Gas chromatographic–mass spectrometric study of metabolites of C₂₁ and C₁₉ steroids in neonatal porcine testicular microsomes

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1. Microsomal fractions obtained from testes of 3-week-old piglets have been incubated, separately, with progesterone, 17-hydroxyprogesterone, 5-pregnene-3 β ,20 β -diol, 16 α -hydroxypregnenolone, 5-androstene-3 β ,17 α -diol and dehydroepiandrosterone. The metabolites, after derivatization, have been separated by capillary gas chromatography and identified by mass spectrometry. Quantification was by selected ion monitoring. 2. Progesterone was shown to be 17-hydroxylated and also converted into 4,16-androstadien-3-one (androstadienone). The major metabolite of 17-hydroxyprogesterone was 4-androstene-3,17-dione (4-androstenedione), but little, if any, androstadienone was formed, indicating that this particular biosynthesis did not require 17-hydroxylation. 3. The metabolites of 5-pregnene-3 β ,20 β -diol were found to be 17-hydroxypregnenolone, 3 β -hydroxy-5,16-pregnadien-20-one (16-dehydropregnenolone) and 5,16-androstadien-3 β -ol. 4. Dehydroepiandrosterone and 5-androstene-3 β ,17 α -diol were interconvertible but neither steroid acted as a substrate for 16-androstene formation. However, dehydroepiandrosterone was metabolized to a small quantity of 4-androstenedione. 5. Under the conditions used, no metabolites of 16 α -hydroxypregnenolone could be detected. 6. The present results, together with those obtained earlier, indicate that the neonatal porcine testis has the capacity to synthesize weak androgens, mainly by the 4-en-3-oxo steroid pathway. Although 16-androstenes cannot be formed from C₁₉ steroids, progesterone served as a substrate and may be converted directly to androstadienone, without being 17-hydroxylated first. The pathway to 5,16-androstadien-3 β -ol, however, involves 17-hydroxypregnenolone and 16-dehydropregnenolone as intermediates.

The mature boar testis is unusual in that it has the capacity to produce androgens, oestrogens and, in particular, the group of steroids known as the 16-androstenes (Claus, 1979; Booth, 1980; Gower, 1984). This group appears to be quantitatively more important than the androgens, as shown by analysis of testis tissue (Booth, 1975) and by studies *in vitro* (Ahmad & Gower, 1968) and *in vivo* (Hurden *et al.*, 1984). Two of the 16-androstenes, namely 5 α -androst-16-en-3-one (5 α -androstenone)

and 5 α -androst-16-en-3 α -ol (An- α), have aroused considerable interest since they elicit both primer and releaser pheromonal effects in pigs (Perry *et al.*, 1980; Kirkwood *et al.*, 1983).

The lipophilic nature of the 16-androstenes has a direct bearing on the quality of boar meat, as the odorous compounds 5 α -androstenone and An- α occur in adipose tissue (Patterson, 1968; Beery & Sink, 1971) and give rise to the problem of boar 'taint'. Booth (1975) has shown that the 16-androstenes are also present in neonatal porcine testis and that the quantities increase progressively with age, particularly after 18 weeks. It seems relevant, therefore, to study pathways of biosynthesis of the 16-androstenes in the immature as well as in the mature boar testis.

Abbreviations used: An- α and An- β , 5 α -androst-16-en-3 α - and 3 β -ols; Andien- β , 5,16-androstadien-3 β -ol; EICP, extracted ion current profile; DHA, dehydroepiandrosterone; MO, methyl oxime; TMS, trimethylsilyl; m.u., methylene units; SIM, selected ion monitoring.

Studies in this laboratory have indicated that the 16-androstenes are formed from pregnenolone and progesterone in the mature boar testis by pathways that do not involve 17-hydroxylation before side-chain cleavage, the first metabolites formed being 5,16-androstadien-3 β -ol (Andien- β) and 4,16-androstadien-3-one (androstadienone), respectively. Further metabolism of androstadienone results in the formation of 5 α -androst-16-en-3 β -ol (An- α) and 5 α -androst-16-en-3 β -ol (An- β) (Cooke *et al.*, 1983; Gower, 1984). Neither 17-hydroxypregnenolone (Katkov & Gower, 1970) nor 17-hydroxyprogesterone (Ahmad & Gower, 1968) could be converted into 16-androstenes, although the two classical pathways of testosterone biosynthesis via these intermediates occurred. The suggestion that 17-hydroxylation of pregnenolone was not a prerequisite for formation of Andien- β was given further support by Shimizu & Nakada (1976). Using [17- 3 H]pregnenolone, these workers showed that the Andien- β formed retained the 3 H at C-17.

