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Studies on the Biosynthesis of 16-Dehydro Steroids

THE METABOLISM OF [4-^{14}C]PREGNENOLONE BY BOAR ADRENAL AND TESTIS TISSUE *IN VITRO*

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1. The metabolism of [4-^{14}C]pregnenolone *in vitro* by boar adrenocortical and testis tissue has been studied. 2. Boar testis tissue formed three labelled Δ^{16}-steroids, 5α-androst-16-en-3α-ol, 5α-androst-16-en-3β-ol and androsta-4,16-dien-3-one. In adrenal tissue very much smaller yields of the same metabolites were obtained. 3. Both tissues produced labelled progesterone, androst-4-ene-3,17-dione and testosterone in varying quantities. The amount of progesterone was about 120 times greater in the adrenal tissue. In testis tissue dehydroepiandrosterone was found only in small quantity. 4. A pathway is suggested for the biosynthesis of Δ^{16}-steroids from pregnenolone in boar testis tissue. The possibility that progesterone may be an intermediate is discussed.

Although the metabolism of pregnenolone (3β-hydroxypregn-5-en-20-one) has been studied both *in vitro* and *in vivo*, there have been only two reports of the formation of Δ^{16}-steroids from this substrate. Burstein & Dorfman (1962) found that, after [4-^{14}C]cholesterol and [7α-^{3}H]pregnenolone had been administered to a woman with a virilizing adrenal adenoma, the An-α* subsequently isolated from her urine contained both isotopes. More recently, Ahmad & Gower (1966) showed that small quantities of two other Δ^{16}-steroids, closely related to An-α, namely 5β-androst-16-en-3α-ol and androsta-4,16-dien-3-one, were formed in the metabolism of [4-^{14}C]pregnenolone in rat testis tissue. Other compounds, including DHA (Gower, 1966), DHA acetate (Gower, 1963) and testosterone (Stylianou, Forchielli & Dorfman, 1961*a*; Stylianou, Forchielli, Tummillo & Dorfman, 1961*b*), can also serve as substrates, but the yields of the Δ^{16}-steroids are fairly low. Since two of these compounds, namely An-α and its 3β-epimer An-β, were originally isolated from pig testes (Prelog & Ruzicka, 1944), it was considered possible that tissue from this species might have a greater capacity for transforming pregnenolone into Δ^{16}-compounds. The present work, part of which has been briefly reported (Gower & Ahmad, 1966*b*) was therefore undertaken to investigate this possibility, both boar testis and adrenocortical tissue being employed. In addition, a number of metabolites of pregnenolone more polar than the Δ^{16}-androstenols have been isolated, including progesterone, androstenedione (androst-4-ene-3,17-dione), testosterone and, from testis only, a small quantity of DHA.

On the basis of these results, a new pathway is suggested for the biosynthesis of Δ^{16}-steroids from pregnenolone, with progesterone as a possible intermediate, but excluding well-known C_{19}-steroids such as testosterone and DHA.

* Abbreviations: An-α, 5α-androst-16-en-3α-ol; An-β, 5α-androst-16-en-3β-ol; DHA, dehydroepiandrosterone.

EXPERIMENTAL

Materials. Benzene was dried over sodium and distilled. Light petroleum (b.p. 80–100°) was shaken with conc. H_2SO_4, washed with water, dried and finally distilled. All other solvents were reagent grade and were distilled before use.

NAD^+, $NADP^+$ and ATP were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

[4-^{14}C]Pregnenolone (specific activity 15mc/m-mole) was obtained from The Radiochemical Centre (Amersham, Bucks.) and checked for purity by thin-layer chromatography with chloroform–acetone (9:1, v/v). A single radioactive spot was observed which corresponded to pure pregnenolone.

Alumina (type H, 100–200 mesh; Peter Spence and Co. Ltd., Widnes, Lancs.) was partially deactivated by the addition of water (3·5%, v/w). Column chromatography was carried out in glass tubes (200mm. long × 10mm. internal diam.) that were fused at the upper end to bulbs of 150ml. capacity. Kieselgel G was obtained from Anderman and Co. Ltd. (London, S.E. 1) and, for the preparation of thin-layer plates, 40g. was mixed with water (78ml.) and a solution (0·0025% in 0·1N-NaOH) of fluorescein (2ml.). Paper chromatography was performed on Whatman 3MM paper that had been washed with methanol and dried before use.

