The Biosynthesis of Some Androst-16-enes from C₂₁ and C₁₉ Steroids in Boar Testicular and Adrenal Tissue

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1. The formation of androst-16-enes from [4-¹⁴C]progesterone has been investigated with long-term incubations and short-term kinetic studies. After 4 hr., 1.7 and 10.3% respectively of 3 α - and 3 β -hydroxy-5 α -androst-16-enes were formed in boar testis minces, but much smaller yields were obtained in boar adrenal. Both tissues formed small quantities of androsta-4,16-dien-3-one. 2. The amounts of androst-4-ene-3,17-dione and testosterone isolated were small, suggesting that androst-16-ene formation may occur preferentially in the boar testis. 3. In the absence of tissue no radioactive androst-16-enes were formed. 4. Incubation of both [4-¹⁴C]pregnenolone and [7 α -³H]progesterone resulted in 3 α - and 3 β -hydroxy-5 α -androst-16-enes containing ³H/¹⁴C ratios of near unity and confirmed that both C₂₁ steroids were precursors. A similar incubation with 17 α -hydroxy[4-¹⁴C]-progesterone and [7 α -³H]progesterone gave the same Δ^{16} -alcohols, but they contained only ³H, indicating that side-chain cleavage of pregnenolone and progesterone occurred before 17 α -hydroxylation. 5. Dehydroepiandrosterone, testosterone, testosterone acetate and 16-dehydropregesterone were not found to be precursors of Δ^{16} -steroids. 6. A pathway is proposed for the biosynthesis of 3 α - and 3 β -hydroxy-5 α -androst-16-enes from pregnenolone and progesterone; this may involve androsta-4,16-dien-3-one as an intermediate, but excludes 17 α -hydroxyprogesterone, testosterone and dehydroepiandrosterone.

The formation of androst-16-enes from pregnenolone* in boar testis tissue has been described (Gower & Ahmad, 1967*a*). The high yield of An- β obtained suggested that the biosynthetic pathway might involve only a small number of intermediates and be one of major importance in this species, in contrast with guinea pigs and rabbits (Gower & Haslewood, 1961) and rats (Stylianou, Forchielli & Dorfman, 1961; Ahmad & Gower, 1966; Gower, 1966). Moreover, the yields of androgens obtained with boar testis from pregnenolone were relatively small and these findings, coupled with the inability

of labelled testosterone to form Δ^{16} -steroids under the same conditions (Gower & Ahmad, 1966), led us to postulate that much of the C₂₁ precursor, pregnenolone, is channelled to the formation of C₁₉ Δ^{16} -compounds rather than to the formation of androgens. Accordingly, the biosynthetic pathway has been studied in more detail, first by the finding that progesterone is also a precursor of Δ^{16} -compounds in a long-term (4 hr.) incubation, and then by short-term kinetic experiments in an attempt to elucidate possible intermediates. These studies have already been briefly reported (Ahmad & Gower, 1967). To find out the relative efficiencies of the pathways leading to Δ^{16} -steroids and to the androgens from progesterone, the metabolism of 17 α -hydroxyprogesterone has been studied as well as that of some C₁₉ compounds, testosterone and DHA. Since testosterone acetate is known to be an intermediate in the transformation of progesterone into testosterone in micro-organisms (Fonken, Murray & Reineke, 1960; Rahim & Sih, 1966; Carlström, 1967) and has been postulated to be one in human tissues (Kase, Forchielli & Dorfman, 1961), this compound was also tested as a precursor of Δ^{16} -steroids.

