Journal of Chemical Ecology, Vol. 18, No. 7, 1992

AN INVESTIGATION OF HUMAN APOCRINE GLAND SECRETION FOR AXILLARY ODOR PRECURSORS

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(Received August 3, 1991; accepted February 20, 1992)

Abstract—Recently completed studies from our laboratories have demonstrated that the characteristic human male axillary odors consist of C_6 to C_{11} normal, branched, and unsaturated aliphatic acids, with (*E*)-3-methyl-2-hexenoic acid being the most abundant. To investigate the mechanism by which the odor is formed, it is necessary to determine the nature of the odorless precursor(s) found in the apocrine secretion which is converted by the cutaneous microorganisms to the characteristic axillary odor. Pooled apocrine secretion was obtained from several male volunteers by intracutaneous injection of epinephrine. Partitioning this secretion into aqueous and organic soluble fractions was followed by hydrolysis of each fraction with NaOH or incubation with axillary microorganisms (cutaneous lipophilic corynebacterium). Analysis by gas chromatography/mass spectrometry (GC/MS) revealed the presence of (*E*)- and (*Z*)-3-methyl-2-hexenoic acid in the aqueous phase hydrolysate and aqueous phase incubated with bacteria; however, only a trace amount was seen in the resultant organic phase mixtures. These results

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0098-0331/92/0700-1039$06.50/0

suggest that a water-soluble precursor(s) is converted by the axillary flora to the characteristic axillary odors.

Key Words—Human axillary odors, human apocrine gland secretion, (*E*)-3-methyl-2-hexenoic acid, axillary odor precursors, androstenol.

INTRODUCTION

Recent reports from our laboratory have shown that the compounds imparting the characteristic odor to axillary secretions consist of C_6–C_{11}, normal, branched, and unsaturated acids, with the most abundant being (*E*)-3-methyl-2-hexenoic acid (3M2H) (Zeng et al., 1991; Leyden et al., 1990). Although organoleptic descriptions (Labows, 1988) and instrumental analysis (Preti et al., 1987; Labows et al., 1981) had previously suggested the presence of C_2–C_{18} acids in axillary secretions and extracts of this fluid, the exact structure of the acids and their relationship to the characteristic axillary odor was only recently determined (Zeng et al., 1991). Prior to this, the compounds suggested as responsible for axillary odor were thought to be volatile androst-16-enes, such as androstenone (5α-androst-16-en-3-one), androstadienone (androst-4,16-en-3-one), and androstenol (5α-androst-16-en-3α-ol) as well as isovaleric acid (Froebe et al., 1990; Labows, 1988, Gower, 1988).

In an attempt to better understand the mechanism by which the characteristic axillary odor is formed, the precursors to the axillary odors have been sought in the apocrine secretion. Our study is the first to look for the precursor(s) to 3M2H since it was not previously reported in axillary sweat or thought to be related to the characteristic axillary odor.

The research of Hurley and Shelly (1960) as well as Shehedah and Kligman (1963) demonstrated that freshly produced secretion is odorless and that the action of the cutaneous microorganisms upon the secretion creates the odor. Most of the published studies in this area have examined the apocrine secretion for steroidal-type molecules, namely, C_{19}-androgen sulfates and/or glucoronides (Labows 1988; Gower, 1988; Froebe et al., 1990). However, several lines of evidence suggested that the precursors to the odor might be lipid in nature. First, the axillary diptheroids, which are generally regarded to produce the more characteristic malodors (Leyden et al., 1981; Labows, 1988), have a lipid requirement for growth (McGinley et al., 1985); second, the structures of the compounds constituting the characteristic odor suggest that they are not formed by catabolism of amino acid substrates but may be secreted bound to glycerol and hydrolyzed to the free acids; third, the odor is formed fairly rapidly when axillary diptheroids are mixed with apocrine secretion (personal observations), suggesting that a rapid process (hydrolysis?) forms the odor; and fourth, other researchers have also alluded to possible nonvolatile, lipid-type molecules such as

triglycerides being present in the apocrine secretion, which could be degraded to give aliphatic acids such as 4-ethyloctanoic acid (Labows et al., 1981; Labows, 1988).

In this paper we report fractionation of apocrine secretion in aqueous and organic phases with subsequent saponification via NaOH and incubation with axillary diphtheroid bacteria. These experiments show that the precursor(s) to the characteristic axillary odors does not appear to be a lipid but is found in the water-soluble portion of the apocrine gland secretion.

METHODS AND MATERIALS

Apocrine Secretion Donors

Several collections of apocrine secretion were made to supply the material necessary for the experiments performed. Healthy male volunteers between 20 and 40 years of age served as paid voluntary donors. Informed consent was obtained prior to each collection. In the first collection, eight donors supplied a total of 8 μl of materia; seven donors supplied 6 μl in the second collection, and seven donors supplied 120 μl for the third collection, and six donors supplied 30 μl for the fourth collection.

The axillae was prepared for sampling by shaving the underarm area on the morning of the collection day (Labows et al., 1979). The donors rested in a supine position, the axillary region was washed with a nonionic detergent (0.1% Triton X-100), rinsed with water, dried, and finally, washed with hexane.

