

Role of Neutral Metabolites in Microbial Conversion of 3 β -Acetoxy-19-Hydroxycholest-5-Ene into Estrone

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Biotransformation of 3 β -acetoxy-19-hydroxycholest-5-ene (19-HCA, 6 g) by *Moraxella* sp. was studied. Estrone (712 mg) was the major metabolite formed. Minor metabolites identified were 5 α -androst-1-en-19-ol-3,17-dione (33 mg), androst-4-en-19-ol-3,17-dione (58 mg), androst-4-en-9 α ,19-diol-3,17-dione (12 mg), and androstan-19-ol-3,17-dione (1 mg). Acidic metabolites were not formed. Time course experiments on the fermentation of 19-HCA indicated that androst-4-en-19-ol-3,17-dione was the major metabolite formed during the early stages of incubation. However, with continuing fermentation its level dropped, with a concomitant increase in estrone. Fermentation of 19-HCA in the presence of specific inhibitors or performing the fermentation for a shorter period (48 h) did not result in the formation of acidic metabolites. Resting-cell experiments carried out with 19-HCA (200 mg) in the presence of α,α' -bipyridyl led to the isolation of three additional metabolites, viz., cholestan-19-ol-3-one (2 mg), cholest-4-en-19-ol-3-one (10 mg), and cholest-5-en-3 β ,19-diol (12 mg). Similar results were also obtained when *n*-propanol was used instead of α,α' -bipyridyl. Resting cells grown on 19-HCA readily converted both 5 α -androst-1-en-19-ol-3,17-dione and androst-4-en-19-ol-3,17-dione into estrone. Partially purified 1,2-dehydrogenase from steroid-induced *Moraxella* cells transformed androst-4-en-19-ol-3,17-dione into estrone and formaldehyde in the presence of phenazine methosulfate, an artificial electron acceptor. These results suggest that the degradation of the hydrocarbon side chain of 19-HCA does not proceed via C₂₂ phenolic acid intermediates and complete removal of the C₁₇ side chain takes place prior to the aromatization of the A ring in estrone. The mode of degradation of the sterol side chain appears to be through the fission of the C₁₇-C₂₀ bond. On the basis of these observations, a new pathway for the formation of estrone from 19-HCA in *Moraxella* sp. has been proposed.

Cholesterol and phytosterols have long been considered potential starting materials for the microbial production of steroid hormones. Studies pertaining to microbial degradation of the hydrocarbon side chain of various sterols have been comprehensively documented (1, 2, 6, 16, 18, 19, 22-24, 26). It was demonstrated that prior to side-chain cleavage in sterols, the organism initially oxidizes the A ring to a 4-en-3-one structure. At this stage, the organism carries out the oxidation of the terminal methyl group of the side chain, followed by β -oxidation. Thus, the hydrocarbon side chain is degraded via C₂₆, C₂₄, and C₂₂ acids to C₁₉ androstane derivatives. These studies have clearly established that the acidic intermediates play an important role in the microbial conversion of sterols to 17-keto steroids (1, 6, 16, 18, 19, 22-24, 26). Studies have also shown that microbes are able to metabolize the bulkier side chain of β -sitosterol in a manner analogous to cholesterol degradation (18, 19).

Sterol-degrading microorganisms are generally known to possess both side chain- and steroid nucleus-degrading activities (16). The nucleus-degrading activity can be prevented either by using specific inhibitor or by chemically modifying the substrate (16). Modified sterols such as cholest-4-en-19-ol-3-one and 3 β -acetoxy-19-hydroxycholest-5-ene (19-HCA) have been shown to be converted into estrone microbiologically (22-24). These studies have clearly demonstrated that the degradation of the C₁₇ side chain proceeds via C₂₂ phenolic acid intermediates (22-24). The three-carbon side chain of C₂₂ phenolic acid is cleaved to estrone and propionic acid, suggest-

ing that the complete removal of the C₁₇ side chain takes place only after the aromatization of the A ring (22-24).

During our attempts to develop a microbial process to selectively eliminate the C₁₇ side chain of cholesterol or its derivatives, a soil microorganism belonging to the genus *Moraxella* was isolated by an enrichment culture technique using a model compound isooctylcyclopentane as the sole source of carbon (2, 11). The organism also accepted cholesterol and 19-HCA (metabolite I) as the sole source of carbon. Earlier, it was shown that this organism converts 19-HCA (I) into estrone (II) (2). However, attempts were not made to establish the degradative sequence in the conversion of I into II.

