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16-ENE-STEROIDS IN THE HUMAN TESTIS

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Summary—Incubation of human testicular homogenates with [4-^{14}C]pregnenolone gave substantial amounts of an unknown metabolite within 1 min, reaching plateau values of 17–23% of total radioactivity added within 5 min. Mass spectrometry of the metabolite showed it to be identical to the boar sex pheromone precursor androsta-5,16-diene-3β-ol (ADL). In cell cultures the major source of ADL and its dehydrogenated metabolite androsta-4,16-diene-3-one (ADN) was the Leydig cell. In rat and monkey testicular homogenates 16-ene-synthetase activity, a prerequisite for the synthesis of ADL and ADN, was completely lacking, limiting the presence of 16-androstenes to boars and men. In contrast to boars, however, in the human testis no 5α-reductase activity was found and consequently no 5α-reduced-16-androstenes, e.g. androstenol (AL, musk like) and androstenone (AN, urine like), known sex pheromones in pigs. As both sex pheromones have been identified in urine, plasma, sweat and saliva of men and (especially hirsute) women we hypothesize that AL and AN are synthesized from ADL via ADN peripherically in tissues rich in 5α-reductase, i.e. skin, axillary sweat glands and probably also the salivary glands. So far, there is some evidence that both sex pheromones may have similar functions in humans as in boars.

INTRODUCTION

In 1944 Prelog and Ruzicka [1] searched for androgens in the pig and isolated three odorous 16-ene-steroids from the testis, the musk-like smelling androstenols (5α-androst-16-en-3α and 3β-ol, ALα and ALβ) and the urine-like ketone derivative androstenone (5α-androst-16-en-3-one, AN) [1, 2]. These 16-unsaturated steroids—which lack androgenic activity despite their A ring resemblance to testosterone and dehydrotestosterone—later appeared to be quantitatively more important than the androgens in pigs [3–5].

Earlier several workers had already commented on the unpleasant smell and flavour of cooked bacon taken from uncastrated mature boars [4]. This "boar taint" was finally isolated by Patterson from fat samples [6]. Its structure was identical to that of AN. Because of its lipophilic properties ketonic AN is readily stored in adipose tissue after its synthesis by the testis and finally transported to the salivary gland, where it is released in saliva unchanged or converted in the parotid gland to the ALβ and less to ALα alcohol. Boar taint is rarely present in boars <43 kg (100 days of age), but increases with age. Signoret and du Mesnil du Buisson [7] and later Patterson [6] demonstrated that the 16-ene-steroids, excreted in the breath via the salivary gland, act as a sex pheromone in pigs, eliciting the characteristic mating stance of the sow in oestrous, when subjected to the usual back pressure test. Sprayed in an aerosol to the snout of recalcitrant females AN and both ALβ and ALα were found to be an aid to the detection of oestrous in pig artificial insemination ("canned boar") [8].

The mainly testicular origin of the 16-unsaturated C^{19}-steroids was demonstrated along several lines. First, castration of mature boars almost completely abolished the boar taint and the accumulation of AN in fat [6]. Secondly, boars show a seasonal variation of AN in blood along with testosterone [9], whereas administration of human chorionic gonadotropin (hCG) [10] increases blood and fat AN concentrations. Thirdly on incubating testis homogenates with pregnenolone, the first 16-ene-steroid formed was androsta-5,16-diene-3β-ol (ADL), followed by androsta-4,16-dien-3-one (ADN) [4, 5]. There was a predominance of the yields of ALβ over ALα after puberty, before puberty rather the reverse was found [4]. By infusing tritiated pregnenolone into the spermatic arteries in mature boars *in vivo* formation by the testis of the 16-androstenes ADN, ALβ and ALα in a ratio of 5:3:1 was demonstrated [11].

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In liver microsomes ADL is oxidized via the 16,17α and 16,17β epoxides to androstenetriols [12].

Remarkably 16-ene-steroids, mainly the "distinguished" AL [13], are also found in truffles, both in the white Italian as in the black Périgord ones in concentrations similar to those in peripheral blood of mature boars [14]. These fungi grow deep beneath the soil and are searched for by female pigs (Italy) or dogs (France) [13]. The more vulgar AN compound has also been detected in parsnips and the roots of celery, which earlier enjoyed a reputation as an aphrodisiac ("the poor man's truffles") [13–15].

