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GENERAL REVIEW

16-UNSATURATED C_{19} STEROIDS A REVIEW OF THEIR CHEMISTRY, BIOCHEMISTRY AND POSSIBLE PHYSIOLOGICAL ROLE

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1. INTRODUCTION

SINCE THE isolation of three 16-unsaturated C_{19} steroids from pigs' testes in 1944 [1] and from human urine in 1950 [2], this group of compounds was not intensively studied until some ten years ago. With their curious musk-like odour (see p. 93) they were considered until recently biochemical oddities that neither fitted into the normal pattern of steroid biosynthesis nor had any physiological activity. This review* surveys current knowledge concerning the chemical synthesis, occurrence, metabolism and physiological role of these compounds, which are now known to be important in the pig and possibly also in humans. Some reference is also made to a phenolic 16-unsaturated steroid, oestratetraenol† since this is related metabolically to androstadienone (see p. 72).

2. OCCURRENCE

The structures of the 16-unsaturated C_{19} steroids, that have so far been isolated from natural sources, are shown in Fig. 1.

(a) *In pigs.* The first report of the presence of 16-unsaturated steroids in pigs' testes was by Prelog and Ruzicka [1] who isolated and characterised relatively large amounts of an-α and an-β. At that time it was difficult to explain why such large quantities of these substances occurred in pigs' testes while the amount of androgenic substances was so small, but more recent work [3–6] has shown conclusively that the 16-unsaturated steroids are quantitatively more important than the androgens in the pig. Indeed, it appears that the metabolism of the C_{21} steroids, pregnenolone and progesterone is biased in favour of the formation of 16-unsaturated steroids rather than androgens [6]. Prelog and Ruzicka [1] were unable to give estimates of the amounts of an-α and an-β present in their pig testes extracts because of analytical losses but later workers [3, 4] have attempted to correct the yields obtained for analytical losses and these values are shown in Table 1.

It is clear that the proportions of the 16-unsaturated steroids in boar testes vary considerably with the age of the animal. Booth (personal communication) has shown that, in 84 day old foetal testes, andien-β is the dominant steroid followed by androstadienone > an-α > an-β > 5α-androstenone. In testes at birth

*This review of the literature was concluded in May 1971.

†Abbreviations and trivial names: an-α: 5α-androst-16-en-3α-ol; ae-α: 5β-androst-16-en-3α-ol; andien-β: 5,16-androstadien-3β-ol; an-β: 5α-androst-16-en-3β-ol; 5α- and 5β- androstenones: 5α- and 5β- androst-16-en-3-ones; androstadienone: 4,16-androstadien-3-one; oestratetraenol: 1,3,5(10),16-oestratetraen-3-ol; 20β-dihydropregnenolone: 5-pregnene- 3β,20β-diol; 16-dehydroprogesterone: 4,16-pregnadiene-3,20-dione.

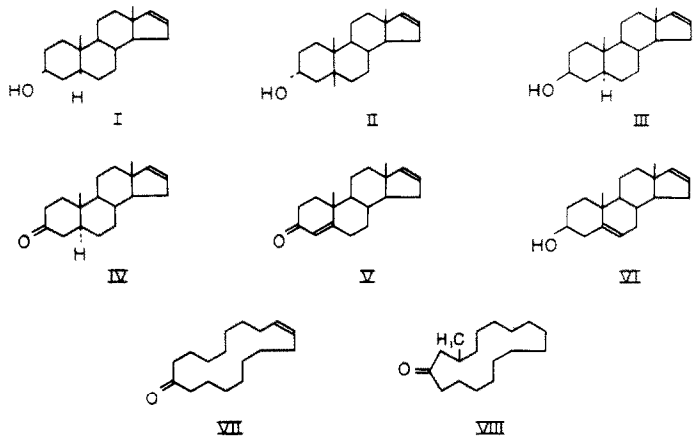


Fig. 1. Structures of some 16-unsaturated C_{19} steroids that have been isolated from natural sources. I, an-α; II, ae-α; III, an-β; IV, 5α-androstenone; V, androstadienone: VI, andien-β. The structures of civetone (VII) and muscone (VIII) are included for comparison.

Table 1. Amounts (μg/g tissue) of 16-unsaturated C_{19} steroids and testosterone present in boar tissues

Tissue	Age of boar (days)	5α-androstenone	an-α	an-β	andien-β	andro-stadienone	testo-sterone	Reference
Testis	175	0·10	0·28	1·35			0·006	
	217	0·27	0·74	4·41			0·021	[4]
	229	0·31	0·89	3·43			0·037	
	252*	0·034	0·78	2·74	0·22	0·006	0·02	W. D. Booth
	252†	0·05	0·34	1·62	0·10	0·006	0·031	(personal com-
	4 years	0·05	0·60	3·29	0·45	0·012	0·007	munication)
Parotid gland	175	0·17	29·9	—				
	217	11·43	82·0	5·69				[4]
	229	1·99	49·9	1·44				
Sub-maxillary	84	0·018	0·12	0·029	trace		trace	[3.61] and
gland	252*	0·063	1·12	0·19	0·032		0·007	W. D. Booth
	252†	0·032	0·35	0·07	0·028		0·013	(personal com-
	4 years	1·08	24·3	0·116	0·098		0·028	munication)
Fat	175	1·03						
	217	7·49						[4]
	229	1·74						

*weight 126 kg.
†weight 100 kg.

androstadienone predominates followed by andien-β > an-α > an-β > 5α-androstenone. The predominance of an-α over an-β continues in the prepubertal animal (to the age of approximately 12 weeks) but by about 18 weeks there is a reversal in the an-α:an-β ratio, an-β then becoming the predominant steroid. Table 1 shows that the ratio of an-β to an-α in animals 175–229 days is 4–6:1. These results clearly demonstrate the development with age of the biosynthetic pathway for 16-unsaturated C_{19} steroids.

In keeping with these findings, recent work [7] has shown that andien-β can be formed from pregnenolone in testes taken from pigs as young as 3 weeks and that

the yield obtained *in vitro* is of the same order as that in adult testis tissue (see p. 66). It also seems likely that andien-β may be the first 16-unsaturated steroid formed from pregnenolone and that it is further metabolised to androstadienone and then to an-α and an-β[8]. The finding of only trace quantities of andien-β sulphate in boar spermatic vein plasma lends support to the idea[5]. The predominance of the yields of an-β over an-α in *in vitro* incubations of mature testis tissue[6, 9] (see p. 56) is in complete agreement with the results of Booth mentioned above. It is of particular interest that the reversal of the ratio of an-α to an-β occurs at puberty in the boar. Since spermatogenesis begins at this time, it is conceivable that the seminiferous tubules may be involved in this transformation. No studies have yet been performed, however, owing to the problems involved in the separation of the tubules from interstitial tissue of boar testis[10] although the seminiferous tubules in other species are capable of some steroid transformations [11].

The data collected in Tables 1 and 2 also show the extent to which 16-unsaturated steroids occur in boar testes compared with testosterone. The ratios of an-β to testosterone are as much as 100–200:1 and strongly suggest that this group of 16-unsaturated steroids may possess a special physiological significance in the boar. The relevant biochemical and physiological studies are reviewed later on pp. 56 and 96.

The amount of testicular 5α-androstenone, a compound with an intense urine-like smell (see p. 93), also increases with age[3, 4]. It is this compound which, once formed in the testes[12] passes into the spermatic venous blood[5] and thence, presumably because of its high lipid solubility, to the adipose tissue. It has long been known that bacon joints, taken from entire male pigs, emit an unpleasant smell on being cooked; Patterson[13] was the first to isolate the so-called "boar taint" from fat samples and to show that its structure was identical with that of 5α-androstenone (see Fig. 2). A quantitative analysis of fat samples from immature pigs and from male castrates, however, showed that there was little if any 5α-androstenone present, a finding in keeping with the testicular origin of this

Table 2. Ratios* of 16-unsaturated C_{19} steroids to testosterone isolated from boar testis and submaxillary glands

Tissue	Age of boar (days)	5α-androstenone / testosterone	an-α / testosterone	an-β / testosterone	andien-β / testosterone	androstadienone / testosterone	Reference
Testis	175	17	47	225			[4]
	217	13	35	200			
	229	9	24	94			
	252†	1·7	39	137	11	0·3	[3]
	252‡	1·6	11	52	3·1	0·2	
	4 years	7	85	470	65	1·7	
Submaxillary gland	252†	9	160	28	4·6		W. D. Booth (personal communication)
	252‡	2·5	27	5·7	2·2		
	4 years	33	870	4	2·5		

*ratios calculated from data in Table 1.

†weight 126 kg.

‡weight 100 kg.

compound[3.4.12]. Fat taken from boars aged 175–225 days contained up to 11·4 μg/g of 5α-androstenone but there was no age-dependence[4] (Table 1). The practical implications of the presence of this steroid for the pig will be discussed later (p. 96).

Until recently the salivary glands in animals were considered as unimportant in steroid metabolism but there is evidence now to implicate them in corticosteroid [14], androgen[15] and 16-unsaturated steroid metabolism. Patterson[16] was the first to isolate an-α from boar submaxillary gland but was unable to demonstrate its presence in the corresponding parotid or sublingual glands. Gower and Katkov [7] were able to show the formation of andien-β from pregnenolone in homogenates of boar sub-maxillary glands (see p. 63). Recently, a gas–liquid chromatographic (g.l.c.) method has been used[4] to analyse parotid glands from boars and has revealed the presence of an-α, an-β and 5α-androstenone (Table 1).

Boar saliva has also been analysed for 16-unsaturated steroids[16.17] and both an-α (approximately 1·0 μg/ml) and 5α-androstenone (approximately 0·05 μg/ml) were shown to be present. The possible implications of these findings for the physiology of the boar will be discussed in a later section (p. 96).

In accord with the finding of an-α, an-β and 5α-androstenone in boar testes, the same three compounds have been detected in boar spermatic vein blood[5] and characterised by column, thin-layer and gas–liquid chromatography and finally by mass spectrometry[18] (Fig. 2). The two alcohols occurred predominantly as sulphates whereas the ketone was obtained in the "free steroid" fraction. The concentration of this ketone has been estimated in peripheral plasma of boars, sows and male castrates[4.19] and a definite age- and sex-dependence was shown to exist (Table 3). There was also a possible correlation with the concentration of testosterone in the same plasma samples (Fig. 3) but so far there is no evidence that the ketone is derived from testosterone[6].

An analysis of boar urine[20.5] revealed the presence of an-β, conjugated as glucosiduronate, at a concentration of approximately 250 μg/l. A corresponding analysis of the sulphate fraction showed that no 16-unsaturated steroids were present. These results, concerning the occurrence of 16-unsaturated steroids in the pig, are summarized in Table 4.

(b) *In man.* In contrast to the pig, the predominant 16-unsaturated steroid in human urine is an-α. This was originally isolated from the hydrolysed glucosiduronate fraction of male and female urine[2]. Using a colorimetric method[21] de-

Table 3. Concentration (ng/ml) of 5α-androstenone and testosterone in the peripheral plasma of pigs (from Ref. 4)

Type	Age (days)	5α-androstenone	testosterone
Boars	175	6·0	9·6
	217	17·6	15·0
	229	22·3	16·1
Sows	175	0·8	0·4
	229	2·0	1·4
	238	2·0	1·9
Male castrates	175	2·7	1·5
	217	1·7	2·1
	236	1·3	2·4

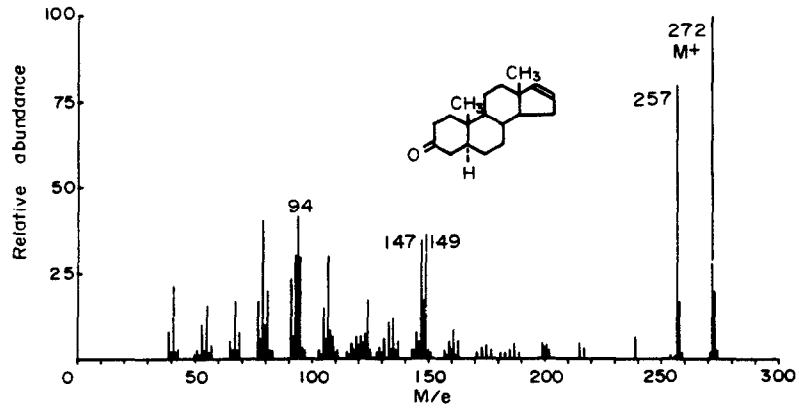
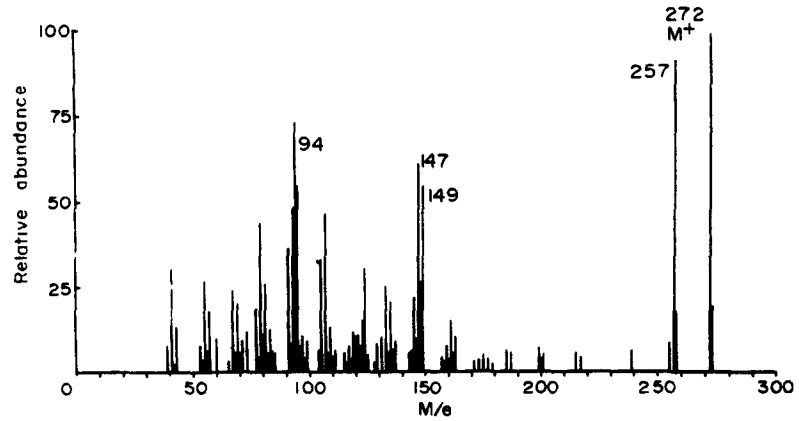


Fig. 2(A).



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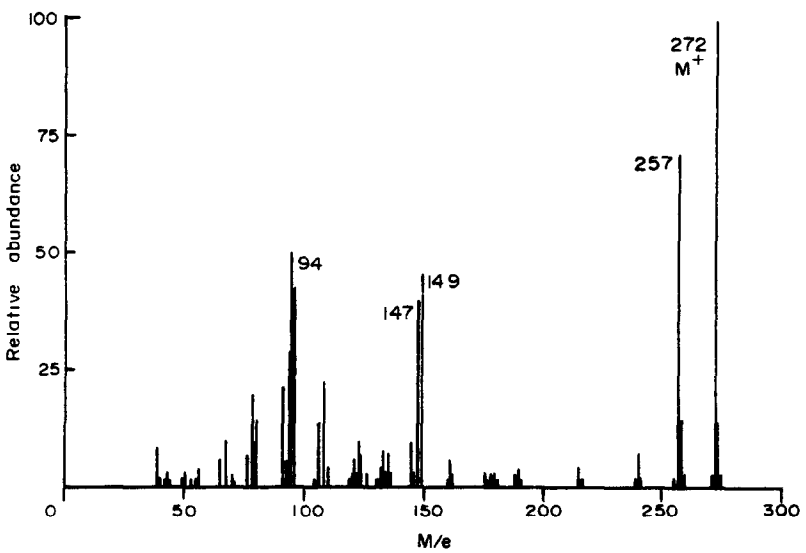


Fig. 2(C).

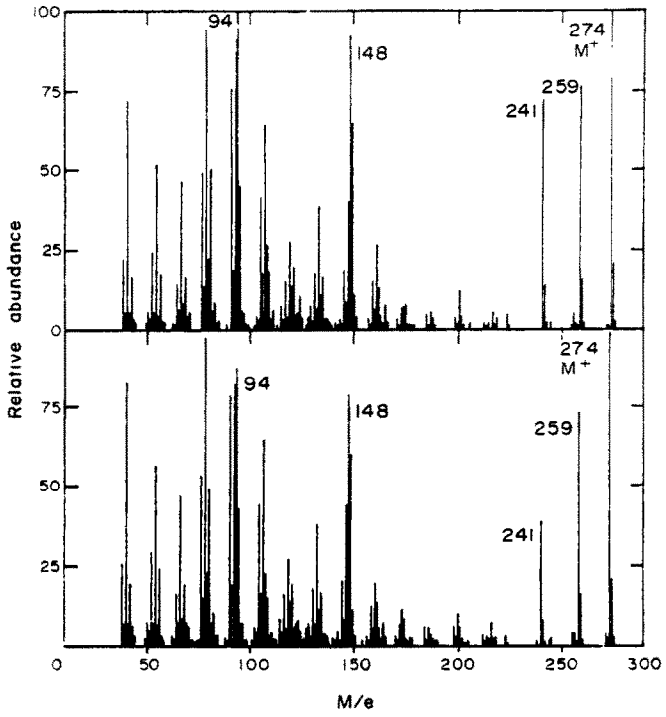


Fig. 2(D upper) and (E).

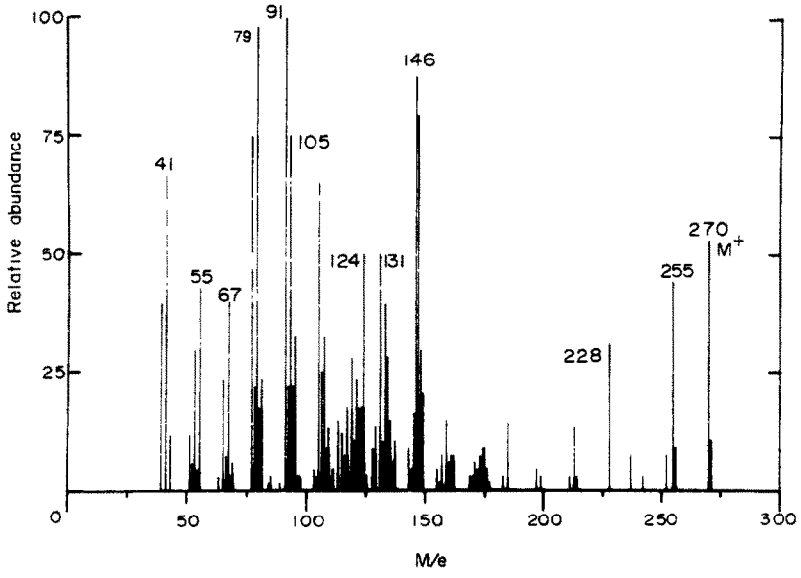


Fig. 2(F).

vised in later work (see p. 86), Brooksbank[24] analysed a large number of 24 h urine samples from healthy men and women of different ages and showed that the mean excretion was 1180 μg/day in men (age range 16–45 yr) and 429 μg/day in women (age range 16–45 yr). These results were confirmed by later investigators

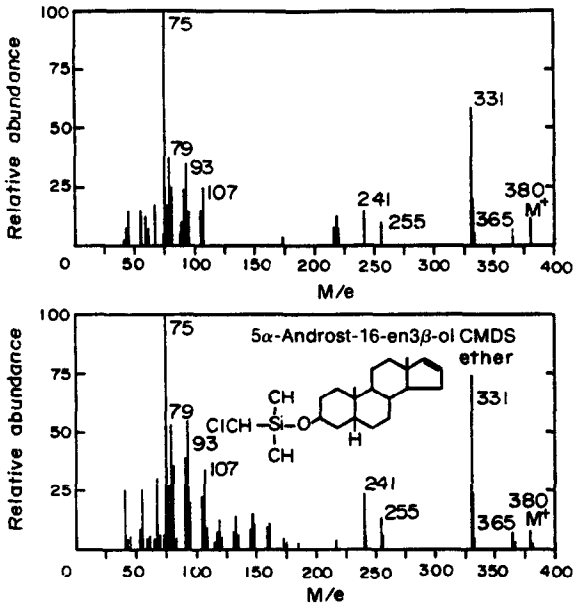


Fig. 2G (upper) and (H).

