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The Intermediary Role of 5-Pregnene-3β,20β-diol in the Biosynthesis of 16-Unsaturated C_{19} Steroids in Boar Testis

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1. The possible involvement of 5-pregnene-3β,20β-diol in 16-unsaturated C_{19} steroid biosynthesis has been investigated. 2. 5,16-Androstadien-3β-ol (andien-β) formation from [4-^{14}C]pregnenolone (3β-hydroxy-5-pregnen-20-one), 5-pregnene-3β,20α-diol and 5-pregnene-3β,20β-diol was studied in homogenates of boar testis and the mean yields obtained were 25.6, 2.7 and 16.0% respectively. 3. Short-term kinetic studies with pregnenolone and 5-pregnene-3β,20β-diol separately and together suggested that the latter compound might be an intermediate in the biosynthesis of andien-β. 4. In agreement with this interpretation, radioactive 5-pregnene-3β,20β-diol has been isolated during andien-β biosynthesis from [4-^{14}C]pregnenolone in the presence of NADPH, more radioactivity being trapped under limiting conditions of andien-β formation with NADH present as cofactor. 5. Further, 5-pregnene-3β,20β-diol and andien-β have been shown to inhibit the formation of the 16-unsaturated C_{19} steroid from [4-^{14}C]pregnenolone, the yield of radioactive 5-pregnene-3β,20β-diol increasing in the presence of added unlabelled andien-β. 6. It is concluded that there may be two pathways leading to 16-unsaturated C_{19} steroid formation from pregnenolone, one of these involving 5-pregnene-3β,20β-diol as an intermediate. Possible mechanisms are presented and discussed.

The biosynthesis *in vitro* of 16-unsaturated C_{19} steroids from progesterone and pregnenolone (3β-hydroxy-5-pregnen-20-one) by boar testis tissue has been described in earlier papers (Gower & Ahmad, 1967; Ahmad & Gower, 1968; Katkov & Gower, 1968; Gower & Katkov, 1969; Katkov & Gower, 1970). Various other steroidal substrates have been tested as intermediates in 16-unsaturated C_{19} steroid biosynthesis from these two C_{21} precursors but all were shown to be ineffective. In continuing these earlier studies the possible involvement of a 20-hydroxy-C_{21} steroid in 16-dehydrosteroid biosynthesis was considered. To investigate this possibility the conversion of pregnenolone into andien-β (5,16-androstadien-3β-ol) has been studied in detail since the latter is formed in high yields and may be the first 16-unsaturated C_{19} steroid formed from pregnenolone (Katkov & Gower, 1970). Some of these results have appeared in a preliminary communication (Loke & Gower, 1971).

Materials and Methods

Materials

Authentic Δ^{16}-steroids, solvents and materials for radioautography, t.l.c. and g.l.c. were as described by Gower & Ahmad (1967). Dry hexane (laboratory grade; Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) was redistilled and dried over sodium. All reagents, unless otherwise stated, were analytical grade. [4-^{14}C]Pregnenolone (specific radioactivity 55.7mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Radioactivity was measured by liquid-scintillation counting as described by Gower & Ahmad (1967) and the weight of andien-β by g.l.c. as described by Gower & Thomas (1968).

Methods

Preparation of the C-20 epimeric 5-pregnene-3β,20β-diols. These were prepared by treating [4-^{14}C]-pregnenolone (0.25–0.5μCi) in methanol (0.1ml) with potassium borohydride (5–10mg) at room temperature. After at least 30min the reaction mixture was diluted with 0.1M-acetic acid and extracted with ethyl acetate. The dried extract was evaporated and the residue subjected to t.l.c. in benzene–acetone (4:1, v/v). After radioautography, two radioactive spots corresponding to authentic 5-pregnene-3β,20α-diol (R_F 0.22–0.27) and 5-pregnene-3β,20β-diol (R_F 0.26–0.31) were seen and these were eluted for use in subsequent incubations. The ratio of the yield of the 20α- to 20β-epimers was

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approximately 1 to 5. Pregnenolone had R_F 0.35–0.45.

Determination of protein. The protein content of tissue homogenates was determined by the method of Lowry *et al.* (1951) with crystalline bovine plasma albumin (Armour Pharmaceuticals Ltd., Eastbourne, Sussex, U.K.) as standard.

