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# ACIDIC STEROID METABOLITES: EVIDENCE FOR THE EXCRETION OF C-21-CARBOXYLIC ACID METABOLITES OF PROGESTERONE IN RABBIT URINE

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SUMMARY

Acidic metabolites of injected progesterone have been isolated from rabbit urine. The acids were chromatographed on alumina and florisil columns, precipitated as salts, methylated and oxidised to give a simplified pure oxosteroid fraction. Gas chromatography on 3% SE-30 gave a major compound that was characterised as 3,6,20-trioxo-5α-pregnan-21-oic acid methyl ester by comparison with the synthesised compound. No evidence for 5β- or dioxosteroids was found indicating that the steroid acids are exclusively trioxygenated-5α-pregnanes. This is the first reported isolation of a C-21-carboxylic acid metabolite of progesterone.

## INTRODUCTION

The first definitive study on the biological formation of steroids bearing a C-21-carboxylic acid function was made by Schneider[1] on the *in vitro* metabolism of desoxycosticosterone by guinea-pig liver. More recently, there has been renewed interest in steroid carboxylic acids with the implication of these compounds in the *in vitro* metabolism of 21-dehydrocorticosteroids by sheep liver microsomal preparations [2] and in the *in vivo* metabolism of fluocortolone [3], cortisol [4–6] and 21-dehydrocortisol [7] by the human.

Acidic metabolites of progesterone were first recognised in rabbit urine by Thomas and coworkers [8] following the administration of the labelled steroid. Subsequently, they provided evidence that oxidation of the progesterone molecule was probably confined to the 17β-acetyl side-chain [9] and obtained indirect evidence for the presence of a 20-oxo-21-carboxylic acid grouping by side-chain cleavage to the 17-aldehydes [10]. Structural analysis of these products indicated that the acidic metabolites were probably 3,6,20-trioxygenated 5α-pregnane compounds. Direct evidence is now presented that the acidic metabolites of progesterone are C-21-carboxylic acids by the isolation and characterisation of 3,6,20-trioxo-5α-pregnane-21-oic acid methyl ester; a major derivative of a novel group of steroid acids isolated from the urine of rabbits injected with progesterone.

## MATERIALS AND SUPPLIES

Absolute ethanol and methanol were distilled once and the middle fractions collected. Diethyl ether was shaken with ferrous sulfate in 5% sulfuric acid [11] and distilled twice. Other solvents of either Certified (Fisher Scientific Co., U.S.A.) or Analar grades (BDH, Canada, Ltd.) were used as received.

The following supplies were obtained from the indicated sources: Methoxyamine HCl; precoated g.l.c. packing (3% SE-30 on 100/120 GCQ) (Applied Science Lab. Inc., U.S.A.); Ketodase (β-glucuronidase; beef liver) (Warner Chilcott, Ltd., U.S.A.); Amberlite XAD-2 resin (BDH (Canada), Ltd.); Florisil (60–100 mesh); Alumina (Neutral, 80–200 mesh) and Whatmann 3MM paper (Fisher Scientific Co., Ltd., U.S.A.): Boron trifluoride in methanol (14% v/v); Progesterone (Sigma Chemical Co., U.S.A.); 3α,6α-Dihydroxy-5β-pregnan-20-one (Ikapharm, Israel); [21-$^{14}$C]-progesterone (55·6 mCi/mmol); [4-$^{14}$C]-progesterone (50 mCi/mmol) (New England Nuclear (Canada) Ltd.).

## METHODS

*Injection mixture.* Progesterone was crystallised from petroleum ether (80–100°) to constant melting point (130–131·5°). The crystalline compound (800 mg) was dissolved in sterile peanut oil (80 ml), containing 10% ethanol to maintain solution, and stored at 37°C. Labelled progesterone was dissolved in 2 ml 50% ethanolic saline immediately before injection.

*Injection and urine collection.* Two female New Zealand white rabbits (av. wt. 3·5 kg) were housed in individual metabolic cages and injected i.m. daily with progesterone (50 mg). Each rabbit also received [4-$^{14}$C]-progesterone or [21-$^{14}$C]-progesterone i.m. (2 μCi each dose; total 6 μCi and 8 μCi respectively),

and excreted 64·9% and 62·8% of the dose respectively. Urines were collected daily for up to 22 days and stored at −17°C until processed.