Fig. 2. Mass spectra of 16-unsaturated C_{19} steroids. A, 5α-androstenone; B, 5α-androstenone from boar fat; C, 5α-androstenone from human axillary sweat; D, an-α; E, an-β (note the different ratio of the intensities of the peaks m/e 241:274); F, androstadienone; G, an-β isolated from boar spermatic vein plasma and converted to the chloromethyl dimethyl silyl (CMDS) ether; H, an-β CMDS ether. (A and B are reproduced by permission of J. & A. Churchill, Ltd. from Ref.[130]; C, data from Ref.[34]; D and E by permission of The Society for Chemical Industry, Ref.[16]; F, from Gower and Patterson, unpublished [cf. Ref. 18]; G and H by permission of Journal of Endocrinology, Ltd., Ref. 18).

Table 4. Summary of 16-unsaturated C_{19} steroids present in boar tissues

Source	16-unsaturated C_{19} steroid present	Reference
Testis	an-α, an-β, 5α-androstenone, andien-β, androstadienone	[1.3.4] and personal communications from W. D. Booth and R. Claus
Salivary glands: submaxillary	an-α, an-β, 5α-androstenone, andien-β	[3.16] and W. D. Booth (personal communication)
parotid	an-α, an-β, 5α-androstenone	[4]
sublingual	none present (as judged by smell)	[16]
Saliva	an-α, 5α-androstenone	[16.17]
Back fat	5α-androstenone	[4.13]
Preputial gland	Only traces present	[65]
Spermatic vein plasma	an-α*, an-β* and andien-β (trace) as sulphates; an-α and 5α-androstenone as free steroids	[5]
Peripheral plasma	5α-androstenone	[4.19]
Urine	an-β (as glucuronoside)†	[5]

*predominantly as sulphate.
†approx. 250 μg/l.

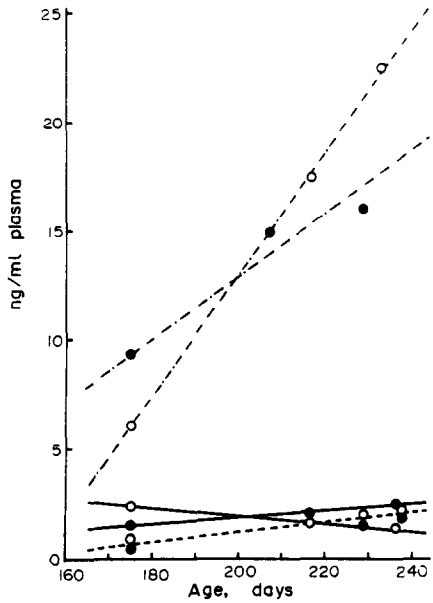


Fig. 3. Correlation with age and sex of 5α-androstenone (○) and testosterone (●) in the peripheral plasma of pigs. Males —·—·—·, male castrates, ———, females ------ (data from Ref. [4] by permission).

using the same colorimetric method [22] although a g.l.c. method devised later [23] resulted in slightly lower mean values for an-α excretion (1052 and 360 μg/day for men and women respectively in the same age ranges as above). Urinary an-α excretion is very small in infants and children [22.24] but increases at puberty to a maximum in the young adult years for both men and women and thereafter decreases to lower values in old age (Fig. 4). In many postmenopausal women an-α excretion was found to be as low as 100 μg/day or less whereas men over the age of 45 yr excreted some 500 μg/day on the average. Although the ranges for an-α excretion are very large (< 100–2630 μg/day for men and < 100–1100 μg/day for women) (Table 5), these changes with sexual development suggest a possible relationship with androgen production. An-α excretion is increased in some men after HCG administration while ACTH causes an increase in urinary an-α in both men and women [22.24] suggesting that the source of an-α may be, in part, the testes in men and the adrenals in women.

In breast cancer patients, a positive correlation was shown to exist between urinary excretion of an-α and that of androsterone, aetiocholanolone and DHA [26]. However, there is no evidence that an-α is derived from testosterone, DHA or other C_{19}-steroids (see p. 64) nor is there any clear correlation in a group of hirsute patients [23] (Table 6).

In 1964, an investigation [27] of 69 male psychiatric patients, aged 18–45 yr, showed that they were excreting significantly less an-α than a control group of healthy men in the same age range and also less than a group of psychiatric patients in whom no signs of schizophrenia were evident. There was, however, no significant difference between the amounts of 17-oxosteroids (17-OS) excreted by the three groups of individuals. The fact that there was a polynomial, or exponential, relationship between urinary an-α and 17-OS in both the healthy subjects and

Table 5. Urinary an-α excretion (μg/24 h) in normal humans

Type	No. of subjects	Age (yr)	Mean	Range	Type	No. of subjects	Age (yr)	Mean	Range	Reference
Males	48	12–80	1008	19–2630	Females	27	15–72	430	62–1010	(21)
Males	82	12–82		< 100–2630	Females	61	12–86		< 100–1100	(24)
	61	16–45	1180 ± 78 (S.E.)†			42	16–45	429 ± 41 (S.E.)†		
Males	17	Adult	950	210–2050	Females	16	Adult	420	0–910	(22)
Males	12	20–40	1052*	17–1659*	Females	8	20–35	360*	230–420*	(23)
Pre-pubertal children	5	4–11	< 20	< 10–53						(21)
Pre-pubertal children	20	4–11	< 50	< 10–90						(24)
Pre-pubertal children	14		0	0–120						(22)

Estimations were performed using the colorimetric method (21) except for those marked * where the g.l.c. method[23] was used.
†Standard Error.

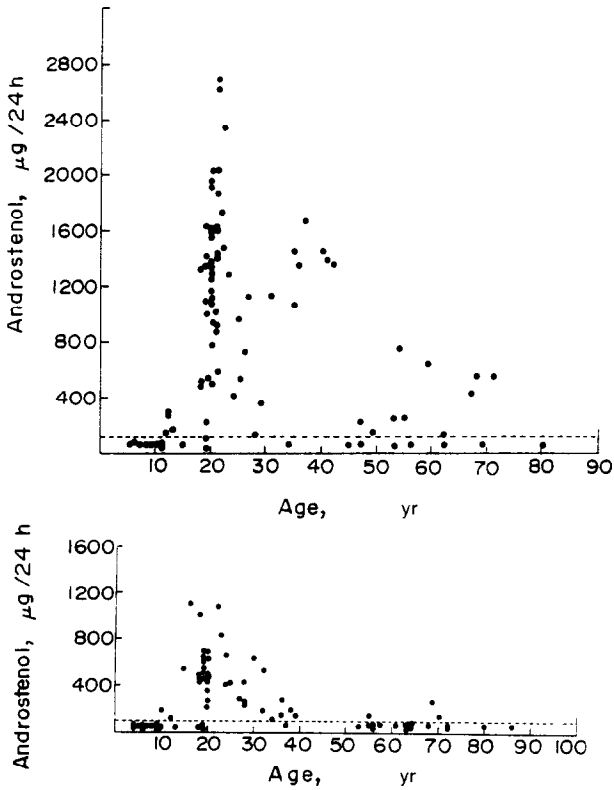


Fig. 4. Variations in urinary an-α with age in men (upper) and women (lower). Redrawn from Ref. [24] by permission of Journal of Endocrinology Ltd. The limit of reliability of the method of estimation (100 μg/24 h) is indicated thus, -------.

Table 6. Urinary an-α and 17-oxosteroids (mg/24 h) in females with simple hirsutism

Patient	Age (yr)	An-α	Androsterone	Aetichol-anolone	DHA
M.C.	32	0·7	4·8	4·8	0·95
J.W.	24	0·9	5·4	6·3	3·1
P.H.	17	0·55	4·0	3·7	6·0
I.B.	27	1·44	5·0	2·3	0·4
D.C.	40	0·43	3·3	3·3	1·75
C.M.	23	0·26	0·3	0·85	0·1
S.S.	19	0·35	2·5	1·1	0·65
P.O.	20	0·42	3·5	2·6	0·33
C.	19	0·47	1·6	1·6	0·4
S.C.	21	0·47	—	—	—
P.W.	19	0·39	—	—	—
Mean normal values (with ranges)		0·36* (0·23–0·42)	1·4† (0·9–2·6)	1·8† (0·6–2·2)	0·56† (0·23–0·42)

*From Ref. [23].

†From Ref. [31] (by permission of the publishers' Periodica, Copenhagen).

the patients was taken to suggest a possible indication of a 'safety-valve' mechanism; that is to say, the production of an-α or other 16-unsaturated steroids may be a side-reaction which becomes of greater importance as androgen production rates increase. At low androgen production rates this mechanism would not be expected to operate. As discussed by Brooksbank and Pryse-Phillips[27] the existence of such a mechanism would imply that an-α excretion would be a more sensitive index of effective androgen secretion than that of 17-OS. In a more recent study[28], however, the urinary an-α levels of a smaller group of schizophrenics (10 individuals) were substantially higher than in the earlier study and there was now no difference between the urinary an-α levels of patients and controls. The reason for the failure to repeat the earlier observations[27] may be the existence of a sub-group of schizophrenics in whom signs of androgen deficiency are present. It is conceivable that, in the earlier study, the patient group contained more individuals of this type.

In addition to an-α, the glucuronoside fraction of human urine contains two more 16-unsaturated C_{19} steroids, ae-α and andien-β, at concentrations of approximately 6 and 25 μg/l respectively[29]. More recent analyses[23] have resulted in mean values (μg/g creatinine) for ae-α and andien-β of 5·88 and 43·4 respectively for men and 4·77 and 20·7 respectively for women. In these studies no an-β has been detected in human urine but recently the presence of a small amount has been demonstrated following the intravenous administration of labelled androstadienone[30]. In a woman with a virilizing adrenocortical carcinoma[31] approximately 30% of the urinary an-α was excreted conjugated as sulphate (see p. 65).

Human plasma has received scant attention as far as 16-unsaturated steroids are concerned. Brooksbank and his colleagues[32] have provided evidence for the presence of small quantities of androstadienone in male peripheral plasma but only approximate values were given by these workers since no correction was made for analytical losses entailed by the method. The amount of androstadienone in the 'free steroid' (ether-extractable) fraction was approximately 0·5–1·0 μg/l, while twice this amount was present in the 'sulphate-conjugated' fraction. From experiments performed subsequently both in Brooksbank's and in the author's laboratories, it seemed likely that some androstadienone existing as 'free steroid' in plasma was not extracted with ether at an alkaline pH and this may thus have contributed to that recovered after solvolysis of sulphates. More recent analyses of pools of plasma from normal men and women, using [^{3}H]-androstadienone to estimate losses, have given values in the free fraction of 0·984 and 0·366 μg/l respectively[33]. The only other reference to 16-unsaturated steroids in human plasma is by Gower and Stern[31] who reported tentative evidence for the presence of an-α in the peripheral plasma of a woman with a virilizing adrenocortical carcinoma; after adrenalectomy, an-α was no longer detectable.

In view of the intense smell of some of the 16-unsaturated C_{19} steroids, especially 5α-androstenone (see p. 93), Gower and Llewellyn[34] felt it worthwhile to analyse axillary sweat obtained from human male subjects to see if these compounds could contribute to underarm smell. Collections of axillary male sweat were made for periods of up to 18h on pads of fat-free cotton-wool strapped in the arm-pits. The pads were changed at intervals of six hours and the combined pads extracted in a Soxhlet apparatus with double-distilled peroxide-free ether. After removal of fatty acids, by washing with 0·1 M NaOH, the ether extract was

dried and evaporated to dryness. The residue was then subjected to sublimation on a micro-scale at 90°C under a vacuum of 0·15 mmHg. The lipid that had condensed on the cold-finger (maintained at −67°C throughout) was subsequently recovered with ether and the solution analysed by g.l.c. and finally by combined gas chromatography-mass spectrometry. 5α-Androstenone was thus characterised in one male axillary sweat sample (Fig. 2), but attempts to detect this same compound in the sweat of other male subjects have failed (B. W. L. Brooksbank, personal communication). It is conceivable that there may be a temporal or individual variation in the excretion by this route of this powerfully-smelling compound. After intravenous administration of [4−^{14}C] androstadienone to men or women, the axillary excretion of ^{14}C was slight and no greater than after administration of labelled 4-androstenedione, progesterone or pregnenolone by the same route (Ref. 35 and B. W. L. Brooksbank, personal communication).

The phenolic 16-unsaturated steroid oestratetraenol is formed from androstadienone (see p. 72) and occurs in pregnancy urine (conjugated as glucosiduronate) at a concentration of approximately 100 μg/l [36].

3. BIOSYNTHESIS OF 16-UNSATURATED STEROIDS *IN VITRO*

(a) *In animals other than pigs.* In 1960 the presence of an-α and an-β in pigs' testes was known [1] and so also was the occurrence of an-α in human urine [2]. At that time, however, nothing was known about the biosynthesis of these compounds although it was generally thought that testosterone was the parent compound. This hypothesis was supported by evidence that testosterone was converted in small yield to androstadienone in a rat testis homogenate [37] and to androstadienone together with an-β in a human liver preparation [38]. At that time an-α was believed to be a specific urinary metabolite of testosterone [39].

In the years following 1960 in the author's laboratory, experiments were designed in order to ascertain the biosynthetic origin of 16-unsaturated steroids in, initially, rats, rabbits and guinea-pigs. An-α was formed in small yield from acetate in testis slices of rabbits and guinea-pigs [40]. In later experiments the metabolism of pregnenolone [41] and DHA [42] to 16-unsaturated steroids was studied in detail in rat testis preparations. Pregnenolone was converted to small yields of androstadienone and ae-α [41] while these compounds as well as an-α were formed from DHA [42]. A kinetic study of the formation androstadienone from DHA showed that the former increased in amount up to 2 h incubation but thereafter remained constant [42]. It should be emphasized, however, that the yields of 16-unsaturated steroids obtained in most of these experiments (summarized in Table 7) were extremely small (approximately 0·1% or less) and it seems unlikely that this group of compounds is of great significance in rabbits, rats or guinea-pigs. In contrast, bullock adrenal slices produced higher yields (1–2%) of an-α from DHA acetate [43].

(b) *Biosynthesis in porcine tissues.* Since 16-unsaturated steroids had been isolated from porcine testis by Prelog and Ruzicka [1], Gower and his collaborators considered it worthwhile to investigate the biosynthesis of these compounds in boar testis preparations. Both the C_{19} steroids pregnenolone [9] and progesterone [6] were metabolised in boar testis preparations to high yields of an-β (10–15%) and also to an-α (1–2%). These yields at once suggested that the 16-unsaturated steroids might be of particular importance in the boar, especially as the yields of androgenic compounds were relatively small in the same incubations [6]

Table 7. Biosynthesis *in vitro* of 16-unsaturated steroids in animals other than pigs

Species	Tissue preparation	Cofactors	Precursor	16-unsaturated steroid formed	Yield (%)	Reference
Rabbit, guinea-pig	testis slices	nicotinamide, ATP	acetate	an-α	0·05–0·1	[41]
Rat	testis homogenate	$NADP^+$, NADPH-generating system	testosterone	an-β androstadienone	~ 0·5 ~ 0·5	[37]
Bull	adrenocortical slices	nicotinamide, ATP	DHA acetate	an-α	1–2	[43]
Rat	testis mince	NAD^+, $NADP^+$, ATP, glucose	pregnenolone	ae-α androstadienone	0·1 0·02	[41]
Rat	testis minces (kinetic study)	NAD^+, $NADP^+$, ATP, glucose	DHA	an-α*, ae-α* androstadienone	0·1	[42]
Rabbit, rat, bull	testis minces	NAD^+, $NADP^+$, ATP	testosterone epitestosterone	none		[45]
Rat	liver 100,000 g supernatant;	NADH or NADPH	testosterone	none		[46]
	liver microsomes	NADH or NADPH	epitestosterone	none		

*tentative identification.

(Table 8). Recent work[3, 5] (already mentioned on p. 47) has also shown that the quantities of 16-unsaturated steroids in boar testis are greater than those of other C_{19} steroids such as testosterone. A more detailed kinetic study[6, 44] of the metabolism of progesterone in boar testis minces (Fig. 5) suggested that androstadienone might be the first 16-unsaturated steroid formed, being subsequently converted to the ring A-saturated alcohols, an-α and an-β, that accumulated later in the incubation period. In the light of more recent work, it seems likely that progesterone gives rise only to traces of androstadienone whereas some ten times as much is formed from pregnenolone under the same incubation conditions[6, 8] (Table 8) and very similar results have been obtained in human testis tissue[71] (Table 13). Evidence has also been provided[8] for the formation of andien-β in high yield (15–20%) from pregnenolone in boar testis

Table 8. Percentage incorporation of ^3H and ^{14}C into 16-unsaturated steroids and other metabolites obtained after incubation of ^3H- and ^{14}C-labelled C_{21} steroids with minces of boar testis

	Incubation 1 ([4-^{14}C]pregnenolone and [7α-^{3}H]progesterone)			Incubation 2 ([7α-^{3}H]progesterone and 17α-hydroxy[4-^{14}C]progesterone)		
Metabolite	^3H(%)	^{14}C(%)	$^3H/^{14}C$ ratio	^3H(%)	^{14}C(%)	$^3H/^{14}C$ ratio
Androstadienone	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
17α-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	—

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

Data from Ref. [6], by permission of The Biochemical Journal.

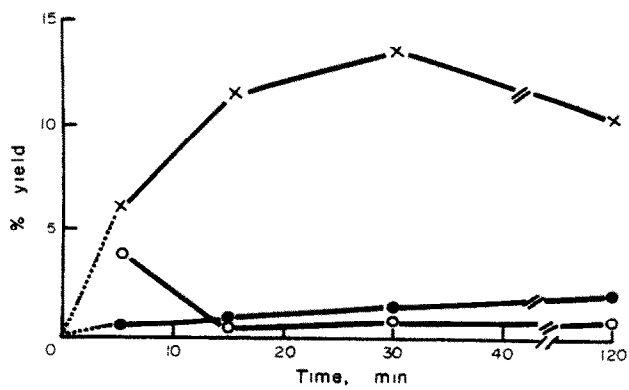


Fig. 5. Kinetic study of the formation of 16-unsaturated C_{19} steroids from [4-^{14}C] progesterone in a boar testis mince. Data from Refs.[6] and [44]. Androstadienone(○), × 100; an-α(●); an-β(x).

homogenates. Since andien-β could be converted to androstadienone if NAD^+ was present[8], it seemed that there were two possible pathways for androstadienone formation from pregnenolone:

(i) pregnenolone → andien-β → androstadienone

and

(ii) pregnenolone → progesterone → androstadienone.

Pathway (ii) however, appeared to be relatively unimportant in view of the minute yields of androstadienone formed from progesterone[6]. (Much larger yields (approximately 3%) of 5α-androstenone are formed from progesterone [12]).

A large number of C_{19}-steroids have been tested as possible precursors of 16-unsaturated C_{19} steroids in boar testis preparations. Testosterone and DHA [6], however, did not serve as precursors. Neither did the former steroid serve as a precursor in testis of rabbits, rats or bulls *in vitro*[45, 46]. In view of the ease of chemical dehydration of epitestosterone compared with testosterone [47], the former was considered a more likely precursor. However, when epitestosterone was incubated with testis preparations from boars[9] and from rats, rabbits and bulls[45, 46] little or no 16-unsaturated C_{19} steroids were formed.

Recently, the metabolism of testosterone has been investigated in the 105,000*g* supernatants of boar testis homogenate[48]. Only small yields (0·15–0·4%) of androstadienone were formed and traces of an-β (tentative identification). These results are therefore essentially in keeping with others in which testosterone was used[6]. However, when this steroid was incubated with the 105,000*g* supernatant of sow ovarian homogenates, good yields of androstadienone (up to 4·7% in some experiments) were obtained[48]. In the same series of experiments[48], both 16α- and 17α-hydroxyprogesterone were converted to 16-dehydroprogesterone but no 16-unsaturated C_{19} steroids were formed. Similar negative results using 16α-hydroxyprogesterone in boar testis homogenate have been obtained by other workers[49].

