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Applications of gas chromatography–mass spectrometry in the study of androgen and odorous 16-androstene metabolism by human axillary bacteria

A. I. MALLET

Institute of Dermatology, U.M.D.S. Guy's and St. Thomas's Hospitals, University of London, London SE1 9RT (U.K.)

K. T. HOLLAND and P. J. RENNIE

Department of Microbiology, University of Leeds, Leeds LS2 9JT (U.K.)

and

W. J. WATKINS and D. B. GOWER*

Division of Biochemistry, U.M.D.S. Guy's and St. Thomas's Hospitals, University of London, London SE1 9RT (U.K.)*

ABSTRACT

The known involvement of axillary microflora with under-arm odour (UAO) production led us to determine whether the odorous 16-androstene steroids are formed in the axilla by bacterial metabolism of an odourless precursor such as testosterone. Axillary bacteria from 34 men were selectively cultured for aerobic coryneform bacteria (ACB), *Micrococcaceae* and propionibacteria. Overnight suspensions of bacteria were incubated separately at 37°C for two weeks with radiolabelled testosterone plus unlabelled testosterone (0.5 mg) and 0.5-mg quantities of 4,16-androstadien-3-one (androstadienone) and 5,16-androstadien-3β-ol (androstadienol). After extraction and purification by Sep-Pak cartridges and thin-layer chromatography, the eluted steroids were derivatised as the pentafluorobenzyl oximes (PFBO) and *tert.*-butyl dimethylsilyl (TBDMS) ethers. Saturated analogues were used as internal standards. Selected-ion monitoring electron-impact mass spectrometry was performed at the *m/z* corresponding to the $M^{+\cdot}$ ion for the PFBO derivatives and the $[M - 57]^+$ ion for the TBDMS ethers. Only ACB produced classical musk-like UAO (UAO +ve) in an *in vitro* odour-producing system with 29% being UAO −ve. ACB (UAO +ve) metabolised far more ($p = 0.001$) testosterone than ACB (UAO −ve), the principal metabolites being 5α(β)-dihydrotestosterone, 5α(β)-androstane-3,17-dione and 4-androstene-3,17-dione (4-androstenedione). No non-polar 16-androstenes were formed. *Micrococcus luteus* (ten strains) metabolised testosterone to 4-androstenedione only; propionibacterium spp. did not metabolise testosterone at all. However, incubation of 16-androstenes with ACB gave evidence for 4-ene-5α(β)-reduction, 3α(β)-oxidoreduction and epimerisation. In general the direction of transformations favoured formation of the more odorous 5α-androst-16-en-3-one (5α-androstenone) and 5α-androst-16-en-3α-ol (3α-androstenol) from less odorous steroids. Such transformations, *in vivo*, would not require *de novo* synthesis of 5α-androstenone or 3α-androstenol and would be consistent with utilisation by ACB of 16-androstenes already present in small quantities in fresh apocrine secretions, which are odourless, to produce a more powerfully smelling mixture on the axillary skin surface.

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INTRODUCTION

Several previous studies have highlighted the important role played by gas chromatography–mass spectrometry (GC–MS) and radioimmunoassay (RIA) in quantification of the odorous components of axillary secretions and axillary hair. We have quantified, simultaneously, five 16-androstenes, some of which possess a powerful odour, in the axillary hair of 24 men. GC–MS was employed using electron-impact (EI) selected-ion monitoring (SIM) of the $M^{+\cdot}$ ion of pentafluorobenzyl oximes (PFBO) for the 3-oxo components and trimethylsilyl (TMS) ethers for the 3-ol species [1]. The following steroids were quantified: 5α-androst-16-en-3-one (5α-androstenone), 4,16-androstadien-3-one (androstadienone), 5α-androst-16-en-3α(β)-ol (3α(β)-androstenol) and 5,16-androstadien-3β-ol (androstadienol). In earlier studies one or more of these steroids had been measured, *e.g.* 5α-androstenone by RIA [2–4] and by GC–MS [5] and 3α-androstenol by GC–MS [6,7]. Likewise, Preti *et al.* [7] have assayed fatty acids from C_2 to C_{18} in human axillary sweat using GC–MS. There appears to be a relatively large number of odorous substances present, bearing in mind the suggestion that *trans*-3-methyl-2-hexenoic acid (a substance originally thought to be present only in schizophrenics [8]) may occur in axillary secretions of normal man [9]. In investigations of the human axillary microflora we [10,11] and others [12] have identified the aerobic coryneform bacteria (ACB) as being predominantly responsible for under-arm odour (UAO). The *Corynebacterium* spp. are able to metabolise testosterone extensively [13] and to bring about a complex series of interconversions of the 16-androstenes [14]. It has long been known that freshly produced apocrine secretions induced by adrenaline injection into the axillary vault are odourless until incubated with certain bacteria, notably ACB.

