Identification of 16-dehydropregnenolone as an intermediate in 16-androstene biosynthesis in neonatal porcine testicular microsomes

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The biosynthesis of 16-androstenes has been studied in neonatal porcine testicular microsomes using 17-hydroxypregnenolone and 16-dehydropregnenolone, separately, as substrates. The metabolites formed after microsomal incubation with these substrates were purified, derivatized as *O*-methyloxime-trimethylsilyl ethers and analysed by capillary gas chromatography–mass spectrometry. In the incubation of 17-hydroxypregnenolone with microsomes, 16-dehydropregnenolone was identified as an intermediate in the biosynthesis of 16-androstenes. Further microsomal incubation of 16-dehydropregnenolone has established the intermediary role of this steroid in the production of 16-androstenes.

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

1. INTRODUCTION

The odorous 16-androstenes are considered to be more important quantitatively than the androgens produced by the porcine testis [1,2]. Because of their lipophilic character, the 16-androstenes readily accumulate in boar adipose tissue and are responsible for the taint of the meat (review [3]). The problem of 'boar taint' has led to research into the biosynthesis of these odorous steroids, in particular to discover at what age, and to what degree, the porcine testis is capable of synthesizing 16-androstenes. It is therefore of considerable interest to study the biosynthetic pathways in the neonatal animal.

The biosynthesis of 16-androstenes in boar testicular microsomes from pregnenolone or progesterone is believed to take place by side-chain cleavage without prior 17-hydroxylation [4,5]. In the young animal, however, the biosynthesis of andien-β from pregnenolone has been reported [6] to involve 17-hydroxypregnenolone as an intermediate. Furthermore, dehydration of this 17-hydroxylated compound is thought to give rise to 16-dehydropregnenolone which undergoes side-chain cleavage to yield andien-β.

Our earlier studies [7] have confirmed the presence of 17-hydroxypregnenolone amongst many other metabolites of pregnenolone in neonatal porcine testicular microsomal incubations. 16-Dehydropregnenolone, however, was not

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Abbreviations: an-α, 5α-androst-16-en-3α-ol; an-β, 5α-androst-16-en-3β-ol; andien-β, 5,16-androstadien-3β-ol; androstadienone, 4,16-androstadien-3-one; androstanediol, 5α-androstane-3α,17α-diol; androstenedione, 4-androstene-3,17-dione; androsterone, 3α-hydroxy-5α-androstan-17-one; 16-dehydropregnenolone, 3β-hydroxy-5,16-pregnadien-20-one; DHA (dehydroepiandrosterone), 3β-hydroxy-5-androsten-17-one; epiandrosterone, 3β-hydroxy-5α-androstan-17-one; 17-hydroxypregnenolone, 3β,17-dihydroxy-5-pregnen-20-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one

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detectable by gas chromatography–mass spectrometry (GC–MS) and it is possible that the 16-dehydro metabolite formed is rapidly turned over. In incubation studies with neonatal porcine testicular microsomes, 17-hydroxy-[4-^{14}C]pregnenolone was biosynthesized from [4-^{14}C]pregnenolone. Subsequent incubation of this biosynthesized 17-hydroxy-[4-^{14}C]pregnenolone resulted in the formation of labelled 16-dehydropregnenolone and andien-β which were detected by radioautography after thin-layer chromatography. Further, microsomal incubation of this 16-dehydro-[4-^{14}C]pregnenolone as substrate yielded labelled andien-β (unpublished).

In this study, unlabelled 17-hydroxypregnenolone and 16-dehydropregnenolone were utilised separately as substrates and were incubated with neonatal porcine testicular microsomes. The metabolites formed were identified by GC–MS and intermediary role of 16-dehydropregnenolone in andien-β biosynthesis was confirmed.

2. MATERIALS AND METHODS

The materials are essentially those given in [7] except that the steroids an-α and an-β were synthesized from androsterone and epiandrosterone, respectively [8]. [1,2,6,7-^{3}H]Androstenedione (spec. act. 82 Ci/mmol) was purchased from Amersham (England).

2.1. *Preparation of neonatal porcine testicular microsomes*

Testicular microsomes from 3-week-old Landrace piglets were prepared and suspended in Tris–sucrose buffer, pH 7.4, as in [7], the homogeneity of the microsomal fraction being checked by 'marker-enzyme' assays electron microscopy. The protein content was determined as in [9].

