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# Axillary malodor production: A new mechanism

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Synopsis

The mechanism proposed by Eigen (1) for the generation of axillary malodor from steroids has been explored. Previous work indicated that axillary odor is largely due to the steroids 16,5α-androstene-3β-ol and 16,5α-androsten-3-one. Following Eigen, we theorize that sterile and odorless apocrine secretions from the axilla contain these steroids as their water-soluble sulfates and glucoronides and that odor is produced only after the volatile free steroids are liberated from these esters by bacterial hydrolytic enzymes such as aryl sulfatase and beta-glucuronidase. Support for this hypothesis was obtained by producing odor from sterile, odorless, apocrine sweat by the addition of either of the two enzymes, or of the corynebacterial strains that produce them. Finally, steroid malodor production can be prevented by inhibitors of beta-glucuronidase and of aryl sulftase.

## INTRODUCTION

The human axilla is populated with two classes of sweat gland: The eccrine glands produce a watery secretion in response to heat. Apocrine glands produce microdroplets of a viscous secretion in response to emotional stress. Apocrine sweat is a complex mixture containing cholesterol, steroids, and other lipids, as well as 10% protein. It was recognized as early as 1956 that axillary odor is generated from apocrine secretion. Although the sterile fluid is odorless, bacterial action on certain components present in sweat produces the characteristic underarm odor (2). Although the initial view of Strauss and Kligman was that any or all of the axillary population could cause odor, within a few years Shehadeh and Kligman found that it was caused chiefly by gram-positive types (3).

Recent work suggests two classes of odorants in the axilla: short-chain fatty acids, e.g. isovaleric acid, and the androgen steroids, especially 5-α-androstenol and 5-α-androstenone (see Figure 1) (4). It must be mentioned that, while these species have been chemically identified in the axilla through gas chromatographic methods, the identification of them as axillary odorants has relied upon organoleptic means. Therefore, the positive identification of these three species as the principal axillary odorants has not yet been made. Indeed, other related compounds may contribute to the complex odor.

A correlation has been found between the composition of the underarm flora and the

nature of the odor produced: Where the axillary microbial population is dominated by coryneform bacteria (lipophilic diphtheroids), the acrid odor of delta-16 steroids is apparent, whereas, if the axillary population is dominated by such micrococci as *Staphylococcus epidermidis,* the odor of isovaleric acid prevails. Pronounced axillary odor is correlated with the occurrence of the coryneform flora (5); it is on the steroid compounds associated with these strains that this research focuses. Many studies have identified axillary steroids and have linked their presence with indigenous bacteria (4–13); however, the mechanism of bacterial action has remained uncertain (9,12).

Since they are not typically water-soluble, steroids are normally transported in body fluids as their water-soluble conjugates with sulfate or glucuronic acid (14). When we began to speculate on the origin of volatile free steroids in the axilla, we hypothesized that apocrine secretion contained the steroids as water-soluble conjugates (1). Conversion *in vivo* to the free steroid generally requires the action of hydrolytic enzymes. It seemed likely that it was the production of these enzymes that represented the contribution of bacteria to the generation of underarm oder (Figure 2). We theorized that sterile apocrine sweat would deposit the water-soluble, odorless conjugates onto hair and skin in the axilla, where enzymes secreted by local bacteria would release the volatile, odorous, free steroids.

The enzymes expected to hydrolyze the steroid esters might be any of several bacterial exoesterases—for example, beta-glucuronidase (beta-G) and aryl sulfatase (AS). These enzymes can be detected with the synthetic substrates 4-methylumbelliferyl glucuronide (4-MUG) and 4-methylumbelliferyl sulfate (4-MUS), respectively, both of which release fluorescent 4-methylumbelliferone (4-MU) upon hydrolysis (Figure 3) (14,15).

