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16-Unsaturated C_{19} 3-Oxo Steroids as Metabolic Intermediates in Boar Testis

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1. The formation of the two 16-unsaturated alcohols 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol from [5α-^{3}H]5α-androst-16-en-3-one has been demonstrated in boar testis homogenates. 2. The optimum yield (23%) of the 3α-alcohol was obtained in the presence of NADPH, whereas that for the 3β-alcohol (74%) was obtained when NADH was the added cofactor. 3. The two alcohols were not interconvertible. 4. Prolonged storage of boar testis tissue at −20°C abolished the ability to form all androst-16-enes except androsta-4,16-dien-3-one from [4-^{14}C]progesterone. 5. The production of 5α-androst-16-en-3-one and the two alcohols from [7α-^{3}H]androsta-4,16-dien-3-one only occurred when fresh tissue was used, whereas reduction of [5α-^{3}H]5α-androst-16-en-3-one was unaffected by storage of testis at −20°C. 6. NADPH was the preferred cofactor for the reduction of androsta-4,16-dien-3-one. 7. The previously established conversion of androsta-5,16-dien-3β-ol into androsta-4,16-dien-3-one was shown to be reversible, NADH and NADPH being equally effective cofactors. 8. Pathways of biosynthesis of 5α-androst-16-en-3α- and 3β-ols, with the C_{19} 3-oxo steroids as intermediates, are presented.

Boar testis tissue has been shown to convert pregnenolone (3β-hydroxypregn-5-en-20-one) and progesterone (pregn-4-ene-3,20-dione) into androst-16-enes *in vitro* (Gower & Ahmad, 1967; Ahmad & Gower, 1968; Katkov & Gower, 1968, 1970). This group of steroids includes an-α (5α-androst-16-en-3α-ol) and 5α-androst-16-en-3-one, both of which have strong odours and may act as pheromones in the pig (Melrose *et al.*, 1971). Previous work, investigating the biosynthesis of androst-16-enes, was hampered by the unavailability of these steroids isotopically labelled. As they have now been synthesized, the present work was undertaken to determine the position of the physiologically active androst-16-enes in the general scheme of androst-16-ene metabolism. A preliminary report of part of this work has appeared (Brophy & Gower, 1972).

Katkov & Gower (1970) showed that andien-β (androsta-5,16-dien-3β-ol) was produced from pregnenolone by boar testis tissue *in vitro* and that this was then converted into androstadienone (androsta-4,16-dien-3-one). Kinetic studies by Ahmad & Gower (1968) also suggested that androstadienone was an intermediate between progesterone and the ring-A-saturated alcohols, an-α and an-β (5α-androst-16-en-3β-ol). By using [7α-^{3}H]androstadienone, [5α-^{3}H]5α-androst-16-en-3-one, [5α-^{3}H]-an-α and [5α-^{3}H]an-β in incubations containing various cofactors, the biosynthetic sequence and the cofactor requirements of each step have now been established.

Materials and Methods

Materials

Authentic androst-16-enes, solvents and materials for column chromatography, t.l.c. and g.l.c. were as described by Gower & Ahmad (1967), Lisboa & Palmer (1967) and Katkov & Gower (1968, 1970). [4-^{14}C]Progesterone (specific radioactivity 60mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [5α-^{3}H]5α-Androst-16-en-3-one (specific radioactivity 17.5Ci/mmol) was generously supplied by Dr. W. Hafferl, Syntex Research, Palo Alto, Calif., U.S.A. [7α-^{3}H]-Androstadienone (specific radioactivity 125mCi/mmol) was prepared by Wilkinson *et al.* (1970). [5α-^{3}H]An-α and [5α-^{3}H]an-β were synthesized by reduction of [5α-^{3}H]5α-androst-16-en-3-one (16μCi) dissolved in methanol (100μl), containing KBH_4 (5mg). The reaction was stopped after 45min by the addition of 0.1M-acetic acid (300μl) and the mixture was extracted with ethyl acetate (5×0.5ml). The reduction products were purified and separated by t.l.c., first in benzene–diethyl ether (9:1, v/v) run twice and then in benzene–methanol (9:1, v/v). The tritiated steroids were located by using a thin-layer scanner, model TRS-1S (Panax, Redhill,

Surrey, U.K.). Two areas of radioactivity were detected corresponding in mobility to authentic an-α and an-β used as markers. These were eluted and their radioactivities measured (see below).