Steroids. For use as carriers and chromatographic markers, a mixture of six Δ^{16}-steroids was prepared in ethanol at a concentration of about 1 mg./ml. each. These were An-α, 5β-androst-16-en-3α-ol, An-β, androsta-5,16-dien-3β-ol, androsta-4,16-dien-3-one and oestra-1,3,5(10), 16-tetraen-3-ol. An-α was prepared by Brooksbank & Haslewood (1961). 5β-Androst-16-en-3α-ol, androsta-5,16-dien-3β-ol, An-β and oestra-1,3,5(10),16-tetraen-3-ol were generously supplied by Dr C. L. Hewett (Organon Laboratories Ltd., Lanarkshire, Scotland). Androsta-4,16-dien-3-one was prepared by oxidation of androsta-5,16-dien-3β-ol (Brooksbank & Gower, 1964).

Preparation of tissue and incubation conditions. Boar testes and adrenals, obtained fresh at the slaughterhouse, were placed in polythene bags, immersed in ice and immediately transported to the laboratory. Small portions of testis and adrenal cortex were rinsed with ice-cold buffer (see below), blotted, weighed and minced quickly. The minced adrenal (1·1 g.) and testis (1·9 g.) were transferred to two flasks containing NAD^+, $NADP^+$, ATP (1 μmole each) and [4-^{14}C]pregnenolone (1630470 counts/min.). A 5 ml. volume of Krebs–Ringer bicarbonate buffer, pH 7·4 (Krebs, 1950), containing glucose (200 mg./100 ml.) was used as the incubation medium. The flasks were incubated for 4 hr. at 37° under air in a metabolic shaker.

Extraction of steroids. The incubation was terminated by the addition of ethyl acetate (5 ml.), after which the mixture of Δ^{16}-steroids (50 μg. each) was added. The contents of each flask were homogenized for 5 min. and then centrifuged. This process was repeated with a further three 5 ml. volumes of ethyl acetate. The combined ethyl acetate extracts were dried over Na_2SO_4, filtered and finally evaporated to dryness. Recoveries of the radioactivity initially added were 68% for adrenocortical tissue and 66% for testis tissue.

Column chromatography. The crude extracts were chromatographed on alumina (5 g.) and benzene–light petroleum (1:1, v/v) was used to elute Δ^{16}-steroids, essentially as described by Gower & Haslewood (1961). For the elution of more polar steroids, however, benzene–ethanol mixtures were used (Table 1) (Ahmad & Gower, 1966).

Paper and thin-layer chromatography. The Bush (1952) system A was used for the initial separation of the metabolites more polar than Δ^{16}-steroids.

Thin-layer chromatography was carried out on Kieselgel G that contained fluorescein. The two systems used for the separation of Δ^{16}-steroids (Gower, 1964) were toluene–ethyl acetate (9:1, v/v) and benzene–ether (9:1, v/v). Acetates of these compounds were run in toluene–ethyl acetate (19:1, v/v). Benzene–acetone (4:1, v/v) was mainly employed in the chromatography of steroids more polar than the Δ^{16}-steroids.

Detection of steroids. The Δ^{16}-steroids and Δ^5-3β-hydroxy steroids were detected by spraying with a saturated solution of iodine in light petroleum (b.p. 60–80°). The presence of Δ^4-3-oxo steroids was revealed with u.v. light (254 mμ).

Radioautography. This was performed with Kodirex X-ray film (Kodak Ltd., London, W.C. 1) after thin-layer chromatography.