2.2. *Microsomal incubation of 17-hydroxypregnenolone*

Microsomal suspensions (3 ml) of known protein content (15 mg) were pre-incubated in air at 37°C for 5 min with 17-hydroxypregnenolone (final concentration 0.16 mmol/l) which was previously dissolved in acetone (30 μl). The reaction was started by the addition of NADPH (final concentration 2.4 mmol/l) and incubation continued for a further 20 min. The enzyme reaction was terminated by the addition of ethyl acetate (5 ml). A control experiment was also set up with boiled (5 min) microsomal suspensions.

A known amount (0.1 μCi) of tritiated androstenedione was added to the incubation mixture before extraction to correct for analytical losses. The metabolites formed were extracted with ethyl acetate and dried down as before [7] after which they were redissolved in ethanol (Na_2SO_4-dried) and purified by column chromatography using Sephadex LH-20 [10] before derivatization for GC–MS analysis.

2.3. *Microsomal incubation of 16-dehydropregnenolone*

16-Dehydropregnenolone (final concentration 0.25 mmol/l), dissolved in acetone (30 μl), was pre-incubated with 2 ml of microsomal suspensions (protein content 10 mg) for 5 min. The reaction was initiated by the addition of NADPH (final concentration 3.6 mmol/l) and the incubation continued for a further 20 min. It was stopped with ethyl acetate and the metabolites extracted and purified as in section 2.2. In the control experiment, boiled (5 min) microsomal suspensions were used.

2.4. *Gas chromatography*

Steroid metabolites were derivatized as the *O*-methyloxime-trimethylsilyl (MO–TMS) ethers as in [7] and were purified on Lipidex-5000 columns [10]. They were analysed on a Becker 409 gas chromatograph equipped with a flame-ionization detector and a glass capillary column (OV-1; 20 m × 0.32 mm; Jaeggi, Trogen, Switzerland). The column oven temperature was programmed from 170–270°C at 2.5°C/min, with helium carrier gas flow (1 ml/min); injector and detector were both kept at 250°C.

The identities of the metabolites were first characterised by their relative retention times expressed in methylene units (MU) [11] when compared with those of the authentic standards. Confirmation was then obtained using GC–MS.

2.5. *Gas chromatography–mass spectrometry*

A Finnigan MAT model 4500 automated gas chromatograph quadrupole mass spectrometer system was used in electron impact mode. It was

equipped with an Incos series 2000 data system. The gas chromatograph housed a fused silica capillary column (stationary phase SE54; length 30 m; i.d. 0.25 mm; Jones Chromatography Ltd., Llanbradach, Mid. Glamorgan, Wales) which was threaded through the transfer line oven and directly into the ion source. Helium was used as carrier gas and the column head pressure was held at 138 kPa (20 lb/in^2). The column oven was temperature programmed from 70–220°C at 20°C/min and then 220–325°C at 4°C/min. A Grob-type capillary injector was used in the splitless mode (sample volume 1 μl; splitless period 36 s). The temperatures for injector, transfer line and ionizer were 250, 260 and 200°C, respectively. The electron energy was 70 eV; emission current, 0.32 mA. The mass range was repetitively scanned from 50–700 a.m.u. at 1.0 s/scan, and mass resolution (10% valley) was 1000.

3. RESULTS

3.1. *Gas chromatographic analysis*

3.1.1. 17-Hydroxypregnenolone incubation

Fig.1 shows the gas chromatogram of the derivatized metabolites from the testicular microsomal incubation of 17-hydroxypregnenolone. A small amount of 16-dehydropregnenolone is indicated at 27.5 MU. Amongst other metabolites formed were andien-β and DHA at relative retention times of 22.6 and 25.9 MU, respectively (table 1). An-α and androstadienone were also