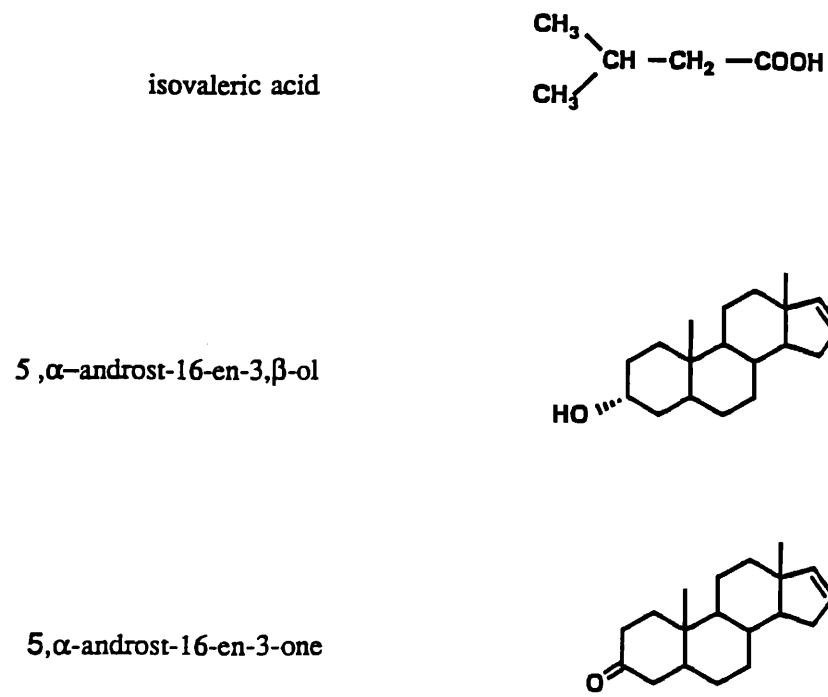


Figure 1. Proposed sources of axillary odor.

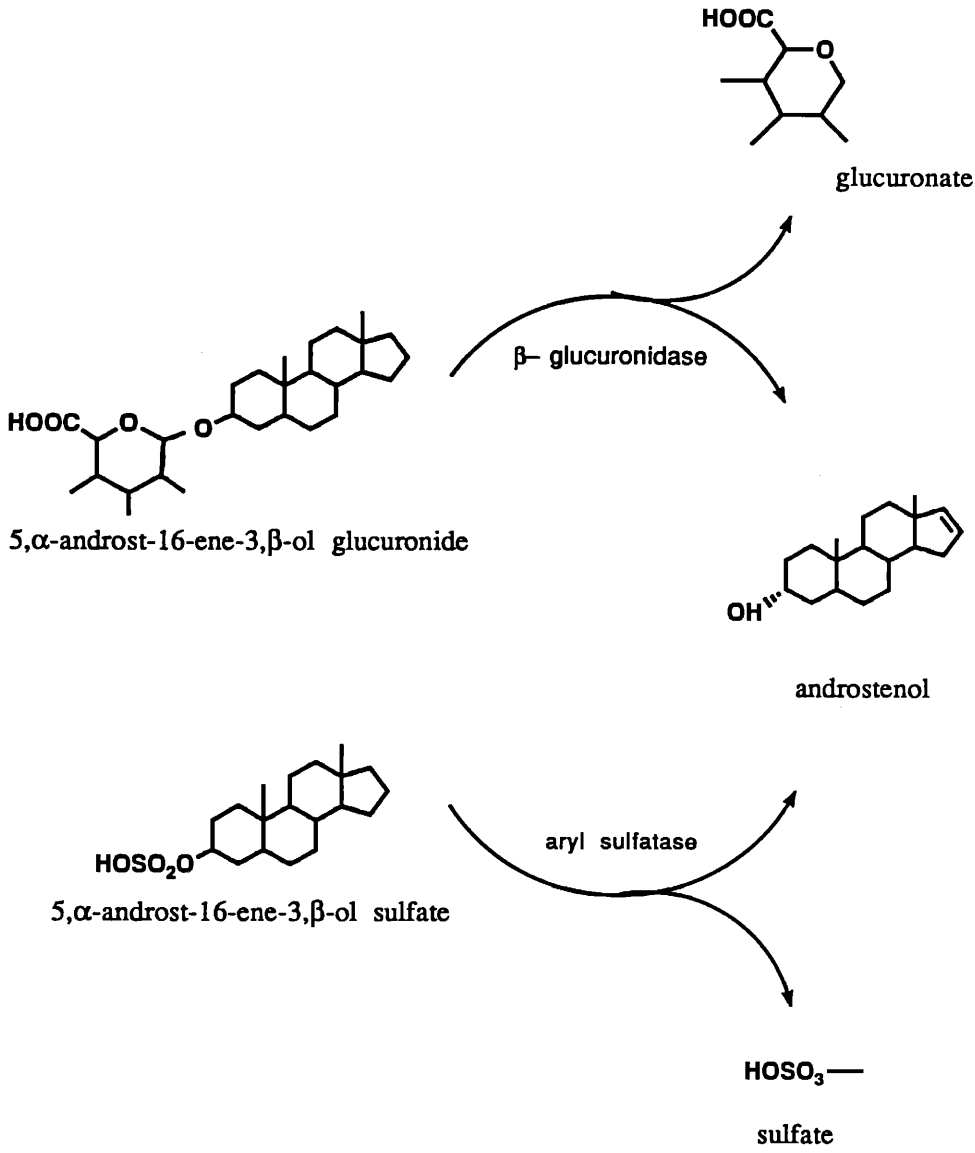


Figure 2. Hydrolysis of steroid conjugates.

These fluorogenic substrates are actually models of the steroid glucuronides and sulfates.