Methods

Preparation of tissue and incubation conditions. Boar testis tissue was obtained fresh from the slaughter-house and either used in experiments immediately or stored at −20°C. Homogenates (10%, w/v) were prepared in 50mM-tris–HCl buffer, pH7.4. Test tubes containing 2ml of buffer, radioactive steroid precursors and suitable cofactors were preincubated for 5min at 37°C. Reactions were then started by the addition of boar testis homogenate (1ml). Unless stated otherwise all incubations were accompanied by controls in which homogenate that had been boiled for 3min was used. Incubations were terminated by the addition of ethyl acetate (2ml) and carrier steroids (50μg each).

Purification of androst-16-enes. Steroids were extracted from the incubation medium into ethyl acetate (4×2ml) and androst-16-enes were separated and purified as shown in Scheme 1. Radiochemical purity of each metabolite was checked by column chromatography either on alumina (Gower & Haslewood, 1961) or on $AgNO_3$-impregnated Kieselgel H (Katkov & Gower, 1970). Radioactivity was measured by liquid-scintillation counting, and the weight of steroids by g.l.c. as described by Ahmad & Gower (1968). Counting efficiencies for ^{3}H and ^{14}C were 52 and 91% respectively. All yields are corrected for analytical losses.

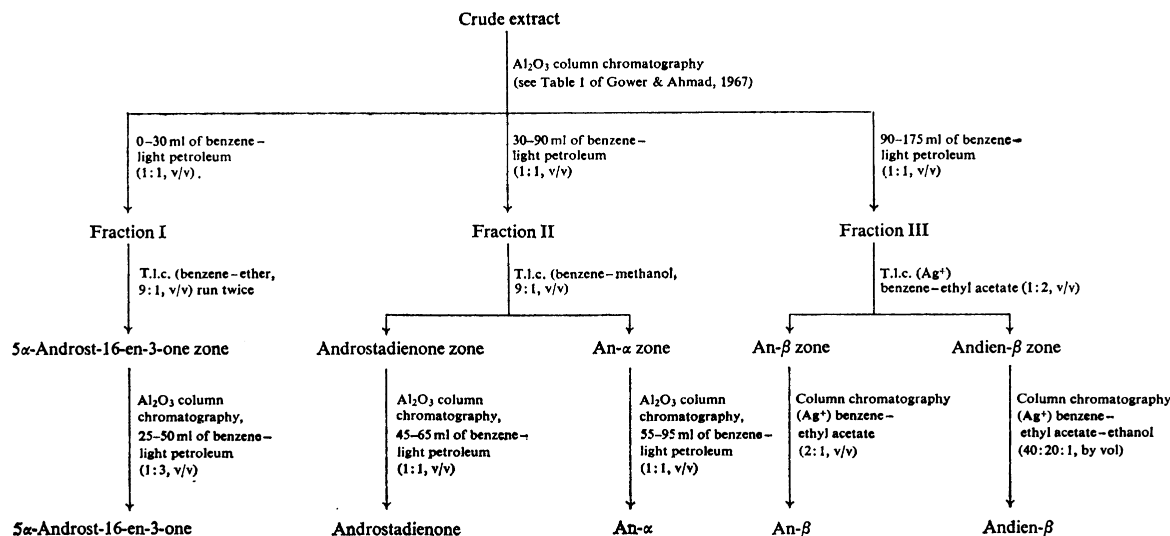
Results

Metabolism of 5α-androst-16-en-3-one

Boar testis homogenate was prepared by using tissue that had been stored frozen and was incubated for 20min with [5α-^{3}H]5α-androst-16-en-3-one (1.75×10^{6} d.p.m.). In the presence of NADH and NADPH (each 0.3mM) the major metabolites an-α and an-β were isolated and characterized (Scheme 1). Figs. 1 and 2 show that these were radiochemically pure.

