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Comparison of 16-Androstene Steroid Concentrations in Sterile Apocrine Sweat and Axillary Secretions: Interconversions of 16-Androstenes by the Axillary Microflora—a Mechanism for Axillary Odour Production in Man?

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The concentrations of five 16-androstene steroids were determined, by a GC-MS method, in freshly-produced apocrine sweat (adrenaline-induced), in 8 men and 2 women. The ranges of concentrations (nmol/μl) in apocrine sweat were: 5α-androst-16-en-3-one (5α-A), 0.1–2.0 and 4,16-androstadien-3-one (androstadienone), 0–1.9. 5,16-Androstadien-3β-ol (androstadienol) was also found in 5 of the subjects (range 0.05–1.05). 5α-Androst-16-en-3α- or 3β-ols [3α(β)-androstenols] were only found in small amounts (< 0.1 nmol/μl) in a few subjects. In the second study, prior to apocrine sweat collection (adrenaline injection), the axillary skin of 6 of the male subjects was washed with diethyl ether on an adjacent site of the axillary vault. The concentrations of 16-androstenes were compared in the ethereal extracts and apocrine sweat. The former contained detectable levels (pmol/cm²) of androstadienone (17.9 ± 2.4), 3α-androstenol (6.9 ± 3.7), 3β-androstenol (1.8 ± 1.0) and androstadienol (1.9 ± 0.5) (means ± SEM) in all 6 subjects. All but 1 subject also had 5α-androstenone, the mean value for the others being 2.5 ± 0.6. The axillary skin levels of 3α- and 3β-androstenols, androstadienol and, in 3 subjects, androstadienone exceeded those in the apocrine sweat obtained from the same subjects, whereas levels of 5α-androstenone in the skin extracts were all lower than in apocrine sweat samples, when related to the corresponding areas of skin sampled. The metabolism of 16-androstenes was studied *in vitro* in the presence of two aerobic coryneform bacteria, previously shown to metabolize testosterone as well as being capable of producing odour from extracts of axillary sweat in an odour-generation test. Although both coryneforms caused complex metabolic reactions and were capable of oxidation or reduction at C-3 and C-4, the overall direction favoured reduction. For example, large quantities of the more odorous 5α-androstenone and 3α-androstenol were formed from androstadienol and androstadienone. In contrast, strains of corynebacteria, unable to produce odour and incapable of metabolizing testosterone, were also unable to metabolize 16-androstenes. We propose that, even without the *de novo* synthesis of odorous 16-androstenes by axillary bacteria, the small quantities of these steroids, including the weakly odorous androstadienol, present in adrenaline-induced apocrine sweat, could be converted by aerobic coryneform bacteria, resident on the axillary skin surface, to a more odorous mixture of 16-androstenes, such that the human olfactory threshold of perception would be exceeded.

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Abbreviations and trivial names: 5α(β)-androstenone, 5α(β)-androst-16-en-3-one; androstadienone, 4,16-androstadien-3-one; androstadienol, 5,16-androstadien-3β-ol; 3α(β)-androstenol, 5α-androst-16-en-3α(β)-ol; 5α-androstanone; 5α-androstan-3-one; 5α-androstanol, 5α-androstan-3β-ol; pregnenolone, 3β-hydroxy-5-pregnen-20-one; DHA, 3β-hydroxy-5-androsten-17-one; testosterone, 17β-hydroxy-4-androsten-3-one; 17-epitestosterone, 17α-hydroxy-4-androsten-3-one; 4-androstenedione, 4-androstene-3,17-dione; 5α(β)-androstanedione, 5α(β)-androstane-3,17-dione; 5α-DHT, 17β-hydroxy-5α-androstan-3-one; ppb, parts per billion.

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INTRODUCTION

During past decades, intensive studies have been directed towards investigating the source(s) of human odour. To a large extent such work has focussed on the human axillae because these have been described as perfect sites for odour dispersal [1]. At least three groups of compounds, present in axillary secretions, have now been described as being contributors to human axillary odour: aliphatic fatty acids, 16-androstene steroids and, most recently, the 2-methyl-3-hexenoic acids. Some members of the first of these groups, the aliphatic fatty acids, have been recognized for many years as giving rise to the 'sweaty' notes of axillary secretions. This applies especially to 2-methylbutanoic acid, but more recently, a whole range of aliphatic fatty acids from C-2 to C-16, have been characterized in human axillary sweat [2], and some of these are quite intensely odorous. The second group, the 16-androstenes, have also been quantified in axillary sweat and axillary hair [3–8] and, bearing in mind the low olfactory thresholds for three of these steroids notably 5α-androst-16-en-3-one (5α-androstenone), 5α-androst-16-en-3α-ol (3α-androstenol) and 4,16-androstadien-3-one (androstadienone) [9], the quantities present could easily contribute to human axillary odour in men [10]. However, freshly produced apocrine sweat is odourless [11] and was thought to contain little or no 16-androstenes [12]. It was therefore postulated that some odourless substrate, initially present in sterile apocrine sweat, was transformed in the presence of axillary bacteria to the odorous 16-androstenes noted above. Numerous intensive studies, involving incubation of putative substrates, such as, testosterone [13], 5α-DHT [13, 14], and pregnenolone [14] with appropriate aerobic corynebacteria have been undertaken without convincing evidence for 16-androstene biosynthesis [14, 15]. There seems to be little doubt that the aerobic *Corynebacterium spp.* are those which are especially associated with axillary odour [16–18].

The third group of substances, the isomeric 2-methyl-3-hexenoic acids [19–21], are said to have an 'unwashed' or 'unhygienic' odour, although the olfactory thresholds for the isomers differ and anosmias have been reported [21].

In the present communication, we have re-examined freshly produced apocrine sweat for the presence of 16-androstenes by a sensitive GC-MS method and have compared the concentrations found therein and in ethereal extracts of the axillary skin of the same subjects. In addition, the metabolism of 16-androstenes by two aerobic corynebacteria previously shown [22] to be able to catalyse the metabolism of testosterone and to be associated with the generation of odour (UAO + ve) [16, 17] has been investigated. Since some 16-androstenes appear to be present even in freshly-produced apocrine sweat, we have discussed the possibility that metabolism of these steroids as substrates by aerobic *Corynebacterium spp.* resident on the axillary skin surface is the only requirement for the odorous steroids to be found in axillary secretions, and that the *de novo* synthesis (as originally predicted) is not required. Such a proposal would explain our earlier failure to produce convincing evidence for the bacterial synthesis of 5α-androstenone from non-odorous precursors [15].