We therefore began a series of studies to test Eigen's view, and this paper describes those experiments.

## MATERIALS

B-glucuronidase from *E. coli* and aryl sulfatase from Aerobacter aerogenes, 4-methyl-

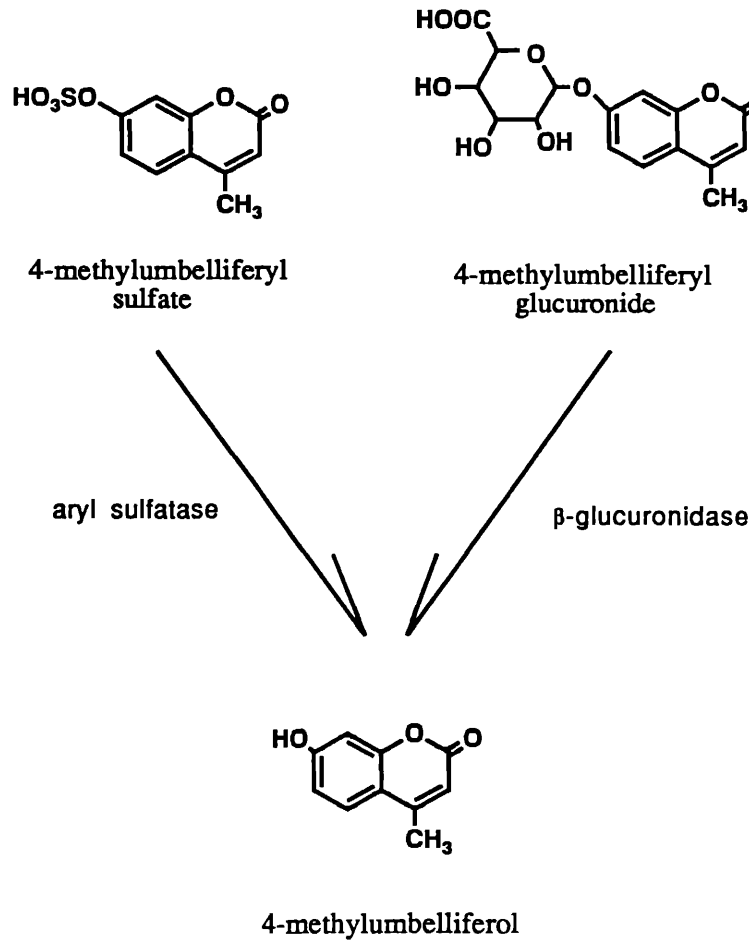


Figure 3. Hydrolysis of 4-MUS and 4-MUG by aryl sulfatase and beta-glucuronidase.

umbelliferyl glucuronide, 4-methylumbelliferyl sulfate, 4-methylumbelliferone, and D-saccharic acid-Δ-lactone were purchased from Sigma Chemical Co. Sodium hexametaphosphate (SPORIX) was obtained from International Sourcing.

Apocrine secretion was obtained from Ivy Research Labs (Philadelphia, PA). The fluid was collected according to the method described by Labows *et al.* (10). Eleven subjects were used. The axillary area was shaved, washed with a 0.1% solution of Triton X-100, rinsed with water, blotted dry, then rinsed with hexane. An intradermal injection of 0.1 ml of 1:2000 adrenaline was performed to stimulate the apocrine glands. Micropipettes (10 μl) were placed directly against the skin to collect the droplets of apocrine secretion. Samples were frozen until ready for use. Samples were eluted from the capillary tubes with 0.1 M Tris buffer, pH 7.0, for use in odor-generation studies. Lipophilic diphtheroid cultures were provided by Dr. John Labows at Monell Chemical Senses Center (Philadelphia, PA); they were cultures of strains originally isolated from human axillae at Monell.

Zinc glycinate was prepared by the method of Dubsky and Rabas (16): 52.5 g (0.6 mol) glycine and 20.35 g (0.25 mol) ZnO were stirred in 300 g water at 75 C until a clear

solution was obtained, and after cooling the product was precipitated with 300 g ethanol. The white crystals were washed three times with absolute ethanol and dried *in vacuo*. Zinc as determined by atomic absorption was 27% (theoretical, 30%), and glycine by Kjeldahl was 68% (theoretical 70%). Solubility of zinc glycinate (Zn-GLY) in water at 20°C and pH 7.2 was found from zinc concentration of a saturated solution to be 5 g/100 ml.

## METHODS

### PILOT STUDY

A double-blind screening study was designed to detect the enzymes beta-G and AS in axillary secretions. Swabs were taken from the axillae of twenty men, classified by professional odor evaluators (Hilltop Laboratories, Cincinnati, OH) as 10 "high-odor formers" and 10 "low-odor formers." Trypticase soy agar plates were prepared containing either 4-MUS (substrate for aryl sulfatase) or 4-MUG (substrate for beta-glucuronidase) at a concentration of 25 ppm. Elutions of the swabs were plated on both substrates, incubated for 24 hrs, and the fluorescence of the plates was estimated visually.