Preparation of tissue and incubation conditions. Boar testis that had been stored at −20°C was used throughout. The preparation and incubation of tissue homogenates were carried out as described by Katkov & Gower (1970). Unless otherwise stated, 0.2ml portions of a 10% (w/v) homogenate in 0.25M-sucrose were used in the incubations and these were found to contain 1200–1400μg of protein. The incubations were terminated by the addition of ethyl acetate (2ml) and carrier steroids.

Isolation and purification of metabolites. Andien-β was isolated and purified as described by Katkov & Gower (1970) and the yield was corrected for analytical losses. Pregnenolone and dehydroepiandrosterone were purified by t.l.c. in benzene–acetone (4:1, v/v). For the isolation of the metabolites 17-hydroxypregnenolone (3β,17-dihydroxy-5-pregnen-20-one) and 5-pregnene-3β,20β-diol, however, which are not separated by t.l.c. in this system, a Girard separation (see below) was first performed on the mixture. Carrier steroids were then added to the appropriate fractions obtained and recrystallized to constant specific radioactivity.

In a typical experiment with [4-^{14}C]pregnenolone as substrate, radioactive 17-hydroxypregnenolone was isolated as above. Carrier (10mg) was added and the specific radioactivities of crystals after successive recrystallizations in ethanol were 3880, 3790 and 3940d.p.m./mg.

Girard separation. The procedure described by Bush (1961) was used except that the reaction with the Girard T reagent was carried out at room temperature.

Results

Formation of andien-β from pregnenolone, 5-pregnene-3β,20α-diol and 5-pregnene-3β,20β-diol in boar testis homogenate

[4-^{14}C]Pregnenolone, 5-[4-^{14}C]pregnene-3β,20α-diol and 5-[4-^{14}C]pregnene-3β,20β-diol (each 9.4–11.0$\times 10^4$d.p.m.) were separately incubated at 37°C for 20min with portions (0.1ml) of boar testis homogenate in 0.05M-tris–HCl buffer, pH7.4, containing 0.4mM-NADPH. After addition of carrier andien-β (50μg), radioactive andien-β was isolated and assayed. The mean yields (four experiments) of andien-β formed from pregnenolone, 5-pregnene-3β,20α-diol and 5-pregnene-3β,20β-diol were 25.6, 2.7 and 16.0% respectively. A control experiment in which boiled homogenate was used did not yield any andien-β from 5-pregnene-3β,20β-diol. The pregnenolone isolated from the incubation of 5-[4-^{14}C]-pregnene-3β,20α-diol contained very small amounts of radioactivity although the 20β-isomer under the same conditions yielded pregnenolone containing approximately 3% of the initial radioactivity (but see also below and Fig. 1).

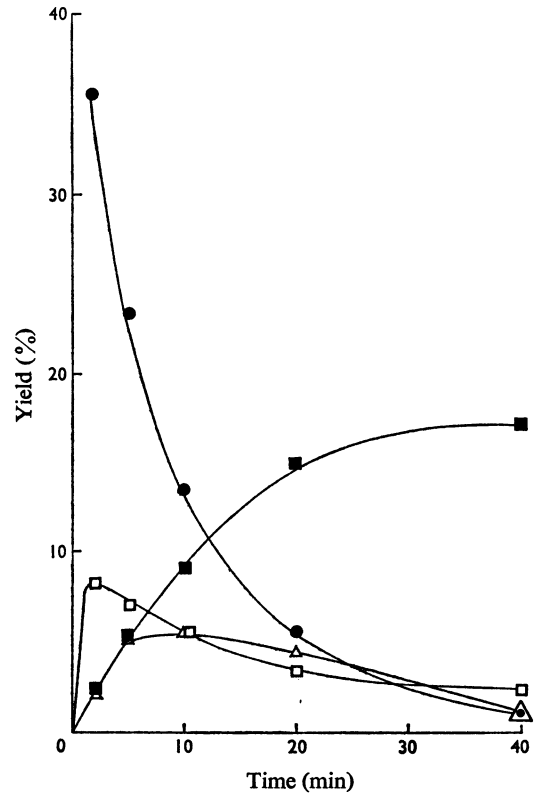


Fig. 1. *Metabolism of 5-[4-^{14}C]pregnene-3β,20β-diol in boar testis homogenate*

Portions (0.1ml) of boar testis homogenate were incubated with 5-[4-^{14}C]pregnene-3β,20β-diol (816000d.p.m.) at 37°C in 1.2ml of 0.05M-tris–HCl buffer, pH7.4, containing 0.4mM-NADPH. The reactions were terminated at various times, carrier steroids were added and metabolites extracted and isolated as described in the Materials and Methods section. ●, 5-Pregnene-3β,20β-diol; □, pregnenolone; △, 17-hydroxypregnenolone; ■, andien-β.