Testosterone has been suggested as a putative substrate, which might subsequently give rise to odorous steroids through microbial action [15]. Testosterone has little or no odour, occurs in apocrine secretions [2] and can give rise to the urinous odorant, adrostadienone, at least by purely chemical modifications [16].

Although we have studied testosterone metabolism by pure strains of *Corynebacterium* spp. [13], we have extended these studies by culturing many other species of axillary microflora and have investigated their ability to metabolise testosterone and 16-androstenes and have attemped correlations of these data with human UAO. Extensive use has been made of GC–MS–SIM to separate and identify metabolites.

EXPERIMENTAL

Materials and reagents

Authentic steroids were either purchased from Sigma (Poole, U.K.) or were provided by the MRC Steroid Reference Collection (Professor D. N. Kirk), Queen Mary College (London, U.K.). [4-^{14}C]Testosterone (specific activity 57

μCi/mol) was obtained from Amersham International (Aylesbury, U.K. and Sep-Pak cartridges from Millipore (UK) (Harrow, U.K.). Pre-coated thin-layer chromatographic (TLC) plates (20 cm × 20 cm, 0.25 mm layer thickness) were obtained from Merck (Anderman, U.K.) and solvents for TLC and extraction were reagent grade, redistilled prior to use, from BDH (Poole, U.K.). For the derivatisation of steroids *tert.*-butyl dimethylchlorosilane and *tert.*-butyl dimethylsilylimidazole were obtained from Aldrich (Gillingham, U.K.) and the O-pentafluorobenzyl hydroxylamine hydrochloride came from Pierce and Warriner U.K. (Chester, U.K.). Triton-X and Tween 80 were obtained from BDH and other media from Oxoid (Hants, U.K.).

Bacterial sampling

Thirty-four healthy men, who were not on medication with antibiotics or other drugs, were used as subjects. They refrained from using deodorants or anti-perspirants for at least five days prior to sampling; washing was performed at normal frequency with non-perfumed soap (Simple Soap, Hampton, U.K.). The last washing was performed 12 h before bacterial sampling. Bacteria were obtained by scrubbing the axillary vault with 3 ml of a solution of 0.1% (w/v) Triton X-100 in phosphate buffer (pH 7.8, 0.1 *M*) [17]. This was contained in a PTFE cylinder. Bacteria were plated out on to media to select for ACB, *Micrococcaceae, Staphylococcus*, spp. and propionibacteria as described by Rennie [18]. Representative strains were grown for 48 h in appropriate liquid media and then centrifuged and re-suspended in 0.1 *M* phosphate buffer (pH 6.0) at a concentration giving an absorbance of 1.0 at a wavelength of 500 nm [18]. The opposite axilla was sampled similarly.

Axillary skin extracts

These were obtained by scrubbing two other areas of the axillary vault, not used for bacterial collection, with diethyl ether (5 ml each) contained in a PTFE cylinder. The opposite axilla was sampled similarly, giving 20 ml ethereal extract which was filtered through a 0.22-μm porosity cellulose acetate filter (Oxoid) in Swinnex filter holders (Millipore U.K.), after which 1-ml portions were aliquoted into 15-ml glass bottles and the solvent was evaporated. A 1-ml volume of bacterial suspension was added to each bottle of axillary extract and incubated for 6 h at 37°C, after which odour was determined organoleptically by two expert odour assessors.

