Intermediates in 16-androstene biosynthesis in neonatal porcine testis

T.K. Kwan, J.W. Honour[+]*, N.F. Taylor[+] and D.B. Gower[†]

Department of Biochemistry, Guy's Hospital Medical School, London SE1 9RT and [+]*Department of Clinical Chemistry, Northwick Park Hospital, Watford Road, Harrow, Middlesex HA1 3UJ, England*

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The metabolism of pregnenolone has been studied in microsomal preparations of testes from 3-week old piglets. Metabolites identified after derivatization by capillary gas-chromatography and further by combined gas chromatography-mass spectrometry were: 17-hydroxy- and 16α-hydroxypregnenolone, dehydroepiandrosterone, 5-androstenediols, 5-pregnenediol and andien-β. Pregnenolone and 16-dehydropregnenolone, previously not separable by other methods, were successfully resolved as methyl oximetrimethylsilyl ethers using capillary column gas-chromatography.

Porcine testis *Neonate* *16-Androstene* *Pregnenolone* *16-Dehydropregnenolone*
Gas chromatography-mass spectrometry

1. INTRODUCTION

There is an increasing interest in the group of steroids known as the 16-androstenes. Firstly, because two members of this group may act both as signalling and primer pheromones in the pig [1,2]. Secondly, the recent finding that the urine-smelling 5α-androstenone occurs also in men in axillary sweat collections [3] and in saliva [4] but to a much lesser extent, or not at all, in women may suggest some role in human social communication [5]. Because of the lipophilic nature of 5α-androstenone and of an-α, these steroids accumulate in boar adipose tissue and give rise to the so-called 'taint' of boar meat which some people, especially women, find unpleasant [6].

The testicular biosynthesis of the 16-androstenes is well-documented [6] and occurs in the mature animal from pregnenolone and progesterone by pathways that are distinct from those for androgens. 17-Hydroxylation of these C_{21} steroids, prior to side-chain cleavage, does not appear to be a requirement in 16-androstene formation [6,7], a finding that is in direct contrast with what occurs in androgenesis. The situation obtaining in the testes of 3-week old piglets has not been studied as extensively as that in the mature male animals. There is, however, one report [8] which indicates the possibility of formation of the 16-androstene,

* Present address: Cobbold Laboratories and Department of Chemical Pathology, The Middlesex Hospital Medical School, London W1N 3AA, England
[†]To whom correspondence should be addressed

Abbreviations: pregnenolone, 3β-hydroxy-5-pregnen-20-one; 16-dehydropregnenolone, 3β-hydroxy-5,16-pregnadien-20-one; progesterone, 4-pregnene-3,20-dione; 17-hydroxypregnenolone, 3β,17-dihydroxy-5-pregnen-20-one; 16α-hydroxypregnenolone, 3β,16α-dihydroxy-5-pregnen-20-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 5α-androstenone, 5α-androst-16-en-3-one; an-α, 5α-androst-16-en-3α-ol; andien-β, 5,16-androstadien-3β-ol; 5-androstenediols, 5-androstene-3β,17α(β)-diol; 5-pregnenediol, 5-pregnene-3β,20β-diol; MU, methylene units; MO, methyl oxime; TMS, trimethylsilyl ether; Ref. Cpd., reference compound

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andien-β, by a pathway involving 17-hydroxypregnenolone as an intermediate, which is then dehydrated and subjected to side-chain cleavage (see scheme 1).

Here, we have studied in more detail the metabolism of pregnenolone in microsomes from neonatal porcine testis.