Kinetic studies of 5-pregnene-3β,20β-diol and pregnenolone metabolism

With 5-pregnene-3β,20β-diol. Portions of boar testis homogenate (0.1ml) were incubated in separate

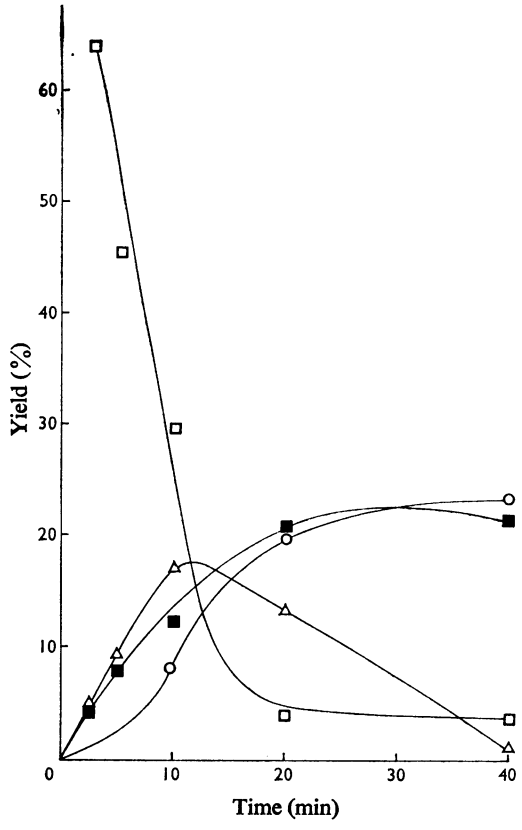


Fig. 2. *Metabolism of [4-^{14}C]pregnenolone in boar testis homogenate*

[4-^{14}C]Pregnenolone (932000d.p.m.) was incubated with portions of boar testis homogenate (0.1ml) under conditions described in the legend to Fig. 1. □, Pregnenolone; △, 17-hydroxypregnenolone; ○, dehydroepiandrosterone; ■, andien-β.

flasks with 5-[4-^{14}C]pregnene-3β,20β-diol (81600 d.p.m.) dissolved in buffer (1.2ml) containing 8mM-NADPH. After 2, 5, 10, 20 and 40min respectively carrier andien-β (50μg) was added to each flask and the metabolites were extracted and separated as described in the Materials and Methods section. Fig. 1 shows the yields of metabolites plotted against time of incubation.

With pregnenolone. The metabolism of [4-^{14}C]-pregnenolone by boar testis homogenate was studied over short periods of time in a manner similar to that described for 5-pregnene-3β,20β-diol. The percentage yields of andien-β, pregnenolone, dehydroepiandrosterone and 17-hydroxypregnenolone were plotted against time of incubation (Fig. 2). Radioactive 5-pregnene-3β,20β-diol was not isolated from this experiment, but in further studies it was possible to demonstrate its formation from [^{14}C]-pregnenolone under the following conditions of andien-β biosynthesis.

(*a*) In NADPH-supported incubations. A portion of boar testis homogenate (0.4ml) was incubated at 37°C with [4-^{14}C]pregnenolone (1670000d.p.m.) and 0.4mM-NADPH for 20min. Radioactive andien-β and 5-pregnene-3β,20β-diol were isolated and purified as described above, the corrected yield of andien-β being 21.0%, whereas that for the 5-pregnene-3β,20β-diol, isolated and characterized by reverse isotope dilution (Table 1), was 0.23%.

(*b*) In NADH-supported incubations. Since Katkov & Gower (1970) have found that NADH was less active than NADPH in promoting andien-β biosynthesis from pregnenolone, two experiments were performed to determine if there was a tendency for the postulated intermediary diol to accumulate under such limiting conditions of andien-β formation. Boar testis homogenate was incubated at 37°C for 20min with [4-^{14}C]pregnenolone, as in the NADPH-supported experiment above, but 0.6mM-NADH was added as cofactor instead of NADPH. The yields of andien-β and 5-pregnene-3β,20β-diol isolated were 5.7 and 0.33% (0.34% in a second experiment) respectively. The use of NADH therefore resulted in only 27% of the andien-β obtained when NADPH was present.