The search for intermediates between C_{21} steroids and 16-unsaturated C_{19} steroids

By comparison with the well-established biosynthetic pathways for C_{19}-steroids such as androstenedione and DHA, Ahmad and Gower[6] considered 17α-hydroxylated C_{21} steroids as likely intermediates, since side-chain cleavage was known to occur only after 17α-hydroxylation in androgen biosynthesis. In a double isotope experiment, however, using 17α-hydroxy-[4-^{14}C] progesterone and [7α-^{3}H] progesterone, the an-α and an-β isolated and purified contained ^{3}H as expected but no ^{14}C (Table 8). This surprising finding was subsequently confirmed and 17α-hydroxypregnenolone likewise excluded as an intermediate [9]. These results strongly suggested that, at least in boar testis, the 16-unsaturated C_{19} steroids are formed from pregnenolone or progesterone by pathways which do not involve the formation of 17α-hydroxylated derivatives before side-chain cleavage occurs. Indeed, it seems likely that such pathways may be unique in the field of steroid biochemistry. Table 8 also indicates the low yields of testosterone and androstenedione relative to those of the 16-unsaturated steroid, an-β. These results are in keeping with analytical results already described (p. 47).

Testosterone acetate is now well-known as an intermediate in the microbial conversion of progesterone to testosterone [50, 51] although it has recently been excluded as an intermediate in mammalian testicular steroidogenesis [52]. When the radioactive material was incubated with a boar testis preparation, however, it was found to be a very inefficient precursor of 16-unsaturated steroids [6]. Ahmad and Gower [6] pointed out the possibility of hydrolysis of the steroid ester to testosterone which, at that time, was already known to give rise only to traces of 16-unsaturated steroids [53]. When a specific esterase inhibitor, phenylmethylsulphonyl fluoride [54] was included in a similar incubation with testosterone acetate [20], only traces of 16-unsaturated steroids were formed and testosterone acetate was largely unhydrolysed. A parallel incubation with ^{14}C-labelled progesterone and an identical amount of the esterase inhibitor gave rise to the anticipated yields of an-α and an-β (Table 9). These experiments thus clearly excluded testosterone acetate as an intermediate in 16-unsaturated C_{19} steroid formation in boar testis.

Using unlabelled 16-dehydroprogesterone with a boar testis preparation [6], no 16-unsaturated C_{19} steroids could be detected suggesting that dehydration of progesterone did not occur prior to side-chain cleavage. Moreover, when unlabelled 5-androsten-3β-ol was used in an incubation together with radioactive pregnenolone [8] no radioactivity was trapped in the 5-androsten-3β-ol isolated even though radioactive andien-β was formed in the expected yield. Taken together, these experiments suggested that andien-β formation occurred from pregnenolone by a concerted series of reactions [8]. Fig. 6 summarizes 16-unsaturated steroid biosynthesis in boar testis *in vitro*.

The conversion of pregnenolone to andien-β

In recent years this step, or series of steps, has been studied intensively in boar testis homogenates. The enzyme or enzyme-system called 'andien-β synthetase' [55] bringing about this conversion requires NADPH and O_2 for full

Table 9. Investigation of testosterone acetate as a precursor for 16-unsaturated C_{19} steroids

Precursor	16-unsaturated C_{19} steroid formed (%)	Other products (%)
Testosterone acetate plus phenylmethylsulphonyl fluoride	None	Testosterone acetate (18·0) Testosterone (none) 5α-Androst-3-one-17β-yl-acetate (5·0)* 5α-Androst-3α- and 3β-diol-17β-yl-acetates (42·0)*
Testosterone acetate	None	Testosterone acetate (17·6) Testosterone (7·5) Androstenedione (0·38)
Progesterone plus phenylmethyl sulphonyl fluoride	An-α (2·0) An-β (12·0) 5α-Androstenone (3·0)	

Mince of boar testis were incubated for 2 h as described earlier [9]. The specific esterase inhibitor, phenylmethylsulphonyl fluoride [54], was added at a final concentration of 8×10^{-3} M.

*tentative identification only.

Yields are expressed as percentages of radioactivity incubated and are corrected for analytical losses. Reproduced from Refs. [20] and [56] by permission of J. & A. Churchill, Ltd.

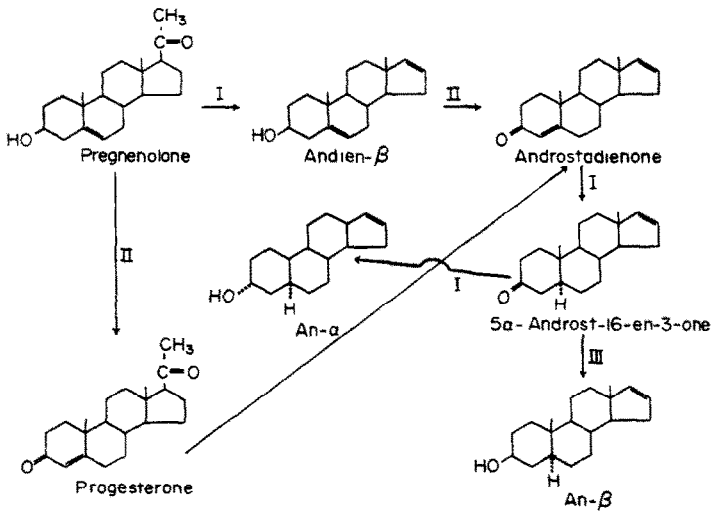


Fig. 6. Pathways of biosynthesis of 16-unsaturated C_{19} steroids in boar testis *in vitro*. I, NADPH; II, NAD^+; (based on results from Refs. 12, 156) III, NADH.

activity, NADH being a rather poor substitute [8, 56]. The 'andien-β synthetase' activity resides in the microsomal fraction of boar testis [55, 159] and enzyme activity is retained for up to three months if stored at −20°C. High yields (corrected for analytical losses) of up to 25% of andien-β have been obtained from pregnenolone in 10 min at 37°C using such preparations [55]. The rate of formation of andien-β from pregnenolone is linear up to 10 min and is also proportional to the amount of boar testis homogenate protein incubated [56, 57] (Figs. 7 and 8). Gower and

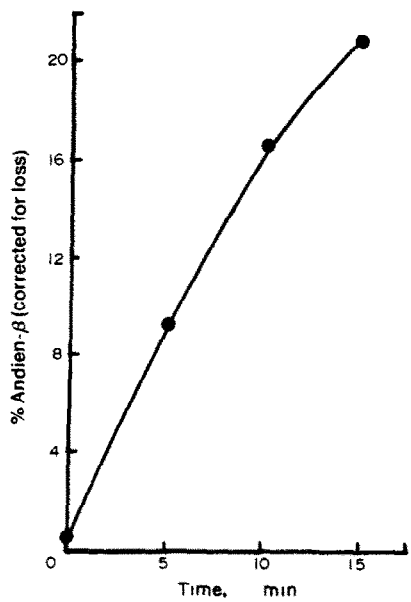


Fig. 7. Rate of formation of andien-β from [4-^{14}C] pregnenolone in boar testis homogenates. For further details see text. Data from Refs. [56] and [57].

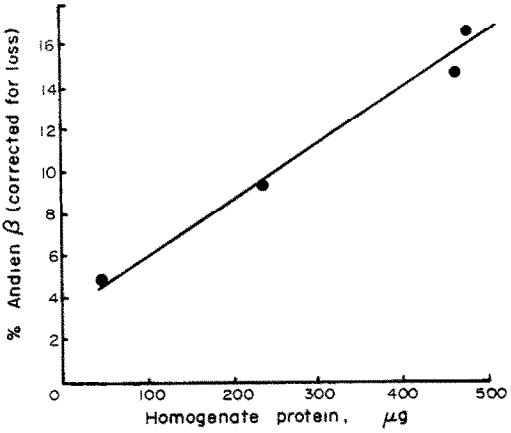


Fig. 8. Influence of the amount of boar testis homogenate incubated on the yield of andien-β formed from [4-^{14}C] pregnenolone. For further details see text. Data from Refs. [56] and [57].

Katkov [57] used as standard conditions, 200–400 μg homogenate protein incubated for 10 min at 37°C with [4-^{14}C] pregnenolone in Tris-HCl buffer (0·05M, pH 7·4) with NADPH (0·4 mM). The radioactive andien-β formed can then be readily isolated by t.l.c. first on Kieselgel G and then on $AgNO_3$-impregnated Kieselgel G, analytical losses being estimated by reverse isotope dilution (see p. 82, 86). In this way a fairly rapid assay for 'andien-β synthetase' activity in various tissues can be performed. The microsomal localisation of the enzyme [55, 159] this strongly indicates that it is distinct from the 'dehydratase' present in the 105,000g supernatant of sow ovaries and, to a lesser extent, in boar testis [48]. Moreover, the 'andien-β synthetase' is very NADPH-dependent.

Although 17α-hydroxypregnenolone has been excluded as an intermediate in this transformation [8] the possibility that dihydroxylated C_{21} steroids might be implicated was considered recently [55]. 16α,20β-Dihydroxyprogesterone had previously been used in a chemical synthesis of 16-unsaturated C_{19} steroids [49] (see p. 77) and 20β-dihydropregnenolone was found to give rise to 15–17% yields of andien-β, and the corresponding 20α-isomer only 2·7% compared with 25% for pregnenolone [55].

Short-term kinetic studies using 20β-dihydropregnenolone as substrate suggested that this steroid might be an intermediate in andien-β formation from pregnenolone and, in keeping with this, the 20β-isomer was isolated during andien-β formation [58]. Moreover, the biosynthesis of this 16-unsaturated steroid was severely inhibited (approximately 90%) if the boar testis preparation was incubated first with 20β-dihydropregnenolone before the usual incubation with pregnenolone took place. On the basis of these findings and a postulated free-radical mechanism for 16-unsaturated steroid biosynthesis [59], a reaction mechanism has been suggested [58] (Fig. 9). Using deoxycorticosterone (DOC) with boar testis preparations, Lippman and Lieberman [59] showed that a very small yield (0·046%) of androstadienone was obtained. This is very similar to the yields of this compound from progesterone [6] and it is conceivable that the 21-hydroxylase present in boar testis may bring about the conversion of progesterone to DOC and that this then forms androstadienone by a free-radical mechanism [59] (Fig.

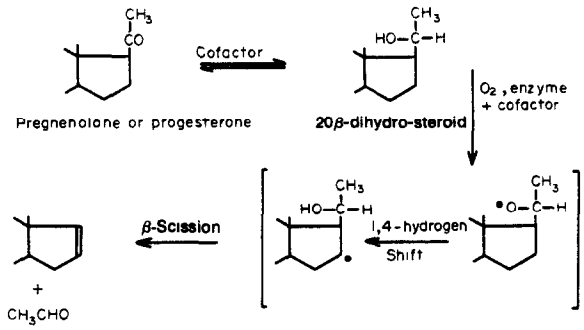


Fig. 9. Postulated free-radical mechanism for the formation of 16-unsaturated C_{19} steroids in boar testis showing the possible involvement of a 20β-dihydro-C_{21} steroid (from Ref. 58).

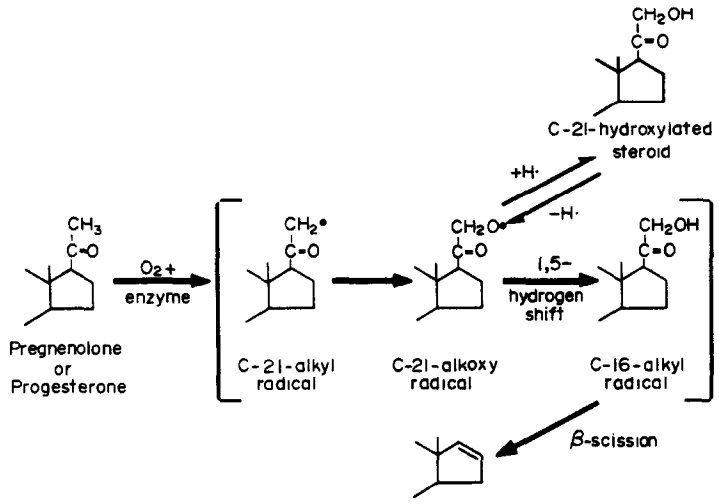


Fig. 10. Postulated free-radical mechanism for the formation of 16-unsaturated C_{19} steroids from deoxycorticosterone. Redrawn from Ref.[59] by permission of National Academy of Sciences, New York.

10). The evidence for the existence of such a sequence of reactions is based on the fact that andostadienone can be synthesized from DOC by free-radical generating reagents such as lead tetra-acetate (see p. 77). There is a somewhat analogous situation in that, when 20α-hydroxycholesterol-3-acetate is treated with this reagent, pregnenolone is formed in good yield [60].

Following the finding of 16-unsaturated steroids in porcine submaxillary glands and in saliva, the biosynthesis of this group of steroids was studied *in vitro* [7, 57]. [4-^{14}C]Pregnenolone was converted to 3·3% of labelled andien-β (Table 10) whereas no such conversion was demonstrated in a similar preparation of submaxillary gland taken from a hog [7, 57]. This strongly suggests that this gland in the pig is under the influence of the testis. Booth [61] considers that the submaxillary gland is a secondary sex organ in the boar, since he has isolated both testosterone and 5α-dihydrotestosterone from this organ, but there is no evidence at present for the actual biosynthesis of these compounds at this site.

Compared with the testes, the adrenal cortex in the boar is an inefficient producer of 16-unsaturated steroids. An-α, an-β and androstadienone were formed from pregnenolone and from progesterone *in vitro* but only in very small quantities [6, 9] (see Table 10).

It has been shown that boar "sex odour" is reduced if the preputial gland is removed surgically [62]. Since there is a high bacterial content in this gland [63] it was considered possible for 16-unsaturated steroids to be formed either by microbial transformation or in the preputial tissue itself. However, it is now clear that the boar preputial gland is histologically similar to keratinizing epithelium with no evidence of steroid secreting cells [20] (Fig. 11). Moreover, when portions of the minced tissue and preputial fluid were incubated with [4-^{14}C] pregnenolone no ^{14}C-labelled 16-unsaturated steroids were formed [20]. These findings are in agreement with those of Patterson who found only traces of 5α-androstenone in boar preputial fluid and considers that this odour is due to phenols, notably p-cresol [64], and long-chain fatty acids [65].

Biosynthesis of 16-*unsaturated steroids in porcine testis in vivo*

When boar testes were infused *in situ* with radioisotopic pregnenolone [101] the testicular venous blood contained labelled 5α-androstenone, an-α and an-β in the ether-extractable fraction. Labelled an-α and an-β were also found in the sulphate fraction but no 16-unsaturated steroids were found conjugated as glucosiduronates, although the urine, collected during the testicular infusion, did contain radioactive an-β glucuronoside. Moreover, analysis of the infused testis revealed the presence of labelled 5α-androstenone, an-α and an-β as free steroids. These results, which are in excellent agreement with earlier work [5], on the occurrence of 16-unsaturated steroids in boar spermatic venous plasma and urine, clearly demonstrate the ability of the boar testis to synthesize these compounds.

4. BIOSYNTHESIS OF 16-UNSATURATED STEROIDS *IN VIVO*

Following the *in vitro* experiments of Dorfman and his colleagues [37, 38], testosterone was at first considered to be the precursor of androstadienone and an-α. A number of workers attempted to show that C_{19} steroids gave rise to 16-unsaturated steroids *in vivo* but without success. Testosterone and DHA [26, 66, 67] and epitestosterone [68] were all excluded as precursors of an-α and other 16-unsaturated steroids. Likewise, 16α-hydroxyprogesterone [69] did not serve as a precursor in human subjects *in vivo*. However, when [7α-^{3}H] pregnenolone and [4-^{14}C]cholesterol were administered to a woman with a virilizing adrenal adenoma [70], the urinary an-α did contain very small amounts of both ^{3}H and ^{14}C.

Recently Brooksbank and Wilson [35] have studied in detail the metabolism of a mixture of [7α-^{3}H] pregnenolone and [4-^{14}C]progesterone and a mixture of [7α-^{3}H] 4-androstene-3,17-dione and [4-^{14}C]progesterone administered intravenously to two men. In the first experiment, the value of the cumulative specific activity of the urinary ^{3}H-andien-β was sufficiently high to indicate that this compound was being derived from the circulating pregnenolone. The percentage conversion of all three precursors into urinary an-α, however, was only 0·026–0·16%, indicating that this was not formed from circulating C_{21} or C_{19} steroids but was produced in a compartment in which these steroids did not come into rapid equilibrium from the general circulation.

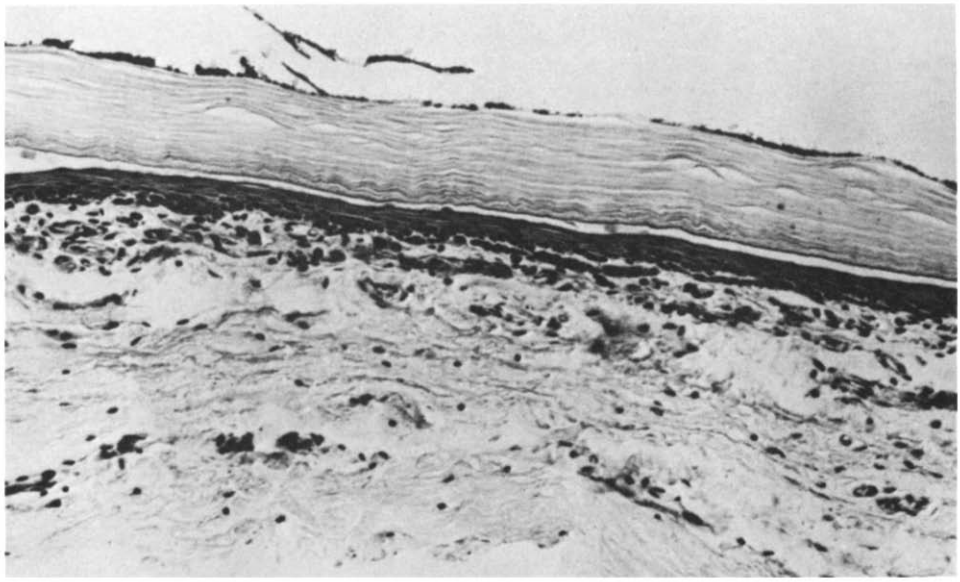


Fig. 11. Photomicrograph of a section (6μ thick) of boar preputial gland. The section was fixed in 4% formaldehyde, embedded and stained with haematoxylin and eosin. Magnification 75 × 50. Reproduced from Ref. [20] by permission of J. & A. Churchill Ltd.

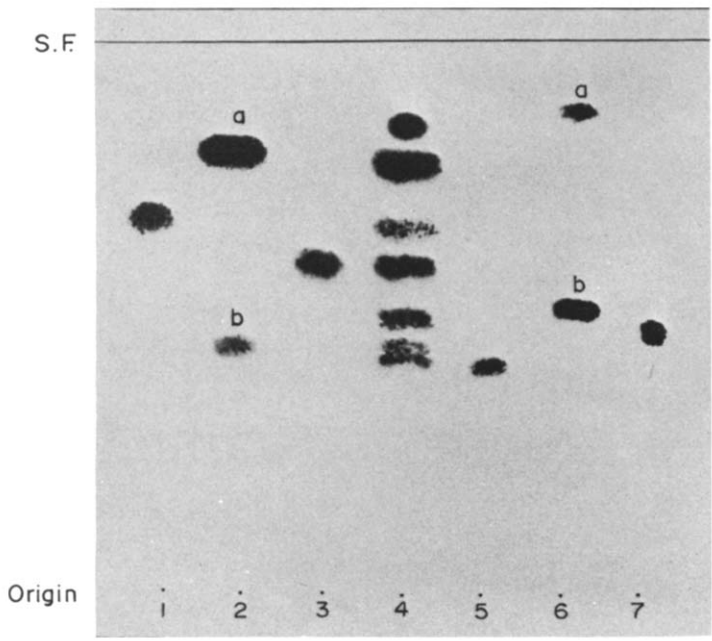


Fig. 19. T.l.c. of 16-unsaturated steroids on Kieselgel G. Lane 1, androstadienone; 2a, oestratetraenol; 2b, andien-β; 3, an-α; 4, mixture of 1, 2, 3, 5, 6 and 7; 5, an-β; 6a, 5α-androstenone; 7, 4,16-androstadien-3β-ol. The plate was run twice in benzene-ether (9:1, v/v).