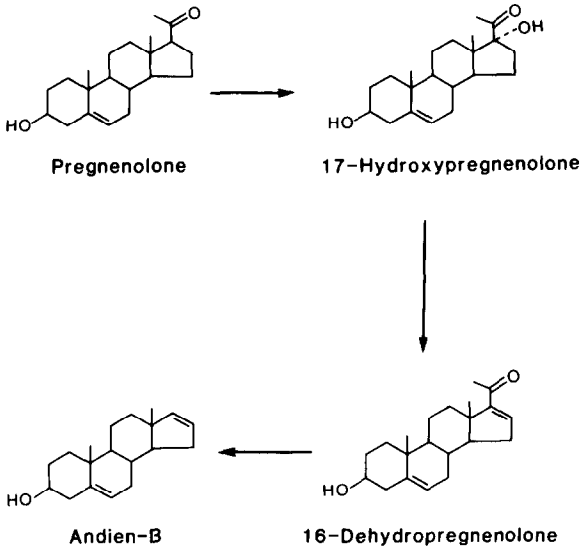
2. MATERIALS AND METHODS

All steroids were obtained from Sigma (Poole, Dorset), except for andien-β which was a gift from Dr G.F. Woods, Organon Laboratories (Lanarkshire). Methoxyamine hydrochloride was purchased from Eastman Kodak Co. [Rochester, NY 14650 (East Organic Chemical)] and hexamethyldisilazane (specially purified grade) from Pierce Chemical Co. (Rockford, IL). Trimethylchlorosilane was obtained from Applied Science Laboratories (State College, PA) and *N*-trimethylsilylimidazole (Regis Chemical Co.) from Phase Separations Ltd (Queensferry, Clwyd). Sephadex LH-20 was purchased from Pharmacia Fine Chemicals AB (Uppsala) and Lipidex-5000 from Packard Instrument Co. (Downers Grove, IL).

2.1. *Preparation of microsomes and incubation conditions*

Testes were obtained by castration of 3-week old Landrace piglets and were transported in ice to the laboratory for use on the same day. Homogenates (10%, w/v) were prepared at 4°C in a Tris–sucrose buffer, pH 7.4 (Tris 0.05 M, sucrose 0.25 M, KCl 0.025 M, $MgCl_2$ 0.005 M and 0.002%, v/v, chlorhexidine) using a Potter homogenizer with a motor-driven Teflon pestle. The homogenate was filtered and centrifuged (MSE MISTRAL 6L, MSE) at $1000 \times g_{max}$ for 20 min and the pellet discarded. The resulting supernatant was then centrifuged (MSE High Speed 18) at $18\,000 \times g_{max}$ for 10 min. This was repeated once more in order to remove any contamination by mitochondria and lysosomes still remaining. The post-lysosomal supernatant was subjected to ultracentrifugation (Beckman L8-70 Ultracentrifuge) at $183\,000 \times g_{max}$ for 40 min.

The microsomal pellet obtained was washed and recentrifuged for a further 40 min and the microsomes homogenized in 30 ml of the buffer. The homogeneity of the microsomal fraction was checked by electron microscopy and by several 'marker-enzyme' assays. Protein content was estimated as in [9].



Scheme 1. The proposed pathway for the biosynthesis of andien-β in immature porcine testis (from [8]).

Portions (3 ml) of microsomal fractions of known protein concentration (5 mg/ml) were preincubated with unlabelled pregnenolone (final conc. 0.17 mmol/l) in air at 37°C for 5 min. The steroid substrate was previously dissolved in 30 μl of acetone. The reactions were initiated by the addition of NADPH (final conc. 3.6 mmol/l) and the mixture incubated for 30 min at 37°C. The incubations were terminated by the addition of ethyl acetate (5 ml). For the control experiment, the conditions used were identical, except that the microsomal suspension was boiled for 5 min prior to incubation.

The steroid metabolites were extracted with a further 2×2 ml of ethyl acetate and the combined extracts dried over anhydrous Na_2SO_4 for 1 h, after which they were evaporated to dryness in a Buchler Vortex-Evaporator (Buchler Instruments, Inc., Fort Lee, NJ) at 37°C under vacuum. To the steroid residue, Na_2SO_4-dried ethanol (Analar, 1 ml) was added and the suspension solubilised in a sonic bath (AB American Beauty S/50). This steroid solution was subjected to chromatographic 'clean-up' procedure using Sephadex LH-20 [10] prior to derivatization for gas-chromatography.

2.2. *Gas-chromatography*

A Becker Model 409 gas chromatograph was fitted with automatic solid injector and a glass capillary Jaeggi column (20 m×0.32 mm, i.d.) coated with OV-1 (Erba Science UK Ltd). Helium was used as carrier gas (1 ml/min) and the injector and detector heater were both maintained at 250°C. Column oven temperature was programmed between 180–270°C at 2.5°C/min followed by an isothermal period of 3 min.

5α-Androstane-3α,17α-diol, stigmasterol and cholesteryl butyrate were added to the samples as internal standards prior to derivatization. Steroid aliquots were derivatized as the *O*-methyloxime-trimethylsilyl ethers as in [10] except that a longer time (overnight at room temperature) was allowed for the oxime formation step. The derivatives were then purified on small Lipidex-5000 columns [10]. The preliminary identification of unknowns was based on retention indices expressed as methylene units, MU [11] obtained by co-injection of the MO–TMS derivatives with a series of *n*-alkanes.