Metabolic studies using testosterone as substrate

Representative strains of UAO producing and UAO non-producing axillary bacteria (0.1 ml overnight culture) were incubated for two weeks in 4 ml of tryptone soya broth containing Tween 80 (0.1%, w/v) and yeast extract (0.1%, w/v) to which had been added [4-^{14}C]testosterone (1 μCi) and unlabelled testosterone (0.5 mg) in 0.1 ml of ethanol. Extraction was performed with ethyl acetate

(3 × 3 ml) and the pooled extracts were purified by passing through a Florisil Sep-Pak cartridge. After evaporation to dryness at 20–25°C (Buchler vortex evaporator, Buchler Instruments, Saddle Brook, NJ, U.S.A.), the residues were dissolved in dichloromethane (0.1 ml) and one half was chromatographed by TLC in benzene–acetone (4:1, v/v) with standards corresponding to the metabolites previously identified by Nixon *et al.* [13]. This was examined by autoradiography (Eastman Kodak, DEF-2 film, Liverpool, U.K.). Control incubations were always performed with no bacteria present. Approximately 10% testosterone was converted by chemical breakdown but the metabolites were polar in nature and did not interfere with those produced in this study.

Preliminary experiments showed that the principal metabolites formed by bacterial action had R_F values which corresponded to those of 5α-and 5β-dihydrotestosterone, 4-androstenedione and 5α-and 5β-androstanedione. The metabolites were quantified by scraping off the radioactive zones from the TLC plate, eluting the silica with ethyl acetate and scintillation counting the activity in each. Purity of fractions was confirmed by crystallisation to constant specific activity as described below.

Steroid metabolites were added separately to the appropriate unlabelled steroid (10 mg) in chloroform (1 ml). This was added to a flask containing hexane (10 ml) and the solutions were evaporated to small volume at 80°C on a water bath. A further 10 ml of hexane was added dropwise and crystallisation was allowed to take place on cooling. The process was repeated several times and samples (1 mg) of the product were removed each time for determination of radioactivity using a Tricarb Model 3255 scintillation spectrometer (Packard Instruments, Downers Grove, IL, U.S.A.). A sample was assumed to be pure when the specific radioactivity of three successive samples of the product did not vary by more than 10%. Further identification and quantification of metabolites was achieved using SIM as described below.

Metabolic studies using 16-androstenes as substrates

Two ACB, which produced UAO in the *in vitro* system described above (UAO +ve), were selected for study. Androstadienone and androstadienol (0.5 mg each) were dissolved in 0.1 ml of ethanol and added separately to tryptone soya broth (4 ml) containing Tween 80 (0.1%, w/v) and yeast extract (0.1%, w/v). Overnight suspensions of the two coryneforms (0.1 ml) were added and incubated without shaking for two weeks at 37°C. Extraction, purification and TLC were performed as described above for testosterone metabolism. In order to locate the unlabelled 16-androstenes on the TLC plates, a sample of radiolabelled metabolites from a previous incubation of [4-^{14}C]testosterone with UAO+ve coryneform bacteria was run on the TLC plate alongside the samples. Radioautography showed that the mobilities of the two testosterone metabolites with R_F values above that of 5α-androstanedione corresponded with those for 5α-androstenone and androstadienone, respectively. Further, the R_F values of 5α-androstanedione

and of androstenedione corresponded to those of 3α-androstenol and androstadienol, respectively. This method for detecting the location of non-radioactive 16-androstenes obviated the need for destructive visualisation methods. Appropriate zones of the plates were scraped off, the metabolites were eluted with ethyl acetate (5 ml), and 10 ng each of internal standards 5α-androstan-3-one (5α-androstanone) and 5α-androstan-3β-ol (3β-androstanol) in ethanol were added. After evaporation to small bulk at 20–25°C to prevent loss of volatile 16-androstenes, the steroids were derivatised and analysed by GC–MS.

Derivatisation and GC–MS of steroids

Ketonic functional groups were converted to the PFBO [1] and alcoholic functional groups to the *tert.*-butyl dimethylsilyl (TBDMS) ethers [19]. GC was carried out with a Varian 3400 gas chromatograph (Varian Assoc., Surrey, U.K.) using an on-column injection system linked to a VG Analytical (Manchester, U.K.) Model 305 mass spectrometer. Data were collected on a Technivent system (Technivent, St. Louis, MO, U.S.A.). Chromatography was carried out on fused-silica columns (25 m × 0.32 mm) coated with SE-30. Samples were injected in decane solution at 175°C and the column was heated from 175 to 310°C at 30°C/min. In order to compensate for the differences in response of the different steroids, calibration curves using 5α-androstanone and 3β-androstanol were obtained for a range of concentrations of each steroid. The PFBO derivatives were monitored at the $M^{+\cdot}$ ion and the TBDMS ions at the ion corresponding to $[M - 57]^+$ (Table I).