(*Facing page* 64)

In keeping with these experiments showing that urinary an-α was not derived from circulating progesterone [35], urinary an-α did not increase during the pregnancies of two women although pregnanediol excretion increased as anticipated (Brooksbank and Gower, unpublished observations).

Possible sites of formation of 16-unsaturated steroids in humans

There is some evidence that andien-β is formed from pregnenolone in human testis *in vitro* [71] (see p. 69), although little or no an-α or ae-α was biosynthesized from this C_{21} steroid. Androstadienone, however, which may be the precursor of an-α and ae-α, is formed in sizeable quantities in the same incubations (Table 13).

Further experiments by Bicknell and Gower [71] indicated the testis as a source of an-α, ae-α and andien-β, or their immediate precursors, since removal of the testis resulted in a decrease in the urinary levels of these compounds (see p. 69).

As urinary an-α excretion is stimulated by administration of ACTH [24] it is conceivable that androstadienone, an-α and andien-β may be secreted by the human adrenals. Hitherto, only diseased adrenals have been studied *in vitro* and these produced no an-α from pregnenolone: androstadienone and especially andien-β were, however, biosynthesized in appreciable yields [25, 31] (Table 14).

Evidently the ovary of the sow has the ability to form androstadienone *in vitro* [48] (p. 59), but no direct evidence for the secretion of 16-unsaturated C_{19} steroids has yet been reported. Thus the origin of the major 16-unsaturated urinary steroid, an-α, still remains a mystery and further work is urgently needed to clarify the situation (see also Section 6).

5. OCCURRENCE AND BIOSYNTHESIS OF 16-UNSATURATED STEROIDS IN ENDOCRINE DISORDERS

Urinary an-α excretion has been measured in a number of women with simple hirsutism or with some abnormality of the endocrine glands. Early reports dealt with the isolation of an-α from the urine of women with adrenal tumours [73], adrenal hyperplasia [74] and luteoma of the ovary [75] but, since the authors made no attempts to estimate analytical losses, it is difficult to obtain accurate data for the urinary an-α. It seems likely, however, that there was a raised excretion of this compound in these patients. The more recent report of a woman with a virilizing adrenal adenoma [70] has already been mentioned (p. 64). In this case the urinary excretion of an-α and 17-oxosteroids was grossly elevated (Table 11).

In more recent work, urinary an-α, ae-α and andien-β have been measured, using a g.l.c. method [23] in a woman and a female infant with virilizing adrenocortical carcinomata [25, 31]. In both patients urinary an-α was grossly elevated, compared with that of normal subjects of the same age; as were the urinary ae-α, andien-β, 17-oxosteroids and testosterone (Table 11). But although there appeared to be a positive correlation in these patients between urinary 16-unsaturated steroids and androgens, no clear correlation emerged in a group of patients with simple hirsutism [23] (see Table 6).

Urinary an-α, ae-α and andien-β have also been measured in some testicular feminization (TF) patients [71]. Only in some individuals were the values raised while in others, normal amounts (for women of the same age) were being excreted (Table 12). In two patients (R.F. and S.D.) urinary an-α, ae-α and andien-β decreased after removal of the testes, indicating that these compounds or their

Table 10. Biosynthesis of 16-unsaturated C_{19} steroids in porcine tissues

Tissue preparation	Cofactor	Precursor	Yields (%) of 16-unsaturated steroids formed					Reference
			an-α	an-β	androstadienone	andien-β	5α-androstenone	
Testis minces	NAD^+, $NADP^+$, ATP, glucose	pregnenolone	0·9	10·0	2·3			[9]
Testis minces	as above	pregnenolone	1·6	10·6	0·06			[6]
Testis minces	as above	pregnenolone		2·0		8–15†		[8]
Testis homogenates	NAD^+, NADPH, ATP	pregnenolone	3–4†	1·9		8–15†		[7]
Testis homogenates:								
3 week old boar	NAD^+, NADPH, ATP	pregnenolone				15·5		[7]
4 week old boar	NAD^+ NADPH, ATP	pregnenolone				8·6		
8 week old boar	NAD^+, NADPH, ATP	pregnenolone				8·5		
Testis microsomes	NADPH	pregnenolone				25·6		[55, 58]
		20α-dihydro-pregnenolone				16·0		
		20α-dihydro-pregnenolone				2·7		
Testis minces	as Ref. [9]	progesterone	1·5–1·8†	10·0–13·0†	0·006–0·008†			[6]
Testis homogenate	NAD^+, NADPH, ATP, glucose	progesterone					3·6	[12]
Testis minces	as Ref. [9]	progesterone	2·0	12·0			3·0	[20]
Testis minces	as Ref. [9]	progesterone			>10% of 16-unsaturated C_{19} steroids			[49]
Testis mince	NAD^+, NADPH, ATP	progesterone		7·0		nil		[8]
Testis homogenate	NADPH	17α-hydroxy-pregnenolone			none formed			[8]
Testis mince	as Ref. [9]	17α-hydroxy-progesterone			none formed			[6]
Testis mince	as above	16α-hydroxy-progesterone			none formed			[49]

Testis mince	as above	16-dehydro-progesterone			none formed		[6]
Testis homogenate		deoxycorticosterone			0·046		[59]
Testis minces	as Ref. [9]	testosterone			none formed		[6, 53]
Testis (105,000 *g* supernatant)	NADPH-generating system	testosterone		trace*	0·15–0·40		[48]
Testis homogenate	NAD^+, NADPH, ATP	epitestosterone		< 1·0 of androstadienone, andien-β and an-β			[8]
Testis minces	as Ref. [9]	testosterone acetate	nil	0·01	0·01		[6]
Testis minces	as above	testosterone acetate			none formed		[20]
	plus phenylmethyl sulphonyl fluoride	testosterone acetate			none formed		[20]
Testis minces	as Ref. [9]	DHA	nil	0·01	0·01		[6]
Adrenocortical minces	as Ref. [9]	pregnenolone	0·03	0·4	0·08		[9]
		progesterone	0·007	0·015	0·04		[6]
		testosterone	nil	nil	nil		[6]
Ovary (no corpora lutea) 105,000 *g* supernatant	NADPH-generating system	testosterone	trace*		0·05–4·7		[48]
Submaxillary gland homogenate	NADPH	pregnenolone				3·3	[7]
Submaxillary gland homogenate (male castrate)	NADPH	pregnenolone				nil	[7]
Preputial gland plus fluid (homogenate)	NAD^+, NADPH, ATP glucose	pregnenolone		none formed			[20]

*tentative identification.
†several experiments.

Table 11. Urinary 16-unsaturated C_{19} steroids and 17-oxosteroids (mg/24 h) in cases of adrenal adenoma and carcinoma

Patient	Clinical condition	Age (yr)	an-α	ae-α	andien-β	andro	aetio	DHA	Reference
R.C.	Adenoma with Cushings' syndrome (hirsute)	29	0·050	—	—	—	—	—	[23]
	Post-operatively (14 days)		0·006	—	—	—	—	—	[23]
S.W.	Virilizing carcinoma	31	2·06 (G 1·46; S 0·6)	0·10	0·157	19·0 (G 13; S 6)	42·5 (G 23·5; S 19)	123·5 (G 46·3; S 77·2)	[23, 31]
	Post-operatively (1 month)		0·11	—	—	0·11	4·0	0·13	
V.D.	Virilizing carcinoma	1‡	0·51	0·004	0·026	2·6 (G 1·3; S 1·3)	1·6 (G 1·4; S 0·2)	21·6 (G 7·5; S 14·1)	[23, 25]
	Post-operatively (14 months)		0·014	N.D.	N.D.	0·03	0·06	0·01	
—	Virilizing adenoma		20‡	—	—	17·0‡	82·0‡	51·0‡	[70]
Mean normal values for adult women (with ranges)			0·36* (0·23–0·42)	0·0048*	0·0207*	1·4† (0·9–2·6)	1·8† (0·6–2·2)	0·56† (0·2–1·1)	
Mean normal values for infants			N.D.*	N.D.*	N.D.*	0·1	0·1	0·1	

*Ref. [23].

†Ref. [31] (by permission of Periodica, Copenhagen).

All values were obtained by the g.l.c method of Brooksbank and Gower[23] except for those marked which‡ were obtained as weights of isolated crystalline material. G = glucuronoside, S = sulphate, Andro = androsterone; aetio = aetiocholanolone. N.D. means not detected.

Table 12. Urinary excretion of 16-unsaturated C_{19} steroids (μg/g creatinine) in testicular feminization

Patient	an-α	ae-α	andien-β
L.R. (pre-operatively)	209 (130–360)*	6·9 (3·0–10·4)*	29·0 (12·5–46·0)*
R.F. (pre-operatively)	1280	11·7	59·0
R.F. (post-operatively)	463	6·3	21·0
S.D. (pre-operatively)	631	1·83	9·7
S.D. (post-operatively two days)	525	0·52	2·5
S.D. (post-operatively seven days)	146	0·0	—
F.N.† (pre-operatively)	339	5·7	25·0
Normal values	230–420‡	4·8‡	20·7‡

*Mean and range of several estimations.
†Male pseudohermaphrodite with histologically normal testes.
‡From Ref. [23]; data from Refs. [71] and [106].

immediate precursor(s) had their origin in these glands. The fact that administration of HCG to another TF patient resulted in 2–4 fold increases in 16-unsaturated steroid excretion[71] (Figs. 12, 13) gave further support to this suggestion; normally the HCG effect occurs only in men. Administration of ACTH also caused an increase in urinary an-α in normal men and women[22, 24] as well as in an-α, ae-α and andien-β in TF syndrome (Figs. 12, 13) thus suggesting an adrenal origin for these compounds[71].

Bicknell and Gower[71], however, were unable to show the formation of more than traces of an-α and ae-α from pregnenolone or progesterone when testis minces from TF patients were incubated *in vitro* (see Table 13); andien-β was formed from pregnenolone in yields of 1–8% but, in contrast to porcine testis (Ref. 6, Table 8), approximately six times as much androstadienone was formed from progesterone than from pregnenolone. This finding in testis tissue from three

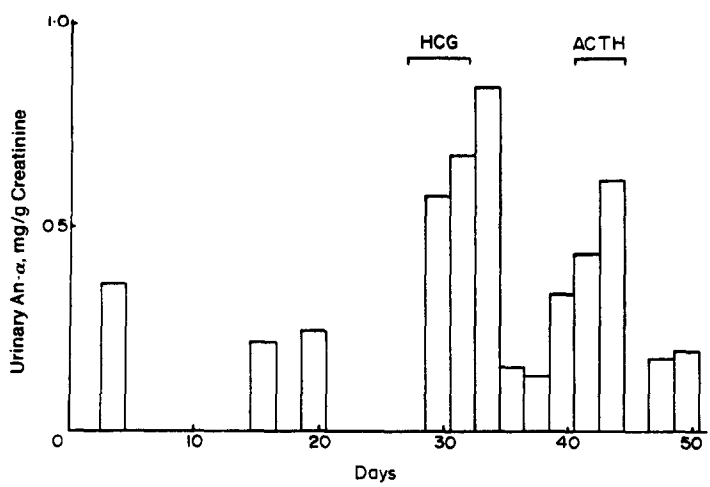


Fig. 12. Effect of ACTH and HCG administration on urinary an-α in a woman (L.R.) with testicular feminization (from Refs. 71 and 106).

Table 13. Biosynthesis of 16-unsaturated C_{19} steroids in human testicular feminization testis preparations

Patient	Precursor	Percentage yields (corrected for analytical losses) Androstadienone	andien-β	an-α†	ae-α†	Ratio of: androstadienone from pregnenolone / androstadienone from progesterone
R.F.	Pregnenolone	0·22	5·1	0·1	nil	0·25
	Progesterone	0·86	nil	0·1	0·2‡	
J.M.	Pregnenolone	<0·1	1·0	nil	nil	<0·5
	Progesterone	0·2	nil	nil	0·2‡	
S.D.	Pregnenolone	0·48	8·54			
	Progesterone	1·33	nil			0·36
	Testosterone	nil	nil			
F.N.*	Pregnenolone	2·8	4·2	—	—	
	Progesterone	0·24	nil	—	—	10·2
	Testosterone	trace	nil	nil	nil	

*Patient F.N. was a male pseudohermaphrodite with histologically-normal testes.
†Tentative identification only.
‡Approximate values only.
Data from Refs. [71] and [106].

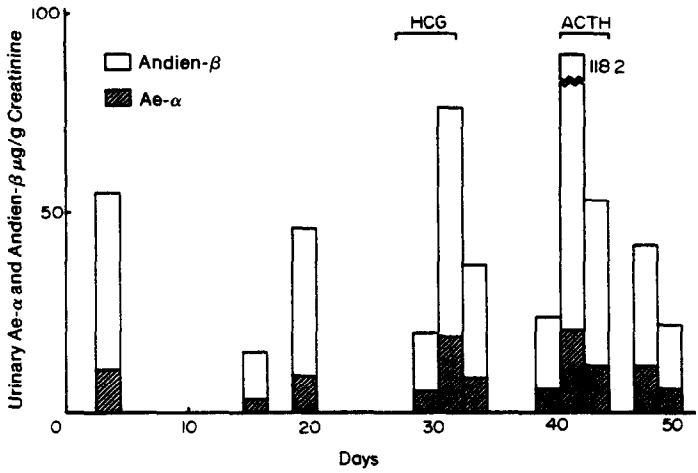


Fig. 13. Effect of ACTH and HCG administration on urinary ae-α and andien-β in a woman (L.R.) with testicular feminization (from Refs. 71 and 106).

TF patients[71] also contrasts directly with results obtained using histologically normal testis from a male pseudohermaphrodite[71]. In this case andien-β (4%) was formed from pregnenolone but considerably more androstadienone (2%) was formed from this precursor than from progesterone (Table 13). The significance, if any, of these differences in 16-unsaturated C_{19} steroid biosynthesis has yet to be determined. Tentative evidence for the formation of andien-β and androstadienone from pregnenolone in TF tissue has been presented in earlier work[76].

Some evidence has been obtained from the biosynthesis of 16-unsaturated steroids in human adrenal carcinoma tissue; pregnenolone and progesterone were metabolised to andien-β and androstadienone but C_{19} steroids such as testosterone or DHA did not serve as precursors[25, 31] (Table 14) thus confirming earlier experiments performed with porcine testis[6].

Only two reports have so far appeared concerning 16-unsaturated steroids in human ovarian tissue. After incubating human follicles with radioactive pregnenolone[76], fast-running metabolites were noted when the tissue extract was subjected to paper chromatography. Subsequent thin-layer chromatography of this material, using 16-unsaturated steroids as markers, resulted in the tentative identification of andien-β and androstadienone. Similar results have been obtained using polycystic ovarian tissue with labelled pregnenolone[77].

Table 14. Yields (as percentage of radioactivity incorporated/g wet wt. of tissue) of 16-unsaturated C_{19} steroids formed in minces of adrenal carcinoma

Precursor	andien-β	androstadienone	an-α	an-β	Reference
Pregnenolone	0·2	N.D.	N.D.	N.D.	[31]
Pregnenolone	4·7	N.D.	N.D.	N.D.	[25]
Progesterone	N.D.	<0·03	N.D.	N.D.	[31]
Progesterone	0·15	N.D.	N.D.	N.D.	[25]
Testosterone	N.D.	N.D.	N.D.	N.D.	
DHA	N.D.	N.D.	N.D.	N.D.	

N.D. not detected.

6. FURTHER METABOLISM OF 16-UNSATURATED C_{19} STEROIDS

(a) *In vitro.* The metabolism of ^{14}C-labelled andien-β, prepared biosynthetically from [4-^{14}C]pregnenolone in a boar testis homogenate [8], has been studied. Andien-β is converted in high yield (31%) to androstadienone when incubated with boar testis homogenate and NAD^+ (0·4 mM) but if both NADPH and NAD^+ are added as cofactors, there is a relatively small yield (4%) of an-β. Recently, 5α-androstenone, labelled with tritium, was shown to be converted to an-α (and an-β) in preparations of porcine parotid gland [4]. Such evidence is in keeping with the relatively large amounts of, particularly, an-α that have been isolated from this source in pigs (see Table 1), and explains the fact that the alcohol occurs in larger quantities than the ketone [3, 4, 17].

(b) *In vivo.* When isotopically-labelled androstadienone became available through a chemical synthesis and purification [78], its metabolism was studied [30, 33] after intravenous administration to two healthy men and one healthy woman (in the follicular phase). The disappearance curves for the purified plasma androstadienone approximated to those of a two-pool distribution [33] and the estimated plasma production rates (PPR) (μg/24 h) were 975 and 1341 in the two men and 456 in the woman. The calculated urinary production rates (UPR) (μg/24 h) of the androstadienone in the men, calculated from the cumulative specific activity of the urinary radioactive an-α were, however, very much higher than the corresponding PPR's and indicate that the urinary an-α does not arise entirely from the circulating androstadienone in men; in the woman the discrepancy between estimated PPR and UPR was very much less. Subsequent investigations of urinary 16-unsaturated steroids after intravenous administration of [^{14}C]-androstadienone to a man and a woman [30] indicated that 95% of the ^{14}C was in the glucuronoside and only 5% in the sulphate fraction. An-α accounted for 79% (in the man) and 51% (in the woman) of the radioactivity of the glucuronoside fraction while some activity was found in ae-α and also in an-β, a compound not previously detected in human urine [29] although present in boar urine [5].

Further analyses [30] of the urines obtained have revealed the presence of polar metabolites including $C_{19}O_3$ compounds that are neither phenolic nor acidic and that behave like androstanetriols chromatographically (B. W. L. Brooksbank, personal communication). These metabolites were formed to a much greater extent in the woman than in the man [30]. Such a metabolic route would be similar to that for oestratetraenol which is known to give rise to a 16,17-glycol [72] (see p. 73). If polar metabolites are also formed from an-α, this might explain the very low recoveries (1·5–11%) of this steroid in urine after an-α was administered to two men [24]. Moreover, the extra an-α did not appear in the urine until the second day after the injection. Such findings are in keeping with a fairly extensive metabolism of an-α rather than this being an end-product of metabolism of other steroids.

7. BIOSYNTHESIS AND FURTHER METABOLISM OF OESTRATETRAENOL

The C_{18} steroid oestratetraenol has weak oestrogenic activity (see p. 97) and is implicated in oestrogen metabolism. As mentioned earlier (p. 56) it occurs in human pregnancy urine [36]. Knuppen and Breuer [79] were the first to show that testosterone could be converted to androstadienone in a human placental microsomal preparation and that this compound was further metabolised to the phenolic 16-unsaturated steroid, oestratetraenol. When the latter was administered to

human subjects intravenously, an increase in the urinary "oestriol" fraction was noticed[80] and a more detailed investigation showed that the oestrogen concerned was 16-epioestriol[81]. The conversion of oestratetraenol to epioestriol may be via the 16,17-epoxide as an intermediate, the epoxide ring subsequently opening to form the 16β,17β-glycol[72] (Fig. 14).