A second pathway was suggested by Loke & Gower (1972) which involved 5-pregnene-3 β ,20 β -diol as an intermediate in the conversion of pregnenolone to Andien- β . As in the earlier studies of Gower & Ahmad (1967) and of Ahmad & Gower (1968), Loke & Gower (1972) confirmed the usual pathways of testosterone biosynthesis. Other metabolites of pregnenolone, including the 17- and 16 α -hydroxy derivatives, DHA, and the 5-androstene-3 β ,17 α - and 3 β ,17 β -diols, were found by Shimizu (1979). The contention that C₁₉ steroids, such as testosterone, 17 α -testosterone and DHA, do not serve as precursors for 16-androstene biosynthesis in the boar testis has received ample support (for references, see Gower, 1984).

In neonatal porcine testis, less information concerning steroid biosynthetic pathways is available. Mason *et al.* (1979) have provided evidence for the involvement of 17-hydroxypregnenolone and 3 β -hydroxy-5,16-pregnadien-20-one (16-dehydropregnenolone) as intermediates in Andien- β synthesis. Our own studies (Kwan *et al.*, 1984a) have shown that metabolites of pregnenolone in testicular microsomes of 3-week-old piglets included DHA, 5-androstene-3 β ,17 α - and 3 β ,17 β -diols, 17-hydroxypregnenolone and 16 α -hydroxypregnenolone as well as Andien- β . 16-Dehydropregnenolone was not detected. More recently, however, Kwan *et al.* (1984b) showed that 17-hydroxypregnenolone was converted to 16-dehydropregnenolone as well as to DHA, An- α , An- β , Andien- β and androstadienone. Incubation of 16-dehydropregnenolone under the same conditions gave rise to large quantities of Andien- β , together with DHA, An- α , An- β and androstadienone. These findings indicate that, unlike

the situation in the boar, 17-hydroxypregnenolone is an intermediate in 16-androstene synthesis in the immature porcine testis.

In order to study in more detail steroid biosynthetic pathways in neonatal porcine testis, we have utilized a number of putative precursors in the present work, namely: progesterone, 17-hydroxyprogesterone, 5-pregnene-3 β ,20 β -diol, 16 α -hydroxypregnenolone, 5-androstene-3 β ,17 α -diol and DHA. The metabolites were purified, derivatized and analysed by capillary g.c.-m.s. and quantified by SIM.

Experimental

Materials

16 α -Hydroxypregnenolone and 5-androstene-3 β ,17 α -diol were kindly supplied by Professor D. N. Kirk from the M.R.C. Steroid Reference Collection, Queen Mary College, London, U.K. An- α and An- β were synthesized from androst-16-en-3-one and epiandrosterone by using a modification of the method of Caglioti & Magi (1962). The purity of the reference steroids was checked before use by t.l.c. in chloroform/ethanol (49:1, v/v), run twice. AnalaR solvents were obtained from BDH (Poole, Dorset, U.K.) and redistilled. Cyclohexane was filtered through an activated charcoal column, followed by double-distillation. Pyridine was stored over NaOH pellets after distillation. The sources of all other materials have been described earlier (Kwan *et al.*, 1984a,b).

Methods

Microsomal incubations. Twelve testes from 3-week-old Landrace piglets were obtained by castration and the microsomes were prepared by differential centrifugation and then suspended in 0.25M-sucrose/0.05M-Tris/HCl buffer, pH 7.4, as before (Kwan *et al.*, 1984a). The protein content of the microsomal suspensions was estimated by the method of Lowry *et al.* (1951) and the homogeneity of the fraction was checked by 'marker-enzyme' assays as in Kwan & Gower (1984).