16-ENE-STEROIDS IN HUMANS

The presence of 16-ene-steroids in humans was first demonstrated by Brooksbank and Haslewood in 1950 [16]. The authors showed that ALα—the main alcohol in man—conjugated as the glucuronide was excreted in the urine of normal men but also, though in lesser quantities, in women and adolescents; not, however, in prepubertal children. Administration of hCG and adrenocorticotropic hormone (ACTH) raised the excretion of ALα in men (hCG, ACTH) and women (ACTH), suggesting a testicular and adrenal source of this 16-ene-steroid [17, 18]. Further evidence for a testicular origin of the 16-androstenes was adduced by Ruokonen [19], who identified and quantified free and monosulphated ALβ, ALα (less) and ADL in human cadaver tissue. Bicknell and Gower [20] showed that the 16-ene-steroids could be synthesized from labelled pregnenolone in testis homogenates from patients with testicular feminization (Tfm), but in much lower yields than in boars. Further evidence was obtained by the finding that removal of the testes from 2 patients with Tfm resulted in a 60–70% decrease of urinary ALα and ADL excretion [20].

Until recently no studies had been performed on testicular 16-ene-steroid synthesis in "normal" men. Studying the early time sequence in [$^{-}$4-^{14}C]pregnenolone metabolism in homogenates of rat and human testes derived from patients with prostatic cancer (56–80 years old), a number of unidentified ^{14}C-labelled metabolites were found, one in rather high yield, comparable to that in boars (17–23% after 1 min), but only in men [21, 22]. Strong evidence was obtained (gas chromatography, mass spectrometry) that this unknown metabolite was identical to the 16-ene-steroid, ADL [22]. This finding was later confirmed by Kohara *et al.* [23]. However, in contrast to boars apart from ADL and to a lesser extent ADN, no sex attractant steroids such as ALα, ALβ or AN could be detected, which confirms the results of Bicknell and Gower [20] with Tfm testes. Studying post mortem testicular tissue (3 men 22–37 years of age), ADL and ADN synthesis was strongly reduced or even completely absent [24]. Again no ALα, ALβ or AN could be detected which contrasts with the data of Ruokonen, *vide supra* [19]. Incubating stroma ovarii tissue with [$^{-}$4-^{14}C]pregnenolone also only showed accumulation of ADL and ADN, no AN, ALα or ALβ. Our inability to find these 16-androstenes fits in with the fact that we (and others) could not detect any 5α-reductase activity in human testicular homogenates (in contrast to rat testis). This enzyme is necessary for the conversion of ADN to AN, which is further metabolized to ALα/ALβ by 3-ketosteroid-oxidoreductase. The presence of AN and ALα in human blood and urine indicates that both 16-androstenes must be synthesized in a 5α-reductase rich compartment outside the testis or ovary (skin?). The discrepancy in blood and urine production rates of AL also points in this direction [25].

Although there is ample evidence of a gonadal source of 16-ene-steroids in boar and men, there is no data so far as to which structure in the testis or ovary is responsible for their synthesis. Incubating cultured human Leydig cells, we demonstrated that these cells are the major source of the sex pheromone precursors ADL and ADN, which account for about 20% of total steroid production [24].

Until recently boars and men were the only species studied synthesizing 16-ene-steroids. Studying labelled pregnenolone metabolism in *Macaca fascicularis* testis homogenates, Kwan *et al.* [26] found evidence that 16-androstene production also occurred in this species, whereas Claus *et al.* [27] reported AN activity in the blood of one male gorilla. We could not find any 16-ene-steroid activity in *Macaca fascicularis* or *Macaca rhesus* testis homogenates incubated with [4-^{14}C]pregnenolone [28].

Remarkably, in our experience the synthesis of ADL and ADN was always accompanied by synthesis of epi-androstenediol (5α-androst-16-ene-3β,17α-diol, epi-A5) [22, 29]. The ratio ADL/epi-A5 was roughly constant during the first 30 min of incubation. All efforts to suppress