Gas–liquid chromatography. Gas–liquid chromatography of three Δ^{16}-steroids was performed with a Series 104 (model 24) dual flame ionization chromatograph (W. G. Pye and Co. Ltd., Cambridge). A coiled glass column (5 ft. long × 3·5 mm. internal diam.) was packed with Gas-Chrom Q (100–200 mesh) coated with cyclohexanedimethanol succinate (CDMS)/methyl silicone gum (JXR) (0·6%/0·75%). At 200° and with carrier gas (argon) flow of 66 ml./min. the retention times relative to cholestane (20·5 min. ≡ 1·0) were: An-α, 0·28; An-β, 0·31; androsta-4,16-dien-3-one, 0·50. Steroids were estimated by dissolving in chloroform, adding cholestane as internal standard and injecting a suitable portion (containing 0·01–0·1 μg. of steroid) on to the column. Areas under the peaks were measured and compared with standard mass-peak area curves.

Determination of radioactivity. A Packard Tri-Carb liquid-scintillation spectrometer (model 3003) was used for radioactive counting. Fractions were dissolved in xylene (8 ml.) containing 2,5-diphenyloxazole (0·3%, w/v). The counting efficiency for ^{14}C was 75% with background about 18 counts/min.

RESULTS

Identification of Δ^{16}-steroids. Alumina column fractions I–III (Table 1) were submitted to thin-layer chromatography with toluene–ethyl acetate (9:1, v/v) together with the mixture of authentic Δ^{16}-

Table 1. *Alumina column chromatography of ethyl acetate-soluble material obtained after the incubation of* [4-^{14}C]*pregnenolone with boar adrenal and testis tissue*

The crude extracts of adrenal tissue (1102000 counts/min.) and testis tissue (1065000 counts/min.) were applied to columns containing alumina (5 g.) partially deactivated with water (3·5%, v/w) and eluted as indicated.

Fraction	Eluent	Vol. of eluent (ml.)	Radioactivity (counts/min). Adrenal	Testis
I	Light petroleum–benzene (1:1, v/v)	21—50	13548	110894
II	Light petroleum–benzene (1:1, v/v)	51—100	2834	121448
III	Light petroleum–benzene (1:1, v/v)	101—150	5724	194486
IV	Benzene containing ethanol (0·2%)	50	511900	90700
V	Benzene containing ethanol (0·5%)	50	63000	79100
VI	Benzene containing ethanol (1·0%)	50	28900	44400
		Total radioactivity ...	625906	641028
		% of radioactivity recovered from alumina column	56·8	60·1

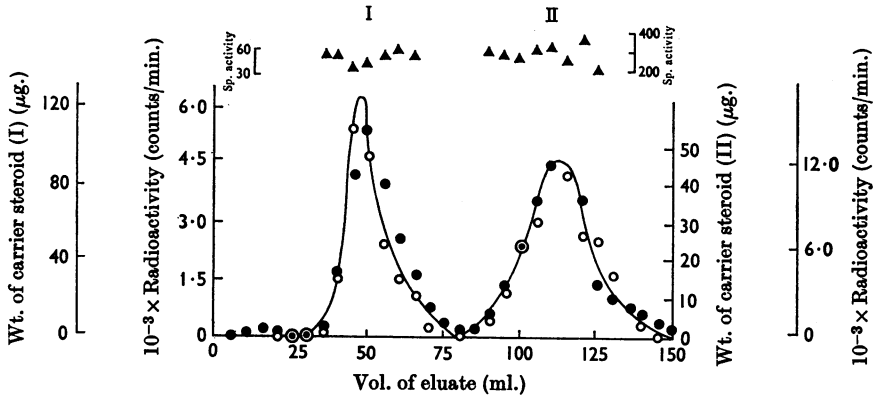


Fig. 1. Alumina column chromatography, with light petroleum–benzene (1:1, v/v) as eluent, of androsta-4,16-dien-3-one (I) and An-β (II) obtained after incubation of [4-^{14}C]pregnenolone with minced boar testis tissue. The weight of steroid (○) was estimated by gas–liquid chromatography and radioactivity (●) by liquid-scintillation counting. ▲, Specific activity (counts/min./μg.).