RESULTS

GC–MS of testosterone metabolites

The principal metabolites formed from testosterone were 5α(β)-dihydrotestos-

TABLE I

STEROIDS MONITORED BY SELECTED-ION MONITORING

Steroid	Derivative	*m/z*
5,16-Androstadien-3β-ol	TBDMS	329 $[M - 57]^+$
5α-Androst-16-en-3α(β)-ol	TBDMS	331 $[M - 57]^+$
5α-Androstan-3β-ol (i.s.)	TBDMS	333 $[M - 57]^+$
4,16-Androstadien-3-one	PFBO	465 $M^{+\cdot}$
5α-Androst-16-en-3-one	PFBO	467 $M^{+\cdot}$
5α-Androstan-3-one (internal standard)	PFBO	469 $M^{+\cdot}$
Testosterone	TBDMS–PFBO	540 $[M - 57]^+$
5α(β)-DHT	TBDMS–PFBO	542 $[M - 57]^+$
4-Androstenedione	PFBO	481 M^+
5α(β)-Androstanedione	PFBO	483 M^+

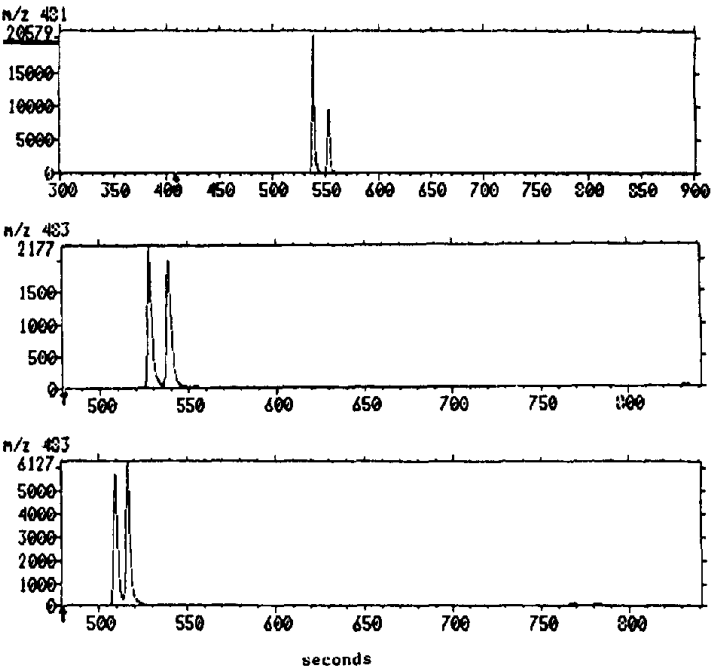


Fig. 1. SIM traces of the PFBO of 4-androstenedione (upper) and 5α- and 5β-androstanedione (middle and lower, respectively).

terone, 4-androstenedione and 5α(β)-androstanedione. Typical SIM traces of the derivatised metabolites are shown in Fig. 1 and from these the clear resolution of the *syn*- and *anti*-forms of the oximes can be seen.

Metabolism of testosterone

Table II summarises the metabolites which were formed by a selected number of aerobic coryneform isolates, previously shown to be UAO +ve or UAO −ve in the *in vitro* odour production test. The recrystallisation studies showed that the metabolites were at least 90% pure. UAO +ve coryneforms metabolised a significantly higher proportion of the testosterone substrate than did UAO −ve coryneforms (*t*-test, $p=0.001$). The percentage of testosterone metabolised ranged from 13.7 to 99.8% for UAO +ve coryneforms and from 0.88% to 4.45% for UAO −ve coryneforms. With the most metabolically active UAO +ve coryneforms the original testosterone substrate was almost depleted after two weeks of incubation. There was significantly greater production of androstenedione ($p=0.02$), 5α-dihydrotestosterone ($p=0.06$) and 5α-androstanedione ($p=0.02$) by UAO +ve aerobic coryneforms compared with UAO −ve ones. There was no significant difference between UAO +ve and UAO −ve coryneforms in the production of 5β-reduced metabolites. A number of minor metabolites, some less

TABLE II

METABOLISM OF TESTOSTERONE BY HUMAN AXILLARY CORYNEFORM BACTERIA

[4-^{14}C]Testosterone (1μCi) plus unlabelled testosterone (0.5 mg) were incubated for two weeks at 37°C with odour producing and non-odour producing axillary aerobic coryneform bacteria. The TLC mobility is related to that of testosterone $R_{test.}$ = 1.0.