The mechanism of the synthesis of
16-Androstenes in human testicular homogenates

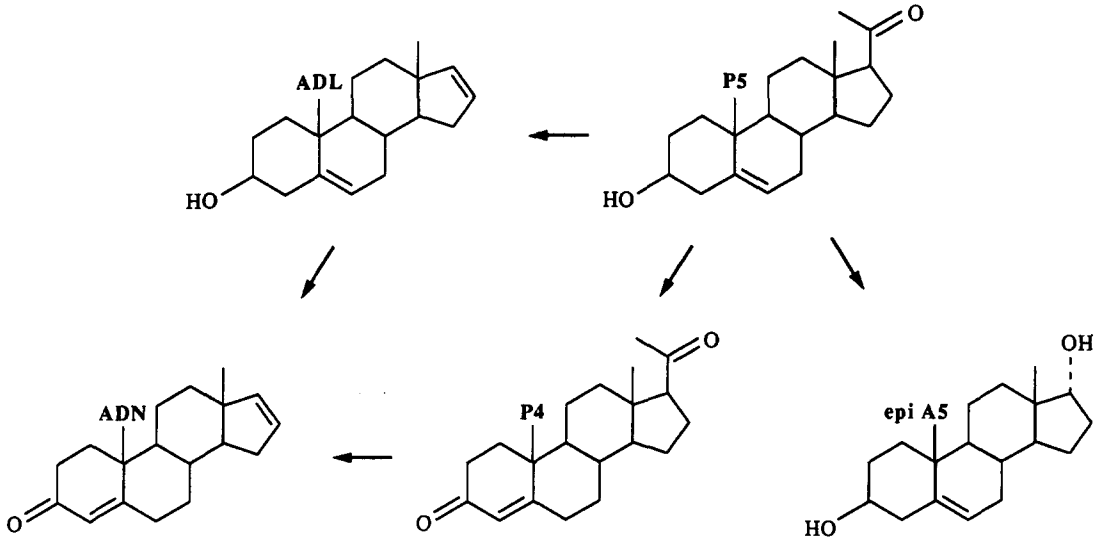

Fig. 1. Pathways in the 16-ene-synthetase process.

the synthesis of one, without affecting the other using various chemical inhibitors of cytochrome *P*450, 3β-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase or side chain cleavage failed [29].

Studying the 16-ene-synthetase reaction in human testicular homogenates, we adduced evidence for the hypothesis, that ADL is synthesized from pregnenolone in a single step, not requiring separate intermediates such as 5-pregnenediol, 21-OH-pregnenolone ($21OHP_5$), $170HP_5$ or 16-dehydroP_5 [29]. Our proposal for the 16-ene-synthetase mechanism also explains why synthesis of ADL is always accompanied by cosynthesis of its satellite epi-A5: both steroids are synthesized as a mere consequence of the fact that the elimination and substitution reaction for the synthesis of ADL and epi-A5, respectively are competitive processes [29]. The 17α-hydrogen of pregnenolone is maintained in the synthesis of both ADL and epi-A5, whereas the 16α-hydrogen is lost in the synthesis of ADL [29, 30]. From these data and those of others [4, 5, 30] the pathways in the 16-ene-synthetase process can be depicted as in Fig. 1.

Smaller amounts of ADN can also be synthesized from progesterone. ADN is then further metabolized as in Fig. 2.

Several authors have demonstrated that the 16-ene-C^{19}-steroid synthetase is a cytochrome *P*450 linked oxygenase system including cytochrome *P*450, cytochrome *P*450-reductase and in particular cytochrome b_5[31–35]. Mason *et al.* [36] demonstrated that the ratio cytochrome $b_5/P450$ in testicular microsomes differs in different animal species but is highest in pigs (ratio 10) and humans (ratio 7) (for comparison, rat 0.3, guinea pig 0.9, cat 0.9, rabbit 1, hamster 1, dog 1.3, all species without major 16-ene-synthetase activity). One may speculate that the high ratio in boar and human testis microsomes may be related to the high yields of ADL in both species [23].

16-ENE-STEROIDS IN HUMAN URINE BLOOD, FAT, SALIVA AND (AXILLARY) SWEAT

In contrast to boars ALα is the major 16-androstene in human urine. Brooksbank *et al.* [17] and Cleveland and Savard [18] found minimal ALα excretion before puberty, peak values in young adults and rather low values at old age. The male/female ratio was about 3 (1180 vs 419 μg/24 h). Exogenous testosterone lowered urinary AL excretion, whereas hCG or ACTH resulted in a distinct increase, *vide supra*. Elevated levels were found in patients with virilism due to adrenal carcinoma [37, 38] and women with hirsutism [4].

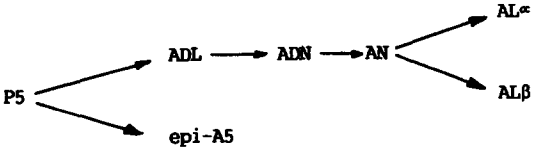

Fig. 2

Table 1

	Plasma (ng/ml)			
	AL[20]	AN[3]	ADN[25]	Testosterone[3]
Men	3.08 (0.3–14.9)	3.3 (2.1–4.4)	0.98	4.4 (2.8–6.7)
Women	0.66 (0.0–2.4)	ND[a]	0.36	—
Boars	30.7 (10.9–58.9)	6.5 (3.0–18.0)	—	5.2 (2.1–15)
Gilts	0.24 (0.08–0.7)	—	—	—

[a]ND, not detected.

