blue (w/v) in the presence of sodium cyanide (7 mg) [9]. The methylene blue was added over 30 min until a permanent blue coloration was obtained. After a further 5 min at room temperature the reaction mixture was acidified with HCl, saturated with NaCl and extracted three times with ethyl acetate. The acid fraction was partitioned into 8% $NaHCO_3$ solution, then back into ethyl acetate at pH1. The extract was dried over Na_2SO_4 and evaporated *in vacuo*. Yield 36.6%. Crystallisation from acidified 8% $NaHCO_3$ solution gave a white solid that gave two spots on t.l.c. in ethyl acetate–isopropyl alcohol–formic acid (90:9:1, by vol.). The major product had a R_F 0.53 and gave a purple color when sprayed with H_2SO_4–ethanol (1:1, v/v). This acid (**V**) could not be adequately purified from a more mobile component that gave a bright green spot with H_2SO_4–ethanol spray (R_F 0.71) despite several chromatographies and crystallisations. The acid was therefore converted to the methyl ester (3α,6α-dihydroxy-5β-pregnan-20-one-21-methyl ester; (**VI**) by methylation of **V** (50 mg; 0.73 mmol) with 14% boron trifluoride in methanol reagent (5 ml) for 5 min in a boiling water bath [10]. The reaction mixture was diluted, saturated with NaCl and extracted twice with ethyl acetate. The extract was washed twice with 8% $NaHCO_3$ solution and water and the extract dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a clear oil. Yield 78.8%. Crystallisation from acetone-petroleum ether (80–100°C) gave a white solid.

t.l.c. Ethyl acetate–ethanol (95.5, v/v) gave a single spot, R_F 0.57. Mp. 178–180°C. GLC: Single peak as TMSE derivative *r* 1.80 (3% SE-30; cholestane internal standard) 2.08 (3% XE-60; cholesterol TMSE internal standard). Elemental analysis: Calc. for $C_{22}H_{34}O_3$; C 69.84, H 8.99, Found; 69.34, 9.84. I.R. (KBr) 3400(*s*), 2875(*s*), 2840(*m*), 1755(*s*), 1730(*s*), 1470(*w*), 1260(*m*), 1230(*w*), 1100(*m*), 1065 (*m*), 1040(*m*). Mass spectrum: M^+ 378; M-59 (—$COOCH_3$ group).

5α-Pregnane-3,6,20-trion-21-oic acid methyl ester (**IX**). **V** (62.7 mg; 0.17 mmol) was dissolved in acetone (5 ml), cooled in an ice bath and oxidised to the 5β-pregnane-3,6,20-trion-21-oic acid (**VII**) by the dropwise addition of chromic acid solution [11]. The crude **VII** (36 mg; 0.1 mmol) was dissolved in methanol (24 ml) containing 0.5 g EDTA/litre in 1.0 ml 2N NaOH, gassed with nitrogen and refluxed for 1 h [12]. The reaction mixture was extracted into ethyl acetate, partitioned into 8% $NaHCO_3$, then back into ethyl acetate at pH1. Evaporation gave a white solid that precipitated from acidic $NaHCO_3$ solution (**VIII**). Yield 74.9%. Methylation of **VIII** with boron-trifluoride–methanol reagent [10] gave 5α-pregnane-3,6,20-trion-21-oic acid methyl ester (**IX**) that corresponded with the compound previously characterised [1]. Yield 85%. Crystallised from aqueous methanol.

Mp. 145–148°C. Elemental analysis; calc. for $C_{22}H_{30}O_5$; C 70.60, H 8.0, Found; 70.48, 7.97.

3α,6α,20ξ-Trihydroxy-5β-pregnane-21-methyl ester (**X**). **III** (2.0 g; 8.0 mmol) was dissolved in methanol (200 ml) and stirred magnetically with cupric acetate H_2O (0.8 g; 4.0 mmol) in an equal vol. of methanol for 120 h at room temperature [13]. The solvent was concentrated by evaporation, diluted with water and extracted three times with ethyl acetate. The extract was washed with $HaHCO_3$ and water and dried over Na_2SO_4. Evaporation gave a yellow oil.