Kinetic study of [7α-^{3}H]pregnenolone plus 5-[4-^{14}C]-pregnene-3β,20β-diol

Portions of boar testis homogenate (0.1ml) were incubated at 37°C as described above with mixtures of [7α-^{3}H]pregnenolone (98000d.p.m.) and 5-[4-^{14}C]-pregnene-3β,20β-diol (66200d.p.m.) for 2, 5, 10, 20 and 40min. NADPH (0.3mM) was included in each tube. Table 2 shows that the yield of [^{3}H]andien-β and [^{14}C]andien-β isolated (corrected for losses) increased progressively up to 30min, comparable with increases depicted in Figs. 1 and 2 when pregnenolone and 5-pregnene-3β,20β-diol were incubated separately. The ^{3}H/^{14}C ratios of the andien-β samples, however, decreased as the time of incubation increased (Table 2).

Studies on the inhibition of andien-β biosynthesis

These experiments were designed to investigate whether andien-β formation from pregnenolone was affected by preincubation of boar testis homogenate with the postulated intermediate, 5-pregnene-3β,20β-diol, the product andien-β or the well-known intermediate in androgen biosynthesis, 17-hydroxypregnenolone. Three tubes containing tris-HCl buffer, pH7.4 (1.2ml), boar testis homogenate

Table 1. *Purification by recrystallization to constant specific radioactivity (d.p.m./mg) of 5-pregnene-3β,20β-diol obtained after incubating [4-^{14}C]pregnenolone with boar testis homogenates*

Values in parentheses are the weights (mg) of crystals. Solvents were: A, ethyl acetate; B, hexane; C, acetone; D, benzene. Boar testis homogenate (0.4ml) was incubated with [4-^{14}C]pregnenolone (1670000d.p.m.) for 20min at 37°C in the presence of 0.4mM-NADPH or 0.6mM-NADH. After the addition of carrier, radioactive andien-β was purified by conventional t.l.c. and by t.l.c. on plates impregnated with $AgNO_3$ (see the text), whereas 5-pregnene-3β,20β-diol was isolated by t.l.c. and by recrystallization to constant specific radioactivity.

In NADPH-supported incubations			In NADH-supported incubations					
			Expt. 1			Expt. 2		
Number of recrystallizations	Solvent	Sp. radioactivity	Number of recrystallizations	Solvent	Sp. radioactivity	Number of recrystallizations	Solvent	Sp. radioactivity
0	—	1330 (10.0)	0	—	915 (10.8)	0	—	1138 (8.5)
1	A,B	638 (4.2)	1	A	637 (2.6)	1	D	559 (—)
2	C,B	384 (3.5)	2	D	513 (1.0)	2	A	559 (2.6)
		199 (5.8)*			149 (2.5)*			229 (3.9)*
3	D	208 (4.3)	3	C,B	156 (2.0)	3	C,B	324 (—)
4	A,B	191 (1.9)						

* After addition of more carrier.

Table 2. *Kinetic study of the formation of 5,16-androstadien-3β-ol from [7α-^{3}H]pregnenolone and 5-[4-^{14}C]-pregnene-3β,20β-diol in boar testis in vitro*

[7α-^{3}H]Pregnenolone (98000d.p.m.) and 5-[4-^{14}C]-pregnene-3β,20β-diol (66200d.p.m.) were incubated with portions of boar testis homogenate (0.2ml) in the presence of 0.4mM-NADPH for various times. Labelled andien-β was then isolated, purified and the ^{3}H and ^{14}C contents of each sample were measured. All percentages are corrected for analytical losses.