The present study was initiated to gain information regarding the aromatization of the A ring with the steroid nucleus intact and cleavage of the C₁₇ side chain. We report here the isolation and identification of neutral metabolites from 19-HCA and propose a new pathway for the microbial conversion of 19-HCA (I) into estrone (II) without the involvement of C₂₂ phenolic acid intermediates. The neutral metabolites III, IV, V, VI, VII, and IX have never been shown as metabolites derived from 19-HCA (I), and among them III appears to be hitherto unknown.

MATERIALS AND METHODS

Materials. 19-HCA (I) was synthesized by following the procedure of Kalvoda et al. (10). Cholesterol, androst-4-en-19-ol-3,17-dione (IV) and α,α' -bipyridyl (α,α' -D) were procured from Sigma Chemical Co. (St. Louis, Mo.). All reference steroids were 100% pure as judged by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

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Methods. Infrared (IR) spectra, UV spectra, nuclear magnetic resonance (NMR) spectra, and mass spectra (MS) were obtained as described previously (17). HPLC analysis was carried out on a Water Associates ALC/GPC 244 series instrument. The analysis was performed on a μ -Porasil normal-phase column with chloroform-methanol (95:5, vol/vol) as the solvent system. Eluents were monitored with a UV detector at 254 nm. TLC analyses were performed on silica gel G plates (0.5 mm thick) developed with ethyl acetate-hexane (1:1, vol/vol, system I; 1:3, vol/vol, system II) as solvent systems. The compounds were visualized by spraying with 50% H_2SO_4 followed by heating at 100°C for 5 to 10 min.

Culture conditions. The organism used in this study was propagated on mineral salts medium (20) containing 0.05% cholesterol and 2% agar (pH 7.2). It was maintained regularly in liquid mineral salts medium containing 0.05% cholesterol as the carbon source. Whenever starter culture was required, an aliquot (5 ml) from the maintenance culture was transferred to 100 ml of a sterilized liquid mineral salts medium (pH 7.2) containing 0.05% cholesterol and incubated on a rotary shaker (220 rpm) at 29 to 30°C for 3 days. These studies were carried out with 3-day-grown culture as the inoculum. Although the organism accepts 19-HCA (I) as the sole source of carbon, the growth rate is slow. Hence, to enhance the growth rate, 0.2% glucose was added to the medium when the fermentation of 19-HCA was carried out.

Metabolism of 19-HCA (I). Flasks containing 100 ml of sterile mineral salts medium (20), 0.2% glucose, and 0.05% 19-HCA (I) were inoculated from a 3-day-old culture (5.0 ml) and incubated at 29 to 30°C on a rotary shaker (220 rpm) for 7 days. At the end of the incubation period, the contents were pooled, acidified to pH 3 to 4, and then extracted with an equal volume of dichloromethane (extracted three times). The pooled extracts were concentrated, washed first with 10% sodium bicarbonate and then with distilled water, and dried over anhydrous sodium sulfate. After evaporation, the methylene chloride extract yielded neutral steroid metabolites. The bicarbonate layer after acidification and extraction with methylene chloride did not contain any acidic metabolites and hence was not processed further. Fermentation of 19-HCA (I) was also carried out for a shorter duration (48 h), and the culture media were processed as already described.

Metabolism of 19-HCA (I) in the presence of inhibitors. The organism was grown in mineral salts medium containing 0.2% glucose and 0.05% 19-HCA for 36 h. After this growth period, α, α' -D (0.6 mM) or *n*-propanol (2%) was added and the incubation continued for an additional 48 h. At the end of the incubation period, the medium was acidified to pH 3 to 4, extracted with dichloromethane, washed with water, and then separated into acidic and neutral fractions. These fractions were subjected to TLC and HPLC analyses. Control experiments were run without the inhibitors.

Metabolism of 19-HCA by resting cells (i) without inhibitor and (ii) with inhibitor (viz., α, α' -D and *n*-propanol). Cells were grown in mineral salts medium containing 0.2% glucose and 0.05% 19-HCA for 48 h. The cells were harvested by centrifugation ($3,000 \times g$ for 20 min) and washed well with phosphate buffer (0.03 M, pH 7.2) aseptically. The washed cells (~1 g) were then suspended in sterile phosphate buffer (50 ml, 0.03 M, pH 7.2). To this cell suspension, (i) 50 mg of 19-HCA was added for the experiment without inhibitors and (ii) for the experiment with inhibitors, the substrate addition was followed by the addition of the respective inhibitors, α, α' -D (0.6 mM) and *n*-propanol (2%). It was then incubated at 30°C for a total of 48 h. Fixed aliquots at definite intervals (12, 24, and 48 h) were withdrawn, extracted with dichloromethane, and sepa-

rated into acidic and neutral fractions as already described. The acid fractions did not contain any steroid metabolites and hence were not processed further. The neutral fractions were subjected to TLC and HPLC analyses.