*Amberlite* XAD-2 *resin column.* Amberlite XAD-2 resin was packed in glass columns (4 × 64 cm. bed) and the steroids recovered from the crude urine as originally proposed by Bradlow[12]. One litre aliquots of urine were processed at a time, the column washed with water (500 ml) and then eluted with methanol (500 ml).

*Alumina adsorption chromatography.* Neutral alumina (100 g) was deactivated with 5% water, packed in a glass column (4 × 25 cm. bed) and eluted as previously described [13] with the following scaled up proportions. The sample was applied in methanol (50 ml) and rinsed in with a further 50 ml of methanol. The column was then eluted sequentially with ether–ethanol (3:1 v/v) (500 ml); ethanol (200 ml); 50% aqueous ethanol (1 litre) and 0·1M followed by 1·0M sodium acetate buffer, pH 5·0 (1 litre of each). Fractions (100 ml) were collected in volumetric flasks and aliquots (0·5 ml) removed for counting.

*Florisil adsorption chromatography.* Florisil (200 g) was activated at 100° for 24 h and packed in a glass column in ethanol–ethyl acetate (1:1 v/v). Urine extracts were applied in the same solvent mixture (50 ml) and eluted sequentially with ethanol–ethyl acetate (1:1 v/v); ethanol (100 ml) and ethanol–water (9:1 v/v) (600 ml). Fractions (100 ml) were collected in volumetric flasks and aliquots (0·5 ml) removed for counting.

*Gas-liquid chromatography.* Steroids were chromatographed on 3% SE-30 columns at 240°C with a Hewlett–Packard high efficiency gas chromatography (model 402) equipped with a hydrogen flame detector. Compounds were injected in tetrahydrofuran solution and the relative retention time ($r$) of each peak was calculated relative to progesterone which was run as the internal standard.

*Hydrolyses.* With β-glucuronidase or 15% HCl as previously described [13].

*Precipitation of steroid salts.* White salts were precipitated from heavily pigmented methanolic solutions of fraction C metabolites (isolated from alumina and florisil columns) by slow concentration in a stream of nitrogen. The white precipitate was removed by centrifugation and additional material was obtained by further concentration. Combined solids were freed of pigmentation with several rinses of cold methanol, and then dried in a vacuum oven.

*Extraction and partition of hydrolysates.* Ketodase and hot acid hydrolysates were saturated with NaCl and adjusted to pH 2 with conc. HCl. The mixture was extracted with ethyl acetate (3×) and the acidic metabolites partitioned into alkali by shaking with 0·1M $NaHCO_3$ (3×). Acidic steroids were re-extracted into ethyl acetate after acidifying with HCl.

*Chemical reactions.* (a) Methylation: Steroid acids were dried in a vacuum oven and heated with a 14% solution of boron trifluoride in methanol (1–5 ml) on a boiling water bath for 5 min [14]. The reaction mixture was diluted with water, saturated with NaCl and extracted with ethyl acetate (3×). Washing with cold 0·1M NaOH (2×), 0·1M $NaHCO_3$ (1×) and water (2×) gave a purified neutral methyl ester fraction.

(b) Oxidation: Steroid methyl ester were oxidised with chromic acid [15] and the neutral benzene extract washed with dilute alkali and water until neutral.

(c) Methoxime derivatives: The steroid (1 mg) was mixed with methoxyamine HCl (3 mg) in pyridine (0·2 ml) and stored overnight in the dark [16]. The pyridine was evaporated and the residue dissolved in a small volume (5–50 μl) of tetrahydrofuran for g.l.c.

*Radioactivity.* The purity of the labelled progesterone were assessed by chromatography and the $^{14}C$ counted as previously described [11].

*Thin-layer chromatography.* Silica gel $GF_{254}$ was coated on glass plates (5 × 20 cm.; 0·25 mm) and developed for 15 cm. in unlined glass tanks. Labelled steroids were located with a Packard Radiochromatogram Scanner (model 7201). Non-labelled steroids were visualised by spraying with ethanolic–$H_2SO_4$ (1:1 v/v) and heating for 10 min at 100°C.

*Paper chromatography.* Whatmann 3MM paper was washed with methanol and the steroids chromatographed in the system A of Bush[17] at 26°C after overnight equilibration in a paper lined tank.

*Mass spectrometry.* Mass spectra were obtained with a Perkin–Elmer–Hitachi (RMU-6E) mass spectrometer with an ionising voltage of 70 V.