By this procedure, desquamated horny cells, most of the cutaneous flora, and skin surface lipids were removed, yielding apocrine secretion with minimal contamination by bacteria, cell debris, and sebum. To stimulate the apocrine glands, 0.1 ml of 1:2000 adrenalin (in physiologic saline) was intradermally injected. Droplets of apocrine secretion, which emerge from the ductal orifices, were collected with 10 μl glass micropipettes.

The secretions collected in the pipettes were pooled in 0.2-ml conical-shaped vials precooled to 0°C (React-a-Vial, Pierce, Rockford, IL). The four collections yielded the amounts detailed above. The third collection was unusual due to the large amount obtained. We think that it was due to several new donors being used. Since they were not familiar with the procedure, it was stressful enough to cause a larger volume of secretion to be expressed. The secretions were stored at −60°C until analysis.

Separation of Apocrine Secretions

The apocrine secretion microdroplets were diluted first with 300 μl distilled water, followed by 400 μl chloroform. The chloroform was mixed well with the apocrine secretions, and the mixture centrifuged for 5 min to form two layers.

The bottom chloroform layer was removed via pipette. The remaining aqueous layer was extracted two more times with chloroform (2 × 400 μl) and subsequently separated by the same procedure as above. This procedure gave a total volume of 1.2 ml of chloroform solution and 300 μl of aqueous apocrine solution.

In the experiment where the large amount (120 μl) of apocrine secretion was employed, aliquots of the aqueous (10 μl) and chloroform layers (10 μl) were set aside for analysis using sodium dodecyl sulfate (SDS)/polyacrylamide gels, as described later.

Aqueous Phase Hydrolysis of Apocrine Secretion

The aqueous apocrine solution was refluxed for 20 min with 1 ml of 5% sodium hydroxide aqueous solution under a stream of nitrogen. The mixture was allowed to cool to room temperature and acidified with 2*M* HCl to pH 2. A slight axillary-like odor developed in the reaction mixture upon acidification. The acidified mixture was extracted with 3 × 150 μl chloroform.

In the first two experiments, the combined chloroform extracts were concentrated to approximately 5 μl for organoleptic evaluation and GC/MS analysis. However, in the experiments employing the larger volumes of apocrine secretion, we concentrated to approximately 12 μl.

Organic Phase Hydrolysis of Apocrine Secretion

In each experiment, the chloroform extract obtained from apocrine secretion was concentrated to approximately 100 μl and refluxed for 20 min with 5% NaOH in 2 ml of a 1 : 1 solution of methanol : water under a stream of nitrogen: the same conditions used for the aqueous phase hydrolysis. After removal of the solvent, the reaction mixture was acidified with 2 *M* HCl to a pH of 2; no odor was detected. This acidic solution was extracted with chloroform (3 × 0.5 ml) and concentrated to approximately 5 μl.

Incubation of Apocrine Secretion Aqueous and Organic Phases with Diphtheroid Bacteria

A combined male apocrine secretion sample (~30 μl) from six donors was separated into aqueous and organic soluble phases as described above. Each phase was transferred to 50-ml, round-bottomed flasks, and the $CHCl_3$ removed from each by rotary evaporation. To each of these flasks was added 200 μl of McFarland Barium Sulfate Standard No. 2 (Balows, 1991) containing ~600,000,000 colony forming units/ml of cutaneous lipophilic corynebacterium (CLC). These are the most abundant species of diphtheroid bacteria found in the human axillae. An equal amount of McFarland Standard No. 2 with CLC

was added to an empty, sterile, 50ml round-bottom flask to use as a blank. All three flasks were sealed with parafilm and incubated at 37°C for 24 hr.

Following this time the contents of each flask were transferred to three centrifuge tubes. Each flask was rinsed with 300 μl doubly distilled water and each rinse was combined with its respective solution. Sodium chloride (20 mg) was added to each centrifuge tube, and the three solutions were extracted separately with chloroform (3 × 500 μl). The extraction procedure was identical to that described above following the hydrolysis with NaOH. Each chloroform extract was concentrated to 10 μl by the vacuum concentration and analyzed by GC/MS.

Sodium Salt of 3-Methyl-2-Hexenoic Acid: Extraction and Hydrolysis

To form the sodium salt, 500 ng of a 10:1 synthetic mixture of *E*- and *Z*-3-methyl-2-hexenoic acid was added to a solution (0.5 ml) of 5% NaOH in a (1:1) water:ethanol mixture. This solution was adjusted to pH 2 by dropwise addition of 6 *N* HCl. The acidified solution was extracted with chloroform (2 × 1 ml). The chloroform was divided into two equal portions. One of these portions as well as the remaining aqueous layer was treated with the 5% NaOH solution and refluxed under N_2 for 20 min. The solutions were cooled to room temperature, acidified to pH 2 with 6 *N* HCl, and extracted with chloroform (2 × 0.5 ml). These two extracts and the unreacted half of the chloroform extract (see above) were each concentrated to approximately 5 μl by vacuum concentration for GC/MS analysis.