### SEMIQUANTITATIVE ASSAYS OF BETA-GLUCURONIDASE AND ARYL SULFATASE

The enzyme assays measure conversion at 37°C of the non-fluorescent substrates 4-MUG (for beta-G) and 4-MUS (for AS) to fluorescent product, 4-MU (Figure 2). The assay mixtures contained:

| For beta-G: | For AS: |
|---|---|
| 0.65 ml water | 1 ml water |
| 0.5 ml 0.1 M Tris buffer, pH 7 | 1.7 ml 0.1 M Tris buffer, pH 7 |
| 0.25 ml 0.01 mg/ml 4-MUG in water | 0.2 ml 0.5 mg/ml 4-MUS in water |
| 0.1 ml 0.001 mg/ml beta-G in Tris | 0.1 ml 0.01 mg/ml AS in Tris |
| (0 mg/ml beta-G/Tris as control) | (0 mg/ml AS/Tris as control) |

For semiquantitative studies, fluorescence was detected by long-wave UV lamp (Black-Light Eastern Corporation, Model XX 15).

The effects of inhibitors on the enzymatic reactions were assessed by substituting aqueous solutions of test inhibitor, at 0.1 mM to 100 mM, for the water. To rule out quenching of the fluorescence reaction by the inhibitors, separate tests were performed comparing the fluorescence from 4-MU at the maximum concentration expected in the enzyme reaction mixtures with either inhibitor solution or water.

### QUANTITATIVE ASSAYS OF BETA-G AND AS

Several inhibitors of each, beta-G and AS, were assayed by quantitative fluorimetry. Studies were carried out with a Perkin-Elmer model MPF-3 with an Osram XBO high pressure Xenon lamp, using quartz curettes only. Prior to the performance of quantitative assays it was necessary to determine absorption and emission maxima of the fluores-

cent product 4-MU, as well as a concentration range for which the fluorescence intensity of this species varies linearly with concentration. A solution of 4-MU, 2 μg/ml, was prepared in 0.1 M Tris buffer, pH 7.0. The absorption spectrum was scanned, giving a maximum at 334 nm. The emission maximum, 444 nm, was obtained by scanning the emission spectrum while irradiating at 334 nm. All quantitative fluorescence studies employed these parameters.

Linear calibration curves (Figure 4) were generated for the concentration ranges, (1) 0.2 μg/ml–2.0 μg/ml and (2) 0.02 μg/ml–0.2 μg/ml. The levels of 4-MU generated in beta-G and AS assays fall into range 1. The conversion factor was 1 fluorescence unit = 0.09 μM 4-MU.

Samples were prepared as described above. All components (except the enzyme) were warmed to 37°C before addition of the enzyme. During the assays, reaction mixtures were maintained at 37°C. Fluorescence intensities were measured as a function of time.

ODOR PRODUCTION IN APOCRINE SECRETION

Sterile, odorless apocrine secretion was eluted from capillaries into 1.5 ml of 0.1 M Tris buffer, pH 7.0. In the first study, apocrine sweat was treated with beta-glucuronidase