steroids. After development, the plates were dried and then developed again in the same direction with the same solvent mixture. This process was repeated once more as described by Gower (1964). Radioautography revealed three radioactive spots, which had the mobility of androsta-4,16-dien-3-one, An-α and An-β in both the adrenal and testicular extracts, although the amount of these steroids seemed to be greater in the testicular extract. The zones containing these compounds were eluted with three 2ml. volumes of methanol as described by Gower (1966) and rechromatographed on plates with benzene–ether (9:1, v/v). The radioactive zones detected by radioautography corresponded exactly to the positions of pure reference steroids (androsta-4,16-dien-3-one, An-α, An-β). The radioactivity of these metabolites obtained from the adrenal tissue was so small that further identification was based only on the preparation of their derivatives. An-α and An-β were acetylated with pyridine and acetic anhydride at room temperature overnight. After thin-layer chromatography with toluene–ethyl acetate (19:1, v/v) and radioautography, the radioactive spots corresponded to the positions of authentic An-α acetate and An-β acetate.

The identity of androsta-4,16-dien-3-one was proved by preparing its 2,4-dinitrophenylhydrazone as described by Stylianou *et al.* (1961*b*). This derivative was subjected to thin-layer chromatography with carbon tetrachloride–ethanol (124:1, v/v) (Ahmad & Gower, 1966) and the radioactivity coincided with the orange–red zone of the dinitrophenylhydrazone.

It was possible, however, to carry out a more detailed study to confirm the formation of An-α, An-β and androsta-4,16-dien-3-one obtained from boar testis tissue in view of the very much greater radioactivity associated with them. The zones corresponding to the $\Delta^{4,16}$-ketone and An-β were mixed, authentic carriers (200 μg. each) were added

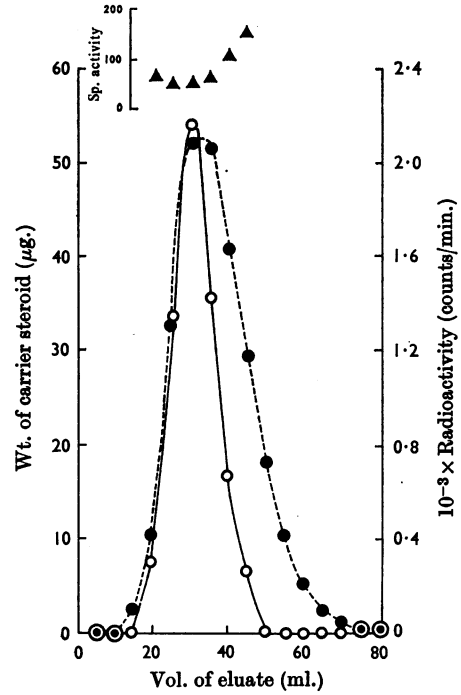


Fig. 2. Alumina column chromatography of An-α obtained after incubation of [4-^{14}C]pregnenolone with minced boar testis tissue. The eluent used and legend are as in Fig. 1.

Table 2. *Purification by recrystallization to constant specific activity of some metabolites of* [4-^{14}C]-*pregnenolone obtained from boar adrenal and testis tissue*

Values in parentheses are the weights (in mg.) of crystals.

	Specific activity (counts/min./mg.)						
		Progesterone		Androstenedione		Testosterone	
No. of crystallizations	An-β Testis	Adrenal	Testis	Adrenal	Testis	Adrenal	Testis
0	2620 (16·9)	2636 (26·8)	20 (30·2)	170 (10·1)	66 (12·8)	24 (24·6)	118 (24·7)
1	2528 (15·6)	2333 (24·0)	19 (28·5)	163 (9·9)	67 (11·9)	25 (24·0)	114 (24·5)
2	2486 (5·1)	2299 (19·2)	15 (20·4)	176 (8·6)	70 (10·1)	23 (10·9)	109 (21·1)
3	—	2175 (16·5)	13 (14·7)	163 (7·3)	62 (8·8)	23 (18·2)	108 (17·3)

Table 3. *Percentage conversion* in vitro *of* [4-^{14}C] *pregnenolone into various 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.