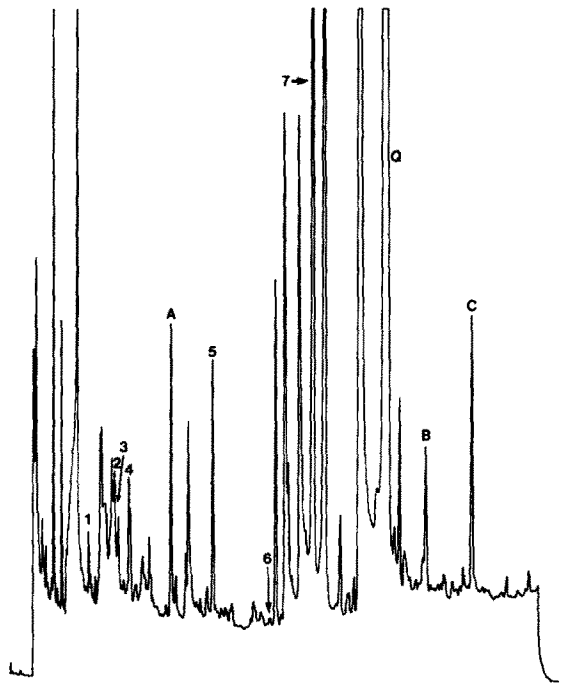


Fig.1. Steroid profile of 17-hydroxypregnenolone metabolites from porcine microsomal incubation. Internal standards A, B and C are androstanediol, stigmasterol and cholesteryl butyrate, respectively. These internal standards and the incubation metabolites were derivatized as MO, TMS or MO–TMS ethers. Q, cholesterol; steroids identified: (1) an-α, (2) andien-β, (3) position of an-β, (4) androstadienone, (5) DHA, (6) position of 16-dehydropregnenolone, (7) 17-hydroxypregnenolone (unmetabolized substrate).

Table 1

Gas chromatographic–mass spectrometric data of metabolites obtained from microsomal incubation of 17-hydroxypregnenolone

Steroid	Derivative formed	Retention time (MU)[a]	Major mass spectral ions[b] *m/z* (%)
An-α	TMS	21.9	346(44); 256(47); 241(100); 148(48); 94(67)
Andien-β	TMS	22.6	344(31); 254(39); 215(47); 129(100); 93(51)
An-β	TMS	22.6	346(22); 241(35); 148(30); 93(46); 75(100)
Androstadienone	MO	22.9/23.0[c]	299(64); 268(22); 147(40); 129(100); 93(69)
DHA	MO–TMS	25.9	389(7); 358(32); 268(45); 260(38); 129(100)

[a] Retention time values were obtained on OV-1 capillary column; in GC–MS, a better separation of andien-β and an-β was achieved with SE54 capillary column

[b] All spectra were normalized to the most intense peak. The first ion of each list is the molecular ion which is followed by the most intense fragment ions (max. 5)

[c] Paired values indicate the *syn* and *anti* forms of the MO derivative of a single compound

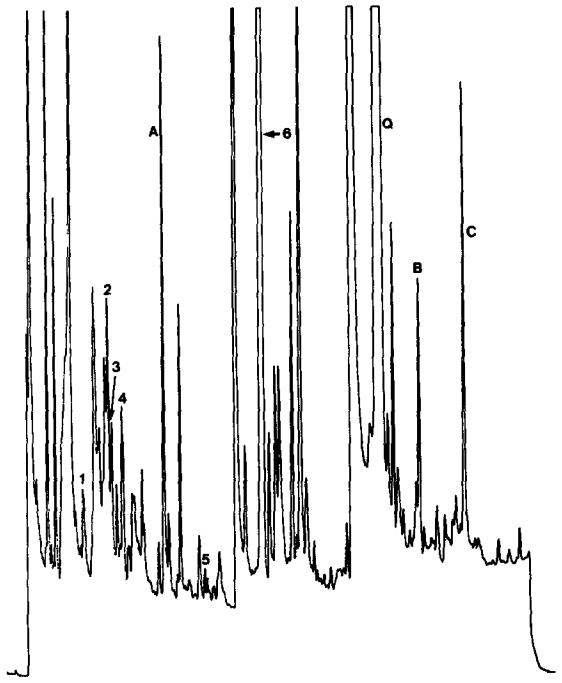


Fig.2. Steroid profile of metabolites (derivatized as MO, TSM and MO–TMS ethers) from microsomal incubation of 16-dehydropregnenolone. A, B, C and Q are as defined in fig.1. The metabolites 1–5 identified follow the scheme in fig.1. 6, unmetabolized substrate peak of 16-dehydropregnenolone.

observed at 21.9 and 22.9/23.0 MU, respectively (table 1).

3.1.2. 16-Dehydropregnenolone incubation

The gas chromatogram of the derivatized metabolites from the microsomal incubation of 16-dehydropregnenolone shows the presence of a large amount of andien-β at 22.6 MU. An-α, androstadienone and DHA were also detected (fig.2).