Microsomal suspensions of 3ml each (protein concn. 0.64mg/ml) were incubated at 37°C in the presence of air and NADPH (1.2mmol/l) separately for 1h with each of the following substrates previously dissolved in acetone (30 μ l): progesterone (0.17mmol/l), 17-hydroxyprogesterone (0.16mmol/l), 5-pregnene-3 β ,20 β -diol (0.17mmol/l), 16 α -hydroxypregnenolone (0.16mmol/l), 5-androstene-3 β ,17 α -diol (0.18mmol/l) and DHA (0.18mmol/l). The reaction was initiated, in each case, by the addition of cofactor and terminated by the addition of ethyl acetate (5 ml). In the control experiments, microsomal suspensions were pre-treated with ethyl acetate (5ml) and cofactor was

omitted before incubation. This is a modification of an earlier method (Kwan *et al.*, 1984a,b) in which boiled microsomes were used in control incubations; boiling did not necessarily inactivate all the enzymes under study. In order to correct for analytical losses, 0.1 μ Ci of [1,2,6,7- 3 H]androstenedione (sp. radioactivity 82 Ci/mmol) was added to each incubation mixture before extraction. The steroid extracts were purified by passing through Sephadex LH-20 columns before derivatization for g.c. (Kwan *et al.*, 1984a). The overall percentage recovery after the derivatization stage and purification by Lipidex (see below) was 70.1 ± 1.9 (S.E.M., $n = 12$). This was considered acceptable in view of the number of steps involved.

Gas chromatography. The steroid metabolites were derivatized as *O*-methyloxime-trimethylsilyl (MO-TMS) ethers and were purified before g.c. by filtration through Lipidex 5000 columns (Kwan *et al.*, 1984a). A glass capillary column (OV-1; 20 m $\times$ 0.32 mm internal diam.; Jaeggi, Trogen, Switzerland) was used and the g.c. conditions were as described earlier (Kwan *et al.*, 1984b). Preliminary identification of unknowns by g.c. was carried out by co-injection of a series of *n*-alkanes with the MO-TMS derivatives of the reference steroids (Kwan *et al.*, 1984a) (Table 1).

Gas chromatographic-mass spectrometric analysis. The g.c.-m.s. analyses were performed using a quadrupole Finnigan (Sunnyvale, CA, U.S.A.) MAT 4500 instrument (operating in the electron impact mode) coupled to an automatic gas chromatograph equipped with an Incos Series 2000 data system as described in detail by Kwan *et al.* (1984b). A silica column (SE 54; 30 m $\times$ 0.25 mm internal diam.; Jones Chromatography, Llanbradach, U.K.) was threaded directly into the ion source.

Once the metabolites from various testicular microsomal incubations of C_{21} and C_{19} steroids were identified by EICP analysis (see Kwan *et al.*, 1984a,b), major metabolites were quantified by SIM. SIM analyses were performed under the following conditions: column oven temperature programmed, 70°C for 1 min followed by 70–325°C at 20°C/min and finally holding at 325°C for 5 min; multiplier voltage, 1.5 kV. Other parameters were as described above. Two ions appropriate to each of ten steroids were monitored. This required specification of 14 different masses, since some occur in common (Table 1). In this SIM system, injections corresponding to 1 ng of starting material gave signal-to-noise ratios of between 6 and 15, reflecting the differing relative abundances of the ions monitored for each steroid derivative. This gave a detection limit of 100–200 pg injected. The internal standard chosen was 5 α -androstane-3 α ,17 α -diol because this does not occur in boar