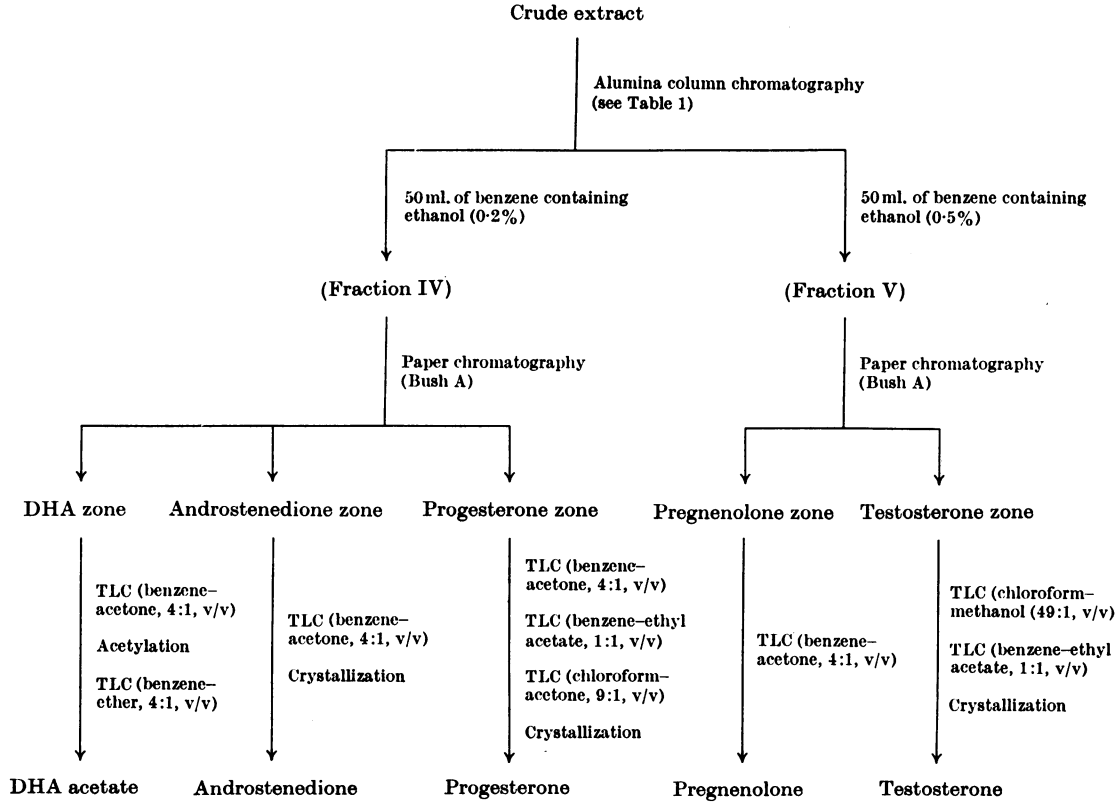
	Percentage conversion	
Metabolite	Adrenal	Testis
Androsta-4,16-dien-3-one	0·08	2·3
An-α	0·03	0·9
An-β	0·4	10·0
Progesterone	12·0	0·1
Androstenedione	0·2	0·1
Testosterone	0·08	0·4
DHA	—	0·03
Unchanged pregnenolone	1·9	1·0

and the mixture was separated on an alumina column with benzene–light petroleum (80–100°) (1:1, v/v) as described above except that fractions (5 ml.) were collected until both steroids were completely eluted. In each fraction the weight of steroid was determined by gas–liquid chromatography and the radioactivity measured. There was coincidence of radioactivity and mass peaks for both compounds (Fig. 1). Similarly, An-α was chromatographed on a separate alumina column and radioactivity and mass peaks were again plotted (Fig. 2). Although there was coincidence of radioactivity and mass peaks, there was some tailing of radioactivity, which suggested the presence of a labelled contaminant only partially resolved from An-α.