Examination of Apocrine Secretion for the Sodium Salt of 3-Methyl-2-Hexenoic Acid

To test if the 3-methyl-2-hexenoic acids are present in apocrine secretion as their sodium salts and therefore remain in the aqueous phase during the partitioning with $CHCl_3$, a sample of combined apocrine secretion from 12 donors (~40 μl) was adjusted to pH 2 by the addition of approximately 50 μl 6*N* HCl. The acidified sample was diluted to 300 μl with doubly distilled water and extracted with 3 × 400 μl of $CHCl_3$. One microgram of D_{19}-decanoic acid was added to each phase as an internal standard to gauge efficiency of recovery. One-half the $CHCl_3$ extract and the remaining aqueous phase were subjected to hydrolysis and analyzed as described above. The portion of extract not subjected to hydrolysis was concentrated to ~10 μl by vacuum concentration and analyzed by GC/MS.

Synthesis of Both 3-Methyl-2-Pentenoic and 3-Methyl-2-Octenoic Acids

The synthesis of these compounds was similar to that employed for (*E*)- and (*Z*)-3-methyl-2-hexenoic acid (Zeng et al., 1991).

To a mixture of NaH (1.33 g, 55.3 mmol) and toluene (80 ml), triethyl

phosphonoacetate (12.4 g, 55.3 mmol) was added dropwise at room temperature for 30 min. The temperature was raised to 40°C. After 1 hr of stirring, 2-butanone (3.98 g, 55.3 mmol) or 2-heptanone (6.31 g, 55.5 mmol) was added dropwise, and stirring was continued at 40°C for 2 hr. The reaction mixture was poured into ice water (70 ml) and extracted with diethyl ether (3 × 40 ml). The extract was dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was added to a solution of KOH (5.0 g) in methanol (30 ml) and water (30 ml). The reaction mixture was refluxed for 2 hr, cooled to room temperature, then poured into ice water (100 ml) and extracted with diethyl ether (2 × 60 ml). The aqueous layer was acidified with dilute sulfuric acid and extracted with *n*-hexane (3 × 100 ml). The extract was dried over anhydrous sodium sulfate and concentrated to give an oil (5.04 g, 80% yield for 3-methylpentenoic acids; and 7.14 g, 83% yield for 3-methyl-2-octenoic acids).

Analysis by GC/MS showed that both products contain the (*E*)- and (*Z*)-isomers at a ratio of approximately 3.6:1. The H^1 and C^{13}-NMR data for these isomers have been previously reported (Ogura et al., 1970; Gahiez et al., 1976; Hagen et al., 1980). The mass spectra and relative chromatographic retention times [ethyl ester unit; EEU (van den Dool and Kratz, 1963)] are given here.

(Z)-*3-Methyl-2-Pentenoic Acid.* Mass spectrum: *m/z* (rel. int.) *m/z* (rel. int.) M+ 114 (100), 99 (42), 98 (16), 97 (18), 95 (20), 85 (13), 81 (19), 70 (31), 69 (25), 67 (32), 59 (10), 53 (23), 45 (9), 43 (50), 42 (13), 41 (45). EEU = 12.27.

(E)-*3-Methyl-2-Pentenoic Acid.* Mass spectrum: M^+ 114 (100), 99 (52), 98 (15), 97 (20), 95 (20), 85 (15), 81 (19), 72 (14), 71 (10), 70 (30), 69 (30), 67 (32), 59 (8), 55 (20), 53 (22), 45 (5), 43 (35), 41 (50). EEU = 12.63.

(Z)-*3-Methyl-2-Octenoic Acid.* M^+ 156 (23), 141 (5), 113 (100), 100 (82), 97 (28), 95 (78), 85 (24), 82 (28), 72 (75), 67 (29), 55 (30), 43 (75), 41 (33). EEU = 14.93.

(E)-*3-Methyl-2-Octenoic Acid.* M^+ 156 (3), 138 (10), 127 (1), 113 (37), 100 (100), 95 (42), 85 (22), 81 (19), 72 (24), 71 (17), 70 (13), 67 (17), 57 (15), 56 (15), 55 (22), 43 (10), 42 (19), 41 (18). EEU = 15.45.

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

A Finnigan 4510 GC/MS data system equipped with a split/splitless injector, a fused silica capillary column, and capabilities for operation in both electron impact and chemical ionization modes, was used for analysis. The column employed was a 30 m × 0.32-mm (I.D.) fused silica column with a 1.00-μm coating of Stabilwax (cross-bonded polyethylene glycol; Restek, Port Matilda, PA). The mass spectrometer is interfaced to a Nova 4X computer, which utilizes the Super Incos software for data acquisition, analysis, and quantitation. The

mass range employed during these analyses was m/z 40–400. This mass range was scanned once each second and a typical run included 4000 scans. The data system also included the NBS library of 42,000 compounds.

Identifications were based on comparison of unknown spectra with both the NBS library and interpretation of the resulting spectrum with mass spectra generated from synthetic standard compounds. In addition, the relative chromatographic retention times of unknown and known standards were compared. A mixture of fatty acid ethyl esters was used to determine the relative retention times (van den Dool and Kratz, 1963).