3.2. *Mass spectrometric analysis*

3.2.1. 17-Hydroxypregnenolone incubation

Fig.3A shows the mass spectrum of authentic 16-dehydropregnenolone MO-TMS. The mass spectrum of the 17-hydroxypregnenolone incubation metabolite at 27.5 MU (fig.3B) shows the presence of the molecular ion for 16-dehydropregnenolone MO–TMS at m/z 415. The characteristic fragment ions for the compound were also observed at m/z 400 (M – 15), 384 (M – 31), 325 (M – 90), 310 (M – 15 – 90), 294 (M – 31 – 90), 286 (M – 129) and 129. The mass spectrum of this metabolite corresponds well with that of the authentic standard compound (cf., fig.3A). A semi-quantification of the metabolite formed was obtained by a computer plot of the ratio of peak heights of the molecular ions for 16-dehydropregnenolone MO-TMS to that of the internal standard, androstanediol TMS. This ratio was then multiplied by the product of the correction factor (for analytical losses) and the amount of internal standard added (i.e., 5 μg). Using this approximate quantification, the level of 16-dehydropregnenolone from the test incubation was 2-times above the control experiment, indicating that the 16-dehydropregnenolone was produced enzymically. The presence of andien-β was confirmed by the mass spectrum at 22.6 MU. Andien-β was produced at a concentration of 14 times above the control level. The characteristic molecular and fragment ions of andien-β TMS are listed in table 1. An-α, an-β, DHA and androstadienone were also detected (see table 1) but were not quantified.

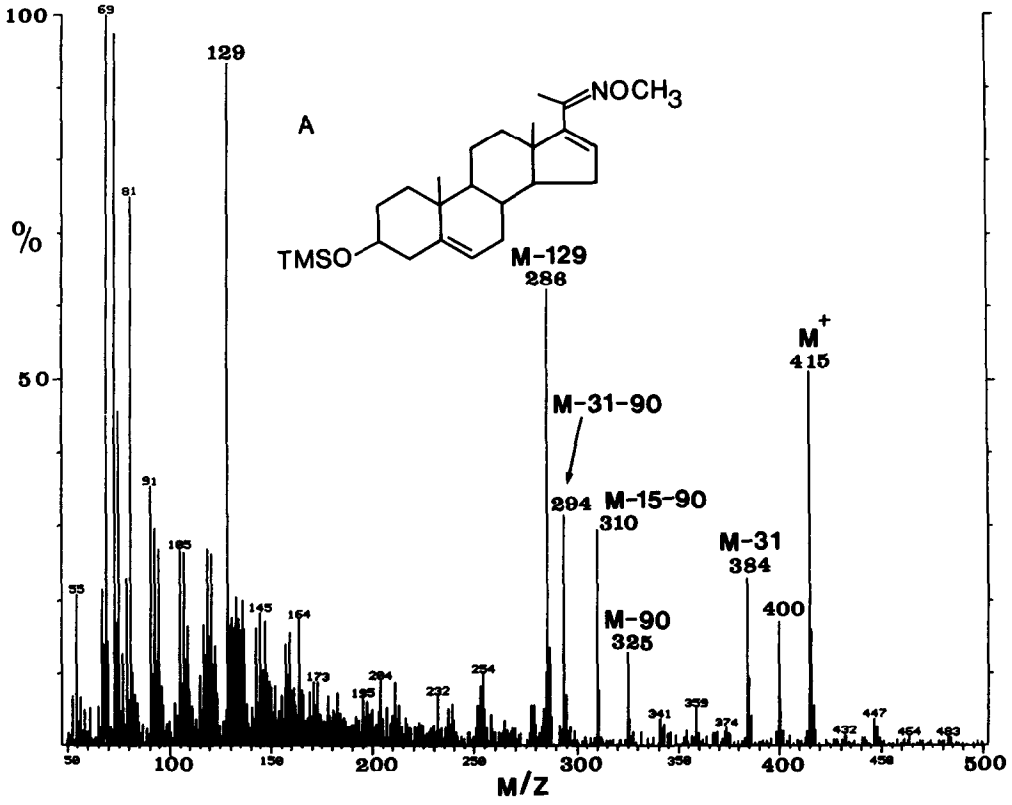
3.2.2. 16-Dehydropregnenolone incubation

The molecular and fragment ions, characteristic of andien-β TMS and having the relative retention time of 22.6 MU, were obtained at the following m/z values (% abundance is indicated in parentheses): M^+, 344 (47); M – 15, 329 (18); M – 90, 254 (63); M – 15 – 90, 239 (40), M – 129, 215 (62); and 129 (100).