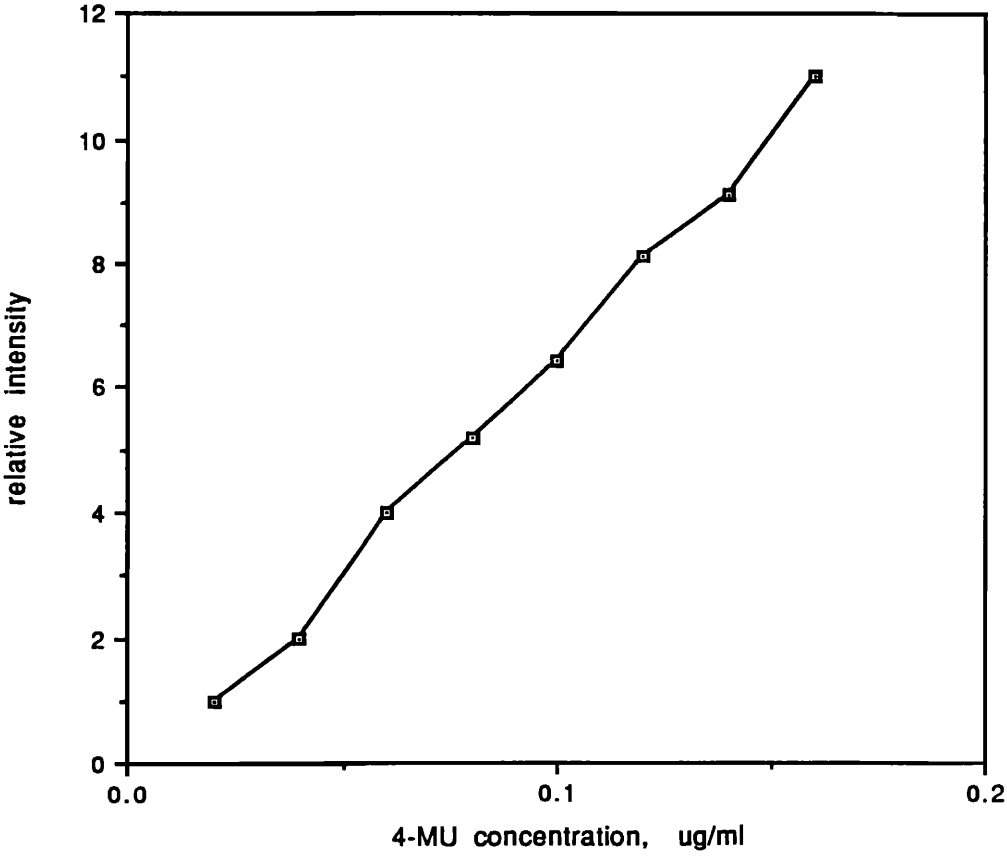


Figure 4. Fluorescence as a function of 4-methylumbelliferol concentration.

(*E. coli*) and aryl sulfatase (*Aerobacter aerogenes*) at 0.01 mg/ml in Tris buffer, 0.1 M, pH 7.0. The apocrine secretion was also treated with the lipophilic diphtheroid and with a mixed culture of axillary bacteria, each in sterile saline suspension.

In the second study, inhibitors of the two enzymes were included in the reaction mixtures—$Zn^{++}$ as ZnGlycinate, saccharic acid lactone, and hexametaphosphate.

Samples were prepared in gas chromatography vials and sealed with crimp caps. Samples were incubated at 37°C. In the first study, odor evaluations were conducted at 16 hr and 40 hr of incubation. In the second study, odor evaluations were conducted at 24 hr and 48 hr. Odor evaluations were performed by a panel of five, including one professional perfumer.

### ENZYME ACTIVITY IN CELL-FREE MEDIA

Lipophilic diphtheroid was grown for 18 hr in brain-heart infusion containing 0.1% Tween 80, centrifuged for 20 min, and the supernatant medium filtered through a 0.22-micron filter. The substrate solution (2 ml of 4-MUG or 4-MUS) was mixed with 1 ml of the filtered medium (or water, as control).

## RESULTS

### PILOT STUDY

A double-blind screening study was designed to detect the enzymes in axillary secretions. On plates designed to test for beta-G, ten sweat samples gave high fluorescence; nine of these came from "high-odor formers." Only one came from a "low-odor former." On plates designed to test for AS, six of eight "high-odor formers" were found to have swabbings containing AS (two samples were lost), and only two of the ten "low-odor formers" had the enzyme. This study shows that at least some axillary bacteria generate steroid esterases and that the presence of these enzymes may be correlated with axillary odor.

### SEMIQUANTITATIVE ASSAYS OF ENZYMES: EFFECTS OF INHIBITORS

Various inhibitors of mammalian beta-Gs and ASs have been reported in the literature (18–32). We tested several of these compounds as well as related materials in our assay. The classic inhibitor of beta-G is saccharic acid-Δ-lactone (glucarolactone) (24). We tested this material as well as the simple sugars, mannose, fucose, and galactosamine, and the polysaccharide pullulan. One of the reported inhibitors of beta-G, EDTA (21), is a strong chelating agent. Thus we also tested o-phenanthroline, citrate, gluconate, nitrilotriacetate, and hexametaphosphate against the enzyme. Table I summarizes the materials tested.

Several materials were found to inhibit each enzyme, but three of the agents tested—$Zn^{++}$, $Cu^{++}$, and hexametaphosphate—inhibited both beta-G and AS.