testis (Ruokonen & Vihko, 1974) and, to our knowledge, has not been detected in neonatal porcine testis. However, the monosulphate of 5 α -androstane-3 α ,17 α -diol has been identified in boar testis (Ruokonen & Vihko, 1974) but, even if this conjugate was present in neonatal testis tissue, it would not have been extracted by ethyl acetate in our experiments. Quantification was achieved by determining the ratios of the internal standard ion, m/z 346, ($M-90$) and one ion per compound, as noted in Table 1. The second ion provided an identification check. Standard curves were prepared by SIM analyses during the same analytical run as the incubation extracts using duplicate determinations of 5 α -androstane-3 α ,17 α -diol (5 μ g) and increasing amounts of pure standard steroids 1–10 as follows: 0.1, 0.5, 5.0 and 25.0 μ g. The conditions for derivatization were identical with those for the incubation extracts. Linear regression equations were calculated (Table 2) and used to compute values for steroids of interest in the incubation extracts based on peak height ratios and corrected for procedural losses. Where the *syn* and *anti* isomers of the MO or MO-TMS derivatives of the steroids were partially resolved on the capillary column, the greater of the peaks was used for quantification purposes (Table 1). With this g.c.-m.s. system, it was not possible to calculate m.u. values owing to limitations of the data system used. Instead, retention times were expressed relative to 5 α -androstane-3 α ,17 α -diol (see Table 1).

Results

Table 1 shows the g.c. retention times of ten standard steroids (derivatized as MO, TMS or MO-TMS) likely to be encountered in the extracts obtained from porcine testicular incubations. The standard curves for the ten reference steroids obtained from SIM analysis are shown in Table 2.

G.c. and m.s. analyses

(i) **Progesterone incubation.** Initial identification of incubation metabolites by g.c. showed the presence of androstadienone MO at 23.13/23.17 m.u. and 17-hydroxyprogesterone MO-TMS at 29.20/29.24 m.u. (authentic standard 29.20/29.24 m.u.) (Fig. 1). The EICP showed the absence of 17-hydroxyprogesterone in the control incubation. The mass spectra of androstadienone and 17-hydroxyprogesterone derivatives are shown in Fig. 2 and are very similar to mass spectra of the corresponding authentic standards, major ions for which are listed in Kwan *et al.* (1984c). Both spectra contain ions at m/z 125, 137 and 153 derived from A ring fragmentation characteristic of 4-en-3-oxo- configuration (Brooks & Middleitch, 1973). The ions at 156, 158 and 188 in the

Table 1. Retention times and major characteristic ions monitored for reference steroid derivatives

Steroid	Derivative	Retention times		Ions monitored‡
		(m.u.)*	Relative†	
1 An- α	TMS	21.88	0.884	346(M^+), 331($M-15$)
2 Andien- β	TMS	22.65	0.923	344(M^+), 329($M-15$)
3 An- β	TMS	22.67	0.928	331($M-15$), 346(M^+)
4 Androstadienone	MO	23.13/23.17	0.965§	299(M^+), 268($M-31$)
5 5-Androstene-3 β ,17 α -diol	TMS	25.66	1.057	344($M-90$), 434(M^+)
6 5-Androstene-3 β ,17 β -diol	TMS	26.12	1.088	344($M-90$), 434(M^+)
7 DHA	MO-TMS	25.85	1.093	268 [$M-(90+31)$], 358($M-31$)
8 4-Androstenedione	MO	26.42/26.47	1.134§	344(M^+), 313($M-31$)
9 16-Dehydropregnenolone	MO-TMS	27.46	1.171	415(M^+), 384($M-31$)
10 16-Dehydroprogestosterone	MO	27.85/27.88	1.217§	370(M^+), 339($M-31$)
Internal standard				
5 α -Androstane-3 α ,17 α -diol	TMS	24.47	1.000	346($M-90$), 436(M^+)

* Retention times as m.u. obtained on an OV-1 column; paired values denote the *syn* and *anti* forms of the MO derivatives of a single compound.

† Retention times relative to 5 α -androstane-3 α ,17 α -diol (retention time 12.39 min = 1.000) obtained using an SE 54 column. Where *syn* and *anti* isomers existed (indicated as §), the greater of the peaks was quantified.

‡ The first ion listed was the one quantified; the second provided an identification check. For further details, see under 'Methods'.

Table 2. Standard curves obtained from SIM analysis of ten reference steroids

Calibration lines, determined by linear regression analysis, are expressed as $y = ax + b$, where y = height ratio of steroid peak to its internal standard, 5 α -androstane-3 α ,17 α -diol, x = amount of steroid in sample (μ g), a = slope, b = intercept, and r = correlation coefficient.