MATERIALS AND METHODS

Most authentic steroids were purchased from Sigma Chemical Co. (Poole, Dorset, England). Androstadienone and androstadienol were obtained from the MRC Steroid Reference Collection, Queen Mary College, University of London (courtesy of Professor D. N. Kirk). Reagents for steroid derivatization were purchased from Pierce and Warriner, Ltd. (Luton, England) and Aldrich Chemical Co. (Dorset, England). Solvents were purchased either from Sigma Chemical Co. or from Fisons, Ltd. (Loughborough, Leicester, England). Ethyl acetate, used for extraction of steroids, and diethyl ether, used in axillary collections, were distilled before use. New, unused glassware was used throughout these studies. Prior to use, glassware was soaked overnight in chromic acid, then rinsed thoroughly with de-ionized water and, finally, with acetone. All closures were polypropylene or, if they were in contact with organic solvents, PTFA-lined. Samples for GC-MS were contained in silanized vials (0.5 dram) with PTFA-lined closures.

Collection of apocrine sweat

Apocrine sweat was collected from human volunteers (8 men and 2 women) using adrenaline injection; 2 of the males were sampled on two separate occasions. Prior to the experiment, informed, written consent was obtained from all volunteers some of whom were drawn from the Staff of Division of Biochemistry, United Medical and Dental Schools (UMDS, Guy's Hospital) and from the authors of this communication. Subjects used non-perfumed soap shown on previous occasions to be devoid of substances which might have interfered with the assay of steroids [4, 5], and refrained from using anti-perspirants or deodorants for at least 5 days prior to sampling. None of them had used any anti-microbial medication for at least 2 weeks prior to the test. The axillae of the subjects were shaved of hair 24 h before sampling. On the morning of the test, subjects washed with non-perfumed soap and were sampled approx. 6 h later.

The subjects lay supine, the right axilla exposed, which was swabbed with 70% aqueous ethanol and allowed to dry. An intra-dermal injection (0.05 ml) of 1:1000 adrenalin BP (Antigen Ltd., Roscrea, Ireland) was made into the centre of the axillary vault, where the hair follicles were most dense. Volunteers were closely monitored with blood pressure measurements at intervals for up to 2 h post-injection. Apart from slight trembling of the hands and fingers, no untoward side-effects were noted in any subject. The Ethical

Committee of Guy's Hospital had previously sanctioned the project.

Droplets of apocrine secretion appeared within 5 min and could be distinguished from eccrine sweat by their association with pilosebaceous ducts and by their milky appearance. The apocrine sweat was collected in sterile 2 μl microcapillary tubes (Shadon Scientific) and the volume determined by measuring the height to the meniscus using a metal ruler with 0.5 mm graduations. The apocrine sweat was then expelled into sterile bijou bottles, which were either empty (for odour determination [16, 17], see also below) or contained 1 ml ethanol in which were dissolved 5α-androstan-3β-ol (5α-androstanol) and 5α-androstan-3-one (5α-androstanone), each 1 ng, as internal standards for GC-MS determination [8]. Finally, the microcapillaries were also dropped into the bottles and crushed with a sterile spatula in order to recover trapped material.

Extraction and purification of steroids

Extraction was performed with 3 × 3 ml of ethyl acetate and the pooled extracts were purified by passing through a two-stage column [8]. The upper part was a Pasteur pipette, containing anhydrous Na_2SO_4, held in place with a plug of fat-free cotton wool. The pipette had the tip end removed so that it could be inserted into a Florisil Sep-Pak cartridge (Waters Associates, Milford, MA, U.S.A.), to form the lower part of the column. The purified extracts were evaporated to dryness under vacuum at room temperature using a Buchler evaporator (Gallenkamp Ltd., London, EC3) and the residues were further cleaned by HPLC [22] before being derivatized ready for GC-MS analysis (see below).

Ethereal extracts of axillary skin

In the second study, prior to apocrine sweat collection (adrenaline-induced), the axillae of six of the male subjects were extracted with diethyl ether (2 × 5 ml) using the cup-scrubbing method [23] on an adjacent site of the axillary vault. Briefly, a sterile Teflon ring (i.d. 2 cm) was placed firmly on the skin and the enclosed area 'scrubbed' for 30 s with diethyl ether, using a Teflon-coated glass rod (care was taken not to damage the skin surface). The ethereal extract was collected into a dry glass tube and the process was repeated, the extracts being pooled and retained pending analysis.

Bacteria

Aerobic coryneforms used in this study were isolated as described previously [17, 24, 25]. The ability of axillary isolates to metabolize testosterone was determined [13, 22] and two aerobic coryneforms F1 and F47, which were active in this respect and also in the production of under arm odour, (UAO + ve) in the standardized odour generation test [16, 17] (see below) were selected for study in the metabolism of 16-androstenes. Isolate F46 was inactive and served as a control.

Odour generation test

This was performed as described previously [16, 17]. In essence ethereal extracts of human axillary skin were filtered through sterile cellulose acetate filters (0.22 μm porosity) and the ether evaporated with a stream of GC-grade, filtered N_2. After addition of phosphate buffer (0.1 M, pH 6.0), incubation was performed at 37°C for 6 h in the presence of the axillary bacterial isolate under investigation. Odours were scored on a scale of 0–5 and were determined organoleptically by a trained panel of experts [16]. Two controls were used, one with no bacterial cell suspension and one with no axillary extract. In the present work the above method was modified slightly to enable odour formation to be assessed when apocrine sweat samples were incubated with UAO + ve coryneform F1 and UAO − ve coryneform F46 for 6 h at 37°C.

Metabolism of four 16-androstenes by aerobic coryneforms F1 and F47

Unlabelled 5α-androstenone, 3α-androstenol, androstadienone and androstadienol (0.5 mg) were separately incubated with bacterial isolates without shaking for up to 2 weeks at 37°C. The steroids were dissolved in ethanol, and 0.5 ml portions added to 9.5 ml of broth [3%, v/v Tryptone Soya Broth (Oxoid CM 129), containing 0.1% (w/v) yeast extract (Oxoid L21 and 0.1% (w/v) Tween 80]. Controls were prepared in the absence of bacteria.