Organoleptic Evaluation of Hydrolyzed Apocrine Secretion Samples

This procedure was performed using a panel of three or four judges in a manner similar to that used for evaluation of chromatographic eluants (Zeng et al., 1991). Judges were presented in a blind manner with the acidified, hydrolyzed aqueous or organic portions of apocrine secretion and asked which had an odor that most resembled axillary odor. A small (2- to 5-μl) aliquot of concentrated axillary extract on a filter-paper strip (in a sealed vial) was on hand for reference if requested by a judge.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Apocrine Secretion and Extracts

SDS-PAGE was done according to Laemmli (1970). The stacking gel was 5% acrylamide and the separating gel was 15%. Samples of interest were dried down to approximately 5 μl, then mixed with 5 μl sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, and 50 m*M* Tris–HCl at pH 6.8. After electrophoresis, the gels were stained with Coomassie blue.

RESULTS

The amount of apocrine secretion collected in each of the collections was highly variable and appeared to be dependent on the nature of the subject rather than their number. Certain individuals yielded as much as 20–30 μl, possibly due to their being tense about the collection procedure.

The first two experiments, performed with small amounts of apocrine secretion (7 and 6 μl, respectively), yielded identical results: the concentrated chloroform extract made from the acidified aqueous layer hydrolyzate had a weak but distinct axillary odor when $\sim$2 μl was allowed to evaporate to dryness on clean, odorless filter-paper strips. The identical extract obtained from the organic layer hydrolyzate had no axillary odor or any other odor quality distinguishable from solvent.

Gas chromatography/mass spectrometry (GC/MS) analysis of the axillary smelling extract did not yield any distinct mass spectra which could point to the presence of odorous components such as (*E*)-3-methyl-2-hexenoic acid (*E*-3M2H) or any of the other acidic components known to be present in the axillary odor bouquet (Zeng et al., 1991). This result is consistent with the olfactory threshold of these compounds being below the instrumental detection level.

The third experiment performed with a large amount of apocrine secretion (~ 120 μl) yielded a chloroform extract from the acidified aqueous layer hydrolysate, which had a strong axillary odor upon concentration (~ 12 μl). One microliter (1 μl) was employed for GC/MS analysis, as was 1 μl of the concentrated extract of the organic layer hydrolyzate.

Figure 1 shows the area in the computer reconstructed chromatogram where previous studies (Zeng et al., 1991) have shown the characteristic axillary odors, (*E*)- and (*Z*)-3M2H elute. Figure 1A is the organic phase hydrolysate, while Figure 1B is the aqueous phase hydrolysate. Above each of the chromatograms is a plot of *m/z* 128, the ion representing the intact, ionized, 3-methyl-2-hexenoic acids, eluting in this area. Only a trace amount of the (*E*)-3M2H, is seen in the organic phase hydrolysate (Figure 1A). From comparison of the area of the ions for *m/z* 128 with injection of known amounts of 3M2H, we estimate that the aqueous phase hydrolysate contained approximately 50.0 ng/μl of the (*E*)-isomer and 4.2 ng/μl of the (*Z*)-isomer. The organic phase hydrolysate contained approximately 0.5 ng/μl of the (*E*)-isomer. We recently reported (Zeng et al., 1991) that the (*E*)-isomer-to-(*Z*)-isomer ratio in a combined male extract was 10:1; the same approximate ratio is also seen here (Figure 1B).

Similar results were obtained from the experiment involving incubation of the diphtheroid bacteria with separate samples of apocrine secretion aqueous and organic phases. Analysis of these samples showed that bacteriolysis of the aqueous phase produced 5.13 ng/μl of the *E*-isomer and 0.12 ng/μl of the *Z*-isomer, while the organic phase yielded only a trace of the *E*-isomer (0.02 ng/μl). Analysis of the blank, which contained only the McFarland medium, did not show any *E*- or *Z*-3M2H. The results of both the NaOH hydrolysis and the bacteriolysis are summarized in Table 1.

Figure 2 shows the portion of the computer-reconstructed chromatogram where previous studies have shown the C_6 to C_{11} acids constituting the axillary "bouquet" elute (Zeng et al., 1991). Each of the compounds is labeled and its identity is given in Table 2. These data show that the aqueous and organic phase hydrolysates contained many of the straight-chained acids which we have reported in the axillary extract (Preti et al., 1987; Zeng et al., 1991), albeit at relatively low levels. In addition, the aqueous phase extract also contained small amounts of three other unsaturated acids. We have identified two of these as 7-octenoic acid (Peak I), (*E*)-3-methyl-2-pentenoic acid (Peak B, a lower homologue of