(*a*) *Cofactor requirement for reduction of 5α-androst-16-en-3-one.* [5α-^{3}H]5α-Androst-16-en-3-one was incubated as described above with various cofactors for 20min; an-α and an-β were isolated and purified from each incubation mixture. The yields obtained, summarized in Table 1, show that the preferred cofactor for the 3α-reductase was NADPH, whereas that for the 3β-reductase was NADH.

(*b*) *Time-course of metabolism of 5α-androst-16-en-3-one.* [5α-^{3}H]5α-Androst-16-en-3-one (1.75×10^{6} d.p.m.) was incubated with boar testis homogenate (1ml) and NADH and NADPH (each 0.3mM) for



Scheme 1. *Flow sheet to illustrate the purification of some androst-16-enes*

For details see the text. Abbreviations: t.l.c. (Ag^{+}), $AgNO_3$-impregnated Kieselgel G (Lisboa & Palmer, 1967); column chromatography (Ag^{+}), $AgNO_3$-impregnated Kieselgel H (Katkov & Gower, 1970). The light petroleum had b.p. 80–100°C.

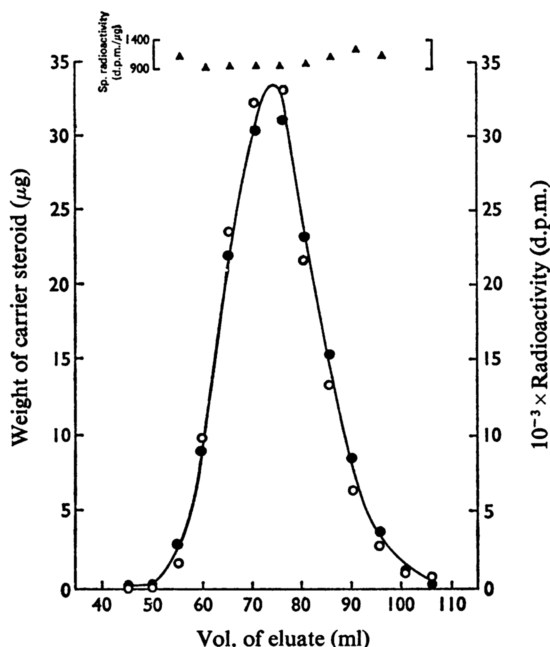


Fig. 1. *Alumina-column chromatography of radioactive an-α*

An-α was isolated after incubation of [5α-^{3}H]5α-androst-16-en-3-one (1.75×10^6 d.p.m.) with boar testis homogenate in the presence of NADH and NADPH (each 0.3mM) for 20min at 37°C. The eluent was benzene–light petroleum (b.p. 80–100°C) (1:1, v/v). The weight of an-α (o), added as carrier, was determined by g.l.c. and its radioactivity (●) by liquid-scintillation counting. ▲, Sp. radioactivity (d.p.m./μg).

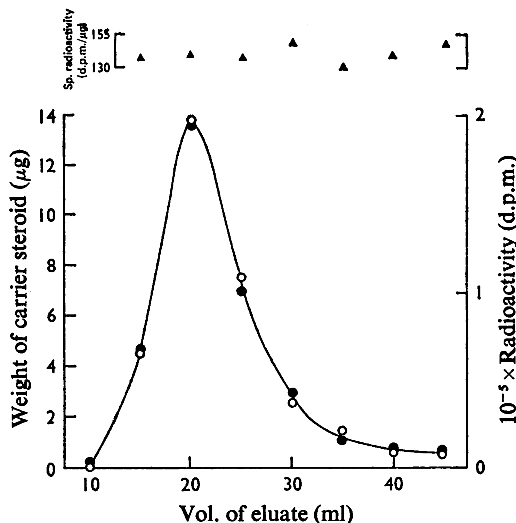


Fig. 2. *Column chromatography of radioactive an-β on $AgNO_3$-impregnated silica gel*