Purification was carried out on a Celite column (200 g; 4.5 × 36 cm) coated with the stationary phase of benzene–formamide (400:20, v/v) at the rate of 0.4 ml/g Celite. The column was eluted with one litre each of benzene–ethanol–formamide in the proportions of 95:5:5, v/v and 90:10:5, v/v, which removed several minor contaminants, and was then stripped with methanol (250 ml). The residue left on evaporation of the methanol was crystallised from acetone–petroleum ether to give a white solid.

t.l.c. Gave two closely running spots, R_F 0.53, 0.55 in ethyl acetate–ethanol (95:5, v/v) and R_F 0.72; 0.77 in benzene–ethanol (8:2, v/v) probably corresponding to the 20-hydroxy epimers [13]. However, GLC of the TMSE derivative on a 3% XE-60 column gave a single clean peak, *r* 1.35 (cholesterol TMSE internal standard).

Elemental analysis; Calc. for $C_{22}H_{36}O_5$; C 69.45, H 9.47, Found; 69.34, 9.84. I.R. (KBr) 3200(*s*), 2970(*s*), 2940(*m*), 1745(*s*), 1470(*m*), 1460(*m*), 1380(*w*), 1290(*m*), 1260(*m*), 1060(*s*), 1040(*s*).

3α,6α-Dihydroxy-5β-androstane-17β-carboxylic acid (**XI**). **III** (178 mg; 0.48 mmol) was dissolved in methanol (3 ml) and oxidised by the addition of an

aqueous solution of periodic acid (4% solution in 0.2 N H_2SO_4) [7]. The mixture was left overnight at room temperature, then diluted with water and extracted with ethyl acetate (2×). The extract was shaken with 8% $NaHCO_3$ and the acidic fraction re-extracted into ethyl acetate at pH1. Evaporation and precipitation from a small vol. of $NaHCO_3$ solution with dilute HCl gave a white solid which was washed with water and dried *in vacuo*. Yield 44%. t.l.c. In ethyl acetate–formic acid (99:1, v/v) gave a single blue-green spot with H_2SO_4–ethanol spray (1:1, v/v) at R_F 0.57.

Mp. sublimed at 300°C. Elemental analysis: Calc. for $C_{19}H_{32}O_4$; C 70.40, H 9.88, Found; 71.13, 9.95. I.R. (KBr): 3240(*s*), 2970(*s*), 1745(*s*), 1465(*m*), 1350(3), 1200(*m*), 1170(*m*), 1040(*s*).

The acid (50 mg) was methylated with boron trifluoride–methanol reagent (5 ml) and the product extracted into ethyl acetate, evaporated, and dried in a vacuum oven. Crystallisation from aqueous methanol gave a white solid (**XII**). Yield 80%.

Mp. 162–164°C. GLC: Single peak as TMSE derivative, *r* 0.40 (3% SE-30; cholesterol TMSE internal standard); *r* 0.67 (3% XE-60; cholesterol TMSE internal standard). Elemental analysis: Calc. for $C_{20}H_{34}O_4$; C 71.0, H 10.06, Found; 72.05, 10.02. I.R. (KBr): 3200(*s*), 2975(*s*), 2950(*m*), 1750(*s*), 1470(*m*), 1360(*w*), 1290(*m*), 1260(*m*), 1170(*m*), 1055(*s*).

RESULTS

Florisil column chromatography

Chromatography of the crude acids on Florisil columns removed considerable pigment and also effected a partial fractionation into three fractions, designated A, B and C. Table 1 gives the distribution of radioactivity between the three fractions. Fraction A contained non-conjugated neutral metabolites, whereas fraction B contained only acidic steroids. Fraction C contained glucuronide conjugates since hydrolysis with β-glucuronidase gave both neutral and acidic metabolites. The fraction B acids from the carrier and carrier-free experiments and fraction C acids from the carrier experiment (representing 24.5% of the fraction C radioactivity) were methylated and chromatographed on Celite columns.