### QUANTITATIVE ASSAYS OF BETA-G AND AS

The effects of five inhibitors of beta-G and three inhibitors of AS identified in semi-

Table I
Compounds Tested for Inhibition of Aryl Sulfatase and beta-Glucuronidase

| | Enzyme inhibition |
|---|---|
| For beta G: | |
| $Cu^{++}$ | + |
| $Zn^{++}$ | + |
| SPORIX hexametaphosphate | + |
| D-glucaro-Δ-lactone | + |
| EDTA | + |
| NTA | + |
| O-phenanthroline | + |
| Citrate | − |
| Sodium sulfate | + |
| Gluconate | − |
| Mannose | − |
| Fucose | − |
| Galactosamine | − |
| Pullulan | − |
| For AS: | |
| $Cu^{++}$ | + |
| $Zn^{++}$ | + |
| SPORIX hexametaphosphate | + |
| Orthophosphate | + |
| EDTA | − |
| NTA | − |

quantitative assays were studied quantitatively in a fluorescence assay. As in the semi-quantitative tests, the progress of the reactions was indicated by the development of fluorescent product 4-MU from the non-fluorescent substrates, 4-MUG and 4-MUS. The data was recorded as fluorescence emission intensity as a function of time. A representative example of the results of these assays is given in Figure 5, which shows the effect of glucarolactone concentration upon beta-G activity. Similar plots were constructed for each inhibitor. At each concentration, the beginning slope indicated initial reaction rate. These rates were then expressed as a fraction of the uninhibited reaction rate and plotted against the log of the inhibitor concentration (Figures 6 and 7).

For beta-G (Figure 6) the glucarolactone was the most effective inhibitor (1−10 μm range). The divalent cations, $Zn^{++}$ and $Cu^{++}$, were effective at approximately 10−100 μM. The sequestering agents EDTA and phenanthroline were significantly less effective, providing inhibition at millimolar levels.

For AS, the $Cu^{++}$ ion was most effective (0.1−10 μM range). $Zn^{++}$ and phosphate were effective at 10−100 μM.

Most microbial AS belongs to the Type I class, which is not inhibited significantly by sulfate or phosphate, but the AS of *Aerobacter aerogenes* (our enzyme) is somewhat more sensitive than that of other bacteria (30). This property is apparent in our study, where phosphate is found to be about as effective as zinc cation.

These studies show that at least some bacterial beta-G and AS can be inhibited by Zn, Cu, chelating agents, or glucarolactone.

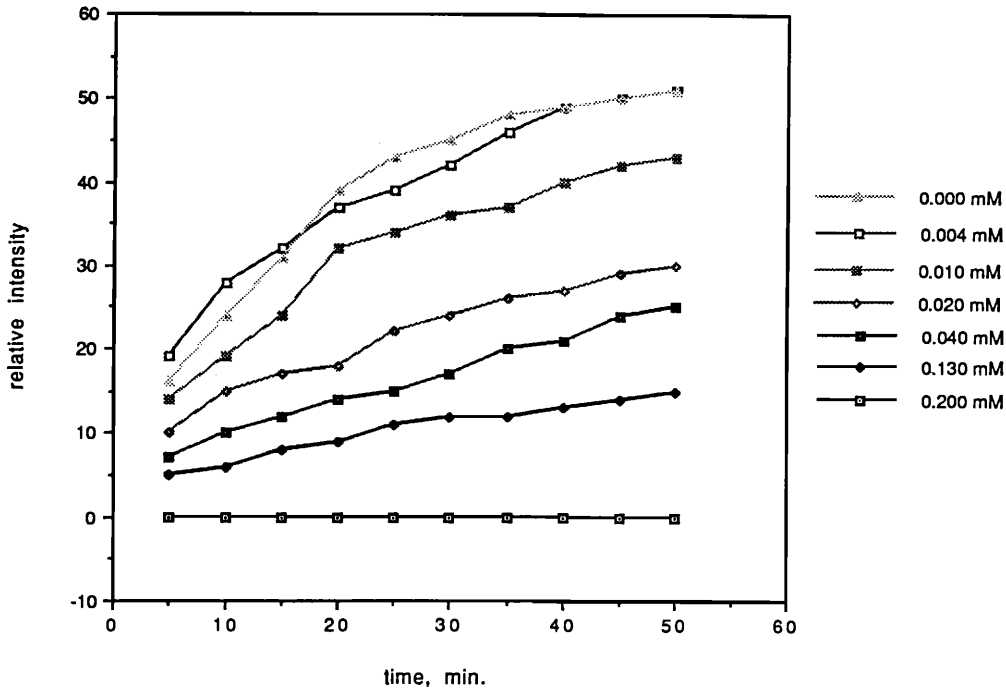


Figure 5. Inhibition of beta-glucuronidase by glucarolactone.