For final identification, the tubes that contained androsta-4,16-dien-3-one were pooled and the dinitrophenylhydrazone was prepared. Radioautography indicated a radioactive spot that coincided with the coloured zone of the authentic derivative. Similarly, the tubes containing radioactivity corresponding to carrier An-α and An-β were pooled separately and the whole of the An-α and a portion of the An-β were acetylated. The acetates were subjected to thin-layer chromatography with toluene–ethyl acetate (19:1, v/v) and the radioactivity associated with the prepared derivatives was eluted. This was subjected to hydrolysis (Bush & Willoughby, 1957) and, after rechromatography in the above system, followed by radioautography, showed radioactive spots having the same mobilities as pure An-α and An-β. To the remainder of the pooled An-β obtained from alumina chromatography was added authentic An-β (15–20 mg.) and recrystallization to constant specific activity was carried out (Table 2).

The results reported in the preliminary account of these experiments (Gower & Ahmad, 1966*b*) were in terms of the total wet weight of adrenal and testis tissue. Table 3 summarizes the percentage yield of metabolites corrected for analytical losses and expressed per g. wet wt. of tissues.

Identification of steroids more polar than Δ^{16}-*steroids.* Column fractions IV and V (Table 1) were chromatographed on paper with pure steroids used as markers in the Bush (1952) system A. The regions corresponding to progesterone, androstenedione and DHA in fraction IV were each eluted with methanol and investigated further as shown in Scheme 1. Purification of progesterone was carried out in various thin-layer chromatography systems and, after each run, radioautography revealed a radioactive spot coinciding with the spot produced in u.v. light by carrier progesterone. Crystallization to constant specific activity confirmed the formation of progesterone in both adrenal and testis tissue (Table 2), although the amount found in the latter tissue was very small. Androstenedione was subjected to thin-layer chromatography with benzene–acetone (4:1, v/v). Radioautography indicated the presence of a single radioactive spot in both incubations overlapping the spot produced in u.v. light by carrier androstenedione. These spots were eluted and, after the addition of carrier (15–30 mg.), crystallized to constant specific activity



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

(Table 2). This steroid seemed to have formed in relatively larger quantity in adrenal than testis tissue. The DHA zone was chromatographed with benzene–acetone (4:1, v/v). There was a small amount of radioactivity associated with the DHA spot formed in the testis incubation, but no radioactivity could be detected in the adrenal tissue incubation. The radioactive zone corresponding to DHA was eluted and acetylated with acetic anhydride and pyridine overnight at room temperature. After chromatography of the product with benzene–ether (4:1, v/v), the spot corresponding to DHA acetate was eluted and counted. No further purification could be carried out owing to the small amount of radioactivity present.

After paper chromatography of column fraction V, only two radioactive zones could be detected, one corresponding in mobility to testosterone and the other to pregnenolone. The testosterone zone was eluted and subjected to thin-layer chromatography with chloroform–methanol (49:1, v/v). A radioactive spot corresponding to 17α-hydroxyprogesterone was found only in adrenal tissue, but further purification failed to confirm its identity. Testosterone, which seemed to be present in both incubation experiments, was subjected to thin-layer chromatography with benzene–ethyl acetate (1:1, v/v). Radioautography again indicated radioactive spots overlapping with the spots produced by carrier testosterone under u.v. light. Further confirmation of the identity of testosterone was provided by crystallization to constant specific activity (Table 2). The pregnenolone zone was purified by thin-layer chromatography (Scheme 1).

The radioactivity in column fraction VI represented 3–5% of the total activity applied to the columns, but no attempts were made to purify and identify the metabolites that might have been present.

DISCUSSION

The suggestion of Neher & Wettstein (1960) that pregnenolone is metabolized to androgens by two pathways has received ample support. The so-