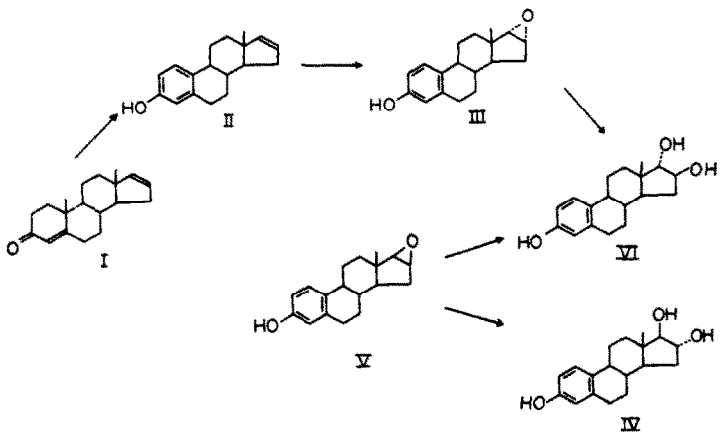
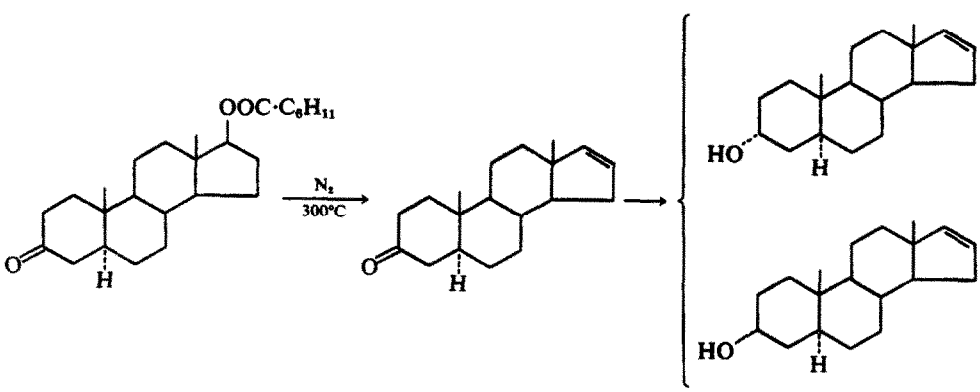


Fig. 14. Biosynthesis and metabolism of oestratetraenol. I, androstadienone; II, oestratetraenol; III, 16α,17α-epoxyoestratrien-3-ol; IV, oestriol; V, 16β,17β-epoxyoestratrien-3-ol; VI, epioestriol.

8. CHEMICAL SYNTHESIS OF 16-UNSATURATED STEROIDS

Many of the methods for the synthesis of this group of compounds have been reviewed recently[78].

In early experiments[82] it was shown that the hexahydrobenzoate of 17β-hydroxy-5α-androstan-3-one (I) could be converted to 5α-androstenone by dry-distillation at 300°C and that a mixture of an-α and an-β was formed from this by reduction:

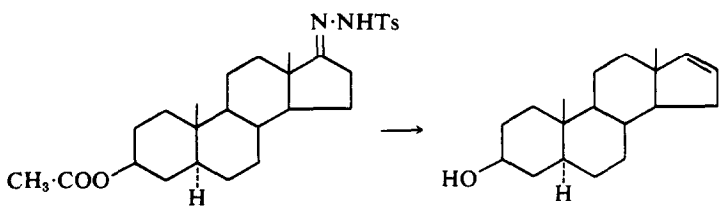


Using the same method of dry-distillation, Fajkos[83] obtained an oil from the hexahydrobenzoate of I which, after saponification, resulted in 5α-androstenone (yield 28%); $LiAlH_4$ reduction then afforded an-β in 78% yield. The Prelog group[84] also showed that the benzoate of 17β-hydroxy-5β-androstan-3-one could be converted by dry-distillation to 5β-androstenone, the latter being reduced

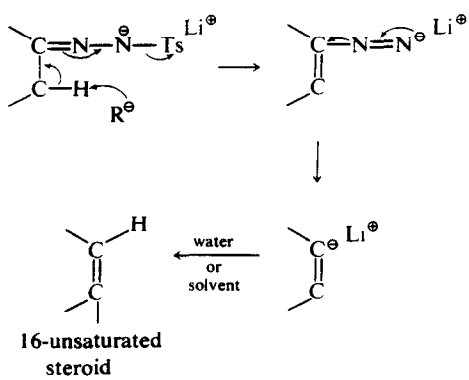
subsequently to a mixture of ae-α and 5β-androst-16-en-3β-ol. Epitestosterone benzoate, under the same conditions, was converted to androstadienone [84]. The dehydration of 17β-xanthates [85] and of 17β-p-toluene sulphonates (tosylates) (cited in Ref. 37) also results in the formation of 16-unsaturated steroids.

Androstadienone is formed in another reaction involving testosterone in which the latter, in pyridine solution, is treated with bromoacetamide [86]. Sulphur dioxide gas is then added until no further reaction to starch-iodide paper is obtained. Extraction and purification is said to yield androstadienone, although later workers [78] were unable to confirm this. In 1962 a study was published [87] of the use of aprotic solvents for nucleophilic substitution at C-3 and C-17 since, under these conditions, structural rearrangements through migration of the C-18 methyl group are inhibited. Thus, when testosterone tosylate was subjected to high temperature acetolysis in N-methyl pyrrolidone with tetra-butyl ammonium acetate at 160°C, the products were androstadienone (57%) and 17α-testosterone acetate (34%). The proportions of these products, however, vary when the reaction is scaled down (see below).

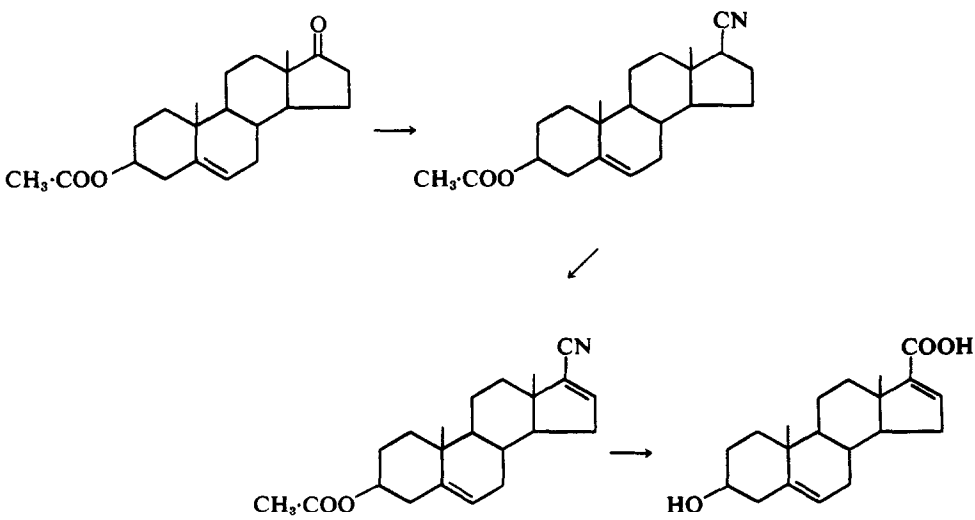
Caglioti and Magi [88] investigated the behaviour of tosylhydrazones of 17-oxosteroids when treated with $LiAlH_4$ in tetrahydrofuran (overnight refluxing). Under these conditions the tosylhydrazone of 3β-acetoxy-5α-androstan-17-one was converted to an-β in 70% yield:



The same derivatives, dissolved in dry ether, also form the corresponding olefins when treated with greater than two equivalents of alkyl lithium compounds at room temperature [89]. The reaction proceeds smoothly even at −20°C and this fact could be particularly useful for volatile or sensitive olefins. A carbanion intermediate is proposed as follows:



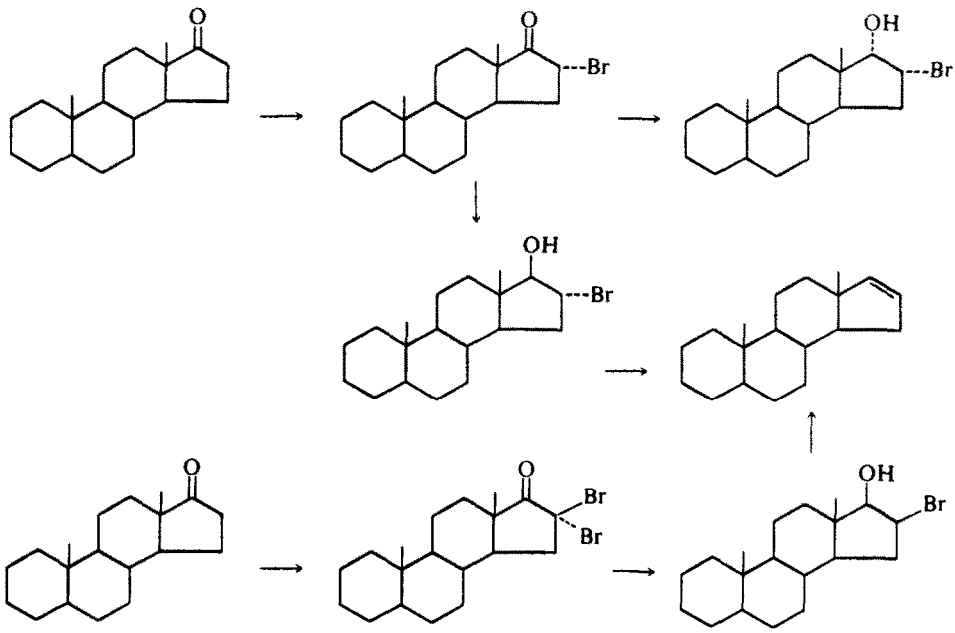
A four-step synthesis of 16-unsaturated steroids has been reported[90] that involves the synthesis of 3β-hydroxy-5,16-androstadien-17β-carboxylic acid from DHA acetate[91]. This was subsequently converted to the cyanohydrin which, on heating with $POCl_3$ in pyridine, resulted in 17β-cyano-5,16-androstadien-3β-yl-acetate (m.p. 210°). Vigorous alkaline hydrolysis afforded 3β-hydroxy-5,16-androstadien-17β-carboxylic acid:–



The unsaturated acid was subsequently refluxed in quinoline in the presence of activated copper chromite as catalyst when andien-β was formed. A similar series of reactions was employed later[21] for the synthesis of an-α starting with androsterone acetate. After 17-cyanohydrin formation, dehydration at C-17 was achieved by heating with $POCl_3$ under pressure to give 17β-cyano-5α-androst-16-en-3α-yl-acetate in 58% yield. Vigorous alkaline hydrolysis under pressure at 180–185° for 5 h resulted in 3α-hydroxy-16-aetioenic acid (79·6% yield). The final step involved decarboxylation in boiling quinoline in the presence of copper chromite [cf. 90] and the crude an-α so obtained was purified by sublimation, the overall yield being approximately 20% without recycling of intermediates.

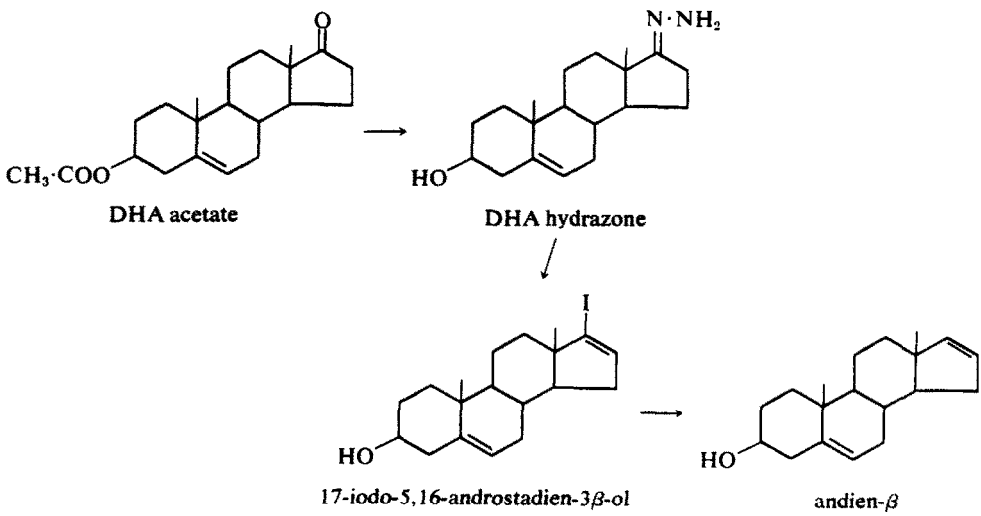
The synthesis of andien-β has recently been described[59] using the methods outlined above[90, 91] except that hydrolysis of the 17-cyano-3-acetoxy-5,16-androstadien-3β-yl-acetate was achieved by refluxing in glycerol in the presence of KOH at 175° for 20 h[92].

The synthesis of the hydrocarbon, 16-androstene, has been described by Shoppee and his collaborators[93] who used androstan-17-one as starting material. Bromination in acetic acid at 15°C resulted in 16α-bromo androstan-17-one and this, on reduction, gave either the 16α-bromo-17α-hydrin ($NaBH_4$ in methanol at 20°C) or the 16α-bromo-17β-hydrin ($LiAlH_4$ in ether at 0°C). A third epimeric bromohydrin was formed by dibromination of androstan-17-one with bromine in ether-acetic acid solution at 36°C, followed by reduction ($NaBH_4$ in methanol at 15°C) giving the 16β-bromo-17β-hydrin:



All three epimeric bromohydrins could be converted to 16-androstene by brief treatment with zinc in acetic acid.

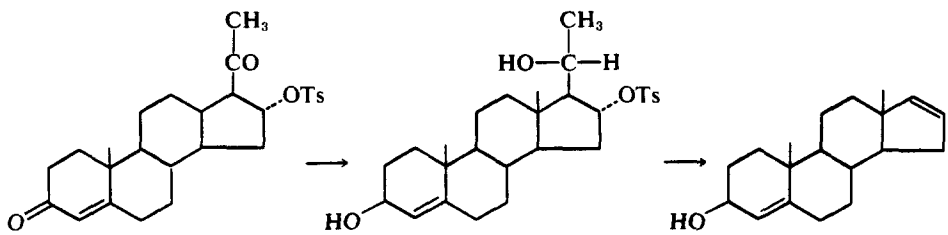
A three-step synthesis of andien-β, also involving a 17-oxosteroid as starting material, was described in 1962[94]. DHA acetate, with hydrazine and triethylamine as catalyst, gave the hydrazone. Oxidation was then achieved using iodine in triethylamine-tetrahydrofuran, resulting in the vinyl iodide. Finally, reduction with sodium and ethanol afforded andien-β:



Using another 17-oxosteroid, epiandrosterone, as starting material, the corresponding 16-unsaturated steroid (an-β) has been obtained by a hydroboration method[95]. The 3β-acetate of epiandrosterone was converted to 17,17-diethoxy-5α-androstan-3β-yl-acetate(I) by treatment with ethyl formate in the

presence of one drop of concentrated sulphuric acid. On heating compound I under N_2, 3β-acetoxy-17-ethoxy-5α-androst-16-en-3β-yt-acetate was formed and this was converted to an-β on treatment with diborane, followed by refluxing with acetic anhydride.

Recently [49] a detailed study has been made of the heterolytic fragmentation of pregnane-16,20-diols and their mesylates, tosylates and sulphates. For example, the tosylate of 16α-hydroxyprogesterone was reduced with $NaBH_4$ to 3β,16α, 20β-trihydroxy-4-pregnene-16-tosylate. On refluxing with potassium t-butoxide in t-butanol, 4,16-androstadien-3β-ol was formed in 29% yield:



The same 16-unsaturated steroid was formed, together with other products, starting with the mesylate of 16α-hydroxyprogesterone. In further experiments [49] it was shown that, when the 16-mesylates of 3β,16β,20β-trihydroxy-5α-pregnan-3β-acetate and 3β,16α,20α-trihydroxy-5α-pregnan-3β-acetate were subjected separately to refluxing with potassium t-butoxide (as described above), among the products were small quantities of an-α (< 1%) (from the 20β-epimer) and an-β (2%).

In a recent paper, Lippman and Lieberman [59] have described the synthesis of androstadienone from deoxycorticosterone (DOC) using the free-radical generating reagent, lead tetra-acetate. Some 4-androsten-3-one was also formed in the reaction. Androstadienone was also formed from DOC using a variety of other hydrogen-abstracting reagents, such as cumene- and t-butyl-hydroperoxides, phenyl azotriphenylmethane, or by photolysis of the C-21 nitrite that was prepared from DOC using NOCl. The implication of this reaction in the biosynthesis of androstadienone and the possible mechanism are discussed in Section 3, page 62.

Chemical synthesis of oestratetraenol

This phenolic 16-unsaturated steroid can be obtained by three of the methods described above:

(i) Dry-distillation of 17β-oestradiol dibenzoate at 300–310°C under water-pump vacuum results in oestratetraenol 3-benzoate [96]. The 16-unsaturated phenol (m.p. 127–129°) can readily be obtained from the benzoate and is almost odourless but has a slight musky smell when warmed.

(ii) Huffman and co-workers [97] selectively benzoylated oestradiol-3,16β to give the 3-benzoate. On refluxing the C-16 tosylate in collidine, oestratetraenol 3-benzoate was formed in 44% yield. These workers discovered that oestratetraenol passes preferentially into the organic phase when partitioned between benzene and 0·1 N NaOH and this property has proved especially useful in the purification of oestratetraenol from uncleaved diol.

(iii) By refluxing the tosylhydrazone of oestrone 3-methyl ether overnight with $LiAlH_4$ in dry tetrahydrofuran, 3-methoxy oestratetraenol was formed in 60-70% yield[88]. A particular feature of this reaction is that no migration of the C-18 methyl group occurs under the conditions used.

Preparation of radioactive 16-unsaturated steroids

Recently some of the available methods for the synthesis of androstadienone were surveyed[78] since this steroid was required by the authors to be labelled with ^{14}C and with tritium. The high temperature acetolysis method from testosterone[87] appeared to be suitable but, on scaling-down the quantities of reagents in a pilot experiment, it was found that the yield of androstadienone was only 38% under very carefully controlled conditions instead of 57% quoted in Ref. [87]. The crystals obtained slowly underwent decomposition and three more recrystallizations were required to give a product that did not subsequently decompose (overall yield approximately 6%). Further problems arose when ^{14}C-labelled and ^{3}H-labelled testosterone were used as starting materials, in that the labelled androstadienone obtained was heavily contaminated with at least three impurities that may have been ring D isomers of androstadienone. The latter was purified using column chromatography on $AgNO_3$-impregnated silicic acid (Fig. 15) and then on alumina (see p. 79) giving a yield of approximately 6% for the pure compound.

Synthesis of 16-unsaturated steroid conjugates

The sulphates of this series of compounds do not seem to have been prepared but presumably the available methods for other steroid sulphates could be employed[98–100].

The glucuronoside of an-α has been synthesized[21] by treating an-α, dissolved in benzene and in presence of Ag_2CO_3, with methyl α-bromotri-O-acetyl glucuronate dropwise over a period of 1·5 h during which time the benzene was continuously distilled. On filtering and evaporating, methyl (androst-16-en-3α-yl-2:3:4-tri-O-acetyl-β-D-glucopyranosid)uronate was obtained in 27% yield. By treating with $Ba(OH)_2$ followed by precipitation of $BaSO_4$ with H_2SO_4, androst-16-en-3α-yl-β-glucosiduronic acid was obtained in 56% yield. This conjugate was employed[21] to estimate losses during the hydrolysis of an-α glucuronoside from human urine.