Celite column chromatography

Figure 2 illustrates the resolution of seven peaks of radioactivity when the Florisil fraction B metabolites from the carrier-free experiment and Florisil fractions B and C from the carrier experiment were chromatographed on Celite columns. Table 2 gives the distribution of radioactivity between the peaks. Similar spectra of peaks were obtained with the Florisil fraction B metabolites from the carrier and carrier free experiments, indicating that the proportions of acids excreted were not influenced by the quantity of progesterone administered. Peak **IV** was the major component of both experiments. The Florisil fraction C acids differed in that peaks **IV** and **V** were absent and peak **VII** was the major peak. Fraction C acids were, however, quantitatively less significant than the fraction B acids, representing only 6.5% of the original acids applied to the Florisil column, compared to an average of 63.5% for the fraction B acids.

Table 1. Percentage distribution of radioactivity between florisil column fractions

Fraction Fraction number	A 3–7	B 12–15	C 16–25
Carrier free expt.	8.2	63.9	26.5
Carrier expt.	7.9	63.9	31.2
(duplicate)	9.1	63.2	36.2

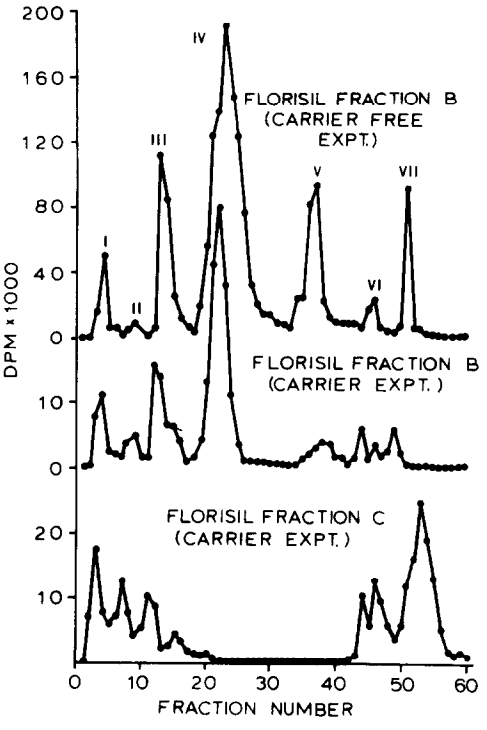

Fig. 2. Resolution of Florisil fractions by Celite partition chromatography

Identification of the major acidic metabolite: Peak **IV**. A single peak was obtained when the TMSE derivative of peak **IV**, isolated from the Celite column, was subjected to GLC on 3% XE-60 and 3% SE-30. Table 3 compares the mobilities of different derivatives of peak **IV** with derivatives of the standard 3α,6α-dihydroxy-5β-pregnan-20-one-21-methyl ester. The standard and peak **IV** had similar mobilities when run singly and admixed as the TMSE and MO-TMSE derivatives. Following oxidation the MO-derivatives differed, peak **IV** giving a duplet characteristic of the 5α-epimer, and the standard a singlet corresponding to the 5β-epimer. The identification of the oxidised peak **IV** as the 5α-epimer was consistent with the reported isolation and characterisation of 3,6,20-trioxo-5α-pregnane-21-methyl ester in a previous study [1]. Admixture of the synthesised stan-

Table 2. Celite partition column fractionation* of the Florisil peak metabolites

Experiment	Fraction number I	II	III	IV	V	VI	VII	Total
Florisil fraction B								
Carrier free	4.0	1.2	14.7	50.6	13.2	2.7	5.5	96.1
Carrier	7.9	2.8	19.0	46.3	6.3	3.2	5.0	79.2
Florisil fraction C								
Carrier	12.6	8.3	8.6	—	—	3.7† 10.6	32.5	89.4

* Percentage of applied radioactivity.
† Double peak.

dard 3,6,20-trioxo-5α-pregnane-21-methyl ester with the oxidised peak **IV** and GLC of the MO-derivatives confirmed the identity of these two compounds. Evidence for the presence of a 20-oxo function in peak **IV** was provided by the shift in mobility of the TMSE derivative from *r* 1.90 to *r* 1.40 following reduction with 20β-hydroxysteroid dehydrogenase. The mobility of the product corresponded to the mobility of the synthesised 3α,6α,20ξ-trihydroxy-5β-pregnane-21-methyl ester.