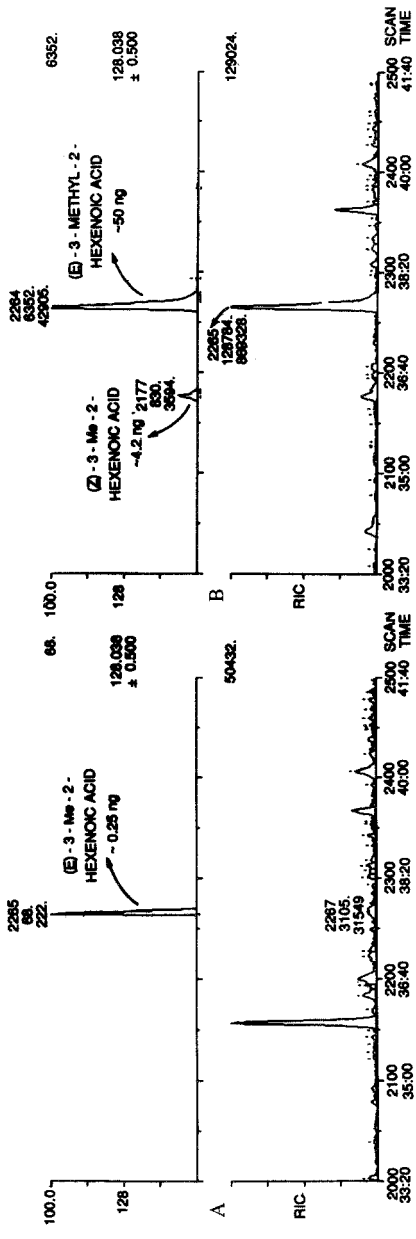

FIG. 1. Reconstructed ion chromatogram showing the area where (Z)- and (E)-3-methyl-2-hexenoic elute. (A) The organic phase hydrolysate; (B) the aqueous phase hydrolysate. The top half of A and B shows a mass chromatogram of m/z 128, the intact, ionized molecular ion of (E)- and (Z)-3-methyl-2-hexenoic acid. The aqueous phase hydrolysate contains approximately 200 times more 3-methyl-2-hexenoic acid than the organic phase hydrolysate.

TABLE 1. AMOUNTS OF 3-METHYL-2-HEXENOIC ACID LIBERATED FROM APOCRINE SECRETION BY NaOH HYDROLYSIS AND BACTERIOLYSIS

	Nanograms per microliter			
	5% NaOH hydrolysis		Bacteriolysis	
	E-Isomer	*Z*-Isomer	*E*-Isomer	*Z*-Isomer
Aqueous phase	50.0	4.20	5.13	0.12 ng/μl
Organic phase	0.25	0	0.02	0

3M2H), and a C_9-unsaturated acid (Peak L). The structure of 3-methyl-2-pentenoic acid was confirmed by comparison (MS and relative retention time) to a synthesized sample. The C_9-unsaturated acid had a mass spectrum and relative retention time identical to that of an unknown C_9-unsaturated acid we reported in our study of male axillary odor components (Zeng et al., 1991). We also synthesized 3-methyl-2-octenoic acid to see if it corresponded to this unknown C_9-unsaturated acid; it did not (see Table 2). No evidence was seen for these three unsaturated acids in the organic phase hydrolysate.

We tested the possibility that 3M2H and other acids might be present in apocrine secretions as their salts and therefore left in the aqueous phase after extraction with $CHCl_3$. To do this we first determined what would result from the $CHCl_3$ extraction and subsequent hydrolysis of a basic solution containing a known amount (500 ng) of *E*- and *Z*-3M2H. Acidification of the basic solution produced the characteristic axillary-like odor of the 3M2H. After extraction with $CHCl_3$ half of this extract (E_1) and the remaining aqueous phase (A_R) were refluxed with 5% NaOH as described above. The other half of the extract (E_2) was concentrated and analyzed by GC/MS. The results of the GC/MS analysis of each sample are given in Table 3. These data show that all the 3M2H were extracted out of the aqueous phase and approximately 58% of the acids extracted into the $CHCl_3$ were recovered.

This experiment was repeated using a 40-μl sample of apocrine secretion. Upon adjusting to pH 2, we noted a weak but distinct odor. The smell was not that of 3-methyl-2-hexenoic acid but a burnt/onion, sulfurous odor. After dilution and extraction of the acidified sample, half the $CHCl_3$ extract (AE_1) and the remaining aqueous phase (AAR) were refluxed with 5% NaOH and analyzed as described above. The other half of the $CHCl_3$ extract (AE_2) was concentrated and analyzed. The results of each analysis are given in Table 4. The recovery of the D_{19}-decanoic acid internal standard for each of the three samples was 44, 52, and 71% for AE, AAR, and AE_2, respectively.