9. CHROMATOGRAPHIC SEPARATION OF 16-UNSATURATED C_{19} STEROIDS

(a) *Column chromatography*. Because of the extremely low polarity of this group of steroids, alumina chromatography has been utilised to great advantage in their isolation provided that low polarity solvents are used for elution. Thus, early workers in this field were able to separate 16-unsaturated steroids from more polar compounds such as DHA and testosterone (e.g. Ref. [1]). Using a single eluting solvent mixture, benzene–light petroleum (1 : 1, v/v), Brooksbank and his colleagues[21], were able to elute an-α from urine extracts and the column fractions so obtained were sufficiently 'clean' for colorimetric or g.l.c. estimation to be carried out[21, 23, 29]. Even for the estimation of the minor urinary 16-unsaturated steroids, ae-α and andien-β, the eluted fractions were suitable for quantitative analysis by g.l.c. without the need for a prior t.l.c. step[23]. Using alumina, partially de-activated with water (4–5%, v/w), Brooksbank and Gower

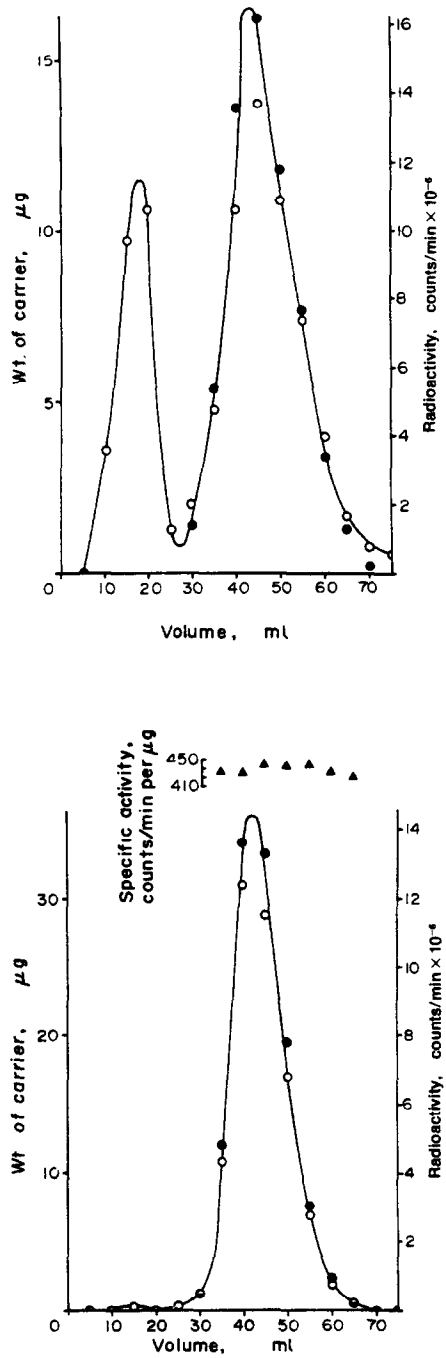


Fig. 15. Purification of synthetic [7α-^{3}H]androstadienone[78] by column chromatography on (a) $AgNO_3$-impregnated silicic acid and (b) Al_2O_3, partially deactivated with water (4–5%, v/w)[9]. The weight (μg) of carrier andostradienone (●) was determined by g.l.c. and radioactivity (○) by liquid scintillation counting. ▲, specific radioactivity (cpm/μg × 10^{-3}). (a) from Gower (unpublished) and (b) redrawn from Ref. [78] by permission. Counting efficiency 41%.

and their colleagues have shown the following approximate pattern of elution with benzene–light petroleum (1:1, v/v): 30–90 ml contains an-α and androstadienone; 90–110 ml contains ae-α; 110–175 ml contains andien-β and an-β. More polar compounds, such as androsterone, DHA and testosterone, can then be eluted from the same column using benzene containing varying proportions of ethanol (Table 15). This technique has been utilised for the separation of 16-unsaturated steroids and 17-oxosteroids in urine extracts[5, 23, 25, 31]. Similar columns have been used extensively by Gower and his colleagues[6, 9, 41–43, 55] in the separation of labelled 16-unsaturated steroids formed in incubations of tissues from various species (Fig. 16). The specific activity of the very non-polar compound 5α-androstenone (labelled with ^{14}C) was determined using an alumina column and benzene–light petroleum (1:9, v/v) as eluent[12]. This very non-polar solvent mixture has been utilised recently[101] to separate fat from 16-unsaturated C_{19} steroids in extracts obtained from boar testis and testicular vein plasma. Using partially deactivated alumina, fat was eluted first with benzene–light petroleum (1:9, v/v) followed by 5α-androstenone. An-α and an-β could be eluted next by increasing the percentage of benzene in the eluent mixture to 50% (as above). In this way, evidence has been obtained for the presence in boar testicular vein plasma of radioactive an-α, an-β and 5α-androstenone biosynthesized *in vivo* through a testicular infusion of isotopically-labelled pregnenolone[101]. These preliminary findings are in accord with the analytical and *in vitro* results already described (Sections 2 and 3).

Column chromatography on silicic acid has also been employed for the separation of mixtures of fat and 16-unsaturated steroids, extracted from porcine peripheral plasma, testes and salivary glands[4]. Fat was eluted first using cyclohexane followed by 5α-androstenone using more polar solvents (Fig. 17). An-α and an-β could be eluted from similar columns with mixtures of cyclohexane and ethyl acetate. It has not been possible to separate andien-β and an-β by alumina column chromatography even though gradient elution has been attempted (Gower,

Table 15. Elution of 16-unsaturated C_{19} steroids from alumina*

Steroids eluted	Eluent	Volume of eluent (ml)‡
5α-androstenone	benzene–light petroleum† (1:9 v/v)	50–100
androstadienone, an-α	benzene–light petroleum (1:1 v/v)	30–90
ae-α	benzene–light petroleum (1:1 v/v)	90–110
andien-β, an-β, cholesterol	benzene–light petroleum (1:1 v/v)	110–175
progesterone, androstenedione, androsterone, pregnenolone	benzene containing ethanol (0·2% v/v)	50
pregnenolone, testosterone, aetiocholanolone, DHA	benzene containing ethanol (0·5% v/v)	50

*Alumina (type H, 100–200 mesh; Peter Spence and Co. Ltd., Widnes, Lancs.) was partially deactivated by the addition of water (4–5% v/w). 5g was used in these experiments.

†Light petroleum (b.p. 80–100°C) was treated with conc. H_2SO_4, washed and dried[9].

‡Volumes given can only be approximate due to differences between batches of alumina.

Data compiled from Refs. [9], [21] and [44].

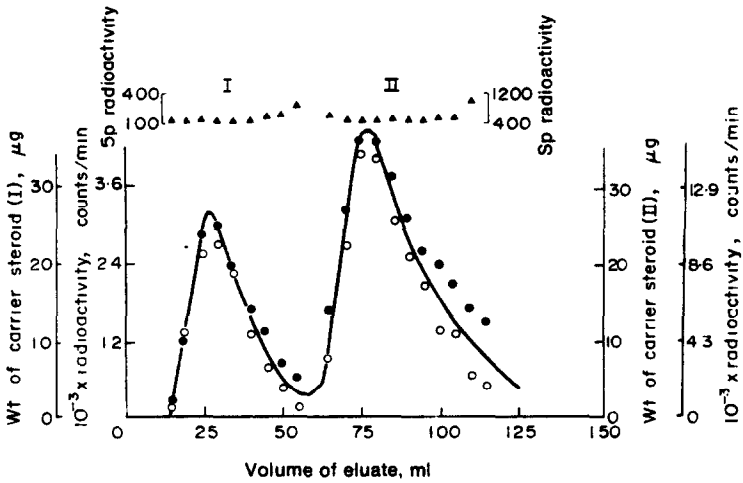


Fig. 16. Alumina-column chromatography, with light petroleum–benzene (1:1, v/v) as eluent, of an-α (I) and an-β (II) obtained after incubation of [4-^{14}C] progesterone with minced boar testis tissue. The weight of steroid (○) was determined by g.l.c. and radioactivity (●) by liquid scintillation counting; △, specific radioactivity (counts/min/μg). Redrawn from Ref. [6] by permission of *The Biochemical Journal*.

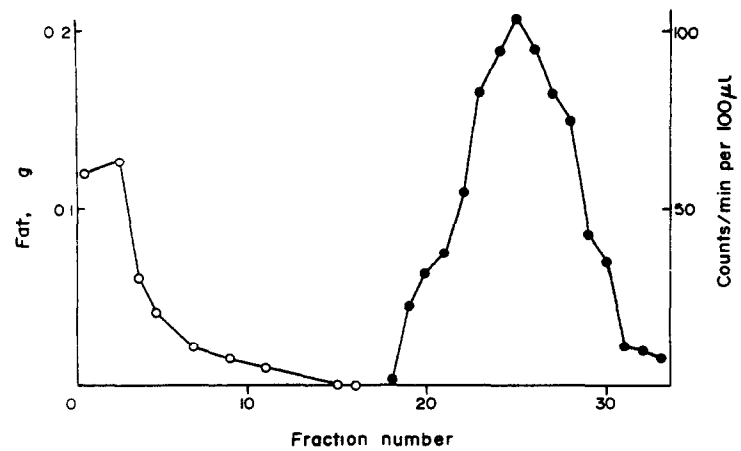


Fig. 17. Separation by silicic acid column chromatography of fat (○) from ^{3}H-labelled 5α-androstenone (●) extracted from boar parotid gland. The column was eluted using the following solvent mixtures (5 ml fractions):—cyclohexane (70 ml); light petroleum–cyclohexane (1:1, v/v) (15 ml); light petroleum (25 ml); light petroleum–ethyl acetate (1:1, v/v)) (10 ml) and ethyl acetate (15 ml). Redrawn from Ref. [4] by permission.

unpublished), but this pair of compounds can be resolved if a column of silicic acid impregnated with silver nitrate is used [8, 25]. An-β can be eluted using benzene–ethyl acetate (2:1, v/v) but the more unsaturated andien-β is avidly retained by the column and can only be eluted using benzene–ethyl acetate–ethanol (40:20:1, by vol). Such a system has been of value in showing that labelled andien-β was formed from labelled pregnenolone in incubations of human adrenal carcinoma tissue [25], human testis [71] and porcine testis [8] (Fig. 18). A positive pressure

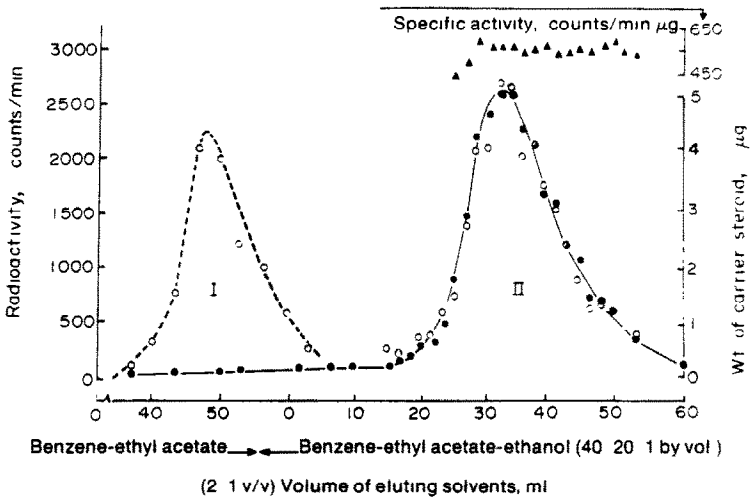


Fig. 18. Purification of radioactive andien-β formed by incubating [4-^{14}C] pregnenolone with adrenocortical carcinoma tissue. Radioactive andien-β was isolated by t.l.c., carrier an-β (I) and andien-β (II) added, and the mixture eluted from a column of Kieselgel H impregnated with $AgNO_3$ (for details see Refs. 8 and 25). The weight (○) of carrier steroids was determined by g.l.c. and radioactivity (●) by liquid scintillation counting. Reproduced from Ref. [25] by permission of Periodica, Copenhagen.

of N_2 (10–20 mm Hg) must be applied to such columns in order to obtain a flow rate of 1 ml/min.

(b) *Paper chromatography.* The 16-unsaturated C_{19} steroids are so non-polar that in the Bush A system[102] they run, as a group, near the solvent front [76, 77]. Gower[103] had previously encountered this problem and employed paper that had been fully acetylated or impregnated with kerosene, liquid paraffin or phenylcellosolve. However, it was only possible to separate an-α from ae-α, andien-β and an-β, these three compounds moving as a group. The situation was thus analogous to that described above for alumina column chromatography. Using silicic acid–impregnated paper, however, an-α and ae-α could be resolved from each other and both were separated from the still unresolved andien-β plus an-β, by ascending chromatography. Non-polar solvents or solvent mixtures, such as light petroleum–benzene (1 : 1, v/v), benzene, toluene and benzene–ether (99 : 1, v/v) were used[103]. The order of mobility for the 16-unsaturated steroids studied was oestratetraenol > an-α > ae-α > andien-β ≥ an-β. Using the descending technique for 18 h on silicic acid–impregnated paper, it was still not possible to resolve andien-β from an-β, although enhanced separations of the other compounds were achieved. The acetates, much less polar than the parent alcohols, were poorly separated in all the systems tried.

(c) *Thin layer chromatography.* When t.l.c. was attempted with 16-unsaturated steroids[103, 104] similar resolutions were achieved as with silicic acid–impregnated paper (Table 16). It was possible to effect reasonable separations of oestratetraenol, an-α and ae-α from andien-β and an-β, if the plate was developed in the same solvent system, e.g. benzene–ether (9 : 1, v/v), two or three times, allowing the plate to dry between runs. Andien-β and an-β were, however, only partially separated. 5α-Androstenone and androstadienone can also be readily

Table 16. R_F values (X100) of some 16-unsaturated steroids on Kieselgel G

	Solvents†																			Colours with detecting reagents‡						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	5	18	19	PMA*	PTA*	RA*	Allen*	Uranyl nitrate*	Anisaldehyde-H_2SO_4*	I_2 reagent*
An-α	8	29	21	34	24	33	66	40	75	61	74	52	68	59	29	51	36	28	48	—	bg	br	m	gr	gr	vb	yb
Ae-α	5	22	19	26	20	27	58	33	62	55	65	45	66	56	24	48	30	23	42	—	g	br	m	gr	bgr	vb	yb
Andien-β	4	18	16	23	17	23	49	28	49	47	59	40	66	53	21	45	27	19	38	—	p	ro	b	m	p	vb	yb
An-β	4	19	15	20	15	20	48	27	46	45	58	38	66	51	20	41	25	18	36	—	b	br	m	gr	p	vb	yb
Oestratetraenol	15	38	37	48	30	51	72	51	93	77	88	80	96§	–	—	—	—	—	—	21§	rp	o	r	bk	r	vb	yb

*PMA, phosphomolybdic acid; PTA, phosphotungstic acid; RA, resorcyaldehyde reagent; I_2 reagent, I_2 in light petroleum[103]; anisaldehyde reagent[104]. Allen reagent[107]; Uranyl nitrate[103].

†Solvents: 1, toluene; 2, methylene chloride; 3, toluene-ethyl acetate (19:1 v/v); 4, toluene:ethyl acetate (9:1 v/v); 5, benzene-ethanol (98:2 v/v); 6, benzene:ether (9:1 v/v); 7, benzene-acetone (85:15 v/v); 8, benzene-methyl ethyl ketone (9:1 v/v); 9, methylene chloride-ethyl acetate (9:1 v/v); 10, ethylene chloride-ethyl acetate (90:12 v/v); 11, di-isopropyl ether-acetic acid (96:4 v/v); 12, di-isopropyl ether-formic acid (99:1 v/v); 13, ethyl acetate-cyclohexane-ethanol (45:45:10, by vol.); 14, ethyl acetate-cyclohexane (1:1 v/v); 15, *n*-hexane-ethyl acetate (75:25 v/v); 16, benzene-ethyl acetate (1:1 v/v); 17, benzene-ethanol (95:5 v/v); 18, chloroform-ethanol (98:2 v/v); 19, benzene.

‡Colours: b = blue: bg = bluish green, bgr = bluish grey; br = brown; g = green; gr = grey; m = mauve; o = orange, r = red; or = orange red; ro = rose; rp = reddish purple; p = purple; pk = pink; vb = violet blue; yb = yellow brown. Data from [36, 103, 104] by permission of the publishers (Elsevier Publishing Co; Academic Press Inc.).

§Detected by Kober reaction[36].

separated by this technique (Fig. 19). Using Kieselgel G layers impregnated with silver nitrate, it is possible to resolve andien-β from an-β, the former being much more retarded[104] (Table 17). In order to separate a range of 16-unsaturated steroids possibly formed in incubations of labelled substrates with testis or adrenocortical tissues, Gower and his colleagues[8,25,31,71] have utilised t.l.c. first on Kieselgel G using the systems benzene-ether (9:1, v/v) or benzene:ether (4:1, v/v) run twice, followed by t.l.c. on Kieselgel G impregnated with $AgNO_3$ in the system benzene:ethyl acetate (1:2, v/v).

However, although the relatively non-polar solvent systems separated the 16-unsaturated steroids, more polar compounds such as pregnenolone, progesterone, 17α-hydroxypregnenolone and testosterone were only partially separated, and had to be eluted 'en bloc' and re-run in systems such as benzene–acetone (4:1, v/v)[31]. In incubations of radioactive pregnenolone or progesterone all of these compounds were likely to be encountered. Thus, in order to separate, on one plate, the 16-unsaturated steroids from C_{21} and other C_{19} steroids, two-dimensional t.l.c. has recently been exploited[105]. Figure 20 shows the separation of some sixteen compounds and it is of particular interest that one curve can be drawn through the positions of all 4-en-3-oxosteroids; a second curve relates the positions of 5-ene-3β-hydroxysteroids. Similar separations have been achieved using extracts of testes from human testicular feminization patients[106] and from rats[105], after incubation with radioactive pregnenolone or progesterone.

(d) *Elution of 16-unsaturated steroids from thin-layer plates.* Using solvents such as chloroform, methylene dichloride, ethyl acetate or ether (4 × 2 ml), it is possible to recover approximately 80% of these compounds from Kieselgel G plates. However, if $AgNO_3$-impregnated Kieselgel G is used, the steroids, particularly the alcohols, are more strongly adsorbed and recoveries of as little as 20% for andien-β have been recorded in the author's laboratory. This difficulty was

Table 17. R_F values (×100) of some 16-unsaturated steroids on silver nitrate-impregnated Kieselgel G

	Solvents							
	1	2	3	4	5	6	7	8
An-α	10	37	6	30	21	15	3	22
Ae-α	6	34	6	15	8	7	3	10
Andien-β	9	32	4	25	14	12	3	17
An-β	14	37	11	31	23	18	4	28
Androstadienone	—	—	—	—	—	—	—	30
5-Androsten-3β-ol	51	67	26	57	50	45	24	63
Cholesterol	—	—	—	—	—	—	—	55
Pregnenolone	—	—	—	—	—	—	—	47
Progesterone	—	—	—	—	—	—	—	58
Oestratetraenol	—	—	—	—	—	—	—	45

Solvents: 1, chloroform-ethanol (95:5 v/v); 2, ethyl acetate-cyclohexane-ethanol (45:45:10 by vol.); 3, ethyl acetate-*n*-hexane (25:75 v/v); 4, ethyl acetate-*n*-hexane (75:25 v/v); 5, cyclohexane-ethyl acetate (1:1 v/v); 6, benzene-ethyl acetate (1:1 v/v); 7, benzene-ethanol (95:5 v/v); 8, benzene-ethyl acetate (1:2 v/v). Data from [104] for solvent systems 1–7 and from [8] for system 8 (by permission of the publishers, Academic Press Inc. and The Biochemical Journal).