An-β was isolated after incubation of [5α-^{3}H]5α-androst-16-en-3-one (1.75×10^6 d.p.m.) with boar testis homogenate in the presence of NADH and NADPH (each 0.3mM) for 20min at 37°C. The eluent was benzene–ethyl acetate (2:1, v/v). The weight of an-β (o), added as carrier, was determined by g.l.c. and its radioactivity (●) by liquid-scintillation counting. ▲, Sp. radioactivity (d.p.m./μg).

5,10, 30 and 60min respectively at 37°C. Fig. 3 shows that an-β was produced in high yield after 5min (49%), but the yield declined to 40% after 30min. In contrast an-α was produced more slowly and rose to a maximum yield of 14% after 30min. These results suggested that an-α might be derived from an-β, and this possibility was investigated in the following experiment.

Investigation of the interconversion of an-α and an-β

Homogenates of boar testis that had been stored at −20°C were incubated for 15min at 37°C with either [5α-^{3}H]an-α (1.7×10^5 d.p.m.) or [5α-^{3}H]an-β (2.6×10^5 d.p.m.). NADH and NADPH (each 0.3mM) were added as cofactors. There was little interconversion of the two alcohols, only 0.12% of an-α being obtained from an-β, and 1.5% of an-β from an-α. However, slight oxidation occurred to 5α-androst-16-en-3-one (2 and 1.1% for an-α and an-β respectively).

Metabolism of androstadienone

Preliminary experiments showed that boar testis that had been stored at −20°C for 4 months was unable to metabolize [7α-^{3}H]androstadienone. In contrast, fresh tissue effected its conversion into andien-β, 5α-androst-16-en-3-one, an-α and an-β. The radiochemical purity of 5α-androst-16-en-3-one was checked as shown in Fig. 4. The cofactor requirements for this metabolism were therefore studied.

(*a*) *Cofactor requirements for the reduction of androstadienone.* Homogenate (1ml) of fresh boar testis was used in incubations for 5min at 37°C with [7α-^{3}H]androstadienone (3.6×10^6 d.p.m.) as substrate. Cofactors were added as indicated in Table 2 and, since 5α-androst-16-en-3-one was a metabolite of androstadienone and was itself rapidly metabolized (Fig. 3), unlabelled 5α-androst-16-en-3-one (20μg) was included in the incubation medium as a trapping agent. Table 2 shows the Δ^4-5α-reductase to be NADPH-dependent. This may be deduced by comparison of the yields of 5α-androst-16-en-3-one

Table 1. *Influence of cofactors on the conversion of* [5α-^{3}H]5α-*androst*-16-*en*-3-*one into an*-α *and an*-β *by boar testis homogenate*

Homogenate of boar testis that had been stored frozen was incubated with [5α-^{3}H]5α-androst-16-en-3-one (1.75×10^{6} d.p.m.) for 20min at 37°C with various cofactors. The steroids were isolated as shown in Scheme 1 and the yields are expressed as percentages of the maximum obtained under optimum conditions (74% and 23% for an-β and an-α respectively).

Metabolite	Conversion (% of maximum)			
	None	NADH	NADPH	NADPH+NAD^{+}
An-α	83	57	100	75
An-β	52	100	42	79