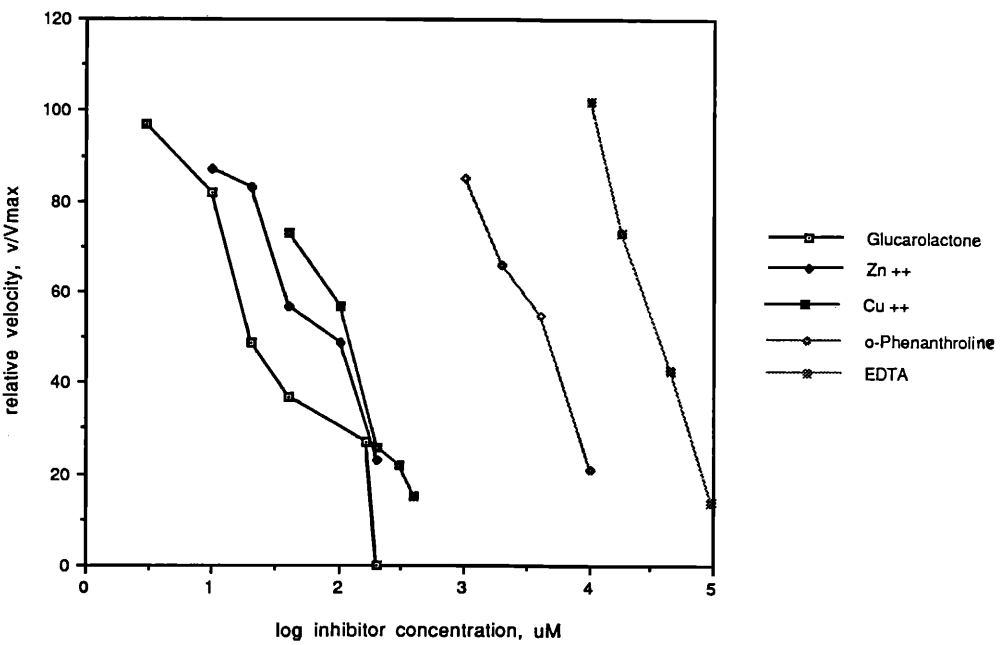


Figure 6. Inhibition of beta-glucuronidase: A decrease in relative reaction velocity with increasing concentration of five inhibitors.

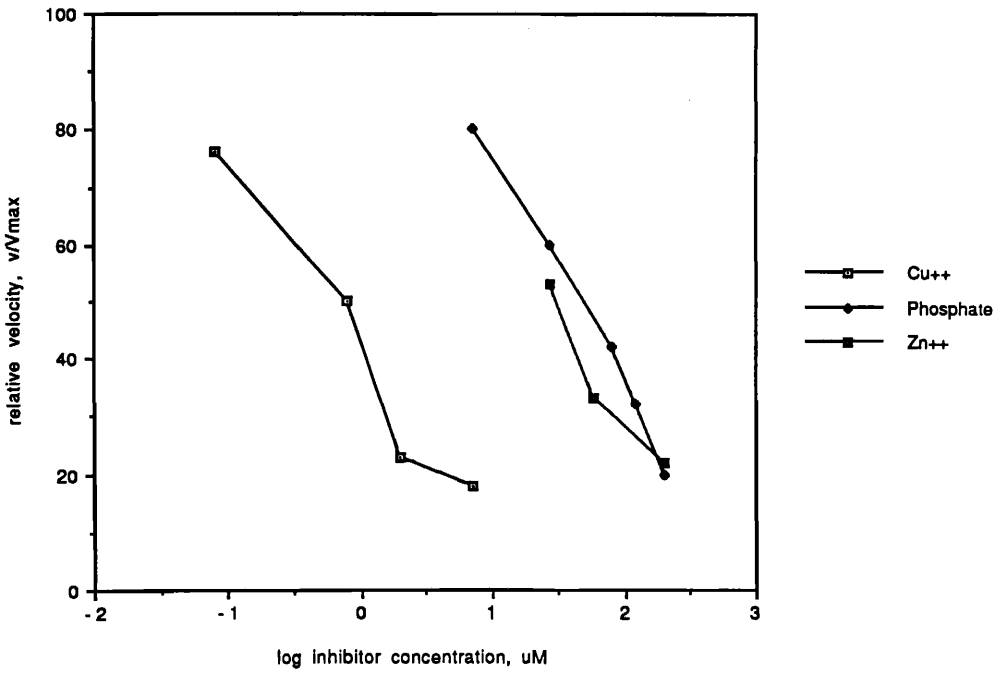


Figure 7. Inhibition of aryl sulfatase: decrease in relative reaction velocity with increasing concentration of three inhibitors.

ODOR PRODUCTION FROM APOCRINE SECRETION

In the preliminary tests, mixed cultures of axillary bacteria converted 4-MUG to 4-MU, as judged from the appearance of visible fluorescence. This result demonstrated that one or more strains of axillary bacteria are generating beta-G. We proposed that the activity of this enzyme upon certain steroid conjugates present in sweat might produce axillary odor. To test this hypothesis, sterile, odorless, apocrine secretion was treated with the individual bacterial enzymes, beta-glucuronidase and aryl sulfatase, and also with lipophilic diphtheroid (1,3) and a mixed culture of axillary bacteria in sterile saline suspension. The odor produced was judged by a panel of five, including an expert, a professional perfumer. Results are given in Table II.