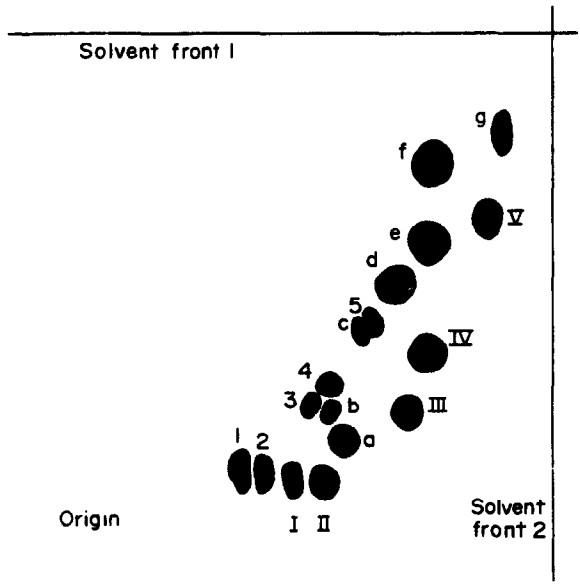


Fig. 20. Two-dimensional t.l.c. of a mixture of steroids using benzene-ether (9:1, v/v), run twice, as the first system and benzene-methanol (9:1, v/v) as the second. 4-en-3-Oxosteroids were I, testosterone; II, 17α-hydroxyprogesterone; III, androstenedione; IV, progesterone; V, androstadienone. 5-ene-3β-Hydroxysteroids were; −1, 17α-hydroxypregnenolone; 2, 5-androstenediol; 3, dehydroepiandrosterone; 4, pregnenolone; 5, andien-β. Other steroids: a, androsterone; b, aetiocholanolone; c, an-β; d, ae-α; e, an-α; f, oestratetraenol and g, 5α-androstenone.

overcome by mixing the appropriate zone of adsorbent material in a test-tube with distilled water (1·5 ml) and 2M-NaOH (0·5 ml) and extracting the steroid with ether (4 × 2 ml). Acceptable recoveries were then obtained[8].

An attempt was made[78] to use t.l.c. on $AgNO_3$–impregnated Kieselgel G to separate synthetic [7α-^{3}H] androstadienone from a reaction mixture containing impurities, possibly ring D isomers of androstadienone (see p. 78). Although an excellent separation of androstadienone was achieved, it seemed likely that the use of NaOH to extract the steroid from the plate caused decomposition (Gower, unpublished observations). As mentioned earlier (p. 78) no decomposition of [^{3}H]- or [^{14}C]-labelled androstadienone occurred on columns of $AgNO_3$-impregnated silicic acid, possibly because the columns were run in an atmosphere of nitrogen.

(e) *Detection of 16-unsaturated steroids on thin-layer plates.* The reagents that have been used to detect 16-unsaturated steroids on thin-layer plates, together with the colours obtained, are given in Table 16. Of the reagents that do not result in destruction of the steroids, iodine in light petroleum spray is undoubtedly the most useful, since it gives yellow-brown colours with oestratetraenol, 5α-androstenone, androstadienone, an-α, ae-α, andien-β and an-β at room temperature[103]. Moreover, the spots disappear after the plate has been allowed to stand at room temperature for approximately two hr. No quenching has been observed if such radioactive spots are eluted and subsequently counted in a liquid scintillation spectrometer. If however, iodine vapour is utilised, colour production is not reversible[103]. U.V. light (254 nm) is also useful in that androstadienone and oestratetraenol can be detected.

16-Unsaturated steroids can be detected on $AgNO_3$-impregnated layers if heated with Allen reagent[107] but radioactive spots (located by radioautography) must be eluted before heating at 110°C[8].

(f) *Gas–liquid chromatography*. The non-polar nature of the 16-unsaturated C_{19} steroids permits their elution from a variety of stationary phases in relatively short times at temperatures in the range 190–210°C. The behaviour of these compounds on g.l.c. columns was first studied by Baker and Gower[108] using a high proportion (10%) of silicone gum as stationary phase. The sequence of elution for the free steroids was ae-α < an-α ≤ andien-β ≤ an-β < oestratetraenol (Table 18). It was not possible to resolve an-α, andien-β and an-β from each other as free steroids. The trimethylsilyl (TMS) ethers, however, were found to be more volatile than the parent alcohols and consequently could be eluted at temperatures some twenty degrees lower. Later studies[29] involved the use of high concentrations (20%) of a more selective stationary phase (QF_1). It was then possible to separate the TMS ethers of andien-β and an-β and to show the absence of the latter compound in human urine. During recent years a variety of stationary phases (summarized in Table 18) have been used to separate and estimate not only the alcohols mentioned above but also the ketones, androstadienone and 5α-androstenone. These compounds are retained longer than the closely related alcohols andien-β and an-α if QF_1 and XE-60 are used. However, if other phases, such as SE 30, are used, the retention times of the ketones are similar to those of the 16-unsaturated alcohols. This large difference in relative retention time on different phases has been useful in suggesting the presence of an oxo-grouping in unknown compounds, in particular, androstadienone[5].

The chloromethyldimethylsilyl (CMDS) and bromomethyldimethylsilyl (BMDS) ethers of the 16-unsaturated C_{19} steroids have been studied recently [25, 109] and, using these derivatives, a separation of an-α and ae-α was achieved on CHDMS*/polysiloxane (JXR) (0·6%/0·75%). The derivatives of andien-β and an-β were, however, not separated on this hybrid phase but only on XE-60 or QF_1 (Table 18 and Fig. 21). These CMDS ethers were especially useful in that they were retained longer on the columns than were the corresponding free alcohols and consequently facilitated the quantitation of an-α from human urine, the impurities being eluted well before the an-α CMDS ether (Ref. [109], Fig. 22). Both the CMDS and the BMDS ethers are less volatile and more stable than the TMS ethers and for this reason, a method for the analysis of this steroid series [23] utilises g.l.c. of the CMDS ethers. The BMDS ethers are more polar than, and are retained longer than, the corresponding CMDS ethers[25] (Table 18) as anticipated by analogy with the C_{19} 17-oxo steroid series[110] and may well be of use in the electron capture determination of nanogram quantities of 16-unsaturated C_{19} steroids.

The pattern of elution of the halogenosilyl ethers on CHDMS/JXR is an-α < ae-α < andien-β = an-β, and on QF_1 or XE-60 is an-α ≤ ae-α < andien-β < an-β.

10. ESTIMATION OF 16-UNSATURATED C_{19} STEROIDS

(a) *Colorimetric methods*. For the estimation of an-α in urine Brooksbank and Haslewood[21] used a modification of the colour reaction described by Miescher [111]. Hydrolysed urinary glucuronoside fractions were purified on alumina and

*Cyclohexanedimethanol Succinate.

Table 18. Relative retention times of 16-unsaturated steroids and their derivatives on various stationary phases

	(MS 2211) Silicone gum					QF_1								
	15%		10%			5%		5%		5%		20%		
	Free steroids 185°C	Free steroids 206°C	Free steroids 185°C	TMS ethers 185°C	Acetates 185°C	TMS ethers 170°C	CMDS ethers 195°C	Free steroids 196°C	CMDS ethers 196°C	CMDS ethers 190°C	CMDS ethers 200°C	Free steroids 205°C	TMS ethers 205°C	Acetates 205°C
An-α	5·37	5·04	2·77	3·05	3·72	0·24	0·59	0·325	0·610	0·606	0·750	3·37	3·05	3·72
Ae-α	4·86	4·47	2·62	2·97	3·76	0·24	0·63	—	—	0·610	0·786	3·45	2·97	3·76
Andien-β	5·25	4·96	2·83	3·53	4·18	0·25	0·73	—	—	0·730	0·910	3·40	3·53	4·18
An-β	5·43	5·11	2·90	3·60	4·21	0·25	0·78	0·410	0·815	0·715	1·16	3·76	3·60	4·21
Oestratetraenol	6·81	5·96	3·63	4·18	5·02	0·26	0·95	—	—	—	—	4·39	4·18	5·02
5α-Androstenone	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Androstadienone	—	—	—	—	—	—	—	1·24	—	—	—	—	—	—
Retention time of standards (min.)	MP	MP	MP	MP	MP	C	C	C	C	C	C	MP	MP	MP
≡ 1·00	4·0		9·35	9·35	9·35	52·0	20·6	18·3	18·3	35·9	33·0	14·1	14·1	14·1
Reference	[108]	[108]	[29]	[29]	[29]	[108]	[108]	[5, 20]	[5, 20]	[25]	[25]	[29]	[29]	[29]
Flow rate (ml/min)	40	35	26	26	26	50	50	50	50	50	50	18	18	18
Column dimensions (length × i.d. in cm)	← 122 × 0·35 →					← 213 × 0·4 →		← 150 × 0·4 →		← 150 × 0·4 →		← 168 × 0·3 →		

Table 18 (*cont.*)

	NPGS 2%			CHDMS 1%		XE-60 1%	XE-60 2%			XE-60 2·5%			
	Free steroids 230°C	TMS ethers 230°C	Acetates 230°C	TMS ethers 170°C	CMDS ethers 195°C	Free steroids 180°C	Free steroids 180°C	TMS ethers 170°C	CMDS ethers 190°C	Free steroids 196°C	Methyl oximes 196°C	CMDS ethers 196°C	BMDS ethers 196°C
An-α	6·17	2·21	5·35	0·15	0·53	—	4·91	1·82	0·62	0·34	—	0·610	0·750
Ae-α	5·80	2·61	5·43	0·15	0·70	—	5·07	2·27	0·715	—	—	0·630	—
Andien-β	6·50	3·11	6·18	0·16	0·87	—	5·60	2·66	0·85	—	—	0·725	—
An-β	6·43	3·10	6·42	0·16	0·87	—	5·57	2·67	0·88	0·39	—	0·792	—
Oestratetraenol	—	4·94	—	0·38	1·72	—	—	—	—	—	—	—	—
5α-Androstenone	—	—	—	—	—	—	—	—	—	0·77	0·344	—	—
Androstadienone	—	—	—	—	—	2·12	—	—	—	1·18	—	—	—
Retention time of standards (min) ≡ 1·00	MP 5·3	MP 5·3	MP 5·3	C 51·6	C 18·1	A 4·6	A 1·95	A 2·66	C 14·1	C 20·75	C 20·75	C 20·75	C 20·75
Reference	[29]	[29]	[29]	[109]	[109]	[32]	[23]	[23]	[23]	[5,20]	[5,20]	[5,20]	[5,20]
Flow rate (ml/min)	17	17	17	50	50	50	50	50	50	50	50	50	50
Column dimensions (length × i.d. in cm.)	← 173 × 0·3 →			← 150 × 0·35 →		150 × 0·4	← 150 × 0·4 →			← 150 × 0·4 →			

Table 18 (*cont.*)

	CHDMS/JXR (0·6%/0·75%)												
	TMS ethers 170°C	TMS ethers 170°C	Free steroids 180°C	Free steroids 190°C	Methyl oximes 190°C	CMDS ethers 190°C	CMDS ethers 195°C	Free steroids 196°C	Methyl oximes 196°C	CMDS ethers 196°C	BMDS ethers 196°C	CMDS ethers 200°C	BMDS ethers 200°C
An-α	0·09	2·02	3·68	—	—	0·415	0·41	0·228	—	0·402	0·532	0·412	0·580
Ae-α	0·09	2·36	3·51	—	—	0·488	0·51	0·210	—	0·437	—	0·475	0·677
Andien-β	0·10	2·84	4·23	—	—	0·580	0·62	0·25	—	0·545	0·748	0·586	0·840
An-β	0·10	2·90	3·95	—	—	0·580	0·63	0·243	—	0·545	0·748	0·591	0·840
Oestratetraenol	0·27	—	—	—	—	—	0·96	—	—	—	—	—	—
5α-Androstenone	—	—	3·99	—	—	—	—	0·254	0·250	—	—	—	—
Androstadienone	—	—	5·97	1·79	5·03† 5·52	—	—	0·357	—	—	—	—	—
Retention time of standards (min)	C	A	A	AO	A	C	C	C	C	C	C	C	C
≡ 1·00	94·5	3·94	2·66	9·3	2·4	32·5	34·3	21·5	21·5	21·5	21·5	17·6	29·8
Reference	[108]	[29]	[23]	[32]	[32]	[25]	[108]	[5]	[5]	[5]	[5]	[23]	[25]
Flow rate (ml/min)	← 50 →												
Column dimensions (length × i.d. in cm.)	← 150 × 0·4 →												

Table 18 (*cont.*)

	JXR/Epon (2%/0·2%)				
	Free steroids 190°C	Methyl oximes 190°C	Free steroids 200°C	TMS ethers 180°C	CMDS ethers 200°C
An-α			2·17	2·05	4·67
Ae-α			2·01	2·22	5·07
Andien-β			2·27	2·69	6·10
An-β			2·29	2·68	6·29
Oestratetraenol			—	—	—
5α-Androstenone			2·60	—	—
Androstadienone	1·44	3·37	3·68	—	—
Retention time of standards (min)	AO	A	A	A	A
≡ 1·00	8·8	3·3	3·61	6·56	3·61
Reference	[32]	[32]	[23]	[23]	[23]
Flow rate (ml/min)	50	50	50	50	50
Column dimensions (length × i.d. in cm.)	← 150 × 0·4 →				

A, 5α-androstane;
AO, 5α-androstan-17-one;
C, 5α-cholestane;
MP, methyl palmitate;
CHDMS, cyclohexanedimethanol succinate;
JXR, polysiloxane gum;
XE-60, cyanoalkyl silicone;
QF_1, fluoralkyl silicone;
NPGS, neopentylglycol sebacate;
Epon, epoxy resin

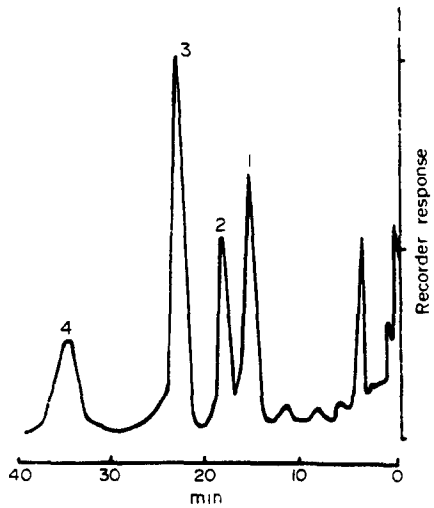


Fig. 21. G.l.c. of CMDS ethers of an-α (peak 1), ae-α (peak 2), andien-β plus an-β (peak 3) and oestratetraenol (peak 4).

Column: CHDMS/JXR (0·6%/0·75%) on Chromosorb W (100–200 mesh) at 195°C and carrier gas flow 50 ml/min. Reproduced from Ref. [109] by permission of Elsevier Publishing Co.

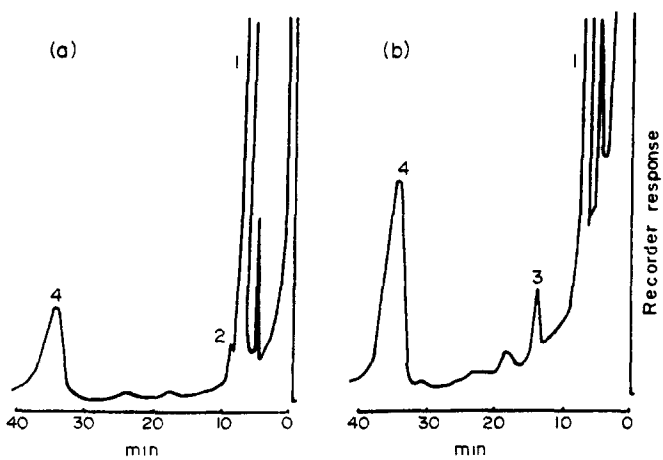


Fig. 22. G.l.c. of the an-α fraction, obtained from alumina column chromatography, of a normal female urine (a) before and (b) after chlorosilanisation (sensitivity twice that in (a)). Peak 1, unknown; peak 2, an-α; peak 3, CMDS ether of peak 2; peak 4, cholestane (internal standard). Column conditions as in Fig. 21 except that temperature was 197°C. Reproduced from Ref. [109] by permission of Elsevier Publishing Co.

an-α eluted with benzene–light petroleum (1 : 1, v/v) (see p. 78). The 'an-α' fraction was heated with resorcylaldehyde and concentrated sulphuric acid in glacial acetic acid, when a purple colour was produced that showed an extinction maximum at 580–585 nm, with an inflexion at 540 nm. A variety of C_{21} and C_{19} steroids were also tested with the same resorcylaldehyde reagent; only 16-unsaturated or 17α-hydroxy C_{19} steroids gave a purple, or in some cases (e.g. andien-β), a blue colour. Other steroids, apart from oestratetraenol (see Fig. 23),

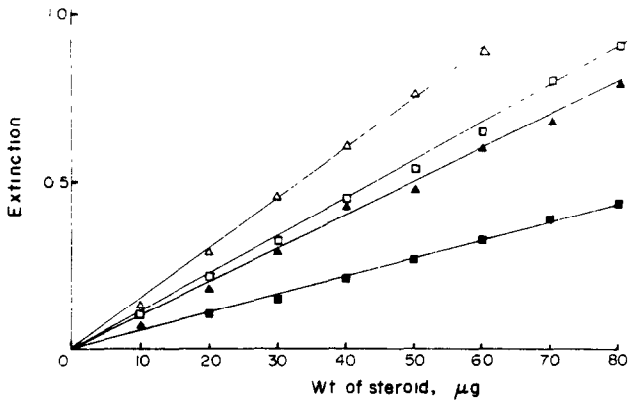


Fig. 23. Calibration curves for some 16-unsaturated steroids using the resorcylaldehyde colour reaction of Brooksbank and Haslewood[21]. Extinctions were measured at the following wave lengths: – ae-α (▲), 585 nm; andien-β (□), 575 nm; an-β (△) 585 nm; oestratetraenol (■), 625 nm. For the sake of clarity the curve of an-α (21) which closely follows that for ae-α has been omitted from the Figure. Data from Gower (unpublished).

gave essentially no colour. Cholesterol in large quantities (100 μg) gave a pink colour but was readily separated from an-α by alumina chromatography (Table 15) so that no interference from it was experienced when a urinary analysis for an-α was performed. However, it was found that the an-α fraction from urine extracts purified on alumina sometimes gave a pinker colour at lower wavelengths (530 nm) than did pure an-α. The observed extinction at 575 nm was therefore corrected for the extinction of interfering chromogens, but for very low titre urines containing 100 μg or less an-α per 24 h, the values obtained were in error even after using the correction factor. The same applies to the quantitative analysis of urinary ae-α and andien-β which occur to a much smaller extent than an-α. Although linear relationships are obtained between the amount of ae-α and andien-β and extinctions at 575 or 585 nm (Gower, unpublished, Fig. 23), the combined values for ae-α plus andien-β obtained by the colorimetric method were some five times greater than the individual values obtained by g.l.c.[29].

Recently, an attempt was made to utilise the same colour reaction for the estimation of 5α-androstenone but the sensitivity was insufficient for measurement of the compound in the peripheral plasma of pigs[4].