*Infra red spectrometry.* I.R. spectra were obtained with a Beckman Aculab Model 4 I.R. spectrometer in carbon tetrachloride solution.

*Melting points.* Melting points were recorded with a Fisher Johns melting point apparatus and are reported as read.

*Synthesis of* 3,6,20-*trioxo*-5α-*pregnane*-21-*oic acid methyl ester.* 3α,6α-Dihydroxy-20-oxo-5β-pregnane was converted to 3,6,20-trioxo-5α-pregnane-21-oic acid methyl ester by a seven stage synthesis involving

3α,6α-Dihydroxy-5β-pregnan-20-one
| Lead tetraacetate [19]
3α,6α-Dihydroxy-5β-pregnan-20-one-21-monoacetate
| Alkaline hydrolysis [20]
3α,6α,21-Trihydroxy-5β-pregnan-20-one
| Cupric acetate oxidn. [21]
3α,6α-Dihydroxy-20-oxo-5β-pregnan-21-al
| Methylene blue oxidn. [22]
3α,6α-Dihydroxy-20-oxo-5β-pregnan-21-oic acid
| Chromium trioxide oxidn. [15]
3,6,20-Trioxo-5β-pregnan-21-oic acid
| Epimerisation [23]
3,6,20-Trioxo-5α-pregnan-21-oic acid
| Methylation [14]
3,6,20-Trioxo-5α-pregnan-21-oic acid methyl ester
|

Fig. 1. Outline of synthesis of 3,6,20-trioxo-5α-pregnane-21-oic acid methyl ester. References refer to the general reactions employed, and not to the synthesis of the particular compounds.

acetoxylation, alkaline hydrolysis, oxidations, epimerisation, and methylation, as indicated in Fig. 1. Details of the preparation and characterisation of the neutral intermediates and acids will be given elsewhere [18]. The properties of the product are given in the results section.

## RESULTS

### *Isolation of steroid acids*

*Column chromatography.* One l. aliquots of urine were percolated through Amberlite XAD-2 resins columns and eluted with methanol (90–95% yield) [12]. Metabolites were either fractionated on an alumina column, or hydrolysed with Ketodase or hot acid. Figure 1 gives a typical alumina chromatogram with the resolution of five distinct peaks. Solvent partition of fractions B and C indicated acids were present in both, in contrast to a previous non-carrier progesterone study with labelled progesterone where the acids were confined to fraction C [13]. Fraction B contained 9·6–32·2% and fraction C, 21·8–37·2% of the applied radioactivity so each was analysed separately. Florisil column chromatography gave a single labelled peak eluted with ethanol–water (9:1 v/v) and effected a considerable purification of the pigmented extracts.

*Recovery of steroid acids.* Concentration of a methanolic solution of the metabolites isolated from the florisil column slowly precipitated a white crystalline solid. The product was soluble in water, sparingly soluble in methanol, did not melt at 330°C and gave a positive sodium flame test. It dissolved readily in $NaHCO_3$ solution and precipitated on acidification. Recovery of free acids extractable into ethyl acetate represented 8·3–15·6% and 15·0–25·7% of the dose and radioactivity excreted in the urine respectively. The yield of acids was not significantly altered when hydrolysis of the urine replaced alumina column chromatography in the early work-up stages.

*Thin layer chromatography of steroid methyl esters.* The methyl esters were prepared and chromatographed on Silica gel $GF_{254}$ in the system chloroform–ethanol (9:1 v/v). The radiochromatogram (Fig. 2) shows two peaks, a major polar peak at $R_F$ 0·25 and a minor peak at $R_F$ 0·47. Oxidation with chromium trioxide simplified the spectrum of metabolites and gave a single peak at $R_F$ 0·63 in the same system. Further studies were therefore confined to the oxosteroid methyl ester fraction.

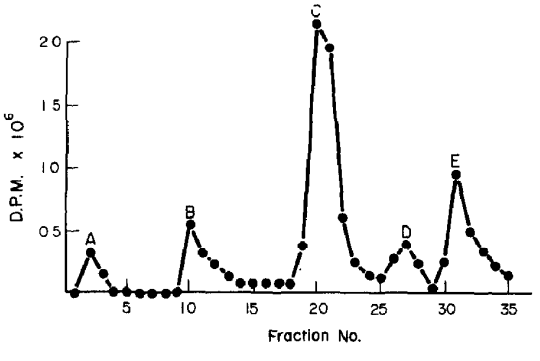


Fig. 1. Alumina adsorption chromatogram of the total urine extract.