* Abbreviations: pregnenolone, 3 β -hydroxypregn-5-en-20-one; progesterone, pregn-4-ene-3,20-dione; 16 α -hydroxyprogesterone, 16 α -hydroxypregn-4-ene-3,20-dione; 17 α -hydroxyprogesterone, 17 α -hydroxypregn-4-ene-3,20-dione; 16-dehydropregesterone, pregn-4,16-diene-3,20-dione; testosterone, 17 β -hydroxyandrost-4-en-3-one; testosterone acetate, 17 β -acetoxyandrost-4-en-3-one; 11 β -hydroxyandrostenedione, 11 β -hydroxyandrost-4-ene-3,17-dione; androstenedione, androst-4-ene-3,17-dione; adrenosterone, androst-4-ene-3,11,17-trione; DHA (dehydroepiandrosterone), 3 β -hydroxyandrost-5-en-17-one; An- α , 3 α -hydroxy-5 α -androst-16-ene; An- β , 3 β -hydroxy-5 α -androst-16-ene.

MATERIALS AND METHODS

Authentic Δ^{16} -steroids, solvents and materials for column, paper, thin-layer and gas-liquid chromatography were as described by Gower & Ahmad (1967a). Isotopically labelled steroids, with the exception of [4- 14 C]testosterone acetate, were purchased from The Radiochemical Centre, Amersham, Bucks. Their specific radioactivities were (mc/m-mole): [4- 14 C]pregnenolone, 24.0; [4- 14 C]progesterone, 21.7; [7- α - 3 H]progesterone, 300; 17 α -hydroxy[4- 14 C]progesterone, 35.9; [4- 14 C]DHA, 27.5; [4- 14 C]testosterone, 29.2.

Preparation and purification of [4- 14 C]testosterone acetate. This was made by acetylating [4- 14 C]testosterone (5 μ C) with pyridine (0.1 ml.) and acetic anhydride (0.1 ml.) and allowing the mixture to stand overnight. After the solvents had been evaporated off and carrier testosterone acetate (30 μ g.) added, the crude acetate was subjected to chromatography on alumina (Gower & Ahmad, 1967a) and eluted with 50 ml. of benzene containing 0.2% of ethanol. Preliminary experiments with unlabelled testosterone acetate had shown that it was eluted in this fraction. Any radioactive testosterone remaining unchanged after acetylation was eluted from the same column with benzene containing 0.5% of ethanol (50 ml.) (cf. Table 1 in Gower & Ahmad, 1967a). The specific radioactivity of the radioactive testosterone acetate was measured as described below and found to be 19.0 mc/m-mole. This material was then submitted to thin-layer chromatography in chloroform-ethyl acetate (9:1, v/v) with testosterone and testosterone acetate as markers. Radioautography revealed only one radioactive spot, whose mobility corresponded with that of testosterone acetate. The specific radioactivity of this steroid after elution (Gower, 1966) was 16.5 mc/m-mole. Thin-layer chromatography of the material eluted from alumina after testosterone acetate showed that <0.1% of the radioactive testosterone was unchanged after acetylation. For specific-radioactivity measurement the weight of carrier testosterone acetate was determined by using gas-liquid chromatography, and the radioactivity was measured as described below. With a 5 ft. column of Gas Chrom Q (100-120 mesh) coated with cyclohexanedimethanol succinate (0.6%)—methyl silicone gum (JXR) (0.75%), testosterone acetate had a retention time of 13.3 min. at 220° and a carrier gas (argon) flow rate of 50 ml./min.

Measurement of radioactivity. This was performed with a Beckman automatic scintillation spectrometer (model 1650). Samples were dissolved in xylene (5 ml.) containing 0.3% of 2,5-diphenyloxazole. Under these conditions counting efficiency for 14 C was 88% with a background of 45 counts/min. When 3 H and 14 C were counted simultaneously the efficiencies were 34% and 67% with backgrounds of 10 and 32 counts/min. respectively. All counts were corrected for cross-over from the 14 C channel to the 3 H channel, which amounted to 22%, and from the 3 H to the 14 C channel, which was 0.3%.