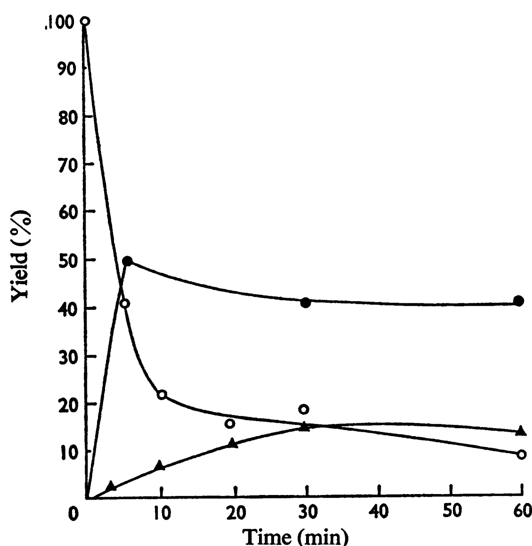


Fig. 3. *Metabolism of* [5α-^{3}H]5α-*androst*-16-*en*-3-*one*

[5α-^{3}H]5α-Androst-16-en-3-one (1.75×10^{6} d.p.m.) was incubated with boar testis homogenate in the presence of NADH and NADPH (each 0.3mM) at 37°C. o, 5α-Androst-16-en-3-one; ●, an-β; ▲, an-α.

obtained from androstadienone in the presence of NADPH (44%) and in the absence of NADPH (25%). Further, if both NADH and NADPH were present, a higher yield of 5α-androst-16-en-3-one was then obtained than in the presence of NADH alone (3 and 2% respectively), but much lower than that with NADPH (44%). It is noteworthy that the yield of an-β obtained with both cofactors present (30%) is approximately equal to the sum of those obtained when NADH and NADPH are incubated separately (17.5 and 11.0% respectively). Since the very active 5α-androst-16-en-3-one 3β-reductase is NADH-dependent, this supports the NADPH-dependency of

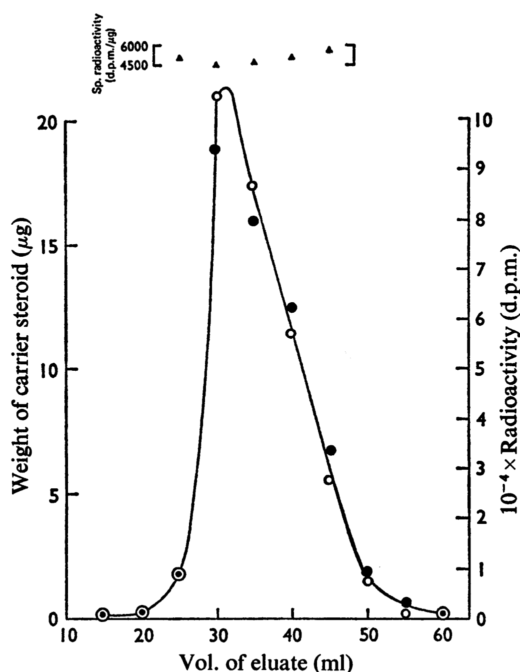


Fig. 4. *Alumina-column chromatography of radio-active* 5α-*androst*-16-*en*-3-*one*

5α-Androst-16-en-3-one was obtained after incubation of [7α-^{3}H]androstadienone (3.6×10^{6} d.p.m.) with boar testis homogenate in the presence of NADH and NADPH (each 0.3mM) for 5min at 37°C. The eluent was benzene–light petroleum (b.p. 80–100°C) (1:3, v/v). The weight of 5α-androst-16-en-3-one (o), added as carrier, was determined by g.l.c. and its radioactivity (●) by liquid-scintillation counting. ▲, Sp. radioactivity (d.p.m./μg).

the Δ^{4}-5α-reductase, which seems to be the next enzyme back along the biosynthetic pathway (Scheme 2). The Δ^{4}-3β-reductase that converted

Table 2. *Influence of cofactors on the production of androst-16-enes from [7α-³H]androstadienone by fresh boar testis tissue*

[7α-³H]Androstadienone (3.6×10^6 d.p.m.) was incubated for 5 min at 37°C with boar testis and with or without cofactors (0.3 mM). Yields of metabolites (corrected for analytical losses) are expressed as percentages of the radioactivity initially added.