Steroid	Derivative	a	b	r
1 An- α	TMS	0.163	-0.047	0.997
2 Andien- β	TMS	0.473	-0.560	0.993
3 An- β	TMS	0.649	-0.876	0.994
4 Androstadienone	MO	0.264	-0.064	0.985
5 5-Androstene-3 β ,17 α -diol	TMS	0.094	-0.021	0.960
6 5-Androstene-3 β ,17 β -diol	TMS	0.541	-0.736	0.986
7 DHA	MO-TMS	1.015	-1.244	0.990
8 4-Androstenedione	MO	0.408	0.020	0.990
9 16-Dehydropregnenolone	MO-TMS	0.070	0.059	0.981
10 16-Dehydroprogestosterone	MO	0.246	-0.034	1.000

mass spectrum of 17-hydroxyprogesterone are specific for a 17 α -hydroxy-20-oxo- configuration and are also prominent in the mass spectrum of the related 17-hydroxypregnenolone derivative (Kwan *et al.*, 1984a). Other prominent ions are interpreted in Fig. 2. The concentration of androstadienone MO determined by SIM in the test experiment was found to be 2.5-fold higher than that in the control incubation (1.1 μ g). No 16-dehydroprogestosterone could be detected. An- α , Andien- β and An- β quantified in the test incubation hardly differed from those in the control (1.3–1.7 μ g). This was found to be true for all the other incubations where these steroid levels varied from 1.1 to 1.9 μ g.

(ii) 17-Hydroxyprogesterone incubation. There was little difference between the levels of androstadienone MO quantified by SIM in the test

(2.0 μ g) and control experiments (1.7 μ g) showing that the synthesis of the 16-androstene, androstadienone, does not require 17-hydroxylation. Instead, the major metabolite identified by g.c. in this incubation was 4-androstenedione MO at 26.42/26.47 m.u. and this was confirmed by the EICP. The concentration was found by SIM to be 31.0 times greater than in the control (0.9 μ g). As with incubation (i) above, no 16-dehydroprogestosterone was detected.

(iii) 5-Pregnene-3 β ,20 β -diol incubation. The g.c. (not shown) of the metabolites showed the presence of 17-hydroxypregnenolone MO-TMS at 28.77 m.u. (authentic standard 28.77 m.u.) and 16-dehydropregnenolone MO-TMS at 27.46 m.u. (see Table 1). These findings were checked by the EICP which also confirmed the presence of 17-

Fig. 1. Capillary g.c. profile of progesterone metabolites from porcine testicular microsomal incubation

Microsomes from testes of 3-week-old piglets were incubated with progesterone (0.17 mmol/l). The extracted metabolites, after purification, were derivatized as MO, TMS or MO-TMS and subjected to capillary g.c. on an OV-1 column. Metabolites tentatively identified were: (1) androstadienone at 23.13/23.17 m.u. and (2) 17-hydroxyprogesterone at 29.20/29.24 m.u. The twin peaks in each case represent the partially resolved *syn* and *anti* forms of the MO derivatives. The internal standards were: A, 5 α -androstane-3 α ,17 α -diol; B, stigmastanol; C, cholesteryl butanoate. S indicates substrate remaining and Ch, cholesterol (endogenous). For further details see under 'Methods'.

hydroxypregnenolone MO-TMS in the test, but not in the control, experiment. The m.s. analysis confirmed the presence of characteristic molecular and fragment ions for 17-hydroxypregnenolone MO-TMS at m/z (% abundance in parentheses) 505, M^+ [the molecular ion] (8); 474, $M-31$ (61); 188 (21); 158 (27); 156 (34); 129 (56) and 73 (100). The concentration of 16-dehydropregnenolone MO-TMS measured by SIM was 2.3 times greater than in the control (0.7 μ g). A small quantity of Andien- β was found by SIM [concn. 1.7 times the control level (0.6 μ g)].

(iv) *16 α -Hydroxypregnenolone incubation.* Amounts of 16-androstenes and other metabolites detected in the incubation extracts of the test were little different from the control experiments (1.1–1.5 μ g). This indicates that 16 α -hydroxypregnenolone did not act as an intermediate.