Time of incubation (min)	2	5	10	20	40
^{3}H in andien-β (%)	5.8	10.9	16.1	20.7	23.8
^{14}C in andien-β (%)	2.2	5.0	7.8	14.6	19.3
^{3}H/^{14}C ratio	2.64	2.18	2.07	1.42	1.23

(0.2ml containing 1250μg of protein N), NADPH (0.4mM) and 5-pregnene-3β,20β-diol (200μg), andien-β (210μg) or 17-hydroxypregnenolone (200μg) respectively were preincubated at 37°C for 5min. A control tube containing buffer, NADPH and homogenate was likewise incubated. [4-^{14}C]Pregnenolone (405000d.p.m.) was then added to each tube and incubation continued for a further 20min. Table 3 shows that andien-β formation was inhibited by preincubation with 5-pregnene-3β,20β-diol (74.9%) and by andien-β (44.3%). Preincubation with 17-hydroxypregnenolone, however, had no effect on the yield of andien-β obtained from pregnenolone. After isolating and recrystallizing the 5-pregnene-3β,20β-diol to constant specific radioactivity, it was calculated that 0.12 and 0.24% of substrate radioactivity was trapped in tubes preincubated with 5-pregnene-3β,20β-diol and andien-β respectively.

The effect of pregnenolone on the metabolism of 5-pregnene-3β,20β-diol to andien-β was studied as follows. Boar testis homogenate (0.2ml) was preincubated (5min) with pregnenolone (200μg) in tris–HCl buffer, pH7.4 (1.2ml), containing 0.4mM-NADPH. 5-[4-^{14}C]Pregnene-3β,20β-diol (66200 d.p.m.) was then added and the incubation continued for a further 20min. The yield of [^{14}C]andien-β isolated was only 0.1% compared with 14.6% (cf. Table 2) in a control incubation without pregnenolone. Moreover, the pregnenolone subsequently isolated was devoid of radioactivity.

Discussion

In the studies testing the capacity of the epimeric compounds 5-pregnene-3β,20α-diol and 5-pregnene-3β,20β-diol to serve as precursors of andien-β in boar testis, and comparing their efficiency in this respect with that of pregnenolone, it was found that 5-pregnene-3β,20β-diol was six to seven times more effective in forming andien-β than the 20α-epimer. This result, although in agreement with the working hypothesis, is by itself not proof for the intermediary

Table 3. *Effect of various steroids on the formation of 5,16-androstadien-3β-ol from pregnenolone in boar testis in vitro*

Boar testis homogenate (0.2ml containing 1250μg of protein N) was incubated for 5min with the unlabelled steroids as indicated. [4-^{14}C]Pregnenolone (405000d.p.m.) was then added and the incubation continued for a further 20min, after which andien-β and 5-pregnene-3β,20β-diol were isolated as described in the text. N.D., not determined.

Steroid added ...	None	5-Pregnene-3β,20β-diol (200μg)	Andien-β (210μg)	17-Hydroxy-pregnenolone (200μg)
Yield of andien-β (%)	25.1	6.3	13.9	26.4
Inhibition compared with yield in control tube (%)	0	74.9	44.3	0
Substrate radioactivity trapped in 5-pregnene-3β,20β-diol	—	0.12	0.21	N.D.

role of the 20β-hydroxy steroid in andien-β biosynthesis, since it is possible that by its preferential oxidation to pregnenolone it could give rise to andien-β.

The subsequent short-term kinetic studies on pregnenolone and 5-pregnene-3β,20β-diol metabolism by boar testis were therefore carried out to shed some light on this problem. As can be seen from Fig. 1 both pregnenolone and andien-β were formed rapidly in the early stages of the metabolism of 5-pregnene-3β,20β-diol, the yields of pregnenolone being greater than those of andien-β. However, after increasing to a maximum, the amount of pregnenolone declined to low values with increasing time of incubation whereas the andien-β in the medium progressively increased and then levelled off with depletion of the substrate. Moreover, it appeared from the kinetic results that the pregnenolone formed was mainly channelled into the biosynthesis of 17-hydroxypregnenolone. When considered together, the results are not inconsistent with the existence of a pathway leading directly from 5-pregnene-3β,20β-diol to andien-β.