Further properties of the isolated peak **IV** metabolite were as follows: Mp 180–182°C; I.R. (KBr): 3275(*s*), 2950(*s*), 1755(*s*), 1725(*m*), 1495(*w*), 1260(*m*), 1220(*w*), 1090(*m*), 1050(*m*), 1001(*m*), 965(*w*). Failure to precipitate from an alcoholic solution of digitonin indicated the absence of a 3β-hydroxyl function. Comparison of the mass spectra of peak **IV** and the synthesised 3α,6α-dihydroxy-5β-pregnane-20-one-21-methyl ester indicated similar spectra (Fig. 3) with M^+ 378 and M-59 corresponding to loss of the $COOCH_3$ unit. Although the appropriate 5α-epimer was not available for comparison it can be concluded from the above data that the major acidic metabolite of progesterone excreted in rabbit urine is a 3α,6ξ-dihydroxy-5α-pregnan-20-one-21-methyl ester. The configuration at C-6 could not be established with certainty in the absence of appropriate reference compounds.

Table 3. Mobilities of peak IV and 3α,6α-dihydroxy-5β-pregnan-20-one-21-methyl ester derivatives on GLC

GLC column Derivative	Peak IV *r* values	3α,6α-dihydroxy-5β-pregnan-20-one-21-methyl ester
3% SE-30 (220°)		
TMSE*	1.83	1.83
TMSE†	0.69	0.69
MO-TMSE†	0.76	0.76
Oxidation product: MO‡	3.0; 3.2§	2.36
3% XE-60 (220°)		
TMSE†	1.90	1.90
20β-Reduction TMSE† product:	1.40	1.40

Internal standards: * 5α-cholestane; † cholesteral TMSE; ‡ progesterone. § Duplet.

Peak **III.** The TMSE derivative of peak **III**, the second major Celite peak, gave a single peak on GLC. The retention time (*r* 0.63) corresponded with that of the TMSE derivative of the standard 3α,6α-dihydroxy-5β-androstan-17β-carboxylic acid methyl ester (*r* 0.65) and was identical when run admixed (*r* 0.66) on 3% XE-60 (210°C; cholesterol TMSE internal standard). The *r* values on 3% SE-30 (240°C) were 0.42, 0.40 and 0.41 respectively. Following oxidation and chromatography of the oxosteroids on 3% SE-30 (240°C) several peaks were obtained with both peak **III** and the standard which corresponded in mobility. Each gave a major peak *r* 0.51 (cholesterol TMSE internal standard). The MO derivatives of the oxidation products of peak **III** and the standard gave major peaks at 4.09 and 4.04 respectively under the same conditions on 3% XE-60 (220°C). No evidence for duplets indicative of the 5α-epimers was observed. Epimerisation of peak **III** and the standard and GLC of the MO derivative however, gave corresponding duplets at *r* 0.62; 0.67 (3% SE-30; 240°C, cholesterol TMSE internal standard) characteristic of 5α-epimers. Reduction of peak **III** with 20β-hydroxysteroid dehydrogenase failed to produce a shift in mobility when the TMSE derivative was chromatographed. These results indicate that peak **III** contains a major metabolite with properties more compatible with a 17β-carboxylic acid than with a 21-oic acid.

Evidence for the predominant excretion of 20-oxo steroidal acids. Peaks **III–VII**, obtained by fractionation of Florisil fraction B, were chromatographed on two GLC columns as TMSE derivatives before and after reduction with 20β-hydroxysteroid dehydrogenase. Table 4 gives the *r* values and indicates that only peak **III** failed to show a shift in mobility. The other peaks showed a shift to shorter *r* values on 3% XE-60 and to longer *r* values on 3% SE-30 indicative of the reduction of a 20-oxo function. The standard 3,20-dione-pregn-4-en-21-oic acid methyl ester showed a similar shift in mobility on 3% SE-30.