Extraction and purification of metabolites

Cultures were extracted with ethyl acetate (3 × 3 ml) and purification was achieved using Florisil Sep-Pak cartridges, as described above, for steroids in apocrine sweat. The eluates from this process were evaporated to dryness (Buchler Vacuum Evaporator) and the residues re-dissolved in dichloromethane (0.1 ml) and 25 μl of each were subjected to TLC on Kieselgel 60 glass plates (BDH, Dagenham, Essex, England) in benzene–acetone (4:1, v/v) run once. In order to locate the 16-androstene metabolites, without having recourse to the use of destructive colour-producing reagents, a sample of radiolabelled metabolites of [4-^{14}C]testosterone (0.25 μCi) was also spotted on to the TLC plates. This mixture was obtained from a previous incubation of the labelled testosterone with coryneform F1 [22] and was known from previous work to contain, 4-androstenedione, 5α-DHT, 5α-androstanedione and 5β-androstanedione. The radiolabelled spots were visualized by radioautography using X-ray film (DEF-2, single-sided; Eastman Kodak, Liverpool, England), placed in contact with the plates for 3 days [24]. In pilot experiments, it had been discovered that the mobilities of 5α-androstanedione (R_f 0.51) and 4-androstenedione (R_f 0.44) corresponded exactly to those of 3α-androstenol and androstadienol,

respectively. In addition the mobilities of two other testosterone metabolites with R_f values above that of 5α-androstanedione (R_f 0.66 and 0.58), corresponded to those for 5α-androstenone and androstadienone, respectively. It was therefore possible to locate the regions of the plate corresponding to the positions of the various 16-androstenes. These zones of Kieselgel were scraped off and eluted with ethyl acetate (5 ml). After addition of internal standards [8], 5α-androstanone and 5α-androstanol (1 μg of each), the solutions were carefully evaporated to dryness, compare Ref. [26] as above, and the steroids derivatized, as described below, prior to GC-MS analysis.

Derivatization of steroids

The extracts of apocrine sweat, axillary skin and the fractions obtained after TLC separation were evaporated to dryness under vacuum usually in silanized glass vials (0.5 dram, Pierce & Warriner). For oxosteroids, the pentafluorobenzyl (PFB) oximes were prepared using Florox (*O*-[pentafluorobenzyl] hydroxylamine HCl) as described previously [8, 22]. For alcohols, the *tert*-butyldimethylsilyl (TBDMS) ethers were preppared [8, 22], using a reagent mixture consisting of acetonitrile, *N*-methyl-*n*-(*tert*-butyldimethylsilyl)-trifluoroacetamide, MTBSTFA and TBDMS imidazole, containing 1% (v/v) TBDMS chloride, 500:100:5, by vol.; 100 μl of reagent were added to the dried-down fractions and heated at 60°C for 30–60 min in a heating block.

GC-MS

GC was performed with a Varian 3400 instrument (Varian Associates, Walton-on-Thames, Surrey, England) equipped with a fused silica column (25 m × 0.33 mm, i.d.), coated with SE-30, and an on-column injection system. The GC was directly interfaced with a VG Analytical (Manchester, England) Model 305 mass spectrometer. Data were collected on a Technivent System (Technivent, St Louis, MO, U.S.A.). Samples, dissolved in *n*-decane (1 μl) were injected onto the column, the temperature of which was 175°C initially and then programmed up to 310°C with a 30°C/min ramp.

Selected ion monitoring (SIM) was performed for the PFB oximes of androstadienone, 5α-androstenone and 5α-androstanone (internal standard) at *m/z* values 465, 467, and 469, respectively. These derivatives, as shown earlier [22, 27] gave intense ions corresponding to the $[M]^+$. In the case of the TBDMS ethers, however the *tert*-butyl group was lost readily to give $[M\text{-}57]^+$ and resulting in prominent ions for androstadienol, 3α(β)-androstenols and 5α-androstanol (internal standard) of *m/z* 329, 331 and 333, respectively [22, 27]. Standard curves for all five 16-androstene with, in each case the appropriate internal standard, were prepared over the range 0–100 ng; the limit of detection was 10 pg.

RESULTS

Apocrine sweat collection

With the exception of one female, all subjects produced tiny droplets of apocrine sweat from the pilosebaceous duct openings, in an area of approx. 4 cm^2 around the adrenaline injection site. The secretions tended to dry quickly so that the volumes given here are only those which could be collected. These ranged from 16–350 nl (mean ± SEM: 149 ± 33, $n = 11$). It was estimated that about 50% of the secreted apocrine sweat was actually collected into the microcapillaries from each subject. In most cases, eccrine sweat was also stimulated but this could be differentiated easily with a magnifying lens, since it was clearer in appearance than the turbid apocrine sweat and was not associated with pilosebaceous duct openings. Two of the male subjects produced coloured sweat, one yellow and the other dark brown. The latter was not a consistent feature, however, since on other occasions this subject produced colourless, milky secretions.

Odour formation following incubation of apocrine sweat with human coryneform isolates

Table 1 summarizes the results of the standard odour generation test [16, 17] in which portions of the

Table 1. Odour formation following incubation of apocrine sweat with UAO +ve coryneform F1 and UAO −ve coryneform F46 for 6 h at 37°C

Subject	Sex	nl of apocrine sweat used	UAO intensity grade		
			Sterile apocrine sweat	Apocrine sweat Plus F1	Plus F46
1	M	57	0	4	0
2	M	47	0	3	0
3 [1]	M	5	0	1	0
3 [2]	M	10	0	3	ND
4 [1]	M	16	0	3	0
4 [2]	M	24	0	5	ND
5	M	16	0	3	0
6	M	38	0	5	0
7	M	167	0	2	0
8	F	58	0	3	ND
9	F	16	0	5	ND

[ ], sampling occasion; ND, not determined.