Table II
Odor Generation in Apocrine Sweat

| | |
|---|---|
| Apocrine sweat | Faint, musky |
| beta-G | No odor |
| Apocrine sweat + beta-G | Strong, musky, and sweaty |
| AS | No odor |
| Apocrine sweat + AS | Moderate, musky, and sweaty |
| Lipophilic diphtheroid | Faint moderate off-odor |
| Apocrine sweat + lipophilic diphtheroid | Strong, musky, and sweaty |
| Mixed bacteria | Moderate malodor |
| Apocrine sweat + mixed bacteria | Strong malodor, sweaty note |

Neither apocrine sweat nor either of the pure enzymes exhibited odor. Cultures of lipophilic diphtheroid, and a mixed axillary population, had the faint-to-moderate malodor characteristic of any bacterial culture, but no particular "sweaty" odor. However, when apocrine sweat was mixed with either enzyme, or with either bacterial culture, a strong, sweaty odor was produced.

To confirm and characterize the implication of lipophilic diphtheroids, the enzyme substrates were exposed to cell-free culture medium. The medium exhibited both aryl sulfatase and beta-glucuronidase activity.

The fact that the odor can be produced by the enzymes alone indicates that it is their activity that is the essential cause of steroidal axillary malodor, confirming the hypothesis from the preliminary tests above. The fact that the odor is produced by lipophilic diphtheroids alone suggests that this strain may be one member of the population that produces the enzymes, and accounts for its implication in odor production by Leyden *et al.* (6) and Jackman *et al.* (5). The presence of enzyme activity in cell-free diphtheroid medium shows that the enzymes are extracellular. Any subsequent intracellular enzymatic conversions of the steroids that may occur seem not to be essential to odor production.

Because the sterile secretion alone generated a faint musky odor after incubation at 37° C, we tested it for indigenous beta-G and AS activity: Addition of 4-MUG, the beta-G substrate, produced the characteristic 4-MU fluorescence, but addition of 4-MUS, the AS substrate, had no effect. The presence of endogenous beta-G in the apocrine gland has been noted separately by Ohkubo and Sano (24).

### INHIBITION OF ODOR FROM APOCRINE SECRETION

Results of the second study are presented in Table III. Again, odor was generated only in those samples containing either apocrine secretion with enzymes or apocrine secretion with bacteria. Zinc glycinate was highly effective in preventing odor; the odor-inhibiting effect of the lactone, although significant, was weaker than that of ZnGLY in both systems; the polyphosphate (SPORIX) had no effect. (It is known that polyphosphates are hydrolyzed in aqueous solution. Possibly SPORIX was degraded to inactive fragments during incubation; it is one of the weakest beta-G inhibitors in Table I.)

**Table III**
*In Vitro* Deodorant Action of Enzyme Inhibitors

| | |
|---|---|
| Enzymes | No odor |
| Apocrine sweat | No odor to faint off-odor |
| Enzymes + apocrine sweat | Faint/moderate musky-sweaty |
| Enzymes + sweat + ZnGLY | No odor |
| Enzymes + sweat + SPORIX | Faint/moderate musky-sweaty |
| Enzymes + sweat + lactone | No odor to faint musky-sweaty |
| Lipophilic diphtheroids | Faint off-odor |
| LDs + apocrine sweat | Strong, sweaty |
| LDs + sweat + ZnGLY | Faint stale musky |
| LDs + sweat + lactone | Moderate sweaty |

## DISCUSSION

It has long been recognized that axillary odor arises from the interaction of bacteria with apocrine sweat. A class of volatile free steroids has been associated with axillary odor and with a particular bacterial population—the lipophilic diphtheroids. We suspected that the enzymes responsible for this process would be those capable of releasing free volatile steroid from odorless steroid conjugates present in sterile apocrine secretion. We showed that a mixed culture of axillary bacteria produces a beta-glucuronidase capable of cleaving a steroid glucuronide. From our preliminary screening tests, we showed that another class of hydrolytic enzyme, aryl sulfatase, was also present in axillary strains.

We have shown that a beta-glucuronidase and an aryl sulfatase, both of bacterial origin, will cleave odorless compounds in sterile secretion to produce distinct axillary odor. Thus, hydrolytic enzymes are implicated in the release of odor.

We have also shown that a lipophilic diphtheroid secretes both of the enzymes necessary to produce steroid malodor from sterile axillary secretion.

We have shown that the generation of odor, from the addition of beta-glucuronidase or aryl sulfatase or lipophilic diphtheroid to apocrine secretion, may be prevented by the inclusion of the enzyme inhibitor $Zn^{++}$ and somewhat reduced by glucarolactone (which inhibits only the beta-glucuronidase but not aryl sulfatase). This further substantiates the role of these bacterial enzymes in axillary odor.

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