Cofactors	Metabolites				
	Andien-β	Androstadienone	5α-Androst-16-en-3-one	An-α	An-β
None	1.3	71	25	4.4	2.9
NADH	3.7	51	2	3.4	17.5
NADPH	4.3	27	44	5.2	11.0
NADH+NADPH	7.0	29	3	5.7	30.0

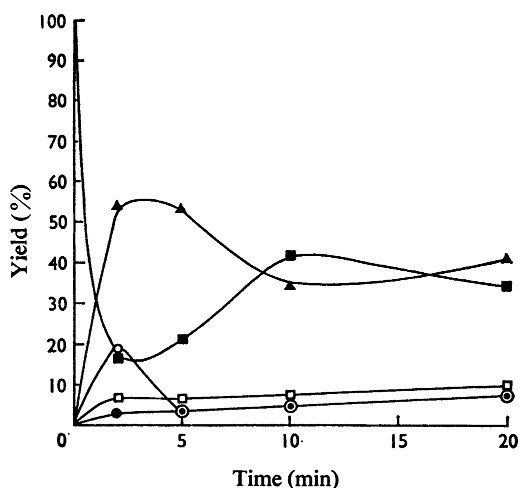


Fig. 5. *Metabolism of [7α-³H]androstadienone*

[7α-³H]Androstadienone (3.6×10^6 d.p.m.) was incubated with boar testis homogenate in the presence of NADPH and NADH (each 0.3 mM) at 37°C. ■, Androstadienone; ○, andien-β; ●, 5α-androst-16-en-3-one; □, an-α; ▲, an-β.

androstadienone into andien-β seems to be both NADH- and NADPH-dependent, since the yields of andien-β obtained in the presence of NADH and NADPH (3.7 and 4.3% respectively) were approximately equal to the yield obtained when both were present (7.0%).

(*b*) *Time-course of metabolism of androstadienone.* The metabolism of [7α-³H]androstadienone (3.6×10^6 d.p.m.) by fresh boar testis tissue was studied in incubations containing NADPH and NADH (each 0.3 mM) for periods of 2, 5, 10 and 20 min at 37°C (Fig. 5). The high initial production of andien-β (18%) demonstrates the reversibility of the andien-β→androstadienone conversion (Katkov & Gower, 1970). However, the lower later yields of andien-β, together with increasing yields of androstadienone, 5α-androst-16-en-3-one and an-α, suggest that the forward reaction is more important. 5α-Androst-16-en-3-one was formed early but in low yields throughout, in keeping with a rapid conversion into an-β (the major product) and an-α. The relative proportions of an-β and an-α were similar to those obtained in Fig. 3 when 5α-androst-16-en-3-one was the substrate.

Effects of storage at –20°C on the metabolism of [4-¹⁴C]progesterone by boar testis tissue

The central position of androstadienone as an intermediate between progesterone (and pregnenolone) and the physiologically active androst-16-enes was confirmed in this experiment. Homogenates of tissues that had been stored at –20°C for periods of 2 weeks and 26 weeks were used in incubations containing [4-¹⁴C]progesterone (2.7×10^6 d.p.m.) and NADH and NADPH (each 0.3 mM) for 10 min at 37°C. Radioautography was used to identify radioactive steroids after t.l.c. Table 3 shows that prolonged storage at –20°C resulted in the loss of ability to metabolize androstadienone to other androst-16-enes with known physiological activity. The total yield (8.2%) of androst-16-enes with 2-week-old tissue was approximately equal to the yield (10%) of androstadienone obtained from progesterone after storage for 26 weeks.