Preparation of tissue and incubation conditions. Boar adrenals and testes were obtained fresh at the slaughterhouse, cooled in ice and immediately transferred to the laboratory. Minces of whole testis and, in some cases, adrenal cortex, were incubated with labelled precursors under conditions described in detail by Gower & Ahmad (1967a), except that the time of incubation was varied in some experiments (see the Results section). Extraction of metabolites was performed with ethyl acetate after the

addition of carrier steroids (50 μ g. each of An- α , An- β , androsta-4,16-dien-3-one, androstenedione, testosterone and 17 α -hydroxyprogesterone). The crude extracts were submitted to column chromatography on alumina and fractions I-VI eluted exactly as described by Gower & Ahmad (1967a).

RESULTS

Formation of androst-16-enes from [4- 14 C]progesterone by boar testis and adrenal cortex. A long-term (4 hr.) incubation of minces of boar testis (1.0 g.) and adrenal cortex (1.2 g.) was performed with [4- 14 C]progesterone (1.3 μ C). Fractions I-III, which would contain androst-16-enes formed during the incubation, were submitted to thin-layer chromatography in toluene-ethyl acetate (9:1, v/v), run three times (Gower, 1964). Radioautography revealed two dense spots from the testis extract with the mobilities of An- α and An- β . The corresponding zones from the adrenal extract, however, contained much less radioactivity. In addition, small amounts of a compound with the mobility of authentic androst-4,16-dien-3-one were seen. The amounts of this metabolite and of An- α and An- β from the adrenal preparation were so small that identification was based only on rechromatography in benzene-ether (9:1, v/v) and on the preparation of the DNP-hydrazone of the $\Delta^{4,16}$ -ketone (Ahmad & Gower, 1966) and of the acetates of the two Δ^{16} -alcohols (Gower & Ahmad, 1967a). Thin-layer chromatography of the parent compounds and of the derivatives gave radioactive spots that, in all cases, coincided exactly with the authentic compounds used as markers.

Purification of the radioactive compounds formed by the testis preparation corresponding to authentic An- α and An- β was more rigorous. After rechromatography in benzene-ether (9:1, v/v) and elution of the zones corresponding to An- α and An- β , these were mixed, authentic carriers were added (200 μ g. of each) and the steroids were eluted from an alumina column in fractions (5 ml.) with benzene-light petroleum (1:1, v/v), as described by Gower & Ahmad (1967a). The radioactivity and the weight of the steroid were measured in each fraction. Coincidence of peaks was obtained for both An- α and An- β , as well as constant specific radioactivity over each peak (Fig. 1). Final identification was achieved by pooling the eluted fractions corresponding to each of the two Δ^{16} -steroids, preparing the acetates and running them in toluene-ethyl acetate (19:1, v/v). Radioautography revealed a single radioactive spot in each case, which corresponded to the mobility of the authentic acetate used as marker. Hydrolysis of the radioactive zones (Bush & Willoughby, 1957) gave products that, on thin-layer chromatography in toluene-ethyl acetate (9:1, v/v), ran with authentic An- α and An- β . The

Fig. 1. Alumina-column chromatography, with light petroleum-benzene (1:1, v/v) as eluent, of An- α (I) and An- β (II) obtained after incubation of [4- 14 C]progesterone with minced boar testis tissue. The weight of steroid (○) was determined by gas-liquid chromatography, and radioactivity (●) by liquid-scintillation counting. ▲, Specific radioactivity (counts/min./ μ g.)

Table 1. Percentage conversion in vitro of various steroids into androst-16-enes and other metabolites by boar adrenal and testis tissue

Results are expressed as percentages of the radioactivity extracted from the equivalent of 1 g. wet wt. of tissue after correction for analytical losses. N.D., Not detected.