Blood levels of AL [20], AN [3] and ADN [25] in men and women measured by radioimmunoassay [39] are given in Table 1. For comparison the (available) data in boars are also depicted. Whereas the AL/AN ratio in men was about 1, this ratio was 5 in boars. It has to be taken into account that due to its lack of polarity AN is avidly taken up by fat [boars: AN concentration fat 530 ng/g, men: 103 (10–170) ng/g, women 10–30 ng/g] [3]. The AN/testosterone ratio in men was of the same order of magnitude as in boars.

The 16-ene-steroids ADL, ADN, AN, ALα and ALβ all have been demonstrated in human axillary sweat [40–43]. Highest mean values were found for ADN and ALα (both about 200 pmol/24 h/armpit) and lower values for ADL and ALβ (both about 100 pmol/24 h) and AN (40 pmol/24 h) [43]. The levels of ADN and AN on one hand and those of ALα and ALβ were closely correlated, suggesting common precursors. Axillary microflora may be responsible for the conversion of ADN to AN via 5α-reductase and of AN to ALα and ALβ via 3-ketosteroid-oxidoreductase [43].

Low concentrations of AN have also been found in human saliva, the values in men being significantly higher than in women [44].

HUMAN SENSITIVITY TO THE ODOUR OF 16-ENE-STEROIDS

Griffith and Patterson [45] demonstrated that 7.6% of women and 44.3% of men are unable to detect the odour of AN. Elsley [46] reported anosmia to AN in 25% of 286 volunteers and Claus in 33% [47].

Anosmia to AL has been found by Kloek in 22% of women and 38% of men [48]. The threshold for normal observers for AN was 0.18 ppb (for ALα 6.2 and for ADN 0.98 ppb) [49]. Anosmia to ADN was present in 5 out of 58 (9%) healthy male and female volunteers from our laboratory [24]. According to Amoore [49] the smell of AL is only musk-like for those observers specifically anosmic to the urinous odour, whereas for normal observers it is still urinous. Menstrual variation to the musk odour of the macrocytic musk pentadecalacton [4, 5, 50, 51] and also AN has been demonstrated [52] with a peak of sensitivity just before ovulation. Amoore, however, did not find any cyclic variation in the sensitivity of women to the musky odour of pentadecalacton [49]. Changes in sensitivity to the odour of AN during adolescence have also recently been reported [53]. A significant increase in the number of males anosmic to AN and the threshold was found between 9 and 14, 15 and 20 years of age. A smaller percentage of females than males become anosmic to the odour of AN during development, and those able to detect it show a decrease in threshold. Griffith and Patterson [45] showed that the hedonic score was significantly higher in women than in men, demonstrating that women judged the smell of AN more unpleasant than men. Gower *et al.* [54] came to the same conclusion, as 70% of the women, but only 15% of men rated the smell of AN as repellent to unpleasant. The greater reluctance of women to the "boar taint" of cooked bacon could be ascribed to their higher sensitivity to this odour, especially before ovulation.

16-ANDROSTENES AS PUTATIVE HUMAN PHEROMONES

There are only a few studies examining the influence of AL or AN on subjects evaluation of others or rating of their own moods. Most reported some positive effect, one no effect [55]. Kirk-Smith *et al.* [56] found photographed women to be rated more sexy and attractive, better, warmer and more friendly during exposition to AL. Cowly *et al.* [52] demonstrated that exposure of females to AL is associated, in the case of male candidates, to assess them more favourably. Filsinger *et al.* [57], however, found female subjects in an AN condition to report themselves to feel less sexy, an effect contrary to the sexual arousal hypothesis. Benton [58] reported a cyclic variation in the influence of AL on mood during the menstrual cycle. In the

middle of the cycle females exposed to AL rated their moods as more submissive in contrast to controls. There was no influence on sexy/unsexy feeling. Clark *et al.* [59] demonstrated that theatre and dental waiting room chairs previously sprayed with AN were occupied mainly by women. Gustavson *et al.* [60] found that men, not women, avoided restroom-stalls treated with AL. The odour had no influence on female stallroom selection. The authors hypothesized that AL acts as a human space hormone primarily affecting males and eliciting avoidance. From the data mentioned above it appears that not only in boars but also in humans 16-ene-steroids may act as putative pheromones in the process of sex attraction [61].

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