Peak **VII** *from Florisil fraction C.* Peak **VII** was the major peak in Florisil fraction C and was recovered after Ketodase hydrolysis and eluted in the most polar solvent from the Celite column. GLC of the TMSE derivative on 3% SE-30 (240°C; cholestane internal standard) and 3% XE-60 (240°C; cholesterol

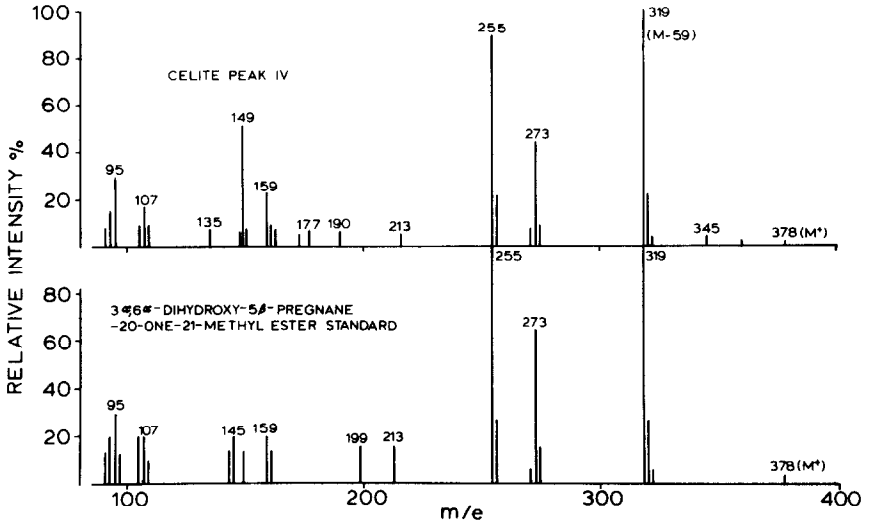

Fig. 3. Comparison of the mass spectrograms of peak IV isolated from a Celite partition column and the standard 3α,6α-dihydroxy-5β-pregnan-20-one-21-methyl ester.

Table 4. Effect of reduction with 20β-hydroxysteroid dehydrogenase on the mobility of the major GLC peaks isolated from the Florisil fraction B carrier experiment

	3% XE-60*		3% SE-30†	
Peak number	Non-reduced r‡	Reduced r	Non-reduced r	Reduced r
III	0.62	0.62	1.14	1.15
IV	1.90	1.40	1.65	2.45
V	2.60	1.87	2.07	2.66
VI	2.28	1.72§	2.09	2.98
	2.51	1.94	—	—
VII	—	—	2.47	2.39§
	2.70	1.91	2.62	3.21
Standard 3,20-dione-pregn-4-en-21-oic acid methyl ester	—	—	1.67	2.07

* 220°C; † 240°C; ‡ Internal standards: Cholesterol TMSE on 3% XE-60; Cholestane on 3% SE-30 except progesterone for peak III and the standard steroid.
§ Duplet peaks.

TMSE internal standard) gave r values of 2.40 and 1.35 respectively which corresponded with the mobilities of the TMSE derivatives of the standard 3α,6α, 20ξ-triol-5β-pregnane-21-methyl ester when run in the same systems.

Chromatography of the MO derivatives of the oxidation products of peak **VII** and the standard on 3% SE-30 (240°C; cholestane internal standard) gave a duplet (r 3.01; 3.22) and a singlet (r 2.36) respectively being indicative of the 5α- and 5β-epimers. Epimerisation of the oxidised 5β-standard to the 5α-epimer and GLC of the MO derivative gave a duplet at r 2.94; 3.17 which when admixed with the MO of the oxidation product of peak **VIII** gave an identical duplet (r 3.06; 3.29). Enzymatic reduction of peak **VII** with 20β-hydroxysteroid dehydrogenase failed to produce a shift in mobility indicating the absence of a 20-oxo function. Consideration of the above data suggests that peak **VII** contains a 3,6,20-trihydroxy-5α-pregnane-21-oic acid. However, this 20-hydroxylated acid only represents an estimated 2.1% of the acids originally applied to the Florisil column.