Table 2. SIM determination of 16-androstenes in apocrine sweat of human subjects

Subject	Sex	16-Androstenes (nmol/μl)				
		5α-Androstenone	Androstadienone	3α-Androstenol	3β-Androstenol	Androstadienol
1	M	1.00	0	0	0	0
2	M	0.2	0.23	0	0	0
3	M	2.0	0	0	0	0
4	M	0.1	0.15	0	0	0
4[a]	M	1.0	1.88	0	0.2	0.7
5	M	0.3	0.48	0	0	0
6	M	1.5	0.33	0	0.1	0.5
7	M	ND	ND	0	0	0.1
8	F	1.2	0.87	0.1	0.03	0.05
9	F	ND	ND	ND	ND	1.05
10	M	0.3	0	0	0	0
Mean ± SEM		0.84 ± 0.2	0.44 ± 0.19	0	0.03 ± 0.02	0.2 ± 0.1

[a]Second estimation; ND, not determined.

apocrine sweat produced in response to adrenaline injection, were incubated with the UAO + ve coryneform F1 and the UAO − ve F46. All samples of apocrine sweat produced odour with coryneform F1 but none with F46 nor with control samples of apocrine sweat which was kept sterile. The odour generated was qualitatively similar for all the apocrine sweat samples and also similar to the acrid/musk-like odour of the ethereal extracts of axillary skin but was purer in that it did not possess the 'acidic' notes of the latter. The data in Table 1 also show that apocrine sweat obtained from some individuals produced a more intense odour than others. For example, apocrine sweat from subject 6 (UAO score, 5) produced a much more intense odour than that from subject 7 (UAO score, 2), even though only one-quarter of the volume was used. Apocrine sweat from female subjects produced UAO which was qualitatively identical and within the same intensity range as the male subjects.

Measurement of 16-androstenes in apocrine sweat

Table 2 summarizes the results of GC-MS analyses (expressed as nmol/μl) in the first study. The principal 16-androstenes present were 5α-androstenone (range 0.1–2.0) and androstadienone (range 0–1.9). Androstadienol (range 0.05–1.05) was also found in 5 of the subjects. Only 1 subject (No. 8, a female) had 3α-androstenol present but this was at a very low (0.1 nmol/μl) concentration. Three subjects (2 male and 1 female) had 3β-androstenol present but, again these were 0.2 nmol/μl or less.

Comparison of concentrations of 16-androstenes in apocrine sweat and axillary skin extracts

The results of the second quantitative study with 6 men are shown in Table 3. For the purposes of comparison of data, the results have been converted into pmol/cm^2 of axillary skin. In the case of apocrine sweat, this was an approximation, since the sampling area was approx. 4 cm^2 around the adrenaline injection site and, as noted above, we estimated that only some 50% of the apocrine sweat was harvested. Axillary skin extracts of all 6 subjects contained detectable amounts of androstadienone (Table 3), 3α- and 3β-androstenols and androstadienol. All but subject No. 4, also had 5α-androstenone present. The skin levels of 3α- and 3β-androstenol, androstadienol and, in three cases, androstadienone exceeded those of the apocrine sweat, whereas the quantities of 5α-androstenone were all considerably lower.

Metabolic interconversions of 16-androstenes in the presence of axillary coryneforms F1 and F47

Figure 1 shows the SIM traces obtained in GC-MS analysis of metabolites of androstadienone. For conciseness, corresponding SIM traces of metabolites of 5α-androstenone, 3α-androstenol and androstadienol

Table 3. Comparison of the 16-androstene content (expressed as pmol/cm^2 of axillary skin) of apocrine sweat and diethyl ether extracts of the axillary vault of 6 male subjects

Subject	16-Androstenes (pmol/cm^2 of axillary skin)									
	5α-Androstenone		Androstadienone		3α-Androstenol		3β-Androstenol		Androstadienol	
	Apocrine sweat	Skin extract	Apocrine sweat	Skin extract	Apocrine sweat	Skin extract	Apocrine sweat	Skin extract	Apocrine sweat	Skin extract
A	18.10	4.1	0	13.2	0	25.6	0	6.9	0	4.1
B	25.9	2.00	0	10.30	0	10.1	0	2.1	0	2.00
C	11.8	2.7	0	26.60	0	1.3	0	0.4	0	1.10
D	32.10	0	0	16.80	0	1.7	0	0.8	0	1.6
E	47.70	4.4	26.50	21.80	0	1.4	0	0.3	0	0.80
F	9.10	2.00	16.80	18.3	0	1.3	0	0.3	0	1.80
Mean ± SEM	28.5 ± 6.4	2.5 ± 0.6	7.2 ± 4.7	17.9 ± 2.4	0	6.9 ± 3.7	0	1.8 ± 1.0	0	1.9 ± 0.5

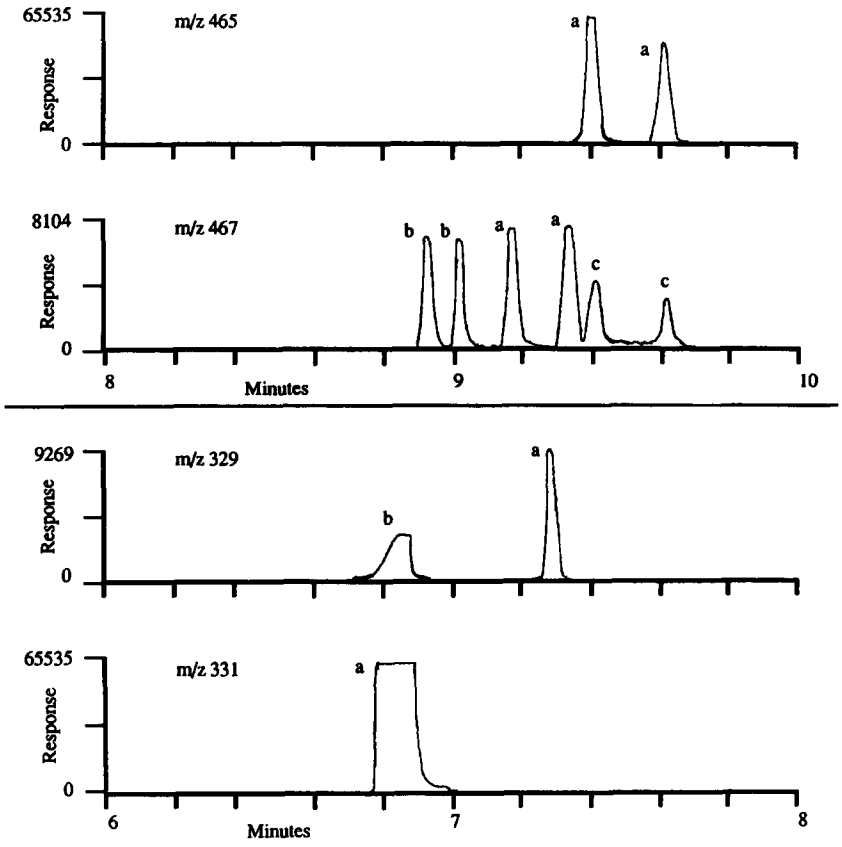


Fig. 1. SIM traces of PFB oximes and TBDMS ethers of metabolites of 4,16-androstadien-3-one formed during a 2 week incubation with UAO + ve coryneform F47 at 37°C. *m/z* 465: *syn* and *anti* forms of androstadienone; *m/z* 467: a, *syn* and *anti* forms of 5α-androstenone; b, *syn* and *anti* forms of putative 5β-androstenone; c, cross-talk from androstadienone; *m/z* 329: a, androstadienol; b, cross-talk from 3α-androstenol; *m/z* 331: a, 3α-androstenol.