called Δ^4 pathway involves progesterone, 17α-hydroxyprogesterone and androstenedione, and has been shown to operate in testis and adrenal tissue from a variety of species including human (Acevedo, Axelrod, Ishikawa & Takaki, 1961), ox (Kahnt, Neher, Schmid & Wettstein, 1961), rat (Ahmad & Gower, 1966) and mouse (Grosso & Ungar, 1964). The second pathway, involving the Δ^5-3β-hydroxy steroids, 17α-hydroxypregnenolone and DHA, has been elucidated by, e.g., Goldstein, Gut & Dorfman (1960), Eik-Nes & Hall (1962), Eik-Nes & Kekre (1963) and Hall, Sozer & Eik-Nes (1964). In kinetic experiments with rat-testis homogenate, Slaunwhite & Burgett (1965) showed that androstenedione and testosterone were formed independently, the former via the Δ^4 pathway and the latter by the Δ^5 pathway. The step between 17α-hydroxypregnenolone and DHA was found to be rate-limiting. In some preparations, e.g. in rat-testis microsomes (Shikita, Kakizaki & Tamaoki, 1964), the Δ^4 pathway occurs preferentially, little or no Δ^5-3β-hydroxy steroids being formed. In the present experiments with boar adrenal and testis tissue, a fixed 4 hr. incubation time was used and a direct comparison of the two pathways is therefore difficult to make. The isolation of progesterone, androstenedione and testosterone at this time (Table 3), but little or no labelled Δ^5-3β-hydroxy steroids, however, suggests that the Δ^4 pathway may be occurring preferentially.

Biosynthesis of Δ^{16}-steroids. The experiments of Stylianou *et al.* (1961*a*,*b*), in which testosterone was transformed into small quantities of androsta-4,16-dien-3-one and An-β, led these workers to suggest that the elements of water were eliminated between C-16 and the 17-hydroxyl group of testosterone, resulting in the formation of the $\Delta^{4,16}$-ketone. Reduction in ring A could then presumably lead to one or more of the Δ^{16}-androsten-3-ols. Dorfman (1961) subsequently postulated An-α as the specific metabolite of testosterone, but experiments *in vivo* performed in a number of Laboratories failed to confirm either this conversion or that of DHA into An-α (Bulbrook, Thomas & Brooksbank, 1963; Ahmad & Morse, 1965; Cleveland & Savard, 1964). In accord with these studies *in vivo*, no Δ^{16}-compounds were found with testosterone as substrate with boar adrenal and testis tissue (Gower & Ahmad, 1966*a*), and only very small yields with DHA acetate with ox adrenal (Gower, 1963) and DHA with rat testis (Gower, 1966). The possibility that the 17β-acetate of testosterone, as opposed to testosterone itself, might give greater yields of Δ^{16}-steroids has been pursued in preliminary experiments in this Laboratory with boar testis tissue because the chemical dehydration of compounds such as testosterone toluene-*p*-sulphonate is known to result in androsta-4,16-dien-3-one (Henbest & Jackson, 1962). These experiments, however, have failed to confirm testosterone acetate as a precursor, although of course the ester may have been hydrolysed by esterase activity before any significant conversion into Δ^{16}-steroids could occur.

The present results (Table 3) provide evidence that pregnenolone is a precursor of Δ^{16}-steroids, in particular of An-β, in boar testis tissue and in adrenocortical tissue. In current experiments in this Laboratory, greater yields of An-α and An-β have been obtained with [4-^{14}C]progesterone as substrate with boar testis tissue, suggesting that progesterone is nearer to Δ^{16}-steroids than is pregnenolone. In the present work, our inability to find androsta-5,16-dien-3β-ol suggests that pregnenolone is oxidized first to the Δ^4-3-oxo steroid progesterone before removal of the C_{20-21} side chain and introduction of the double bond at C_{16-17}. The compound(s) that may be intermediate between progesterone and Δ^{16}-steroids are as yet unknown, but kinetic studies to investigate the early stages in the metabolism of progesterone may help to elucidate the problem.

The results presented in this paper are in accord with those of Burstein & Dorfman (1962), who showed that both cholesterol and pregnenolone gave rise to An-α *in vivo*. They are also in agreement with the studies of Brooksbank (1962) and of Cleveland & Savard (1964), in which the administration of adrenocorticotrophic hormone to normal human subjects increased their urinary An-α excretion. Adrenocorticotrophic hormone is now known to act specifically at the steps in steroidogenesis between cholesterol and pregnenolone (Karaboyas & Koritz, 1965) and would therefore be expected to increase the formation of Δ^{16}-steroids if cholesterol and pregnenolone were precursors.

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