$R_{test.}$	Identity by GC–MS	Principal metabolites (%)												
		UAO +ve coryneforms							UAO −ve coryneforms					
		F1	F17	F20	F21	F25	F32	F47	F5	F7	F9	F13	F46	F50
0.0	Origin	0.02	0	0	0	0	0	0	0	0	0	0	0.02	0
0.64	Unknown	0	0	0	0.57	0	0	0.15	0	0	0	0	0	0
0.71	Unknown	0	0	0.26	0	0.20	0	0	0	0	0	0.28	0.18	0
0.84	Unknown	0	0	0	0.07	0	0	0	0	0	0	0	0	0
1.00	Testosterone	0.76	69.9	86.3	1.48	1.19	1.13	0.19	99.1	95.6	96.0	99.0	97.0	98.3
1.21	5β-Dihydrotestosterone	0.47	0	0	0	0	0	11.4	0	1.23	3.99	0.70	1.79	0.54
1.25	5α-Dihydrotestosterone	14.7	1.29	3.22	0.83	48.2	86.8	68.3	0	0.23	0	0	0.90	0.80
1.45	Androstenedione	76.7	24.2	10.1	93.8	47.6	2.80	30.1	0.43	2.34	0	0	0	0
1.72	5β-Androstanedione	1.84	0	0	0	0	0	0.41	0	0.09	0.03	0	0	0.23
1.78	5α-Androstanedione	5.51	3.97	0.06	2.69	2.18	8.93	0.78	0	0.09	0	0	0	0
2.10	Unknown	0	0.24	0	0.30	0.30	0.32	0.07	0.42	0.44	0	0	0	0.42
2.22	Unknown	0	0.39	0	0.22	0.30	0	0	0	0.03	0	0	0	0
Testosterone metabolised (%)		99.2	30.1	13.7	98.5	98.8	98.9	99.8	0.88	4.45	4.02	0.99	3.05	1.69

TABLE III

METABOLITES OF [4-^{14}C]TESTOSTERONE PRODUCED BY HUMAN AXILLARY *M. LUTEUS* ISOLATES

[14-^{14}C]Testosterone plus unlabelled testosterone were incubated with the bacterial isolates for two weeks at 37°C, TLC mobility is related to that for testosterone as in Table II.

$R_{test.}$	Identity by GC–MS	Principal metabolites (%)									
		A4	A17	A26	A41	A47	A60	A61	A62	A76	A82
1.00	Testosterone	46.31	39.92	47.35	41.46	49.68	39.14	42.89	33.12	37.54	38.22
1.25	5α(β)-DHT	0	0	0	0	0	0	0	0	0	0
1.45	Androstenedione	56.69	60.08	52.65	58.54	50.32	60.86	57.11	66.88	62.46	61.78
1.78	5α(β)-Androstanedione	0	0	0	0	0	0	0	0	0	0
Testosterone metabolised (%)		56.69	60.08	52.65	58.54	50.32	60.86	57.11	66.88	62.46	61.78

polar and some more polar than testosterone, were also formed by the majority of UAO +ve and UAO −ve coryneforms. The amount of these metabolites was generally too small for them to be reliably identified. Overall recovery of material through incubation, extraction, purification, etc. ranged from 44 to 62%.

Ten isolates of *Micrococcus luteus* were also incubated with testosterone but, with remarkable consistency between strains, produced only 4-androstenedione (Table III). The percentage conversion of testosterone ranged from 50 to 67%; no other metabolites were detected. Other bacteria tested for their ability to metabolise testosterone were 21 staphylococci isolates and 5 propionibacteria isolates. None of these produced any metabolites above those produced by spontaneous breakdown.