2.3. *Gas-chromatography-mass spectrometry (GC-MS)*

A varian MAT 112 double-focussing mass spectrometer was coupled to a Varian 1400 gas chromatograph housing a capillary column (CP Sil 5 CB, 25 m×0.32 mm, i.d., Chrompack UK). Helium flow rate was 1.8 ml/min and the injector and transfer line temperatures were 230 and 250°C, respectively. Column oven temperature was programmed between 180–250°C at 2°C/min. The scanning was performed over the mass range of 50–800 a.m.u. at 1.4 s/decade at an ionising voltage of 70 eV; accelerating voltage was 800 V and the resolution was 1000 (10% valley definition). Data were processed with a Finnigan MAT SS 200 data system.

3. RESULTS AND DISCUSSION

3.1. *Gas-chromatography (GC)*

Using the conditions in section 2.2 we were able to resolve standard pregnenolone from 16-dehydropregnenolone by capillary GC, the retention times (as MO–TMS derivatives) being 27.8 and 27.5 methylene units, respectively. Previous attempts to separate these underivatized compounds by thin-layer chromatography were unsuccessful (unpublished). The relative retention times of TMS or MO–TMS derivatives of the reference steroids concerned were used for preliminary identification of peaks for the expected compounds in the incubation extracts. Confirmation was then sought using GC-MS.

3.2. *Identification of metabolites*

3.2.1. Pregnenolone

In the incubation of neonatal porcine testicular microsomes, the unmetabolized substrate pregnenolone, after derivatization, gave a mass spectrum at MU 27.8 that was almost identical to that of the standard pregnenolone MO–TMS derivative (27.8 MU). This was included for comparison and amongst the ions present were *m/z* 417 (M^+), 402 (M-15), 386 (M-31), 312 (M-15-90), 296 (M-31-90) and 288 (M-129). In addition to these characteristic ions, the fragment ion at *m/z* 100 arises from fragmentation of ring D of the steroid nucleus with hydrogen transfer [12]. This ion, which forms the base peak here, is generally prominent and seems to be diagnostic for many substituted 20-oxo-steroids, except for 16-unsaturated steroids where 16-unsaturation causes hindrance to its formation.

3.2.2. 17-Hydroxy- and 16α-hydroxypregnenolone

The mass spectrum at MU 28.8 (cf. Ref. Cpd. 28.8) showed that ions characteristic of 17-hydroxypregnenolone were present at *m/z* 156, 158, 188, 294 (M-31-2×90), 384 (M-31-90), 474 (M-31) and 505 (M^+) (fig.1). In the control experiment, however, the molecular ion and a fragment ion at *m/z* 505 and 294, respectively, were absent although the other characteristic ions for the 17-hydroxypregnenolone MO–TMS appeared at much lower relative abundances. This indicates that the formation of 17-hydroxypregnenolone from pregnenolone occurred enzymically. The ion of mass 188 is thought to be comprised of the side-chain, C-16 and C-17 with substituents, and one additional hydrogen atom [12,13]. Apart from the absence of an ion peak at *m/z* 158 in the mass spectrum of 16α-hydroxypregnenolone MO–TMS at MU 29.5 (cf. Ref. Cpd. 29.4), there are many fragment ions common to both the 17-hydroxy- and 16α-hydroxypregnenolone derivatives. The fragment ion at *m/z* 129 probably consists of a trimethylsilyl group, the carbon atoms from posi-