DISCUSSION

The rabbit metabolises progesterone to both neutral and acidic metabolites. Whereas the structures of the majority of the neutral metabolites have been well characterised [14–17] there is a paucity of data on the structures of the acidic metabolites. Studies on the latter have been hampered by the heavy pigmentation that accompanies them in alkaline extracts and the lack of appropriate reference compounds. This has resulted in indirect studies being devised to analyse the structures of the acids. Thus, Thomas *et al.* [18] proposed the presence of a 20-oxo-21-oic acid function on the results of sodium bismuthate oxidation of the acids which cleaved the acid side-chain only after a preliminary reduction with potassium borohydride. The products were partially characterised as 3,6-dioxygenated-androstane-17-als. The basic structure of the acids was confirmed in this laboratory by the isolation of 3,6,20-trioxo-5α-pregnane-21-oic acid after oxidation of the acidic metabolites of progesterone excreted in rabbit urine [1]. In the present study, evidence is presented for the excretion of a 3α,6ξ-dihydroxy-5α-pregnane-20-one-21-oic acid as

the major acidic metabolite. The configuration at C-6 could not be established with certainty, but by analogy with the isolated neutral metabolites it is most likely to be the 6α-epimer [19, 20]. The properties of the second major metabolite eluted from the Celite column in peak **III** are more consistent with those of a 17-carboxylic acid than a 21-oic acid. The lesser polar metabolites eluted in peaks **I** and **II** also appear to be 17-carboxylic acids which gave complex spectra on GLC and possibly represent breakdown products.

Evidence for the predominant excretion of 20-oxo-21-oic acids was indicated by the effect of enzymatic reduction with 20β-hydroxysteroid dehydrogenase on the GLC mobilities of peaks **III–VII** isolated from Florisil fraction B. Peak **VII** from Florisil fraction C had the properties of a 20-hydroxylated acid but was quantitatively less significant than the 20-oxo-acids. These results contrast with the metabolism of cortisol by the human where 20-hydroxylated cortoic acids were the major acidic urinary metabolites [21]. However, several *in vitro* studies on the metabolism of 17-deoxysteroids indicate that 20-oxo-acids are formed in preference to 20-hydroxylated acids by the human [5], sheep [22] and guinea pig [23] liver preparations. A recent study from this laboratory also identified 20-oxo acids as the major *in vitro* metabolites of progesterone and deoxycorticosterone formed by rabbit liver microsomes [24]. The identification of a 20-oxo-21-oic acid function in the acidic metabolites of several steroids may be of significance for the elucidation of the mechanism of side-chain oxidation of 17-deoxysteroids that are metabolised to steroidal carboxylic acids.

The trivial name, pregnanoic acid, is proposed to describe the ring A saturated-21-oic acids of the 17-deoxypregnane series. By analogy, the ring A unsaturated acids would be termed pregnenoic acids.