Precursor tested	Conversion (%)						
	Progesterone		17 α -Hydroxy-progesterone	Testosterone		DHA	Testosterone acetate
	Adrenal	Testis	Testis	Adrenal	Testis	Testis	Testis
Metabolite							
Androsta-4,16-dien-3-one	0.04	0.008 0.006 0.007	N.D.	N.D.	N.D.	<0.01	<0.01
An- α	0.007	1.7	N.D.	N.D.	N.D.	N.D.	N.D.
An- β	0.015	10.3	N.D.	N.D.	N.D.	<0.01	<0.01
17 α -Hydroxyprogesterone	0.10	0.15					
Androstenedione	0.37	0.04	0.63	0.02	0.01		0.38
Testosterone	1.3	0.24	0.60				7.5
11 β -Hydroxyandrostenedione				0.024	N.D.		
3 α ,17 β -Dihydroxy-5 α -androstane				0.013	0.125		
3 β ,17 α -Dihydroxy-5 α -androstane				0.75	0.23		
16 α -Hydroxyprogesterone	0.024						

* Results from three experiments.

yields of the androst-16-enes, corrected for analytical losses, that were obtained in this experiment are shown in Table 1.

Formation of other metabolites of progesterone in boar adrenal and testis tissue. From preliminary experiments, fraction IV from the alumina column was known to contain androstenedione and testosterone acetate, if they were formed in the incubation, as well as any unchanged [4- 14 C]progesterone, and fraction V would contain any testosterone and 17 α -hydroxyprogesterone. These fractions were chromatographed on paper in the Bush (1952)

system A together with authentic marker steroids, and the zones corresponding to progesterone, androstenedione and testosterone were eluted. It was known that testosterone acetate moved with progesterone in this system, so that thin-layer chromatography in benzene-ethyl acetate-methanol (66:33:1, by vol.) was used to separate them. Radioautography revealed radioactive zones coinciding with the spots produced in u.v. light (254 m μ) by carrier progesterone and testosterone acetate. Rechromatography in chloroform-ethyl acetate (4:1, v/v) confirmed the presence of

Scheme 1. Flow-sheet for the isolation and purification of metabolites of $[4\text{-}^{14}\text{C}]$ progesterone more polar than Δ^{16} -steroids. TLC, Thin-layer chromatography.

progesterone but not that of testosterone acetate. Androstenedione was confirmed by thin-layer chromatography in benzene-acetone (4:1, v/v), when the radioactive zone coincided exactly with the position of carrier androstenedione as revealed by u.v. light (Scheme 1).

The testosterone zone eluted from paper might have also contained 17α -hydroxyprogesterone, which was not separated in the Bush (1952) system A. Thin-layer chromatography in chloroform-methanol (49:1, v/v) also failed to resolve them. The mixture therefore was acetylated and run in benzene-ethyl acetate (3:2, v/v), which easily separated testosterone acetate from unchanged 17α -hydroxyprogesterone. Radioautography revealed radioactive zones corresponding to both compounds from the adrenal and the testis extracts. The identity of the testosterone acetate formed was proved by hydrolysis and chromatography of the product in benzene-ethyl acetate (1:3, v/v). There was only one radioactive spot, which coincided exactly with the spot produced in u.v. light by carrier testosterone.

Fraction VI from the alumina column was known

from preliminary experiments to contain 16α -hydroxyprogesterone. Accordingly, carrier 16α -hydroxyprogesterone was added and the fraction was chromatographed in chloroform-ethanol (9:1, v/v) together with the authentic steroid as marker. Radioautography revealed a radioactive zone from the adrenal-tissue extract that coincided exactly with the spot produced in u.v. light by the authentic carrier. There was no evidence of the formation of this metabolite in the testis preparation. The yields, of the compounds formed from progesterone, corrected for analytical losses, are given in Table 1.