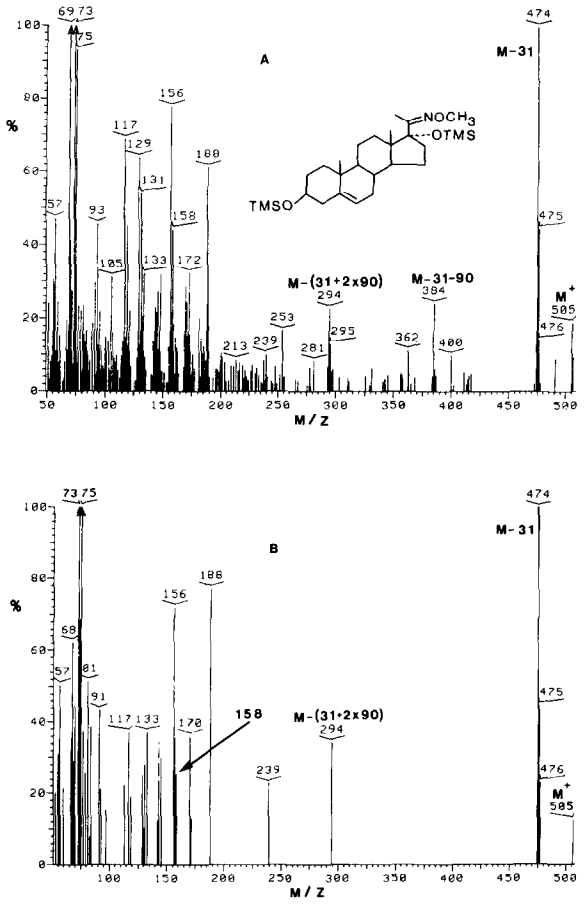


Fig. 1. Mass spectra of 17-hydroxypregnenolone (as MO-TMS derivative): (A) biosynthesized from pregnenolone (0.17 mmol/l) by incubation (30 min) with testicular microsomes from 3-week old Landrace piglets; (B) authentic standard.

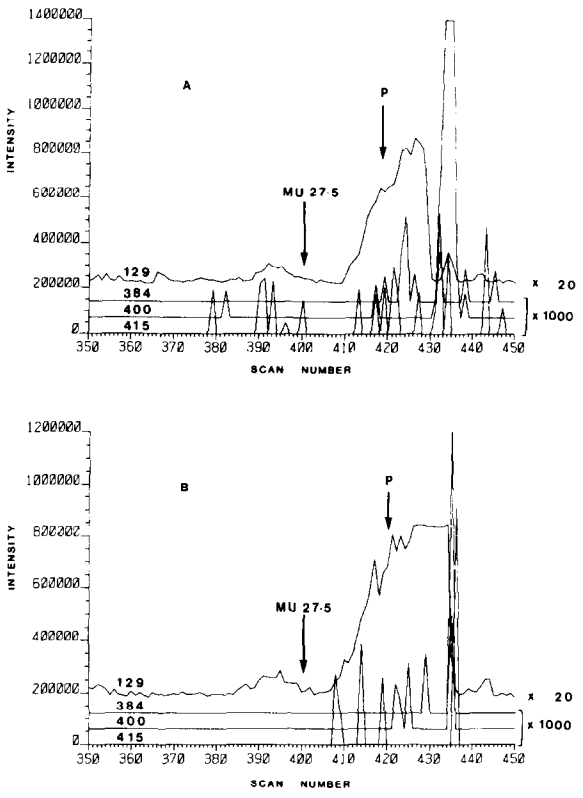


Fig. 2. Extracted ion current profiles: (A) to show that the MO-TMS derivative of 16-dehydropregnenolone is absent at retention time 27.5 MU (no coincidence of ion peaks), and implying the lack of biosynthesis of the steroid from pregnenolone; (B) as (A), except that microsomes were boiled prior to incubation with pregnenolone. P indicates the large peak of ion 129 belonging to unmetabolized pregnenolone (as MO-TMS derivative). See legend to fig. 1 for further details.

tions 2, 3 and 4 and four hydrogen atoms [14–16] and is given by all 5-en-3β-ol TMS ethers.

3.2.3. 16-Dehydropregnenolone

The intensities of selected ions characteristic of 16-dehydropregnenolone MO-TMS were plotted on the ordinate and the spectrum number on the abscissa (by computer). This constitutes essentially the extracted ion current profile (EICP). The ions chosen were of m/z 415 (M^+), 400 (M-15), 384 (M-31) and 129 (fig.2). As there was no coincidence of characteristic ion peaks in the EICP of the test or control experiment, the presence of this compound cannot be confirmed.

3.2.4. Andien-β

The mass spectrum at MU 22.6 (cf. Ref. Cpd. 22.7) showed that ions characteristic of andien-β were observed at m/z 344 (M^+), 329 (M-15), 254 (M-90). 239 (M-15-90), 215 (M-129), 129 and 93. Although these ions were also detected in the control experiment, they were present in much lower relative abundances, indicating that a small amount of endogenous andien-β was present initially (fig.3). This would be in keeping with [17]. It is noteworthy that the 16-en-3-ol TMS ethers all show the prominent ion of m/z 93. This was noted earlier [18] and is now known to be characteristic of ring D unsaturation [19,20].