are not shown. The results of the incubations with the four substrates (Table 4) clearly show that the two UAO + ve coryneforms caused a complex series of interconversions (Figs 2 and 3). Both F1 and F47 had a similar effect in that large yields of 5α-androstenone and 3α-androstenol were produced from androstadienone and androstadienol, respectively. In most cases, yields were at least 10% of the original substrate, corresponding to at least 50 μg. Androstadienol also produced some 3β-androstenol but androstadienone did not serve as a substrate in this case; reversible transformations occurred (Figs 2 and 3). For example, large quantities of 5α-androstenone were produced from 3α-androstenol, and conversely, similar yields of 3α-androstenol resulted when 5α-androstenone was utilized as a substrate. Other reversible reactions noted included the interconversion of androstadienone to androstadienol, conversion of 5α-androstenone to androstadienone and androstadienol, and 3α-androstenol to androstadienol. When 3α-androstenol was used a substrate evidence for the formation of its 3β-epimer was obtained.

The SIM data (Fig. 1) for the products of the androstadienone incubation gave evidence for

Table 4. SIM determination of 16-androstene metabolites following incubation with aerobic coryneforms F1 and F47 for 2 weeks at 37°C

	Quantities of metabolites formed expressed as percentages of substrates							
	5α-Androstenone		Androstadienone		3α-Androstenol		Androstadienol	
Substrate	F1	F47	F1	F47	F1	F47	F1	F47
5α-Androstenone	—	—	>10	>10	>10	>10	>10	>10
5β-Androstenone[a]	0	0	>10	>10	0	0	<1	<1
Androstadienone	<1	1–10	—	—	0	0	<1	1–10
3α-Androstenol	>10	>10	>10	>10	—	—	1–10	>10
3β-Androstenol	1–10	>10	0	0	<1	>10	<1	1–10
Androstadienol	<1	1–10	<1	<1	1–10	1–10	—	—

[a]Presumptive identification.
— Indicates substrate.

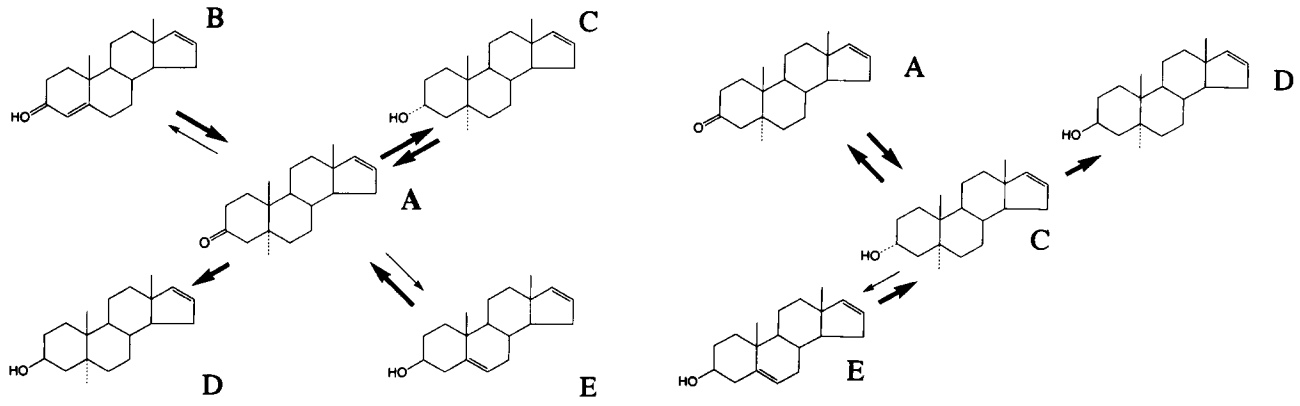


Fig. 2. Pattern of 16-androstene metabolites formed during incubation of UAO + ve coryneform F47 with 5α-androst-16-en-3-one (left) and 5α-androst-16-en-3α-ol (right). A, 5α-androstenone; B, androstadienone; C, 3α-androstenol; D, 3β-androstenol; E, androstadienol. Large arrows indicate >10% conversions, small arrows indicate <10% conversions.

considerable quantities of a compound which (a) possessed a ketonic function because it was converted to the PFB oximes (*syn*- and *anti*-forms being separated), (b) it was detected at the same m/z as that for 5α-androstenone; (c) had a R_f similar to that of the 5α-isomer on TLC, but (d) was eluted appreciably earlier from the GC column. These data suggest that the metabolite may be 5β-androstenone but, as neither the authentic 5β-androstenone nor its precursor, 5β-androst-16-en-3α-ol (which could be oxidized chemically to the ketone) are available.

A more detailed study of the metabolism of androstadienone was undertaken in the presence of five UAO + ve and four UAO − ve aerobic coryneforms, previously isolated and tested as described in Materials and Methods. Quantitative results (Table 5) obtained by SIM clearly show that the UAO + ve strains, F1, F20, F25, F32 and F47 possess the ability to reduce androstadienone to 5α-androstenone, while the UAO − ve strains F5, F9, F13 and F46 have no such ability. In addition, F47 produced some 5.5% of the metabolite, suggested as being 5β-androstenone.

DISCUSSION

In the present work 16-androstenes were quantified in freshly-produced apocrine sweat. Other workers [12] have analysed apocrine sweat for steroids but were only able to measure androgens. All subjects, including one female, tested had detectable levels of 5α-androstenone in their apocrine sweat (Table 2). Androstadienone and androstadienol were also detected in the majority of cases. Most subjects had no 3-androstenols, and when present, these were only just above the limit of detection.

In order for the apocrine sweat to develop odour, bacterial intervention was essential (Table 1). This in turn suggests that if the 16-androstenes have a role in UAO formation, they must be precursors of the odorous compounds. The relatively low levels of 3α- and 3β-androstenol in apocrine sweat, in most cases below the limit for detection, means that these steroids are more likely to be the odorous compounds in axillary sweat than 5α-androstenone. This is supported by the results of the analysis of diethyl ether extracts of the axillary skin, where all 6 subjects had detectable levels of both 3α- and 3β-androstenol (Table 3).