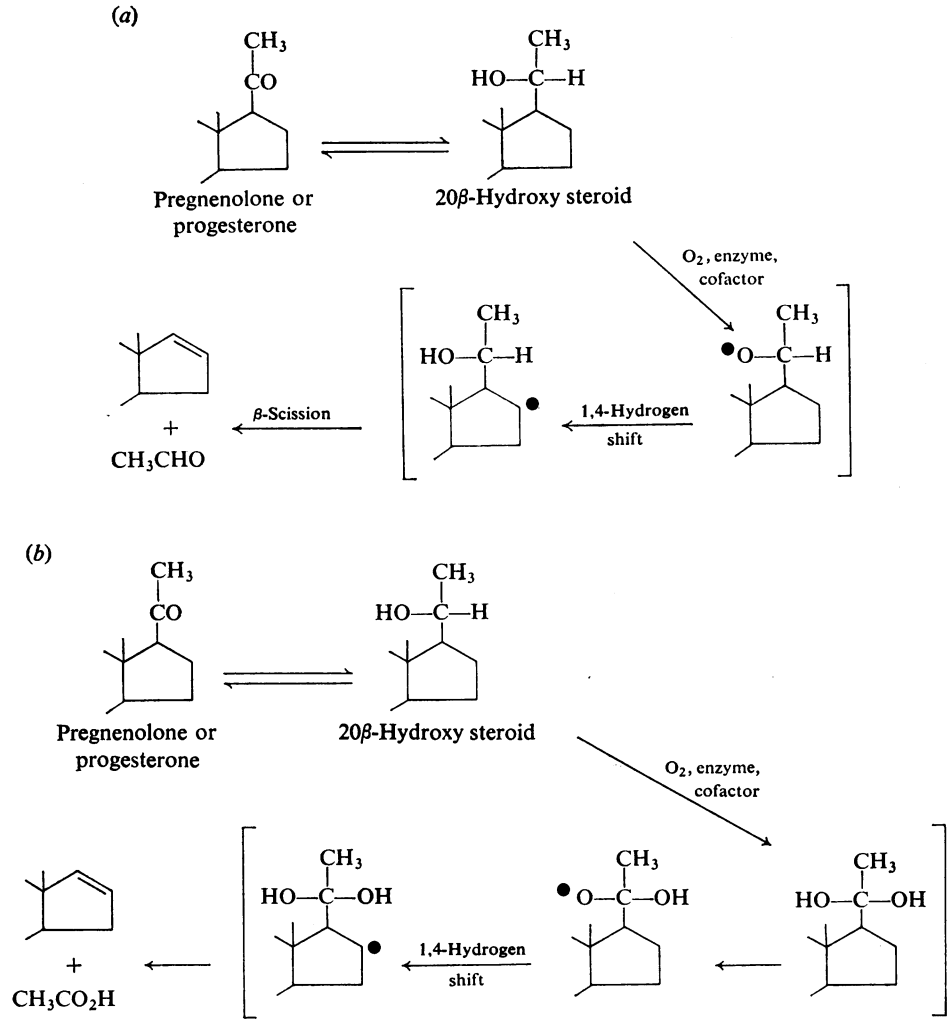
In the kinetic study with pregnenolone as substrate (Fig. 2), 17-hydroxypregnenolone was formed that bore a precursor–product relationship with pregnenolone and, moreover, was almost certain to be a precursor of dehydroepiandrosterone in boar testis.

The case for the intermediacy of 5-pregnene-3β,20β-diol in andien-β biosynthesis would be considerably strengthened if this compound could be isolated under the conditions of andien-β biosynthesis from pregnenolone. As reported in the Results section, attempts to isolate the intermediate from NADPH-supported incubations, which have been shown earlier by Katkov & Gower (1970) to result in optimum yields of andien-β, were successful. The yields obtained were generally low (0.2%) and this fact would seem to indicate a rapid utilization of the postulated intermediate as soon as it was formed. This could also be isolated from the incubations with NADH as cofactor and it may be significant that there was a greater yield of the 20β-hydroxy steroid under these experimental conditions than when andien-β synthesis was occurring in optimum amounts. The finding that only 27% of andien-β was formed in the presence of NADH compared with the yield in the presence of NADPH confirms the results of Katkov & Gower (1970), who obtained a value of 21.3%.

If the 20β-ol were involved in andien-β biosynthesis, it seems reasonable to expect that preincubation of the enzyme system with 5-pregnene-3β,20β-diol would result in an inhibition of the formation of andien-β from pregnenolone. This was indeed found to be the case (Table 3) and, moreover, consistent with its intermediary role, substrate radioactivity was trapped by the added inhibitor. Further, the presence of excess of andien-β also caused inhibition, and twice as much radioactivity was trapped in the postulated intermediate (Table 3).