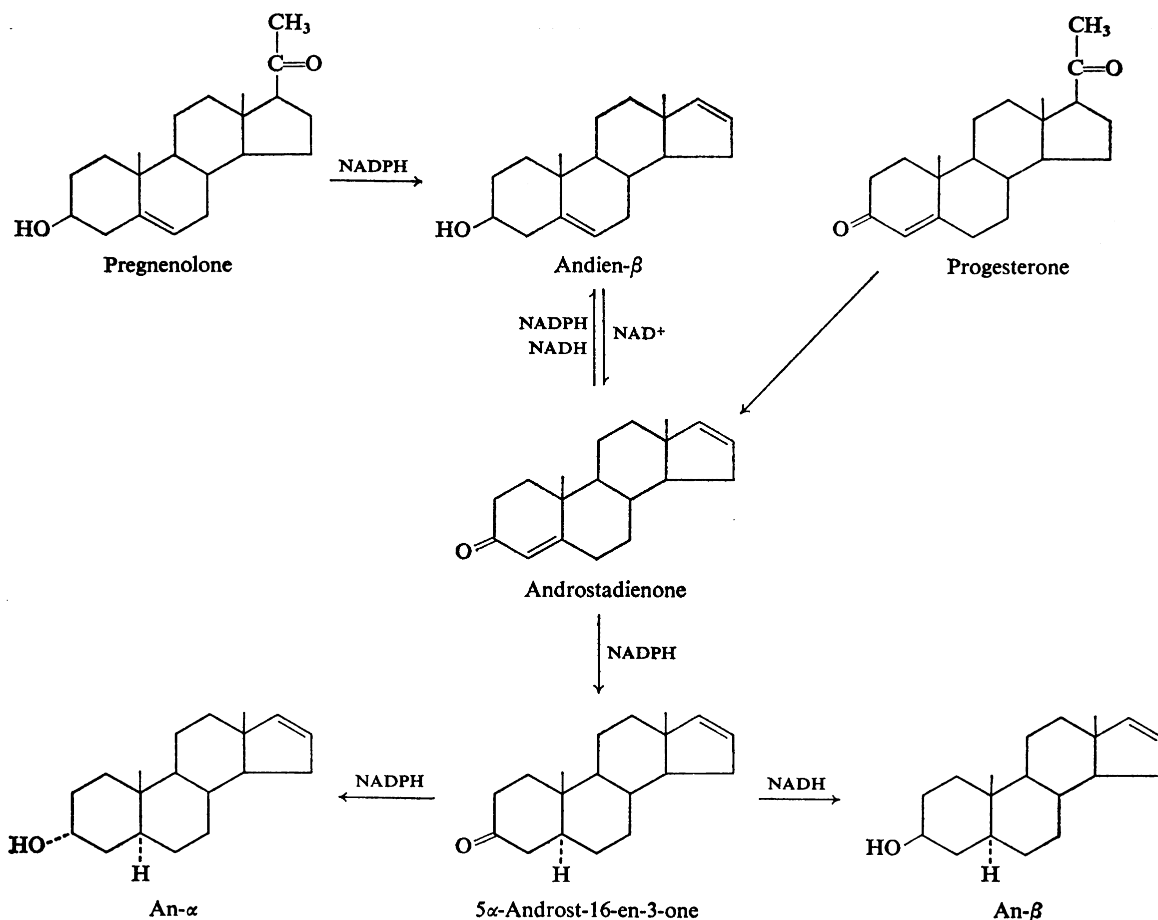
Discussion

The conversion of pregnenolone into andien-β by boar testis homogenates is now well established (Katkov & Gower, 1970; Gower & Loke, 1971; Loke & Gower, 1972). In the same preparations

Table 3. *Effects of storage at −20°C on the metabolism of* [4-^{14}C]*progesterone by boar testis tissue*

[4-^{14}C]Progesterone (2.7×10^6 d.p.m.) was incubated for 10min at 37°C with boar testis homogenate and NADH and NADPH (each 0.3mM). Yields of metabolites (corrected for analytical losses) are expressed as percentages of the radioactivity initially added.

Length of storage time (weeks)	Metabolite				
	Androstadienone	5α-Androst-16-en-3-one	Andien-β	An-α	An-β
2	2.4	0.4	0	0.8	4.6
26	10	0	0	0	0



Scheme 2. *Pathways of biosynthesis of androst-*16*-enes from* C_{21} *steroids in vitro*

This scheme is deduced from the present work and from Katkov & Gower (1970).