(v) *5-Androstene-3 β ,17 α -diol incubation.* DHA MO-TMS was the main metabolite identified at 25.85 m.u. by g.c. (see Table 1). The molecular and fragment ions characteristic of this compound and

occurring at the same relative retention time were obtained as follows (m/z , % abundance in parentheses): M^+ [the molecular ion], 389 (3); $M-31$, 358 (14); $M-(31+90)$, 268 (22); $M-129$, 260 (24) and 129 (100). The fragment ion, m/z 268, [$M-(90+31)$] monitored for this derivative in the test sample was 3.3-fold greater in abundance when compared with the control (0.7 μ g).

(vi) *DHA incubation.* Both 5-androstene-3 β ,17 α -diol and 4-androstenedione were identified as metabolites of this incubation by EICP at their relative retention times of 25.66 and 26.42/26.47 m.u., respectively. Only the mass spectrum of the diol was selected for plotting and showed the following characteristic ions at m/z (%): 434, M^+ [the molecular ion] (30); 344, $M-90$ (55); 329, $M-(15+90)$ (33); 305, $M-129$ (48) and 239, $M-[15+(2 \times 90)]$ (100). SIM showed that the amount of the diol in the test incubation was 2.2 μ g but it was not detected in the control, whereas 4-androstenedione was present at twice the concentration of that in the control (1.2 μ g).

Discussion

In this study we have demonstrated that capillary g.c. coupled with automated SIM analysis is a powerful tool for investigating steroid metabolic pathways. Whereas cyclic scanning g.c.-m.s. is initially necessary for absolute identification of metabolites, EICP data derived from this is imprecise quantitatively since only one or two data points may be recorded across an eluting capillary g.c. peak. By monitoring a carefully selected set of ions, precision of quantitative measurement is greatly improved, as ten or more data points can be collected across each g.c. peak and the increased measuring time per ion results in approximately 50-fold increase in sensitivity compared with scans across a large mass range. The detection limit obtained in this study could be improved by monitoring fewer ions, but in this instance we wished to retain the extra degree of certainty in compound identification afforded by monitoring two ions per compound. Coupling SIM with a powerful dedicated data system, we have shown that, from a single injection of a derivatized incubation extract, up to ten possible products may be quantified by the method of internal standards, and enzymically produced metabolites may be sensitively distinguished from endogenous material.

Although it is not possible to draw firm conclusions concerning predominant pathways based on relative amounts of metabolites recovered, the present results and those of Mason *et al.* (1979) and Kwan *et al.* (1984a,b) have highlighted certain major differences in the biosynthetic path-

Fig. 2. Mass spectra of (a) androstadienone MO and (b) 17-hydroxyprogesterone MO-TMS. After initial identification by capillary g.c. with an OV-1 column, the derivatized metabolites were characterized by taking mass spectra at their relative retention times on an SE 54 column. For further details see legend to Fig. 1 and under 'Methods'.

ways that occur in the neonatal, compared with the mature, porcine testis. In the former, pregnenolone appears to be converted into Andien- β via a pathway involving 17-hydroxypregnenolone and 16-dehydropregnenolone. Further metabolism of Andien- β must also occur since androstadienone, An- α and An- β were found (Kwan *et al.*, 1984b). When 5 α -androstene is incubated with testicular preparations from 3-week-old piglets, the conversion to An- α is always greater than to An- β (Kwan

& Gower, 1984). This finding is consistent with analytical data (Booth, 1975) which shows that the testicular concentration of An- α is some three times higher than that of An- β . In contrast, in the mature boar testis, the concentration of An- β is higher than that of An- α . Incubation of testicular homogenates of 3-week-old piglets with pregnenolone results in the formation of all the 16-androstenes mentioned above but only to the extent of 1–2% *in toto* (D. B. Gower & C. Orengo, unpublished

work) whereas the yield in mature boar testis is approx. 20% *in toto* (see Gower, 1972; Shimizu, 1979).