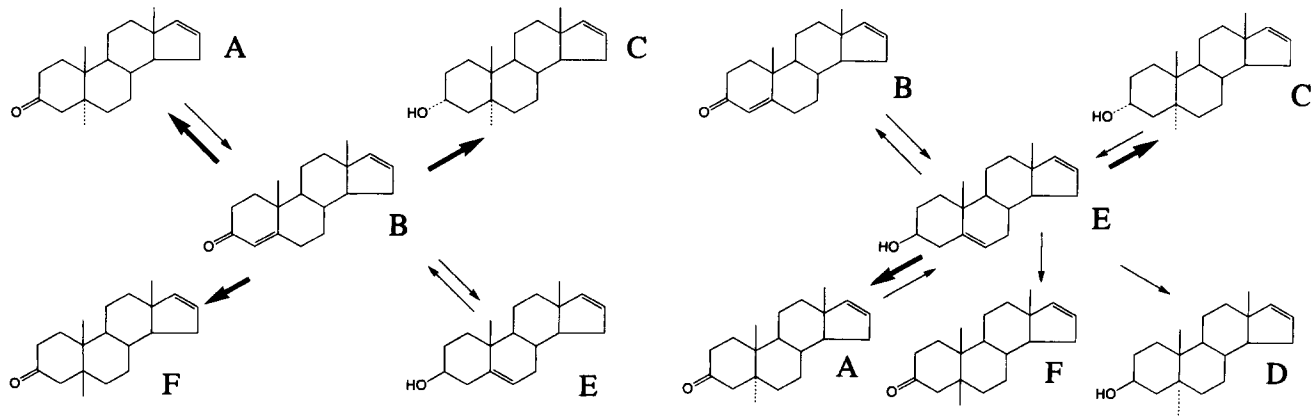


Fig. 3. Pattern of 16-androstene metabolites formed during incubation of UAO + ve coryneform F47 with 4,16-androstadien-3-one (left) and 5,16-androstadien-3β-ol (right). F, 5β-androstenone. For abbreviations, see legend to Fig. 2.

Table 5. Production of 5α- and 5β-androstenone from androstadienone by UAO +ve and UAO −ve axillary aerobic coryneforms during a 2 week incubation at 37°C

Coryneform	UAO	Metabolite ng (% conversion in parentheses) 5α-Androstenone	5β-Androstenone[a]
F1	+	222.0 (0.45)	0
F20	+	30.8 (0.54)	0
F25	+	15.7 (1.09)	0
F32	+	21.7 (0.67)	0
F47	+	9.1 (3.36)	15.2 (5.50)
F5	−	0	0
F9	−	0	0
F13	−	0	0
F46	−	0	0

[a]Presumptive identification.

Assuming that the adrenaline injection had caused complete emptying of the apocrine tubules (a reasonable assumption on the basis of Hurley and Shelley's work on individual apocrine gland responses and refractory periods, [28]), it was possible to relate the output of 16-androstenes from apocrine sweat, to the axillary skin levels of the same subjects. Even with the most generous estimate of apocrine sweat contribution, the axillary skin levels of 3α- and 3β-androstenol and androstadienol could not be accounted for. This means that the androstenols on the axillary skin surface are probably the result of bacterial conversion of 5α-androstenone and androstadienone secreted in apocrine sweat by the transformations shown in Figs 2 and 3.

From the analyses of apocrine sweat it is possible to conclude that the secretion may be the source of 16-androstenes on the axillary skin surface. It also appears that the levels of these steroids in the axilla were higher than the average human threshold of detection [9] (5α-androstenone, 0.2 ppb; androstadienone, 1 ppb; 3α-androstenol, 6.2 ppb) and therefore sufficient to account for axillary odour [10].

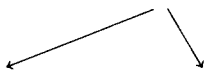
It was unfortunate that all of the subjects pre-selected for this stage of the study fell into the medium or low odour groups, and there was no opportunity to examine the apocrine sweat or axillary skin 16-androstenes of high odour subjects. Due to the low number of subjects and the similarity in their odour scores, it was not possible to establish whether there was a relationship between apocrine sweat or axillary skin levels of 16-androstenes and UAO intensity. It is probable that high odour subjects have higher 16-androstene levels in their axillae (see also below).

The experiments, using 16-androstenes as substrates for coryneform metabolism have demonstrated clearly that bacteria resident in the human axilla can interconvert the highly odorous 16-androstenes believed to play a role in UAO (Figs 2 and 3). Moreover, this work has confirmed a link between steroid metabolism by axillary aerobic coryneforms and UAO formation, seen in earlier experiments with testosterone. Only UAO+ve aerobic coryneforms produced detectable levels of 5α-androstenone when incubated with androstadienone (Table 5).

More detailed studies of the UAO+ve axillary aerobic coryneforms, F1 and F47, showed that such microorganisms are capable of a complex array of interconversion between 16-androstenes. These two coryneforms were chosen for the 16-androstene experiments because of their potent testosterone-metabolizing enzymes, and the intensity of UAO formed from axillary extracts noted in our earlier work [16, 17, 22]. The testosterone experiments showed that both of these isolates possessed 4-ene 5α(β)-reductases, and 3α(β)-hydroxysteroid oxidoreductases [22]. The pattern of transformation of 5α-androstenone, 3α-androstenol, androstadienone and androstadienol suggested that these enzymes may also be involved in 16-androstene metabolism, together with a 5-ene-3β-hydroxysteroid dehydrogenase/4,5-isomerase (involved in the androstadienol/androstadienone reaction). This appeared to be reversible in our studies with coryneforms F1 and F47. In this context, the metabolic activity of other species related to skin bacteria may be compared. For example, *C. mediolanum* can transform DHA to 4-androstenedione [29], while *C. simplex* has the ability to catalyse the reversible conversion of the 5-ene-3β-hydroxysteroid, 5-androstenediol to the 4-en-3-oxosteroid, 4-androstenedione [30]. In contrast, in mammalian species, there is only slight evidence for reversibility of such reactions [31].