(b) *Gas–liquid chromatographic methods.* The behaviour on g.l.c. columns of 16-unsaturated C_{19} steroids has been described earlier (p. 86). The responses of some of these compounds and their derivatives to flame ionization detectors have been found to be linear. Calibration curves for some alcohols, silyl ethers and ketones are shown in Fig. 24. A number of compounds have been employed as internal standards in the g.l.c. estimation of 16-unsaturated steroids e.g. methyl palmitate, 5α-androstane, cholestane, 5α-androstan-17-one, 5α-androstan-3β-ol and 5α-androstan-17β-ol. The latter alcohols are particularly useful if TMS ethers or halogenosilyl ethers have to be prepared during the method since their use can correct for the losses that may be incurred during the silylation. An-α, ae-α and andien-β have thus been estimated in both normal and abnormal human urine [23–25.31.71] and an-β has been estimated in boar urine[5]. G.l.c. has also been helpful in the estimation of an-α, ae-α, andien-β and an-β as well as androstadienone and 5α-androstenone, added as carriers to *in vitro* incubations[6.8.9.12].

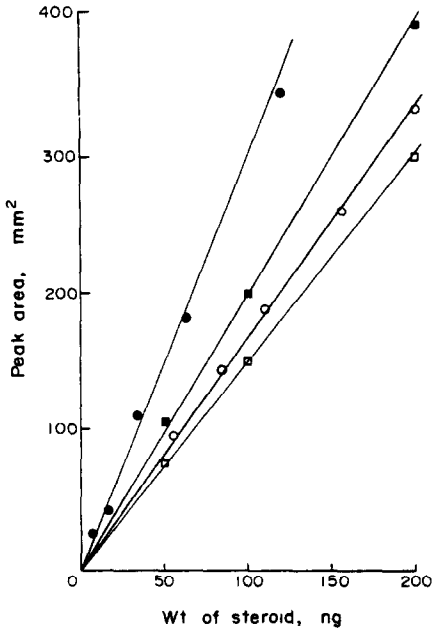


Fig. 24. Responses of some 16-unsaturated steroids and derivatives to flame ionization detection:– an-α (○), an-β (□), androstadienone (■), ae-α TMS ether (●). The gas chromatograph used was the dual flame ionization, model 24 (Pye-Unicam Ltd. Cambridge) with attenuation $\times 50$ (full scale deflection 5×10^{-11} A). Data from Gower (unpublished).

G.l.c. methods have been utilised for the measurement of some 16-unsaturated steroids in pig peripheral plasma, boar testis and salivary glands[3,4]. The concentrations of an-α, an-β and 5α-androstenone have been determined in boar spermatic vein plasma[5] and the concentration of androstadienone in human peripheral plasma has likewise been measured by a g.l.c. method[32].

(c) *Protein-binding methods.* Attempts to estimate 5α-androstenone in pig peripheral plasma by protein-binding have so far been unsuccessful[4]. It seems likely, however, that an-α may be able to displace testosterone from testosterone binding globulin in bovine plasma[4].

11. PRACTICAL IMPLICATIONS OF THE ODOUR OF 16-UNSATURATED C_{19} STEROIDS

Prelog and Ruzicka[82] commented upon the musk-like odour of an-α and an-β and described that of an-α as being more intense than that of an-β. Subsequent studies[84] showed that the corresponding 5β-isomers were odourless and that the ketones, androstadienone and 5α-androstenone, possessed pronounced urine-like or perspiration-like smells. These authors also drew attention to the superficial structural similarities between 5α-androstenone and civetone (Fig. 1). The characteristic odours of this group of compounds have been useful in detecting the presence of very small quantities in tissue, plasma or urine extracts. For example, 5α-androstenone was detected in boar fat extracts as it emerged from a g.l.c. column[13]; Brooksbank and Haslewood[2] were able to smell the musk-like odour of a compound (later identified as an-α) in hydrolysed urine

extracts; and other workers[5] described how the smell of 5α-androstenone could be detected on the hot syringe needle after a boar spermatic vein plasma extract had been injected on to a g.l.c. column. However, the characteristic musky smell is not restricted to 16-unsaturated alcohols; the 3-hydroxy-5α-androstanes also possess a musk-like odour[84], although the 5β-epimers are odourless and, out of 33 steroids investigated by Beets[112], the musk odour was detected in thirteen derivatives of androstane. The olfactory properties of the 16-unsaturated steroids has been referred to in a number of papers[113–116] and has been reviewed recently[56]. The olfactory response of men, women and children to the musky odour of the macrocyclic ketone, exaltolide, has been intensively studied[117, 118]. The ability of women to smell this compound increases during the follicular phase of the menstrual cycle, reaches a maximum at ovulation but declines during the progestational phase. Le Magnen[117] has also shown that children, men and post-menopausal or oophorectomized women can only smell exaltolide faintly. Administration of oestrogens, however, causes a recovery in the olfactory acuity. In a more recent study of 73 female student nurses[118] two peaks occurred in olfactory acuity, one just preceding ovulation (17 days before the menses) and the other during the luteal phase (8 days before the menses). Such experiments[117, 118] suggest that there may be a link between oestrogens and the ability to smell musky odours.

In 1948, an interesting case of anosmia to an-α was described[119] and a more detailed study of 200 men and women[120] revealed that 29% of the men were anosmic to an-α, 38% described the smell as faint and 33% as strong. The corresponding percentages for women were 22, 36 and 42%. The results of a similar study using 5α-androstenone were more clear cut[121]. In this investigation records were made of the olfactory response of 50 men and 50 women to 800 ng. of the pure compound that had been applied (in ethereal solution) to 5 cm^2 of a watch-glass. It was found that 44·3% of the men were unable to detect the odour in contrast to only 7·6% of the women. Most of the female subjects rated the smell as extremely unpleasant. A high proportion of women also find the smell of roasting boar meat unpleasant[122] since the odour of the 5α-androstenone in the fat (see p. 47) is especially noticeable when the meat is hot. This has important practical implications, as discussed recently[121], since women, rather than men, are most often involved in the preparation and cooking of pork or bacon and will decide whether it is acceptable. However, when the meat has reached the table and cooled somewhat, the intensity of the odour may have decreased and the meat may then be more palatable. In this connection, it is of interest that the German cartoonist Wilhelm Busch (1832–1908) has alluded to the great sensitivity of women to the smell of pigs. In a cartoon entitled "Ebergeruch" (pig smell), a herd of pigs is shown being to market. The woman near them holds up her hands in horror at the smell (depicted as a spirit), while the man walks on, apparently unconcerned.

The unpleasant smell and flavour of cooked meat taken from an uncastrated or partially castrated boar was described many years ago[123, 124]. One investigator [124] considered that the submaxillary glands possessed the unpleasant taste and recommended their removal; another described the parotid glands as possessing the bad odour even when the fat and meat were considered as negative. By smelling the meat and melted fat separately at different times after castration, it was evident that the smell persisted for two months. Cryptorchid animals have

also been shown to possess the smell[124, 125]. As discussed earlier (p. 63) the situation regarding boar salivary glands and 16-unsaturated steroids has still not been clarified. That the so-called 'sex-odour' was sex-dependent was shown by Williams and his collaborators[126] who found that 64% of males possessed the 'sex-odour' substances but only 1–5% of females and castrates. Experiments[62] performed in 1959 led to the suggestion that boar 'sex-odour' emanated from the preputial gland, since its surgical removal reduced the odour. However, the gland possesses no 16-unsaturated steroid biosynthetic activity[20] (see p. 63) nor does it contain more than traces of these steroids[65]. Patterson therefore believes that the smell of the gland is due to the presence of phenols, especially p-cresol[64] and fatty acids[65] (see p. 63).

Although the sex-odour-producing substances were known to be water-insoluble and ether-soluble and to be present in the unsaponifiable fraction of boar fatty tissue extracts[127.128], attempts to characterize the odorous principle were unsuccessful[129]. It was not until 1968 that 5α-androstenone was isolated and characterized as the 'boar taint' ketone[13] (see p. 47). The high vacuum distillation apparatus used in these experiments, however, did not give quantitative yields of the ketone. For this reason, a pot-still was used in later experiments [130] in which the ketone and other volatile compounds, removed from the molten fat under high vacuum, were trapped on a glass surface (cooled by liquid nitrogen at -196°C). The distance between the heated fat and the cooled surface was made as short as possible to increase the efficiency ($92 \pm 5\%$) of the process. A clean-up procedure with aqueous alkali, followed by t.l.c., was found to be necessary before the non-saponifiable material could be subjected to g.l.c. for final analysis (cf. the method used for human axillary sweat[34]).

Since boar 'sex-odour' is thought to originate principally in the testis, castration is normally performed during the first few weeks of life. Recent results[131] show that partial castration is as effective as complete castration in eliminating sex-taint in pork meat. In one group of animals, only testicular parenchymal tissue was removed; from another group, the testes were removed but the epididymis left intact. In both groups, little or no taint could be detected. However, although the principal site of formation of the odorous 16-unsaturated steroids is removed by such procedures, the formation of testicular androgens is also eliminated and it has long been known[132] that this results in a smaller percentage of lean meat, more back-fat, lower live-weight and a reduction in the quality of the meat. Another point worth consideration is that castration will result in lack of an-α (see p. 46), a compound that has a definite myogenic effect (Table 19). For these reasons, some attempts have been made to suppress the odour of the live boar without resorting to castration. Administration of stilboestrol and of 17α-methyl testosterone[133–135] were indeed shown to reduce the 'sex odour' in boars and a more recent study[136] confirmed that implantation in boars of 96 mg diethyl-stilboestrol at a live weight of 70·3 kg reduced boar odour even though plasma androgen levels were unaffected. Chlormadinone acetate (20 or 30 mg daily for periods of 33–70 days) also significantly decreased the taint of boar meat (tested by smell and taste) but the fat content of the meat doubled[137]. Such methods, however, are expensive and tedious to perform during the life of the pig up to the time of slaughter, and, as mentioned above, castration of most males is normally performed at an early age. Whether stilboestrol and 17α-methyl testosterone have an effect on the biosynthesis of 16-unsaturated steroids has not yet been estab-

lished but such an investigation is at present being undertaken in the author's laboratory. The problems connected with the taint in pork have been the subject of a number of recent reviews and papers [4, 138–140]. In particular the work of Elsley and Livingstone [140] has shown that the taint of boar fat is rarely detected in immature pigs of less than 43 kg body weight (< 100 days old) but thereafter it becomes increasingly common and there is a correlation between taint and age between 135 and 365 days, i.e. in animals weighing more than 92 kg (cf. Table 1).

Possible physiological role of 16-unsaturated steroids in pigs

In 1961 Signoret and du Mesnil du Buisson [141] showed that boar odour was necessary to elicit the characteristic mating stance of the sow *in oestrus* when subjected to the usual back-pressure test [142]. Over 81% of oestrus females so tested responded when kept in a pen previously occupied by a male. Further experiments [143] showed that the stimulus was an olfactory one, since surgical removal of the olfactory bulbs of the female abolished the response and, moreover, interfered with maintenance of the genital tract and inhibited the release of FSH from the anterior pituitary.

Since the period during which the female is *in oestrus* is relatively short, it is obviously of great importance for pig breeders to ascertain with the minimum delay if the sow is ready for artificial insemination without having to expose her to boar odour. Both boar urine and seminal fluid [143, 144] have been rubbed on to the snouts of sows and have been shown to induce the mating stance even in a proportion of recalcitrant animals (i.e. those that did not respond immediately to the normal back-pressure test of Altmann [142]). Such procedures, although effective, are not hygienic.

The penetrating smell of the 16-unsaturated steroids and their possible relationship to boar 'sex-odour' led Sink [145] to propose that these steroids might be acting as sex-attractants. Shortly afterwards [16] an-α was discovered in boar saliva and it was suggested that, as the boar becomes sexually excited and salivates profusely, the smell of the alcohol on the breath of the males reaches the female and elicits the characteristic immobilization response. The male is then able to mount and copulate. an-α and 5α-androstenone are the compounds that elicit the response in the female, was shown by Melrose, Patterson and Reed [146, 158] who sprayed 5α-androstenone and an-α separately, in aerosol form, towards the snouts of recalcitrant females and found that approximately 50% then responded to the back-pressure test. Artificial insemination can thus be carried out more efficiently and expeditiously than would otherwise be the case.

It is of interest that the musk-smelling an-β occurs in boar urine [5] and this may explain the effect (mentioned above) of the smell of such urine on sows *in oestrus*. It is also possible that 16-unsaturated steroids may occur in boar seminal fluid, their smell eliciting the characteristic response in the female.

Recent work reviewed by Signoret [147] has revealed that the reproductive behaviour of pigs is extremely complex. The female *in oestrus* requires not only the odour of a boar to elicit the mating stance; the sound of its 'courting song' and the sight of it are also important. Even the frequency of the males' grunts is important, since a tape-recording of a "song" with the frequency of grunts reduced by 50% causes a much lower frequency of characteristic responses when played back to the female.

16-unsaturated steroids as androgens

Since an-α is structurally related to the androgens and is also excreted in human urine in milligram quantities, this compound was for many years considered as possessing androgenic properties. The physiological effects of an-α, an-β and androstadienone have now been studied by bio-assay [148] (Table 19) and summarized recently [32.56]. It is clear that, at least in rats, rabbits and mice, an-α, an-β and androstadienone, show little or no androgenic activity. Recently, 5α-androstenone was also shown to have no androgenic activity compared with testosterone in the chick comb test [4]. Androstadienone was, however, shown to be weakly oestrogenic and this is explained on the basis of its possible conversion to oestratetraenol (p. 73) and of the latter's oestrogenic activity (see below). The physiological role of this group of compounds in these species and in humans therefore remains enigmatic. However, it is of interest that the powerfully-smelling 5α-androstenone has been found in the axillary sweat of a man (Ref. [34], see p. 55) but whether this compound plays any role as a sex-attractant remains to be elucidated. In this connection it is of particular interest that in some folk dances performed in Mediterranean countries, the male dancers stimulate their female partners by waving in front of them handkerchiefs that have been held for some time under the armpits of the men [149]. The nature of the active principle so obtained on the handkerchiefs is unknown but it is tempting to speculate that a blend of 5α-androstenone and other secreted substances may play a role in this respect. The smell of the ketone itself would presumably not produce the required stimulation in the women since many find the smell unpleasant [121].

Oestrogenicity of oestratetraenol

The relationship of this compound to epioestriol has already been described

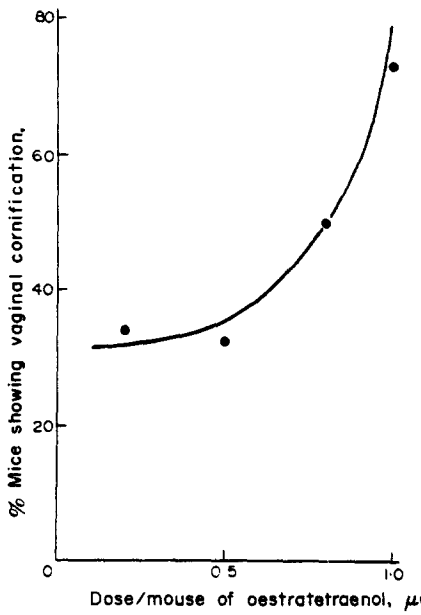


Fig. 25. Dose-response curve for oestratetraenol. Ovariectomized mice were given a single sub-cutaneous injection of oestratetraenol in sesame oil and vaginal cornification recorded.

Table 19. Physiological activity of 16-unsaturated steroids

	Androgenic	Anti-androgenic	myogenic***	Anti-myogenic	progestational	Anti-progestational	Uterotropic†	Anti-uterotropic†	oestrogenic	glycolytic, mineralocorticord, thymolytic, anti-inflammatory, anti-ACTH
Androstadienone	none*	none	none	none	none	trace (at 1 and 20 mg/3 days)	none	trace (at 0·01 mg/3 days against 0·32 μg oestrone)	moderate†† (9 mg/3 days)	none
an-α	none*	very slight	100% as effective at 6 and 60 mg/7 days against 1–2·4 mg testosterone	moderate	none	none	none	none	none	none
an-β	none*	none	none	none	slight (at 8 mg/5 days against 0·32 μg oestrone)	none	none	slight (at 9 and 10 mg/3 days against 0·32 μg oestrone)	none	none
5α-androstenone	none**									

*wt. of accessory sex organs of castrated male rats, **chick comb test, ***wt. of levator ani of castrated male rats.
†wt. of uteri of immature female Swiss albino mice, ††ovarian wts. of immature New Zealand white rabbits
Data from refs. 148 and 4.

(p. 73) and its oestrogenicity in rats has been studied briefly by a number of workers[96. 150]. More recent investigations[151] have shown that, in mice, oestratetraenol was much less oestrogenic than oestradiol-17β, the threshold dose being 0·2–0·5 μg per mouse. A dose of 1·0 μg caused 76% of mice injected to go into oestrus and caused a significant increase in uterine weight compared with a control group. The dose-response curve (Fig. 25) was similar to that for oestradiol, approximately 1·0 μg oestratetraenol per mouse being equivalent to 0·1 μg of oestradiol-17β.

12. SUMMARY AND CONCLUSIONS

The experiments described here, that have been performed by workers in the disciplines of chemistry, biochemistry, physiology and agriculture, have made it abundantly clear that the 16-unsaturated C_{19} steroids are of great physiological importance, at least in pigs. The question as to whether these compounds may act as pheromones in humans, through excretion in the sweat or urine, has been discussed and, for the present, remains open. Further problems still exist, such as the significance, or otherwise, of the increased amounts excreted in endocrine disorders such as virilizing adrenal carcinoma and especially the source of an-α. Undoubtedly, further research will help to solve the mystery that still pervades this fascinating group of compounds.

NOTES ADDED IN PROOF

p. 47. Recent work [152. 153] has now revealed that boar fat contains an-α in addition to 5α-androstenone.

p. 48. Analyses of parotid glands taken from Landrace boars (Ref. 154; D. B. Gower and Y. A. Saat, unpublished observations) have shown the presence of only very small quantities of 16-unsaturated C_{19} steroids. These results contrast markedly with those of Claus[4] (see Table 1). Boar parotid glands, however, contain a high proportion of fat and, if this is separated and analysed, it is found to contain a much higher concentration of 16-unsaturated C_{19} steroids than the parotid gland tissue itself (D. B. Gower and Y. A. Saat, unpublished observations). It is possible that the discrepancy between the available analytical results may be explicable in the light of these recent findings.

p. 64. More detailed analyses[155] of boar testes that had been infused with [4-^{14}C]pregnenolone have shown the presence in these tissues of the sulphates of an-α and an-β (labelled with ^{14}C) in addition to the unconjugated alcohols[101]. In keeping with analytical results (Table 1), the amount of ^{14}C-an-β sulphate found exceeded that of the 3α-compound.

pp. 61 and 72. The reduction of [7α-^{3}H]androstadienone and [5α-^{3}H]5α-androstenone has recently been studied in preparations of boar testis[156. 157] and salivary glands[154]. In boar testis homogenates, kinetic studies revealed that androstadienone was converted first to 5α-androstenone (a reduction requiring NADPH) and that this was subsequently metabolised to a mixture of an-β (the major product) and an-α. The formation of an-α from 5α-androstenone was shown to be NADPH-dependent whereas the formation of an-β was NADH-dependent. These results are in excellent agreement with studies *in vivo*[101] and clearly establish androstadienone and 5α-androstenone as intermediates in 16-unsaturated C_{19} steroid biosynthesis (Fig. 6). By incubating minces of boar submaxillary and parotid glands, mixtures of an-α and an-β were also formed

from both ^{3}H-androstadienone or ^{3}H-5α-androstenone[154]. Using submaxillary gland tissue, the reduction of 5α-androstenone to an-α was shown to be NADPH-dependent, although NADPH or NADH seemed to be equally as effective in promoting the reduction when parotid gland was used. In contrast to earlier results (Ref. 7, see p. 19) no 16-unsaturated C_{19} steroids were formed when [4-^{14}C]pregnenolone was incubated with boar submaxillary gland minces[154]. Testosterone and DHA were likewise ineffective precursors *in vitro*[154].

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