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REFERENCES

1. Senciall I. R. and Dey A. C.: Acidic steroid metabolites: Evidence for excretion of C-21-carboxylic acid metabolites of progesterone in rabbit urine. *J. steroid Biochem.* **7** (1976) 125–129.
2. Senciall I. R., Harding C. A. and Dey A. C.: Acidic steroid metabolites: Species differences in the urinary excretion of acidic metabolites of progesterone. *J. Endocr.* **68** (1976) 169–170.
3. Kelly W. G., Bandi L., Shooler J. N. and Lieberman S.: Isolation and characterisation of aldosterone metabolites from human urine; two metabolites bearing a bicyclic acetal structure. *Biochemistry* **1** (1962) 172–181.
4. Senciall I. R.: Acidic steroid metabolites: Application of alumina adsorption chromatography in the differentiation of an acidic progesterone-^{14}C metabolite fraction in rabbit bile and urine. *Biochem. Med.* **8** (1973) 423–431.
5. Martin K. O. and Monder C.: Oxidation of corticosteroids to steroidal-21-oic acids by human liver enzymes. *Biochemistry* **15** (1976) 576–582.
6. *Organic Reactions in Steroid Chemistry* (Edited by J. Fried and J. A. Edwards). Van Nostrand Reinhold, New York (1972) Vol. 2, p. 207.
7. Bush I. E.: *The Chromatography of Steroids.* Pergamon Press, Oxford (1961) p. 360.
8. Monder C. and Martinson F. A. E.: Partial purification of a NADPH-dependent 21-hydroxysteroid dehydrogenase from human placenta. *Biochim. biophys. Acta* **171** (1969) 217–228.
9. Monder C.: Synthesis of steroids containing the α-keto acid side-chain. *Steroids* **18** (1971) 187–194.
10. Morrison W. R. and Smith L. M.: Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoride-methanol. *J. Lipid Res.* **5** (1964) 500–608.
11. Eneroth P., Gordon B. A. and Sjovall J.: Characterisation of trisubstituted cholanoic acids in human feces. *J. Lipid Res.* **7** (1966) 524–530.
12. Chatterton R. T. Jr, Chatterton A. J. and Hellman L.: Metabolism of progesterone by the rabbit liver. *Endocrinology* **87** (1970) 941–952.
13. Lewbart M. L. and Mattox V. R.: Conversion of steroid-17-yl glyoxals to epimeric glycolic esters. *J. Org. Chem.* **28** (1963) 1779–1986.
14. Senciall I. R. and Thomas G. H.: Urinary and biliary excretion of [4-^{14}C]-progesterone, [4-^{14}C]-20α- and [4-^{14}C]-20β-hydroxypregn-4-en-3-one metabolites in the rabbit. *J. Endocr.* **48** (1970) 61–71.
15. Cooke A. M., Rogers A. W. and Thomas G. H.; The urinary metabolites of progesterone labelled with tritium and carbon-14 in the rabbit. *J. Endocr.* **27** (1963) 299–315.
16. Knights B. A., Rogers A. W. and Thomas G. H.: 5α-Pregnane-3α,6α,20α-triol. A metabolite of progesterone in the rabbit. *Biochem. biophys. Res. Commun.* **8** (1972) 253.
17. Chatterton R. J. Jr, Chatterton A. and Hellman L.: Metabolism of progesterone by the rabbit kidney. *Endocrinology* **84** (1969) 1089–1097.
18. Allen J. G., Cooke A. M. and Thomas G. H.: The structure of some reaction products of the acidic urinary metabolites of progesterone. *J. Endocr.* **40** (1968) 153–163.
19. Fotherby K.: Metabolism of progesterone to oxygenated steroids *in vivo* and *in vitro*. Advances in Biosciences **3** (1968) 43–50. Shering Workshop in Steroid Metabolism, Berlin.
20. Dey A. C. and Senciall I. R.: C-21- and 6-hydroxylation of progesterone by rabbit subcellular fractions *Can. J. Biochem.* **55** (1977) 602–608.
21. Bradlow H. L., Zumoff B., Monder C., Lee H. J. and Hellman L.: Isolation and identification of four new carboxylic acid metabolites of cortisol in man. *J. clin. Endocr. Metab.* **37** (1973) 811–819.
22. Monder C. and Wang P. T.: Oxidation of 21-dehydrocorticosteroids to steroidal 20-oxo-21-oic acids by ketoaldehyde dehydrogenase of sheep liver. *J. steroid Biochem.* **4** (1973) 153–162.
23. Schneider J. J.: *In vitro* conversion of deoxycorticosterone to some acidic metabolites. In *Hormonal Steroids* (Edited by L. Martine and A. Pecile). Academic Press, New York, Vol. 1 (1964) pp. 127–135.
24. Dey A. C. and Senciall I. R.: Unpublished data.