Kinetic studies of the formation of androst-16-enes from $[4\text{-}^{14}\text{C}]$ progesterone by boar testis tissue. Portions of a mince of boar testis (1.0, 1.3, 1.4 and 1.7 g.) were incubated in separate flasks with $[4\text{-}^{14}\text{C}]$ progesterone (Gower & Ahmad, 1967a), and the reaction was terminated with ethyl acetate after 5, 15, 30 and 120 min. respectively. After extraction, recoveries of the radioactivity initially added were 87, 80, 70 and 86% respectively. Preliminary purification was achieved by alumina-column chromatography, and fractions I-III were chromatographed on thin layers in toluene-ethyl acetate

EXPLANATION OF PLATE 1

Radioautographs of thin-layer chromatograms of fractions II (upper) and III (lower) from the alumina column obtained after incubation of [4- 14 C]progesterone with minces of boar testis tissue. The incubation was terminated at (a) 5 min., (b) 15 min., (c) 30 min. or (d) 120 min. The positions of some authentic Δ^{16} -steroids run as markers on the same plates are shown on the right: 1, oestra-1,3,5(10),16-tetraen-3-ol (10.0 cm.); 2, androsta-4,16-dien-3-one (8.7 cm.); 3, An- α (7.5 cm.); 4, 3 α -hydroxy-5 β -androst-16-ene (6.6 cm.); 5, 3 β -hydroxyandrost-5,16-diene (5.4 cm.); 6, An- β (4.8 cm.); O, origin. The elution system used was benzene-ether (9:1, v/v), the solvent being allowed to run to 16 cm. three times.

Table 2. *Percentage conversion of [4-¹⁴C]progesterone into various metabolites by minces of boar testis at different time-intervals*

Results are expressed as percentages of the radioactivity extracted from the equivalent of 1 g. wet wt. of tissue after correction for analytical losses.

Metabolite	Time (min.)	Conversion (%)			
		5	15	30	120
Androsta-4,16-dien-3-one		0.037	0.003	0.002	0.002
An- α		0.26	0.49	1.2	1.8
An- β		6.1	11.5	13.5	10.3
Androstenedione		0.85	0.07	0.07	0.07
Testosterone		0.10	0.05	0.03	0.04
17 α -Hydroxyprogesterone		0.07	0.02	0.02	0.03
Unchanged progesterone		3.63	0.54	0.17	0.08

Table 3. *Percentage incorporation of ³H and ¹⁴C into androst-16-enes and other metabolites obtained after incubation of ³H- and ¹⁴C-labelled C₂₁ steroids with minces of boar testis*

Results are expressed as percentages of the radioactivity extracted from the equivalent of 1 g. wet wt. of tissue, after correction for analytical losses.

Metabolite	Incubation 1 ([4- ¹⁴ C]pregnenolone and [7 α - ³ H]progesterone)			Incubation 2 ([7 α - ³ H]progesterone and 17 α -hydroxy[4- ¹⁴ C]progesterone)		
	³ H (%)	¹⁴ C (%)	³ H/ ¹⁴ C ratio	³ H (%)	¹⁴ C (%)	³ H/ ¹⁴ C ratio
Androsta-4,16-dien-3-one	0.007	0.064	0.1	0.006	0	—
An- α	1.92	1.62	1.1	1.5	0	—
An- β	11.1	10.6	1.1	10.0	0	—
Androstenedione	0.46	0.86	0.5	0.21	0.33	0.63
Testosterone	0.37	1.23	0.3	0.32	0.53	0.6
17 α -Hydroxyprogesterone	0.014	0.005	0.3	0.07	0.75	0.1
Progesterone	2.17	0.011	197	1.9	0	—
Pregnenolone	0	9.0	—	0	0	—
DHA	0	0.007	—	0	0	—

(9:1, v/v), followed by benzene-ether (9:1, v/v). As in the long-term experiment with [4-¹⁴C]-progesterone described above, radioautography gave evidence of the formation of androsta-4,16-dien-3-one, An- α and An- β . Visual examination of the series of radioautographs (Plate 1) showed that the $\Delta^{4,16}$ -3-ketone was formed from progesterone after 5 min., but thereafter the amount declined to extremely low values; in contrast, the amounts of An- α and An- β increased as the time of incubation increased. After further purification and final identification of these metabolites had been achieved as described above, and the yields had been expressed in terms of 1 g. wet wt. of tissue (Table 2), it was found that An- β was formed in as much as 6% yield in 5 min., increasing to some 14% by 30 min. and thereafter decreasing to 10%. An- α was formed more slowly and to a smaller extent, reaching 2% after 120 min. Androst-4,16-dien-3-one was presumably formed very early in the incubation, so that by the time of the first analysis (5 min.) the amount was already low, and it decreased even further as the incubation proceeded,

suggesting that this ketone was a precursor of An- α and An- β .