Using the computer plot as described in section 3.2.1, the concentration of andien-β produced was found to be approximately 20 times higher than that in the control experiment. Other metabolites such as an-α, an-β, androstadienone and DHA were also detected (but not quantified) at their respective relative retention times by a computer plot [i.e., extracted ion current profile (EICP)] of

→

Fig.3. Mass spectra of 16-dehydropregnenolone MO–TMS: (A) authentic standard; (B) biosynthesized from 17-hydroxypregnenolone (0.16 mmol/l) by incubation (20 min) with neonatal porcine testicular microsomes (protein, 15 mg).


100
%
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A
NOCH3
TMSO
M-129
286
M+
415
M-31-90
294
M-15-90
310
M-31
384
400
M-90
325
129
69
81
91
105
55
145
164
173
195
204
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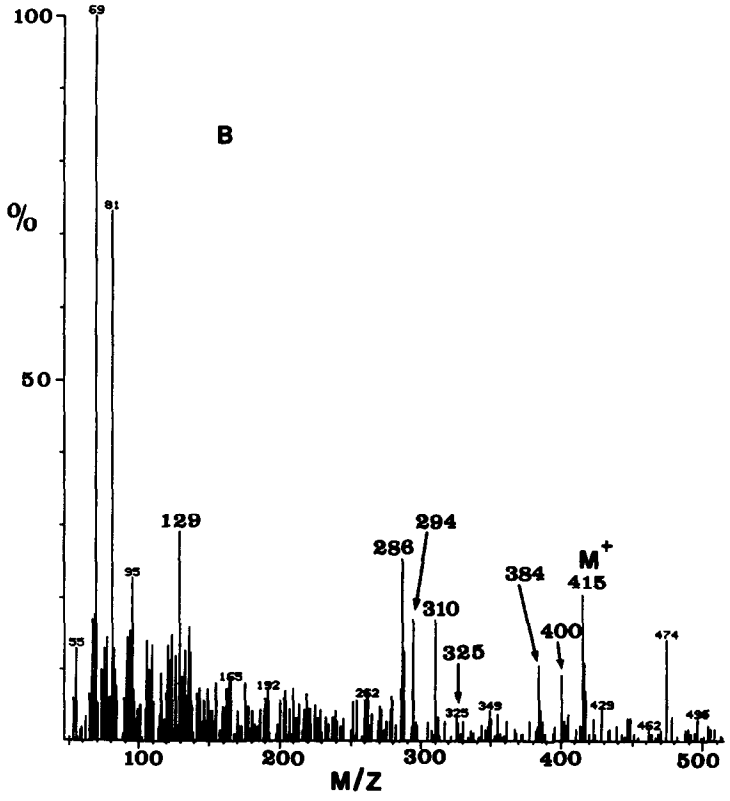
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294
286
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325
349
384
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415
400
429
462
474
496
100
200
300
400
500
M/Z

their individual characteristic molecular and fragment ions against scan number. Coincidence of these ion peaks was obtained in all cases, thus indicating their presence as metabolites of 16-dehydropregnenolone incubation. The EICP also showed that an-β eluted very soon after andien-β.

4. DISCUSSION

In our earlier studies [7], incubation of neonatal porcine testicular microsomes with pregnenolone did not produce 16-dehydropregnenolone in amounts detectable by GC–MS. This is probably due to the rapid turning over of this intermediate. It is also conceivable that the 17-hydroxypregnenolone formed in the incubation was channelled to other products, one of which is DHA. Consequently, little 17-hydroxypregnenolone was left for conversion to the 16-dehydropregnenolone compound.

Here, the use of 17-hydroxypregnenolone as a substrate in the microsomal incubation has permitted us to identify the presence of 16-dehydropregnenolone (fig.3B) and to semi-quantify its concentration. The low ratio of 2 (above the control level) for 16-dehydropregnenolone could be due to its rapid conversion to andien-β and other metabolites. The intermediary role of 16-dehydropregnenolone in the production of 16-androstenes was examined by its incubation with testicular microsomes. A 20-fold increase in andien-β production when compared with the control incubation is a clear indication of the intermediary role played by 16-dehydropregnenolone in the biosynthesis of andien-β and other 16-androstenes such as androstadienone, an-α and an-β.

The identification of 16-dehydropregnenolone is significant to the understanding of the metabolic pathway of andien-β biosynthesis in the neonatal piglets. Whether other routes of its metabolism are important remains to be established. It poses the question of how much of the 16-androstenes is biosynthesized by the pathway: pregnenolone $\longrightarrow$ 17-hydroxypregnenolone $\longrightarrow$ 16-dehydropregnenolone $\longrightarrow$ andien-β and whether this occurs also in the adult animal. Attempts to clarify these issues are currently being undertaken.

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