Metabolism of 16-androstene by human axillary aerobic coryneform bacteria

Two aerobic coryneforms F1 and F47 (Table II), previously identified as UAO

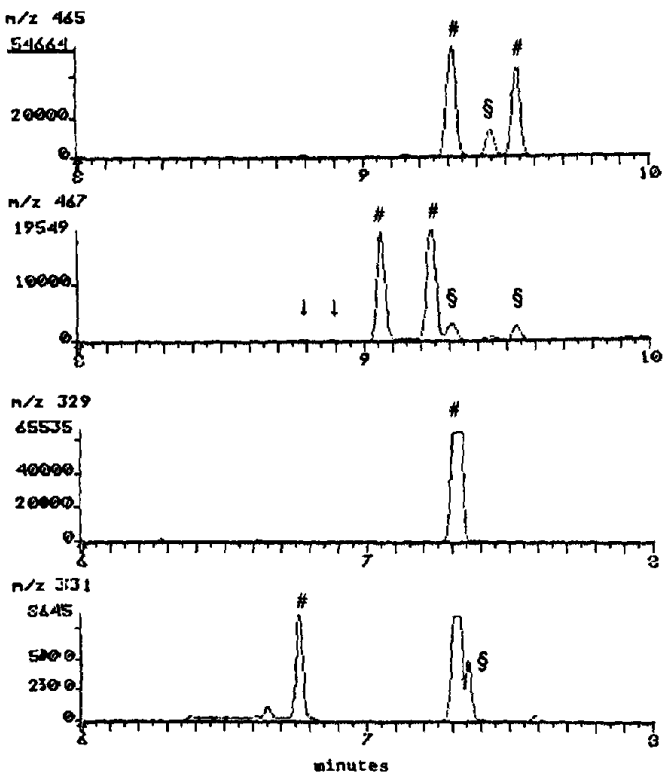


Fig. 2. SIM of PFBO and TBDMS ethers of metabolites of androstadienol. UAO +ve ACB F47. *m/z* 465: § = unidentified compound; # = *syn/anti*-androstadienone; *m/z* 467: ↓ = possible 5β-androstenone; # = *syn/anti*-5α-androstenone; § = cross-talk; *m/z* 329: # = androstadienol; *m/z* 331: # = 3α-androstenol; § = 3β-androstenol.

+ve, were selected for metabolic studies with 16-androstene substrates as described above. Both isolates produced large yields (>10%) of 5α-androstenone and 3α-androstenol from androstadienone and androstadienol. Fig. 2 shows the SIM traces of these incubation products. As noted earlier [1] it was possible to resolve the *syn-* and *anti*-forms of the PFBO derivatives of 5α-androstenone and androstadienone. The latter steroid gave rise also to appreciable (3–4%) yields of a compound tentatively identified [20] as 5β-androstenone. This had a similar R_F to the 5α-isomer on TLC, formed a PFBO which was resolved into two peaks on GC and was detected by SIM at the same *m/z* as that for 5α-androstenone, but it eluted appreciably earlier from the GC column. Further identification waits upon the availability of the authentic steroid.

DISCUSSION

The present studies on steroid metabolism in human axillary bacteria have relied heavily on the use of GC–MS with SIM for identification of the metabolites formed and for the quantitation of sub-nanogram quantities of derivatised steroids. The use of PFBO derivatives of steroidal ketones has been reported before, especially in negative-ion MS [21,22], but we have had good response in the EI mode [1]. Earlier studies used TMS ethers [1], but we have obtained better signal-to-noise ratios with the TBDMS ethers employed in these studies. The pair of sterols, androstadienol and 3β-androstenol, differing by one double bond at C-5–C-6, have been particulary difficult to separate and the TBDMS ethers were no different in this respect, with only a few seconds difference in elution time of these isomers. Highly specific stationary phases have been used earlier [23] but, with SIM, the mass specificity helps to distinguish the isomers (Fig. 2).

It is of particular interest that the metabolic activity of ACB with regard to testosterone as substrate correlates well with their ability to produce odour in the *in vitro* test. We [10,11] and others [12] have shown that it is the aerobic coryneforms which are largely responsible for UAO in men; *Micrococcacea* and propionibacteria produced "off-odours" significantly distinct from typical UAO [10].

Fig. 3 summarises the pathways of metabolism of testosterone in UAO +ve coryneforms which have been identified in the present work. The formation of the metabolites is consistent with the presence in these bacteria of 4-ene-5α(β)-reductases and 17β-hydroxysteroid oxido-reductase. The presence of 3α(β)-hydroxysteroid oxido-reductases would also be expected by analogy with pure strains of *Corynebacterium* spp. [13] but the products (androstanediols) were not investigated in the present work. The indications are, that there is a relationship between possession of potent steroid 5α-reducing enzymes by axillary bacteria and the formation of true "musky" UAO. In this respect it is noteworthy that strains of *M. luteus* (which are not associated with UAO production) could only produce 4-androstenedione from testosterone (consistent with 17β-hydroxysteroid oxido-reductase activity), rather than 5α(β)-dihydrotestosterone.