A number of other metabolites were isolated from these incubations and tentatively identified as above (Table 2). Except for androstenedione (0.85% yield at 5 min.) the yields were small and became even lower as the time of incubation increased.

Metabolism of [4-¹⁴C]pregnenolone plus [7 α -³H]-progesterone and [7 α -³H]progesterone plus [4-¹⁴C]-17 α -hydroxyprogesterone by boar testis tissue. These experiments were designed (a) to confirm that both pregnenolone and progesterone were precursors of androst-16-enes and (b) to determine whether these compounds were formed from 17 α -hydroxyprogesterone by the usual Δ^4 pathway, already well known for C₁₉-steroid biosynthesis, or from progesterone by a hitherto unrecognized pathway. A brief report has appeared (Gower & Ahmad, 1967b).

Two flasks containing portions of a boar testis mince (1.8 g. and 1.9 g.) were incubated under conditions identical with those described by Gower & Ahmad (1967a), with [4-¹⁴C]pregnenolone (0.66 μ C)

Scheme 2. Flow-sheet for the isolation and purification of some metabolites of [4-¹⁴C]DHA, testosterone and testosterone acetate more polar than Δ^{16} -steroids. TLC, Thin-layer chromatography.

plus [7 α -³H]progesterone (0.86 μ C) and [7 α -³H]-progesterone (0.86 μ C) plus 17 α -hydroxy[4-¹⁴C]-progesterone (0.55 μ C). A third flask contained

[4-¹⁴C]progesterone (0.86 μ C) but no tissue. After incubation for 2 hr. and extraction with ethyl acetate, radioactive androst-4,16-dien-3-one, An- α ,

An- β , androstenedione, testosterone and 17 α -hydroxyprogesterone were isolated and purified as described above. The percentage of ^3H and ^{14}C incorporated and the ratios of ^3H radioactivity to ^{14}C radioactivity found for each compound isolated are given in Table 3. In the control experiment, when no tissue was present, no radioactivity was found in fractions I–III from the alumina column; it was recovered entirely in fraction IV. Purification of this radioactive material proved it to be progesterone, showing that the three Δ^{16} -compounds formed when tissue was present were not artifacts.

In the experiment with labelled pregnenolone and progesterone, the $^3\text{H}/^{14}\text{C}$ ratios of near unity for An- α and An- β confirm our earlier findings that both C_{21} compounds are precursors. In the experiment with [^3H]progesterone and 17 α -hydroxy[^{14}C]progesterone, the androsta-4,16-dien-3-one, An- α and An- β isolated were devoid of ^{14}C radioactivity, and the incorporations of ^3H were close to the values obtained with [^3H]- or [^{14}C]progesterone in other experiments (Tables 1 and 2). These findings indicated that the removal of the progesterone side chain occurred before 17 α -hydroxylation. The experiment gave no indication, however, whether the C-16–C-17 double bond was introduced into the molecule before or after the removal of the side chain. Accordingly, unlabelled 16-dehydroprogesterone (2mg.) was incubated with a boar testis mince (2.2g.) under the same conditions as before. Fractions I–III from alumina-column chromatography of the extracts were chromatographed on a thin layer in toluene–ethyl acetate (9:1, v/v) and the zones corresponding to androst-4,16-dien-3-one, An- α and An- β were eluted. Gas-liquid chromatography of these eluates revealed no peaks with the retention times of authentic Δ^{16} -steroids, thus indicating that 16-dehydroprogesterone was not an

intermediate in the formation of androst-16-enes from progesterone and that the C-16–C-17 double bond was not formed before side-chain cleavage.