andien-β can be converted into androstadienone in the presence of NAD$^+$ or into an-β if both NAD$^+$ and NADPH are present (Katkov & Gower, 1970). The results given in the present paper have shown the andien-β → androstadienone step to be reversible, NADH and NADPH being equally effective co-factors. On this basis, it may be possible to explain the conversion of progesterone into andien-β in a

human adrenal carcinoma preparation (Gower *et al.*, 1970), if progesterone were converted first into androstadienone (as shown by Ahmad & Gower, 1968, and in the present work) and the latter was then converted into andien-β. There is growing evidence for the conversion of Δ^4-3-oxo steroids into Δ^5-3β-hydroxy steroids; this has been shown in rabbit testis (Rosner *et al.*, 1965), sheep adrenals (Ward & Engel, 1964, 1966) and boar testis (P. J. Brophy, unpublished work).

The metabolism of progesterone to 5α-androst-16-en-3-one was demonstrated by Katkov & Gower (1968), and short-term kinetic studies (Ahmad & Gower, 1968) suggested that androstadienone was first formed from progesterone and that this was subsequently reduced in ring A and at C-3 to form an-α and an-β. The present results, especially those of Figs. 3 and 5, now clearly establish the ketones androstadienone and 5α-androst-16-en-3-one as intermediates in the formation of these 16-unsaturated alcohols and, taken together with the results of Katkov & Gower (1970), suggest the sequence of reactions shown in Scheme 2.

The kinetic results of Ahmad & Gower (1968) also suggested that, after an-β was formed from progesterone in boar testis minces, a small proportion may have been converted into an-α. Similar results were obtained in the present experiments (Fig. 3), since the yield (43%) of an-β formed from 5α-androst-16-en-3-one after incubation for 5min declined slightly during the subsequent 20min. However, it is now clear that an-β and an-α are not interconvertible.

If an-β was not converted into an-α, it is conceivable that it was metabolized to some extent to more polar androstane-3,16,17-triols by C-16,C-17-glycol formation, or by sulphation at C-3. Androstanetriol formation from androstadienone *in vivo* has been reported (Brooksbank & Wilson, 1971); however, the possible sulphation of 16-unsaturated steroids has not been investigated *in vitro*, although an-α and an-β sulphates are formed in boar testis *in vivo* (Gower *et al.*, 1972).

In the present results the formation of both an-α and an-β has been demonstrated from 5α-androst-16-en-3-one by an NADPH-dependent 3α-reductase and an NADH-dependent 3β-reductase respectively. Since the 3β-reductase is not NADPH-dependent, the production of an-β from andien-β in the presence of NADPH (Katkov & Gower, 1970) may be explained by the conversion of androstadienone into 5α-androst-16-en-3-one by an NADPH-dependent Δ^4-5α-reductase. This enzyme has a similar cofactor requirement to the enzyme found in liver responsible for reduction of 17α,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone) to 17α,21-dihydroxy-5β-pregnane-3,11,20-trione (Tomkins & Isselbacher, 1954).

The Δ^4-5α-reductase of boar testis was shown in the present work to be more sensitive to prolonged storage at −20°C than the enzyme system involved in the formation of androstadienone from progesterone or the 5α-androst-16-en-3-one 3-oxo reductases involved in an-α and an-β formation. The requirement of the Δ^4-5α-reductase for the production of other androst-16-enes was demonstrated by using tissue that had been stored at −20°C. This tissue produced androstadienone from progesterone (Table 3), but was unable to metabolize it to an-β and the physiologically active steroids, 5α-androst-16-en-3-one and an-α.

Recent work (Katkov *et al.*, 1972) has shown that an-α and an-β are formed by reduction of 5α-androst-16-en-3-one in boar submaxillary glands. The cofactor requirements of the 3α- and 3β-reductases were found to be similar to those of the testicular enzymes. However, there is little evidence for the conversion of C_{21} steroids into 16-unsaturated C_{19} steroids in the submaxillary glands (Katkov *et al.*, 1972) and it seems likely that the latter compounds, once formed in the testes, are then transported to the salivary glands where reduction of the ketones occurs. This would explain the occurrence of 5α-androst-16-en-3-one and an-α in boar saliva (Gower, 1972).

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