The data in Table 4 shows that the hydrolysis procedure liberated 3-methyl-

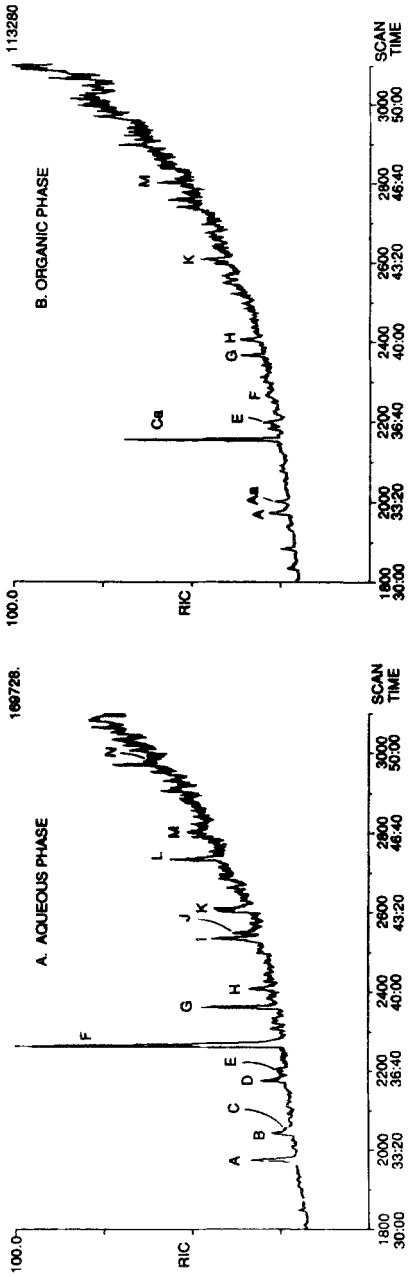

FIG. 2. Reconstructed ion chromatogram showing the region where C_6–C_{11} acids elute in both the aqueous phase hydrolysate (A) and the organic phase hydrolysate (B). Identity and data for labeled peaks are given in Table 1.

TABLE 2. IDENTIFICATION OF COMPONENTS IN FIGURE 2[a]

	Peak	MW	Relative retention time (EEU)[b]
A	Hexanoic acid (C)	116	12.20
Aa	Unknown[c]	—	12.14
B	(*E*)-3-Methyl-2-pentenoic acid (C)	114	12.55
C	3-Methylhexanoic acid (C)	130	12.60
Ca	Unknown[d]	—	12.80
D	(*Z*)-3-Methyl-2-hexenoic acid (C)	128	13.05
E	Heptanoic acid (C)	130	13.23
F	(*E*)-3-Methyl-2-hexenoic acid (C)	128	13.50
G	Unknown[e]	—	14.00
H	Octanoic acid (C)	144	14.30
I	7-Octenoic acid (C)	142	14.91
J	Unknown[f]		15.00
K	Nonanoic acid (C)	158	15.31
L	C_9-Unsaturated acid[g]	156	15.96
M	Decanoic acid (C)	172	16.30
N	Undecanoic acid (C)	186	17.30

[a] C—correspondence of mass spectrum and relative chromatographic retention times with commercially available or synthetic sample; T—tentatively assigned from mass spectral and relative retention time data.

[b] Ethyl ester units.

[c] Unknown at 12.14 EEU found only in organic phase hydrolyzate: *m/z* (relative intensity) 43 (95), 56 (20),70 (27), 71 (100), 96 (8), 100 (5), 111 (8).

[d] Unknown at 12.80 EEU found only in organic phase hydrolyzate: *m/z* (relative intensity) 45 (20), 59 (15), 74 (100), 87 (8), 107 (7), 139 (12), 141 (6). Although this contains some salient mass spectral characteristics of a methyl ester, it was not one.

[e] Unknown at 14.00 EEU found only in aqueous phase hydrolyzate: *m/z* (relative intensity) 41 (10), 43 (45), 55 (7), 56 (11), 57 (21), 69 (8), 71 (100).

[f] Unknown at 15.00 EEU found only in aqueous phase hydrolysate: *m/z* (relative intensity) 45 (100), 55 (100), 56 (12), 60 (8), 67 (11), 68 (15), 70 (8), 71 (9), 72 (15), 81 (18), 82 (21), 87 (9), 95 (5), 110 (3), 142 (5).

[g] Mass spectrum of unknown, C_9-unsaturated acid: *m/z* (relative intensity) M^+ 41 (70), 43 (85), 45 (92), 55 (100), 56 (55), 58 (35), 67 (55), 68 (70), 69 (72), 70 (22), 71 (22), 72 (25), 81 (45), 83 (35), 85 (25), 88 (25), 96 (57), 97 (38), 114 (35), 138 (3), 156 (10). EEU = 16.04. Does not compare with mass spectrum of 3-methyl-2-octenoic acid in Methods and Materials or 8-nonenoic acid reported previously (Zeng et al., 1991).

2-hexenoic acid from the aqueous phase; this is in contrast to A_R in Table 3, where no 3-methyl-2-hexenoic acids were found.