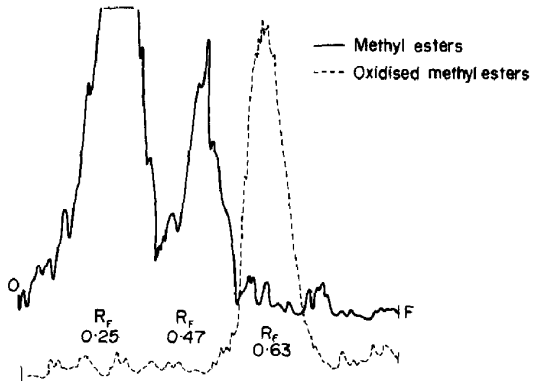


Fig. 2. Thin layer chromatogram of steroid methyl esters before and after oxidation.

*Gas chromatography of oxosteroid methyl esters.* The derived oxosteroids were sufficiently pure for direct gas chromatography on a 3% SE-30 column. Simple gas chromatograms were obtained consisting of a single major peak ($r$ 2·24) and a minor peak ($r$ 1·35) representing less than 5% of the combined peaks. Both fractions B and C from the alumina column gave similar gas chromatograms, and no significant changes were evident when the metabolites obtained after enzyme, and hot acid hydrolysis were compared.

*Characterisation of oxosteroid methyl esters.* Based on the indirect evidence of a previous study [10] it was expected that the acids were probably 3,6,20-trioxygenated-5α-pregnanes. The C-21-carboxylic acids of 3,20-dioxo- and 3,6,20-trioxo-5ξ-pregnanes were therefore synthesised for comparison [18]. No evidence was obtained in the gas chromatograms for the presence of the methyl esters of 3,20-dioxo-5α-pregnane-21-oic acid ($r$ 1·29) or 3,20-dioxo-4-pregnen-21-oic acid ($r$ 1·73). However, the major peak in the gas chromatograms corresponded in mobility with 3,6,20-trioxo-5α-pregnane-21-oic acid methyl ester ($r$ 2·26). Both isolated and synthetic derivatives also gave a minor peak at $r$ 1·35, which preliminary evidence indicates may be a 17β-etianic acid. The latter was separated by chromatography on Whatmann 3MM paper in the system A of Bush[17]. The major compound had an $R_F$ 0·32–0·36 and gave a primrose-yellow fluoresence under U.V. when sprayed with dilute alkali, which is characteristic of 3,6-dioxy-steroids [24]. Three crystallisations from aqueous methanol gave a single peak ($r$ 2·24) on a 3% SE-30 g.l.c. column. The isolated product was also crystallised to constant S.A. (3468 d.p.m./mg). Methoxime derivatives of synthetic and isolated compounds gave symmetrical duplets at $r$ 3·57; $r$ 3·87 in the same g.l.c. system, being indicative of the 5α-epimer [25].

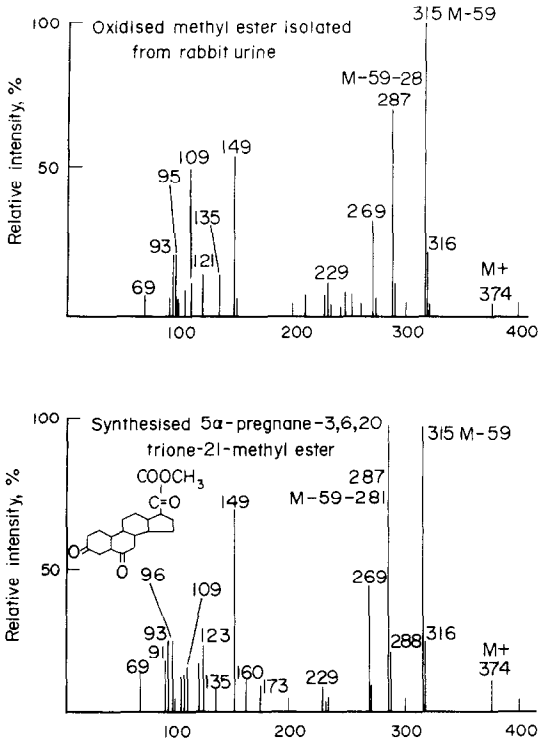


Fig. 3. Mass spectrograms of isolated and synthesised 3,6,20-trioxo-5α-pregnane-21-oic acid methyl esters.