In contrast with the results obtained with pregnenolone, progesterone appears to be converted into androstadienone (as found also in the mature animal; Ahmad & Gower, 1968) but not via 17-hydroxyprogesterone or 16-dehydropregesterone as intermediates. Side-chain cleavage of 17-hydroxypregnenolone (Kwan *et al.*, 1984b) and 17-hydroxyprogesterone (the present work) undoubtedly occurs and yields, as expected, DHA or 4-androstenedione, respectively. Since our main interest was in the 16-androstenes, the production of testosterone was not measured in the present work, but we have shown the formation of weak androgens.

The finding that the concentration of biosynthesized 4-androstenedione was 31 times than that in the control incubation may indicate that the 4-en-3-oxo pathway is important. If a high proportion of 17-hydroxyprogesterone was metabolized by side-chain cleavage, then it may help to explain why it is not available for conversion to 16-dehydropregesterone and thence to androstadienone. The validity of such an explanation also depends on the assumption that the '4,16-androstadienone synthase' (Brophy & Gower, 1974; Kaufman & Schubert, 1980) would be active in catalysing the direct conversion of progesterone to the 16-androstene in neonatal porcine testis. However, it is not possible to ascertain this from the present data.

Loke & Gower (1972) suggested that 5-pregnene-3 β ,20 β -diol was an intermediate in Andien- β synthesis from pregnenolone in boar testis homogenates and that the diol might give rise directly to Andien- β , although such a pathway might well be much less important quantitatively. The present results indicate that 17-hydroxypregnenolone, 16-dehydropregnenolone and Andien- β were formed, but it is difficult to explain this unless the diol was first converted back to pregnenolone (Scheme 1). This could then be metabolized to Andien- β as shown by Mason *et al.* (1979) and Kwan *et al.* (1984b). The 20 β -hydroxysteroid oxidoreductase (EC 1.1.1.53) required for the formation of pregnenolone from 5-pregnene-3 β ,20 β -diol is usually considered to be a cytosolic enzyme (Jeffery, 1980) and would not be expected in the microsomal fractions used and which were free of cytosolic contamination (Kwan & Gower, 1984). Thus, our present results may imply that a microsomal 20 β -hydroxysteroid oxidoreductase occurs in the microsomal fraction. The work of Kwan *et al.* (1984b) also indicates that some of the 17-hydroxypregnenolone formed in this way would be utilized for DHA formation, which would be further metabolized to 4-androstenedione and/or 5-androstene-3 β ,17 α -diol.

The fact that 5-androstene-3 β ,17 α -diol and DHA were not converted into 16-androstenes is in keeping with earlier studies (see Gower, 1984) in which several C₁₉ steroids were used as substrates but without success. It is of interest that the 3 β ,17 α -

Scheme 1. Biosynthetic pathways of 16-androstenes and other C₁₉ steroids in immature porcine testicular microsomes. The major pathways are denoted by bold arrows. The arrow (→) indicates no metabolites found. Postulated pathways are indicated as ---->. Data are obtained from the present work and from Gower (1984), Kwan *et al.* (1984b) and Kwan & Gower (1984).

diol and DHA could be interconverted, whereas the $3\beta,17\beta$ -diol was not detected as a metabolite of DHA and may have been metabolized rapidly. The boar testis microsomal 17β -hydroxysteroid oxidoreductase (EC 1.1.1.64) is well-documented (Inano & Tamaoki, 1974; Cooke & Gower, 1977) but the 17α -hydroxysteroid oxidoreductase (EC 1.1.1.148) is a cytosolic enzyme occurring predominantly in liver and kidney of a number of species, including the rabbit (Williamson, 1979). The present results suggest that there may be a microsomal 17α -hydroxysteroid oxidoreductase (albeit weakly active) in neonatal porcine testicular microsomes.

When 16α -hydroxypregnenolone was used as a putative precursor, no 16-androstenes were formed. A parallel finding was reported by Matsui & Fukushima (1970) who used 16α -hydroxyprogesterone and boar testis preparations.

Administration of human chorionic gonadotrophin or lutropin to boars is known to increase plasma 5α -androstene levels (Andresen, 1975; Claus & Giménez, 1977). Whether gonadotropins are involved in increasing the testicular biosynthesis of other 16-androstenes, and in causing the switch to An- β as opposed to An- α production as testicular development occurs, requires further investigation.

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