All of the reactions detected here for coryneforms F1 and F47 have been detected in porcine testis [32], including the reversibility of the 5,16-dienol to the 4,16-dienone [33] reaction, viz:

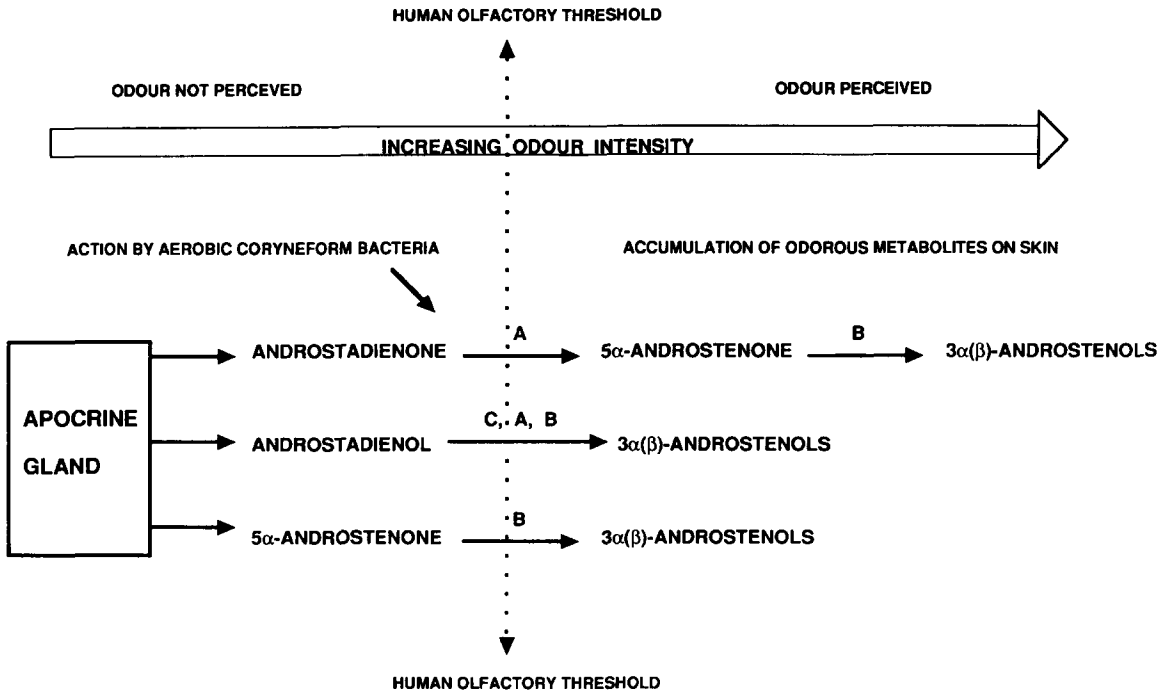
androstadienol ⇄ androstadienone → 5α-androstenone



3α-androstenol 3β-androstenol

A similar sequence has also been shown recently for human testis [34], although the possibility of reversibility of the initial reaction was not investigated in our studies.

Two other reactions, shown in Figs 2 and 3, deserve comment: first, the conversion of 3α-androstenol to its 3β-epimer, presumably by isomerization at C-3 (Fig. 2). A similar isomerization, but at C-17, was reported for incubations of some *Corynebacterium spp.* with the conversion of 17β-testosterone to its 17α-epimer (17-epitestosterone) [13, 14]. The second reaction of potential interest is the presumed 5β-reduction of the 4,16-dienone to 5β-androstenone (Figs 2 and 3). For reasons stated in the Results, further investigation of this metabolite (which occurs in fairly high yield in some incubations) must await the authentic standard, which is not available commercially. Nonetheless, 5β-reduction is well-known in microbiological transformations of steroids, e.g. androgens in the presence of *Corynebacterium spp.* [13, 14].



Scheme 1. Postulated mechanisms for the role of 16-androstenes in odour production in the axillae of men. Presumed enzymic reactions: A, 5α-reductase; B, 3α(β)-hydroxysteroid dehydrogenases; C, 5-ene-3β-hydroxysteroid dehydrogenase/steroid 4,5-isomerase.

If the results of the present work are taken together, i.e. the comparison of 16-androstene contents of fresh apocrine sweat with axillary skin and the metabolic transformations of these steroids, it is possible to propose a mechanism (Scheme 1) for the involvement of 16-androstene steroids in the formation of human axillary odour. Consistent with earlier findings [18], it is suggested that intensity of an individual' axillary odour is primarily a function of the population density of UAO + ve aerobic coryneform bacteria. The source of odour precursors is apocrine sweat which contains 5α-androstenone, androstadienone and androstadienol at levels which are below the human threshold of detection. The latter two are converted to the more odorous musk-smelling 3α- and 3β-androstenols by the action of 5-reductases of specific strains of aerobic coryneform bacteria, under conditions of oxygen limitation. In the case of androstadienone, this would also require conversion of the 3-ketone to an alcohol group by the action of 3α(β)-hydroxysteroid oxidoreductase. The two isomers of 5-androstenone may also be formed from androstadienone or androstadienol by the same mechanism or from 3α-androstenol. It is suggested that axillary odour becomes perceivable by humans once the androstenols and/or the androstenones have accumulated on the axillary skin surface to levels above the threshold of human olfactory detection. The rate at which androstenols or androstenones accumulate on the axillary surface is a function of the population density of aerobic coryneforms and the activity of their steroid-reducing enzymes. This was true in the case of a patient with Trichomycosis axillaris [10], who had a very pronounced personal odour (reminiscent of odorous 16-androstenes), greatly increased axillary microflora, relative to our normal values [17], and grossly raised 16-androstenes in his axillary hair, including 3α-androstenol and 5α-androstenone. In the case of low odour subjects, however, the threshold of human olfactory detection is rarely reached, not because the odour precursors are lacking, but because the population density of UAO + ve coryneform is too low.