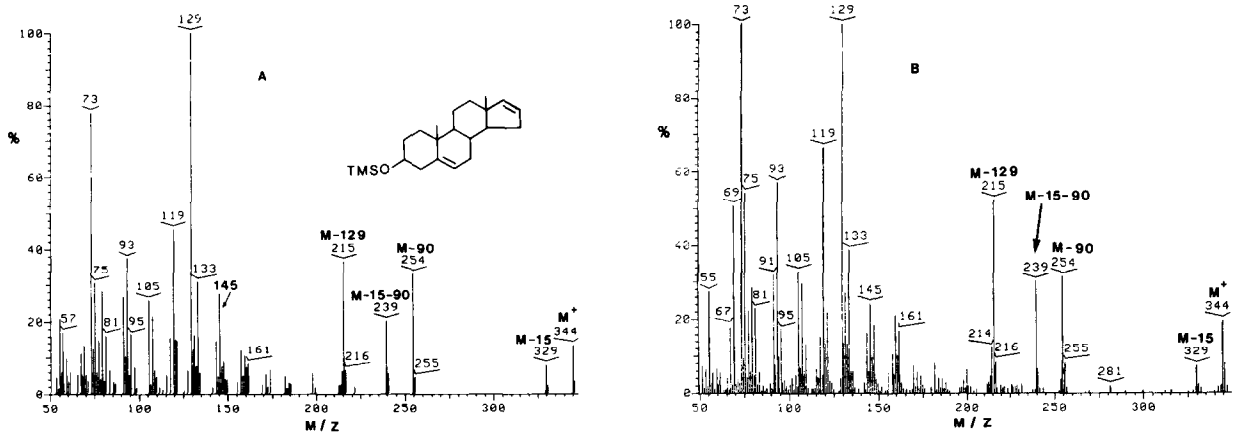


Fig. 3. Mass spectra of andien-β (as TMS ether): (A) biosynthesized from pregnenolone; (B) authentic standard. See legend to fig. 1 for further details.

3.2.5. Other metabolites

A number of other metabolites were also identified but only 4 of these with their relative retention times and characteristic fragment ions are summarized in table 1.

In an earlier study [21] of pregnenolone metabolism in boar testicular homogenates, the intermediary role of 5-pregnene-3β,20β-diol was proposed. Further, using adult porcine microsomal fractions of testis, 5-androstene-3β,17α- and

Table 1

Relative retention time values of TMS or MO-TMS (where indicated) derivatives of steroids isolated from neonatal porcine testicular microsomal incubation with pregnonolone and those of corresponding derivatives of reference compounds

Steroid identified	Retention time (MU)		Base[a] peak	Characteristic mass spectrum ions present in metabolites m/z (relative intensity in parentheses)
	Compound from incubation	Reference compound		
Dehydroepiandro-[b] sterone	25.9	25.9	129	M^+, 389(5); M-15, 374(6); M-31, 358(19); M-90, 299(9); M-31-90, 268(30); M-129, 260(35)
5-Androstenediols; -3β, 17-α-diol	25.7	25.7	129	M^+, 434(10); M-90, 344(30); M-15-90, 329(25); M-129, 305(23); M-2 × 90, 254(37); M-15-2 × 90, 239(29); M-129-90, 215(56)
-3β, 17β-diol	26.1	26.1	117	434(–); 344(9); 254(–); 239(22); 215(21), 129(54)
5-Pregnene-3β, 20β-diol	28.0	28.0	169[c]	M^+, 462(31); M-15, 447(15); M-90, 372(26); M-15-90, 357(20); 129 and 117 (very intense)

[a]Normalized above m/z 100

[b]As MO-TMS derivative

[c]Normalized above m/z 150 (–) ions thus marked may be absent or relative intensity too low. For details of combined GC-MS, see section 3.1

-3β,17β-diols were identified in pregnenolone metabolism [22]. Our results have demonstrated the presence of the same metabolites in neonatal porcine testicular microsomal incubations. In contrast to [8], where 16-dehydropregnenolone was produced from pregnenolone in 27.6% yield, our studies have shown that 16-dehydropregnenolone was not present in detectable quantities (fig.2). The reason for these contradictory findings is currently under investigation using short-term kinetic studies and specific ion monitoring to quantify the metabolites formed.

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