The cumulative evidence therefore suggests an intermediary role for 5-pregnene-3β,20β-diol in andien-β biosynthesis. However, at present it is uncertain if the formation of andien-β from pregnenolone must compulsorily involve the 20β-hydroxy derivative. The finding that the yield of andien-β from the 5-pregnene-3β,20β-diol was about 65% that from pregnenolone would seem to indicate the existence of another pathway by-passing the 20β-hydroxy derivative. The fact that preincubation with unlabelled pregnenolone almost eliminates andien-β synthesis, even though the pregnenolone subsequently isolated was devoid of radioactivity, suggests that andien-β formation is not occurring from 5-pregnene-3β,20β-diol via pregnenolone. This view is supported by the kinetic results shown in Figs. 1 and 2.

Taking into consideration the results obtained from the double-isotope experiment (Table 2), it seems likely that, since the $^{3}H/^{14}C$ ratios fell progressively as incubation time increased, the con-



Scheme 1. *Postulated mechanisms (a and b) for 16-unsaturated C_{19} steroid biosynthesis from pregnenolone or progesterone involving 20β-hydroxy-C_{21} steroid intermediates*

version of pregnenolone into andien-β is faster than that of 5-pregnene-3β,20β-diol into andien-β. The intermediary role of a 20β-hydroxy-C_{21} steroid in 16-unsaturated C_{19} steroid synthesis may be rationalized by one or both of the mechanisms shown in Scheme 1. Both mechanisms are based on the postulate of Lieberman *et al.* (1969), which stated that, in those steroid biosyntheses utilizing oxygen, free-radical intermediates might be involved. In agreement with this postulate Lippman & Lieberman (1970) have reported the conversion of deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione) nto low yields of 4,16-androstadien-3-one by boar testis homogenate and by the action of free-radical-generating reagents. On the basis of their findings these workers have suggested a possible mechanism for 16-unsaturated C_{19} steroid biosynthesis involving a C-21 alkoxy radical.

The reaction of 20β-hydroxy steroids with lead tetra-acetate (a free-radical-generating reagent) has been extensively used in the introduction of functionality into angular methyl groups and is well documented in chemical literature (Erikson & Forbess, 1963). However, the finding of Cainelli *et al.* (1961) that a ring D-saturated C_{19} steroid was among the products of the reaction between the 3-ethylene ketal derivative of 20β-hydroxy-4-pregnen-3-one and lead tetra-acetate is particularly noteworthy. Thus by analogy it is conceivable that, under the influence of NADPH, oxygen and the enzyme system, the 20β-

hydroxy steroid would give rise to a C-20 alkoxy radical, that would then undergo a 1,4-hydrogen shift to the C-16 radical. Subsequent β-scission of the C-16 radical would result in the formation of a 16-unsaturated C_{19} steroid and acetaldehyde (Scheme 1*a*).

Mechanism (*b*) is a variant of (*a*) and its key feature is the hypothetical C-20 dihydroxylated intermediate, which would either form the 20-ketone or undergo fragmentation to a 16-unsaturated C_{19} steroid and acetic acid as shown. As observed in the kinetic experiment with 5-pregnene-3β,20β-diol, pregnenolone was formed early in the incubation and, since its formation occurred in the presence of NADPH and oxygen, the mechanism may be considered as analogous to that proposed by Skinner & Akhtar (1968) to account for the conversion under similar conditions of 19-hydroxy-4-androstene-3,17-dione by placental microsomes into 19-oxo-4-androstene-3,17-dione.

Skinner & Akhtar (1969) have emphasized that the removal of C-19 in oestrogen biosynthesis must occur at the oxidation state of a 19-aldehyde with the liberation of formic acid. By analogy it is possible that the 20-ketone or its probable equivalent, the C-20 dihydroxylated intermediate, may undergo fragmentation to the 16-dehydrosteroid and acetic acid.

It is noteworthy that 16-unsaturated C_{19} steroids have been chemically synthesized from the mesylates or tosylates of pregnane-16,20-diols (Matsui & Fukushima, 1970). The possibility that such transformations may occur in boar testis tissue has yet to be investigated.

It should be possible to distinguish the mechanisms from one another by a study of the intermediates involved or the nature of the C_2 fragment liberated or both; preliminary results, obtained by using [21-^{14}C]-progesterone with boar testis homogenates, indicate that acetaldehyde may be liberated during the reaction.

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