Metabolism of [4- ^{14}C]testosterone acetate, [4- ^{14}C]testosterone and [4- ^{14}C]DHA by boar testis and adrenal tissue. [4- ^{14}C]Testosterone (1.8 μC) was incubated for 3hr. with a mince of boar testis (1.65g.) and with adrenal cortex (3.0g.); [4- ^{14}C]DHA (1.5 μC) and [4- ^{14}C]testosterone acetate (0.4 μC) were incubated with 1.5g. and 1.6g. of boar testis mince respectively.

Initial purification was achieved by chromatography of the ethyl acetate extracts on alumina followed by thin-layer chromatography of fractions I–III. In the experiment with labelled testosterone, radioautography revealed the presence of a compound with the mobility of An- α in the testis extract only, but acetylation failed to confirm its identity. Similarly, both radioactive testosterone acetate and radioactive DHA were shown to give rise to only trace amounts of compounds that had the mobility in thin-layer-chromatographic systems of the $\Delta^{4,16}$ -3-ketone and An- β . Some metabolites of labelled testosterone, DHA and testosterone acetate more polar than Δ^{16} -steroids were isolated from fractions IV–VI from the alumina column and purified further according to Scheme 2. Androstenedione was isolated from fraction IV by thin-layer chromatography in various systems and its specific radioactivity determined (Table 4), either by recrystallization or by the method described by Gower (1966). The presence of unchanged testosterone in fraction V was confirmed; this fraction also contained 11 β -hydroxyandrostenedione from the adrenal extract only. Final identification of this metabolite was achieved by oxidation (Chamberlain, Jagarinec & Ofner, 1965) and rechromatography, after which the radioactive zone corresponded exactly with the spot produced in u.v. light by

Table 4. *Purification of radioactive androstenedione and testosterone obtained after the incubation of [4- ^{14}C]DHA and [4- ^{14}C]testosterone with boar adrenal or testis tissue*

Purification was achieved either by recrystallization to constant specific radioactivity or by thin-layer chromatography (see the text). Values in parentheses are the weights (in mg.) of crystals, or the weights (in $\mu\text{g.}$) recovered from thin-layer plates. Specific radioactivity values are in counts/min. per mg. or $\mu\text{g.}$

Incubation with [4- ^{14}C]testosterone			Incubation with [4- ^{14}C]DHA		
No. of crystallizations	Sp. radioactivity and wt. (mg.) of androstenedione recovered		No. of runs	Sp. radioactivity and wt. ($\mu\text{g.}$) of product recovered	
	Adrenal	Testis		Androstenedione (testis)	Testosterone (testis)
0	175 (10.1)	127 (11.2)	0	1101 (25.1)	1720 (23.4)
1	115 (7.8)	106 (8.4)	1	843 (20.0)	1600 (20.7)
2	105 (5.3)	118 (5.5)	2	852 (16.7)	1420 (18.4)
3	91 (4.4)	116 (4.8)	3	790 (11.0)	—

marker adrenosterone. The zone that might contain androstenediols and that separated from 11β -hydroxyandrostenedione in chloroform-ethanol (19:1, v/v) (Scheme 2) was shown to contain both $3\alpha,17\beta$ - and $3\beta,17\alpha$ -dihydroxy- 5α -androstane by thin-layer chromatography followed by oxidation; this gave a radioactive compound that had the same mobility as 5α -androstane-3,17-dione. The yields of these metabolites are given in Table 2.