The apocrine secretion aqueous and organic phase hydrolysates were also investigated for the presence of volatile steroids and/or steroid sulfates by GC/MS using previously established procedures employing bonded methyl silicone columns (Preti et al., 1987; Zeng et al., 1991). No evidence of these compounds was found in the organic phase hydrolysate. In contrast, the aqueous

TABLE 3. RECOVERY OF SODIUM 3-METHYL-2-HEXENOATE AFTER HYDROLYSIS

Sample	Nanograms of 3-methyl-2-hexenoic acid
E_1	160.7
E_2	130.6
A_R	0

TABLE 4. RECOVERY OF 3-METHYL-2-HEXENOIC ACID FROM ACIDIFIED APOCRINE SECRETION AND SUBSEQUENT HYDROLYSIS

Sample	Nanograms of 3-methyl-2-hexenoic acid
AE_1	46.1
AE_2	106.2
AA_R	136.5

phase hydrolysate contained detectable amounts of the steroid sulfates; 17-oxo-5-androsten-3α-yl sulfate (dehydroepiandrosterone sulfate) and 17-oxo-5α-androstan-3α-yl sulfate (androsterone sulfate) were seen as their pyrolysis products (Labows et al., 1979; Preti et al., 1987). The free steroids, androsterone and dehydroepiandrosterone, were also present. No evidence of the volatile steroids (e.g., androstenone and androstenol) was seen in these samples.

Similar analyses were carried out on the aqueous and organic phase following bacteriolysis. No evidence was found for the presence of C_{19}-steroids in the organic phase. However, the aqueous phase contained both dehydroepiandrosterone sulfate and androsterone sulfate. In addition, androsternol was found in this sample along with dehydroepiandrosterone and androsterone. Although quantitation was not done, the level of androstenol was comparable to the levels of dehydroepiandrosterone sulfate.

To determine how much of the steroid sulfates would be converted to the free steroids by the hydrolysis procedure, we performed an experiment employing 100 μg of dehydroepiandrosterone sulfate and 50 μg of androsterone sulfate. These are the approximate amounts expected in 120 μl of apocrine secretion as per previous studies (Labows et al., 1979). One milliliter of 5% aqueous NaOH was added to 0.12 ml of doubly distilled water containing the above sulfate levels and the hydrolysis performed as described under Methods and Materials. Workup and analysis of the resultant hydrolysate showed that only 17% of the sulfates were cleaved by the procedure. Previous studies have shown that these

sulfates do not appear to be precursors for androstenol (Labows, 1988; Froebe et al., 1990).

Further investigation of the nonhydrolyzed organic and aqueous layers was performed using SDS/polyacrylamide electrophoresis. Figure 3 shows the Coomassie blue-stained gel on which were loaded comparable aliquots of organic soluble compounds (lanes 1 and 2) and aqueous soluble compounds (lane 3) prior to hydrolysis. These were saved from the experiment which utilized the large amount (~ 120 ml) of apocrine secretion (as noted above).

The aqueous portion of the apocrine secretion appears to be extremely rich in proteins, while little or no protemaceous material is seen in the organic phase. Several protein bands can be seen in lane 3 including major protein components found at apparent molecular weights of 66,000, 40,000, and 26,000.

DISCUSSION

The finding that the water-soluble components of apocrine secretion contained nonodorous molecules that could be hydrolyzed to yield (*E*)- and (*Z*)-3M2H by both hydrolysis with NaOH and incubation with axillary diphtheroids was unexpected. It suggests that an important, characteristic human axillary odor is not being synthesized on the skin surface. Possibly, this is made elsewhere in the body, stored in the apocrine gland, and secreted in the apocrine secretion with the water soluble components. Consequently, this axillary odor appears on the axillary skin surface bound to a precursor and is liberated by the action of the cutaneous microflora.

The experiments in which the sodium salts of *E*- and *Z*-3M2H and apocrine secretion were acidified, extracted, and hydrolyzed further support our conclusion of an aqueous soluble precursor. Acidification of the basic solution containing the sodium 3M2H yielded the distinct characteristic odor of the 3M2H; subsequent extraction of the aqueous phase with $CHCl_3$, hydrolysis with NaOH, and analysis by GC/MS showed no 3M2H in the resulting hydrolysate (Table 3, Ar). This suggests that the 3M2H was removed from the acidified aqueous phase. However, such a result was not seen when the similar procedure was performed with apocrine secretion. The odor produced by the acidified apocrine secretion was not that of the 3M2H but had a distinct burnt, sulfur/onion odor, which suggested that a reaction was occurring which liberated 3M2H as shown in Table 4. However, a larger amount of 3M2H remained in the aqueous phase of the acidified apocrine secretion than in its $CHCl_3$ extract.

These data are consistent with the 3M2H being associated, in some fashion, with a water-soluble precursor molecule. Addition of HCl to the apocrine secretion either cleaves a precursor–3M2H covalent bond or disrupts the intermolecular forces holding the precursor–3M2H together. If the precursor molecule is