Mass spectrometry gave identical fragmentation patterns (Fig. 3). The molecular ion indicated the correct Mwt of 374. Loss of 59 units was associated with loss of the —$COOCH_3$ group, and loss of 59 + 28 units with cleavage of the —$COCOOCH_3$ side-chain attached at C-17.

Further physical properties are summarised in Table 1 establishing the identity of 3,6,20-trioxo-5α-pregnan-21-oic acid methyl ester.

## DISCUSSION

Studies on progesterone metabolism by the rabbit have revealed a complex situation in terms of both the variety of metabolites formed and the number of possible metabolic pathways. The early studies suggested that the rabbit resembled the human by forming 5β-pregnane-3α,20α-diol as an end product of metabolism, whether by a pathway involving ring A reduction before reduction at C-20 [26] or the reverse [8]. The dioxygenated-5β-pregnanes are however quantitatively less significant than the more polar 3,6,20-trioxygenated pregnanes [8, 27, 28] of which 5α-pregnane-3α,6α,20α-triol is a major component [29].

A third class of compounds, the acidic metabolites, are also of significance. These compounds resemble the polar neutral triols in being 5α-pregnanes with oxygen functions at C-3,6 and 20. In addition, they were suggested to be C-21-carboxylic acids [10], and this has been confirmed in the present study by the isolation of 3,6,20-trioxo-5α-pregnan-21-oic acid methyl ester; a major derivative of the acidic metabolites of progesterone excreted in rabbit urine. Identity has been established by comparison with the synthesised compound. Isolation of the oxosteroid fraction facilitated the purification and provided a simplified spectrum of metabolites for identification. Oxidation of the complex mixture of polar acids gave a remarkably simple gas chromatogram consisting of a single major compound. Most notably, no 5β-epimers were present and no evidence was obtained for the presence of 5α- or 5β-epimers of the 3,20-dioxy-pregnanes.

The oxosteroid methyl esters that were individually isolated from the B and C alumina fractions proved to be identical compounds. The reasons for their separate elution is uncertain, but might involve dimeric cyclisation to a neutral compound which is eluted in fraction B. Such a reaction is known to occur with α-hydroxy-carboxylic acids.

Identification of a C-21-carboxylic acid metabolite of progesterone, a 21-deoxy steroid, is novel since to date these compounds have only been isolated as metabolites of corticoids with a C-21-hydroxyl- [1, 3, 5, 6]. Current studies on the mechanism of formation of C-21-carboxylic acids of cortisol by the

Table 1. Comparison of the properties of the synthesized and isolated 3,6,20-trioxo-5α-pregnane-21-oic acid methyl ester

| | SYNTHETIC | ISOLATED |
|---|---|---|
| Mpt. | 145-148° | 147-149° |
| Mixed Mpt. | 146-149° | |
| Mwt. (M.S.)[1]. | 374 | 374 |
| GLC(3%SE-30) | r2.26 | r2.24 |
| Methoxime deriv. | r3.57; r3.83(duplet) | r3.58;r3.84(duplet) |
| IR[2].($CCl_4$) | 2960(m), 2930(m), 2860(w), 1725(s), 1715(m) 1270(s), 1100(s), 1015(m) | 2975(m), 2940(m), 2860(w), 1730(s), 1715(m), 1270(s), 1100(s), 1015(m) |
| Elemental analysis[3]. | | |
| Calcd. | C, 70.58; H, 8.02 | C, 70.58; H, 8.02 |
| Found | C, 70.48; H, 7.97 | C, 70.36; H, 8.00 |

[1]. Mass spectrometry

[2]. Infra red peaks designated as weak(w), medium(m), strong(s).

[3]. Galbraith Laboratories, Tennessee, U.S.A.

human also suggest that a C-20-oxo-21-hydroxyl function is required to undergo an intramolecular rearrangement to the 20-hydroxy-21-oic acid [7]. Although 21-hydroxylase activity has been demonstrated in rabbit liver with large scale incubations with 15α-hydroxyprogesterone [30], the yield of 21-hydroxylated metabolites was low. Furthermore, 21-hydroxylated metabolites have not been detected as *in vitro* metabolites of progesterone with rabbit liver [23, 26] or kidney [31]. Whether this is a result of further metabolism to C-21-carboxylic acids remains to be determined. In this context it is of interest that acidic metabolites of progesterone and desoxycorticosterone have recently been identified following *in vitro* incubations with rabbit liver microsomal fractions [32].

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