DISCUSSION

The experiments described in the present paper confirm earlier results in which pregnenolone was found to serve as a precursor of Δ^{16} -steroids in boar testis tissue and further reveal that progesterone is a precursor. Moreover, the inability of 17α -hydroxyprogesterone to yield Δ^{16} -steroids indicates that the removal of the side chain from pregnenolone or progesterone occurs before 17α -hydroxylation. This is in marked contrast with earlier experiments on C_{19} steroid biosynthesis, the results of which have been consistent with the view that 17α -hydroxylation of pregnenolone and progesterone occurs before cleavage of the side chain and oxidation at C-17, to give the 17-oxo steroids, DHA or androstenedione. A pathway for the conversion of C_{21} into C_{19} steroids without 17α -hydroxylation has also been postulated by Kase *et al.* (1961), who used human ovaries. The biosynthesis of both androstenedione and testosterone appeared to take place from progesterone without 17α -hydroxyprogesterone as an intermediate. This type of biosynthesis has not been confirmed by experiments with other mammalian tissues but is well known in some strains of micro-organisms, and testosterone acetate has been demonstrated as the intermediate in the conversion

of progesterone into testosterone (Fonken *et al.* 1960; Rahim & Sih, 1966; Carlström, 1967). In the present experiments, however, radioactive testosterone acetate could not be isolated from incubations in which labelled progesterone was used as precursor, even after short periods, and testosterone acetate, when incubated with boar testis tissue, did not give rise to Δ^{16} -steroids (Table 1).

16-Dehydropregesterone was also considered as a possible intermediate in the conversion of progesterone into Δ^{16} -steroids. After incubating this with boar testis tissue, however, we were unable to isolate An- α , An- β or androsta-4,16-dien-3-one, and this may indicate that the double bond at C-16-C-17 is inserted in the molecule after, and not before, 17α -hydroxylation.

The kinetic study of progesterone metabolism (Plate 1 and Table 2) suggests that androsta-4,16-dien-3-one is formed rapidly but is thereafter metabolized. It is possible that reduction takes place first to 5α -androst-16-en-3-one and then to An- α and An- β . The ketone has recently been isolated from the fat of mature boars (Patterson, 1968) but it has not been found in the fat of hogs or immature pigs, suggesting that it has a testicular origin.

The yields of androstenedione and testosterone obtained from progesterone in the present experiments (Tables 1 and 2) and from pregnenolone (Gower & Ahmad, 1967*a*) were smaller with testis tissue from boars than with the same tissue from rats (Ahmad & Gower, 1966, and unpublished work). Moreover, yields of Δ^{16} -steroids in rat testis preparations were only 0.1% or less and these results suggest that, in the boar, the biosynthesis of Δ^{16} -steroids occurs preferentially. It is noteworthy that An- α and An- β were first isolated from pigs'

Scheme 3. Possible pathways of metabolism of pregnenolone and progesterone in boar testis based on results given by Gower & Ahmad (1967*a*) and in the present paper. $\rightarrow$, possible intermediate; $- - \rightarrow$, no conversion found.

testes (Prelog & Ruzicka, 1944). Suggested pathways of metabolism of pregnenolone and progesterone in boar testis are shown in Scheme 3.

The large difference in the yields of androsta-4,16-dien-3-one obtained from pregnenolone (Gower & Ahmad, 1967a) and progesterone (Tables 1 and 3) has been confirmed in a number of experiments. The use of double-isotope labelling has shown that ten times more pregnenolone than progesterone is converted into the $\Delta^{4,16}$ -3-ketone. The reason for this is unknown at present, but it may be that pregnenolone is converted first into 3 β -hydroxy-androsta-5,16-diene and then into androsta-4,16-dien-3-one, thus bypassing progesterone. The $\Delta^{5,16}$ -3 β -ol was formed from pregnenolone in 0.2% yield by a virilizing adrenal tumour (Gower & Stern, 1967) but has not so far been detected in our studies with boar testis and adrenal gland.

The physiological significance of androsta-4,16-dien-3-one, An- α , An- β and 5 α -androst-16-en-3-one is still unknown but, on the basis of the intense musk-like odour of these compounds, Sink (1967) has proposed an androstenol theory of sex odour and chemical communication.

Note added in proof. Since this paper was submitted for publication evidence has been obtained in this Laboratory for the formation of 5 α -androst-16-en-3-one from progesterone in boar testis homogenate.

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