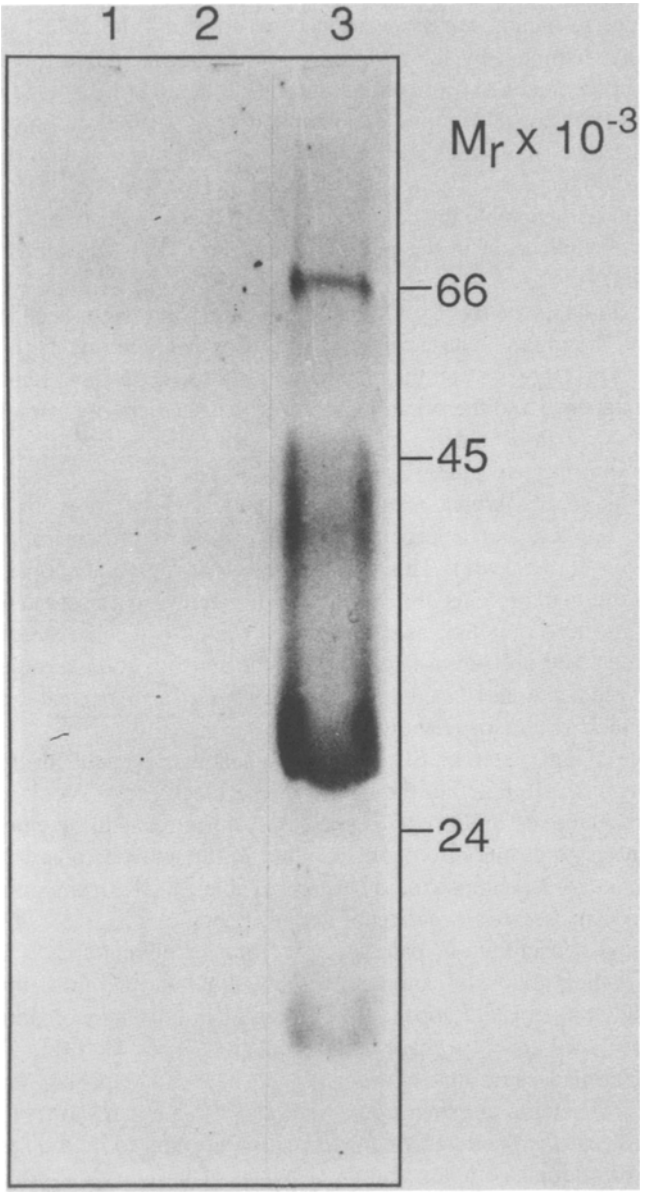

FIG. 3. The SDS–polyacrylamide gel electrophoretic profile of the unhydrolyzed organic phase (lanes 1 and 2) and aqueous phase (lane 3). The aqueous soluble molecules contain a number of proteins as shown by Coomassie blue staining. No proteinaceous material is seen in lanes 1 and 2. Molecular weight markers (not shown) were used to generate the scale at the right.

proteinaceous in nature, the association between it and the 3M2H may be one that is easily disrupted by the addition of acid or base. Some proteins, under acidic conditions, even at room temperature, will begin to hydrolyze and/or lose their three-dimensional structure (Hunkapillar et al., 1983; Kopple, 1966). In the latter instance, molecules such as 3M2H intercathelated within the protein's structure would be released. The odor noted when HCl was added to the apocrine secretion did not resemble the acid but may have been indicative of bond cleavage (thioester linkages?) in the putative precursor. These are areas for further investigation.

Small amounts of the C_6–C_{11} straight-chain acids were seen in both the aqueous and the organic phase mixtures following bacterial incubation and base hydrolysis. This suggests that the precursors for these acids have some solubility in both the aqueous and the organic phases or that they are present at low levels (below olfactory threshold) in the collected apocrine secretion and partition themselves into the two phases, prior to hydrolysis.

Previous studies which examined the apocrine secretion for its various components suggest that a large amount of protein is present: approximately 10% (Labows et al., 1981). This percentage seems high and should be reevaluated. In addition to proteins, the steroid sulfates were also present in the aqueous portion of apocrine secretion as expected. However, a volatile steroid (androstenol) was detected only upon interaction of the aqueous soluble molecules with the diphtheroid bacteria; consequently, axillary bacteria are needed to metabolize them from their precursor (Labows, 1988).

Our initial data based on SDS-PAGE suggest that the aqueous phase is rich in proteinaceous material, but the nature of the precursor molecules associated with the characteristic axillary odor (i.e., 3M2H) remains to be elucidated.

One interesting minor component found in the aqueous phase hydrolysate was (*E*)-3-methyl-2-pentenoic acid (Peak B, Table 2). This compound does not appear in axillary secretions collected in pads (Zeng et al., 1991). This may be due to its low concentration, precluding it from instrumental detection and its coelution, in the previously examined combined extract with 3-methylhexanoic acid. The latter compound appears to be present in only trace quantities in the freshly hydrolyzed aqueous phase component (i.e., Peak C, Table 2).

Our data also show that hydrolysis with base accomplishes much of the same effect as bacterial enzymes do *in vivo*, albeit in a much shorter time frame. The main role of the bacteria in odor formation appears to be the disruption of the precursor/odorant complex and/or cleavage of the covalent bonds holding the acid molecules to the precursor.

Acknowledgments—The authors wish to thank Mr. John Chicchi of K.G.L./Ivy Labs for recruiting apocrine secretion donors and aiding Dr. Leyden in secretion collection. We also thank Mrs. Linda Robinson and Mrs. Janice Blescia for preparing the manuscript. This study was supported in part by a postdoctoral fellowship awarded to the Monell Chemical Senses Center by Takasago International Corporation.

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