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REFERENCES

1. Montagna W. and Parakkal P. F.: *The Structure and Function of Skin*. Academic Press, New York (1974).
2. Preti G., Cutler W., Christensen C., Lawley H., Huggins G. and Garcia C. R.: Human axillary extracts: analysis of compounds from samples which influence menstrual timing. *J. Chem. Ecol.* 13 (1987) 717–737.
3. Claus R. and Alsing W.: Occurrence of 5α-androst-16-en-3-one, a boar pheromone, in man and its relationship to testosterone. *J. Endocr.* 68 (1976) 483–484.
4. Bird S. and Gower D. B.: The validation and use of radioimmunoassay for 5α-androst-16-en-3-one in human axillary collections. *J. Steroid Biochem.* 14 (1981) 213–219.
5. Bird S. and Gower D. B.: Axillary 5α-androst-16-en-3-one, cholesterol and squalene in men: preliminary evidence for 5α-androst-16-ene-3-one being a product of bacterial action. *J. Steroid Biochem.* 17 (1982) 517–522.
6. Labows J. N.: Odour detection, generation and etiology in the axilla. In *Antiperspirants and Deodorants* (Edited by C. Felgen and K. Laden). Marcel Dekker, New York (1988) pp. 321–343.
7. Gower D. B., Nixon A. and Mallet A. I.: The significance of odorous steroids in axillary odour. In *Perfumery: The Psychology*

and Biology of Fragrance (Edited by S. Van Toller and G. H. Dodd). Chapman-Hall, London (1988) pp. 47–76.
8. Nixon A., Mallet A. I. and Gower D. B.: Simultaneous quantification of five odorous steroids (16-androstenes) in the axillary hair of men. *J. Steroid Biochem.* **29** (1988) 505–510.
9. Amoore J. E., Pelosi P. and Forrester L. J.: Specific anosmias to 5α-androst-16-en-3-one and ω-pentadecalactone: the urinous and musky primary odours. *Chem. Senses Flav.* **2** (1977) 401–425.
10. Mallet A. I., Nixon A., Ruparelia B. and Gower D. B.: Analysis of odorous 16-androstene steroids in human axillary hair and saliva. In *Advances in Steroid Analysis '87* (Edited by S. Gorog). Akademiai Kiado, Budapest (1988) pp. 287–291.
11. Shehadeh N. and Kligman A. M.: The effect of topical antibacterial agents on the bacterial flora of the axilla. *J. Invest. Dermat.* **40** (1963) 61–71.
12. Labows J. N., Preti G., Hoelzle E., Leyden J. and Kligman A. M.: Steroid analysis of apocrine secretion. *Steroids* **34** (1979) 249–258.
13. Nixon A., Mallet A. I., Jackman P. J. H. and Gower D. B.: Testosterone metabolism by isolated human axillary *Corynebacterium spp.*: A gas chromatographic-mass spectrometric study. *J. Steroid Biochem.* **24** (1986) 887–892.
14. Gower D. B., Nixon A., Jackman P. J. H. and Mallet A. I.: Transformation of steroids by axillary coryneform bacteria. *Int. J. Cosmet. Sci.* **8** (1986) 149–158.
15. Nixon A., Jackman P. J. H., Mallet A. I. and Gower D. B.: Testosterone metabolism by pure and mixed cultures of human corynebacteria. *FEMS Lett.* **41** (1987) 53–58.
16. Rennie P. J., Gower D. B., Holland K. T., Mallet A. I. and Watkins W. J.: The skin microflora and the formation of human axillary odour. *Int. J. Cosmet. Sci.* **12** (1990) 197–207.
17. Rennie P. J., Gower D. B. and Holland K. T.: In vitro and in vivo studies of human axillary odour and the cutaneous microflora. *Br. J. Dermat.* **124** (1991) 596–602.
18. Leyden J. J., McGinley K. J., Hoelzle E., Labows J. N. and Kligman A. M.: The microbiology of the human axilla and its relationship to axillary odor. *J. Invest. Dermat.* **77** (1981) 413–416.
19. Zeng X. N., Leyden J. J., Lawley H. J., Sawano K., Nohara I. and Preti G.: Analysis of characteristic odour from human male axillae. *J. Chem. Ecol.* **17** (1991) 1469–1492.
20. Wysocki C. J., Zeng X. N. and Preti G.: Specific anosmia and olfactory sensitivity to 3-methyl-2-hexenoic acid: a major component of human axillary odour. *Chem. Senses*. In press.
21. Baydar A. E., Petrizilka M. and Schott M. P.: Perception of characteristic axillary odours. *Perfurmer Flavorist* **17** (1992) 1–9.
22. Mallet A. I., Holland K. T., Rennie P. J., Watkins W. J. and Gower D. B.: Applications of gas chromatography-mass spectrometry in the study of androgen and odorous 16-androstene metabolism by human axillary bacteria. *J. Chromat.* **562** (1991) 647–658.
23. Williamson P. and Kligman A. M.: A new method for the quantitative investigation of cutaneous bacteria. *J. Invest. Dermat.* **45** (1965) 498–503.
24. Marshall J., Lemming J. P. and Holland K. T.: The cutaneous microbiology of normal human feet. *J. Appl. Bacteriol.* **62** (1987) 139–146.
25. Rennie P. J.: The skin microflora and the formation of human axillary odour. Ph.D. Thesis, University of Leeds (1989).
26. Kaufmann G., Ritter K. and Schubert K.: Quantitative determination of the boar taint substance 5α-androst-16-en-3-one in fat. *J. Steroid Biochem.* **7** (1976) 593–597.
27. Kwan T. K., Gower D. B., Trafford D. J. H. and Makin H. L. J.: Gas chromatographic-mass spectrometric characteristics of 16-androstenes, saturated analogues and derivatives. *Biol. Mass Spec.* **21** (1992) 160–164.
28. Hurley H. J. and Shelley W. B.: *The Human Apocrine Sweat Gland in Health and Disease*. Charles C. Thomas, Springfield (1960).
29. Mamoli L.: Uber biochemische Dehydrierungen in der Cortungruppe. *Ber. Deutsch. Chem. Ges.* **72** (1939) 1863–1865.
30. Charney W.: Transformation of steroids by Corynebacteriaceae. *J. Appl. Bact.* **29** (1966) 93–106.
31. Gower D. B.: The role of cytochrome P-450 in steroidogenesis and properties of some of the steroid-transforming enzymes. In *Biochemistry of Steroid Hormones* (Edited by H. L. J. Makin). Blackwells Scientific Publications, Oxford, 2nd Edn (1984) pp. 230–292.
32. Gower D. B.: Biosynthesis of the androgens and other C_{19} steroids. In *Biochemistry of Steroid Hormones* (Edited by H. L. J. Makin). Blackwells Scientific Publications, Oxford, 2nd Edn (1984) pp. 170–206.
33. Katkov T. and Gower D. B.: The biosynthesis of androst-16-enes in boar testis tissue. *Biochem. J.* **117** (1970) 533–538.
34. Kwan T. K., Makin H. L. J., Trafford D. J. H. and Gower D. B.: The use of GC-MS with selected ion monitoring in elucidation of human testicular androgen and 16-androstene biosynthesis. *J. Endocr.* **135** (suppl.) (1992) P16 (Abstr.).