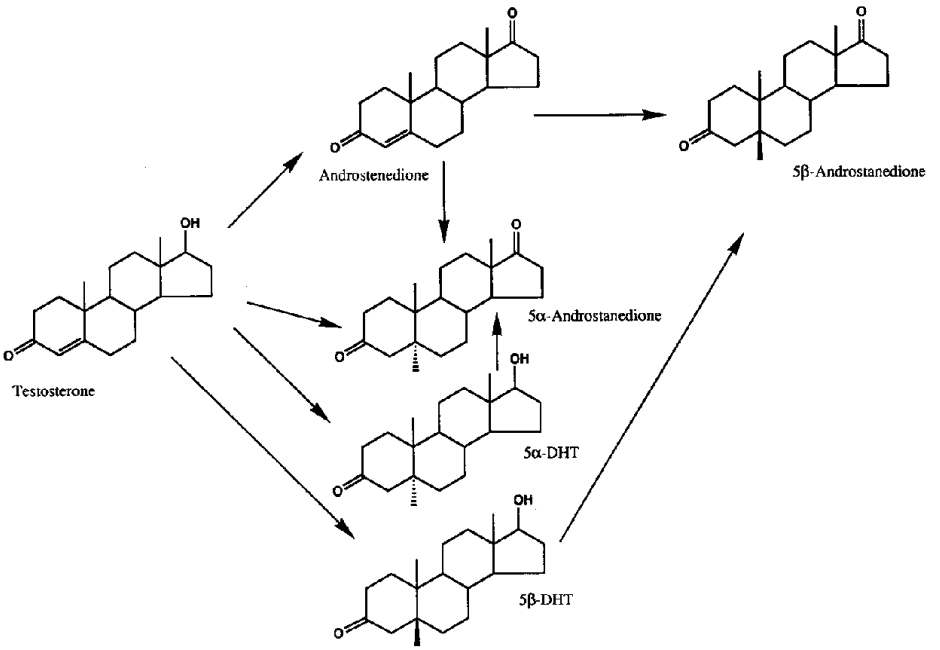
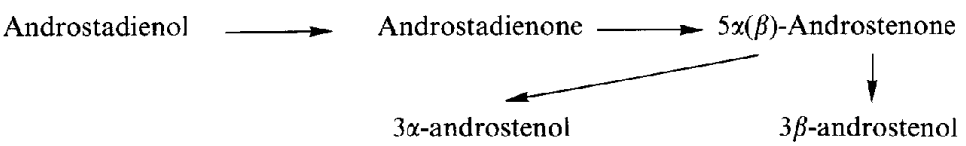


Fig. 3. Pathways of transformation of testosterone by aerobic coryneform F1.

Despite numerous attempts in this and earlier work [15], we have been unable to show the bacterial transformation of testosterone to the odorous 16-androstenes. Nevertheless these and previous studies [14] show that human ACB possess the enzymes necessary for converting androstadienol and androstedienone into appreciable yields of 5α-androstenone and 3α-androstenol. By analogy with the biosynthetic pathway of 16-androstenes in porcine testis [24], it is likely that transformations in axillary coryneform bacteria may be as below:



Recent results, currently being prepared for publication elsewhere, suggest that the metabolism of 16-androstenes in human coryneform bacteria is more complex involving numerous interconversions. Overall, the formation of the more odorous 5α-androstenone and 3α-androstenol from less odorous androstadienone and androstadienol is favoured, rather than the reverse. This would be entirely consistent with the ability of the ACB in converting low-odour 16-androstene precursors to a blend of more odorous steroids and would not require their *de novo* synthesis. Recent work from this laboratory suggests that small

quantities of 16-androstenes with little or no odour occur in sterile adrenaline-induced apocrine sweat [25]. We propose that these can be modified microbially on the axillary skin surface to a mixture of the more powerfully smelling members of the series, thus contributing, together with other substances such as fatty acids, to UAO.

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