Large-scale resting-cell experiment. A batch of 20 flasks containing glucose (0.2%) and 19-HCA (0.05%) was inoculated with *Moraxella* cells as already described. At the end of the incubation period (48 h), the cells (4 g) were harvested, washed, and suspended in phosphate buffer, as described earlier. To this cell suspension (200 ml), 19-HCA (I; 200 mg) and α, α' -D (0.6 mM) were added and incubation at 30°C continued for 48 h. From the culture medium, acidic and neutral metabolites were separated as described earlier. The acidic fraction did not contain any steroid metabolites and hence was not processed further.

Metabolism of androst-4-en-19-ol-3,17-dione (IV) by resting cells. Cells grown (48 h) on 19-HCA were washed with phosphate buffer (0.03 M, pH 7.2) aseptically and then suspended (1 g) in the same buffer (25 ml). To this cell suspension, IV (25 mg) was added and incubated for 48 h. Transformation products formed were monitored by taking fixed aliquots (5 ml) at different time intervals for extraction and analysis by HPLC. This experiment was repeated with 5 α -androst-1-en-19-ol-3,17-dione (III).

Preparation of cell extract. Steroid-induced cells grown for 48 h were washed twice with ice-cold phosphate buffer (0.03 M, pH 7.2) and suspended in Tris buffer (0.03 M, pH 7.5) containing 10% glycerol (2 ml/g [wet weight] of cells). The cell suspension was sonicated in a Branson B-30 sonifier with cooling for 10 30-s intervals at maximum output. The sonicate was centrifuged at $105,000 \times g$ for 2 h, and the supernatant (650 mg) was applied to a DEAE-cellulose column (3 by 12 cm) previously equilibrated with 0.03 M Tris-HCl buffer (pH 7.5) containing 10% glycerol (buffer A). After the column was washed with buffer A (500 ml), it was successively eluted with buffer A containing 0.1 M KCl (250 ml) and 0.2 M KCl (250 ml). The 0.2 M KCl eluate contained most of the 1,2-dehydrogenase activity. This fraction, upon concentration by ultrafiltration (P-10 membrane) and dialyzing (10 to 12 h) against buffer A, yielded nearly sevenfold purified enzyme (60 mg).

Enzyme assay. 1,2-Dehydrogenase activity was determined by measuring the rate of 2,6-dichlorophenol-indophenol (DCIP) reduction at 600 nm in a mixture consisting of phosphate buffer (0.03 M, pH 7.5), enzyme (1 mg), phenazine methosulfate (0.4 μ mol), DCIP (0.45 μ mol), and progesterone (1.2 μ mol in 6 μ l of acetone) in a total volume of 3 ml. The reaction was initiated by the addition of substrate (progesterone). The reduction of DCIP was measured using an extinction coefficient of $21 \times 10^3 M^{-1} cm^{-1}$ (25). The enzyme readily converts progesterone to 1,2-dehydroprogesterone. The protein determinations were made by the method of Lowry et al. (12).

Conversion of androst-4-en-19-ol-3,17-dione (IV) to estrone (II) by 1,2-dehydrogenase. Partially purified enzyme (50 mg) was incubated with IV (16.5 μ mol) and phenazine methosulfate (20 μ mol) in phosphate buffer (75 ml, 0.03 M, pH 7.5) at 30°C for 1 h. The assay was terminated by the addition of 2 N HCl, and the suspension was centrifuged. One half of the supernatant was extracted twice with methylene chloride, and the enzymatic product formed was isolated and characterized. To the other half, 2,4-dinitrophenylhydrazine reagent was added and the derivative formed was isolated, purified, and characterized.

Manometric studies. Manometric studies were performed with a Gilson 5/6 Oxygraph at 30°C. The 48-h-grown cells adapted to 19-HCA were harvested, washed, and suspended in

Tris-HCl buffer (0.03 M, pH 7.2) to give a final A_{660} of 1.1. From this, 0.5 ml was used for the oxygen uptake studies. 6-Methylheptan-2-one (25 μ g in 5 μ l of acetone) was used as the substrate. The reaction mixture consisted of 1.5 ml of Tris buffer (0.03 M, pH 7.2) and 0.5 ml of the cell suspension. After the endogenous oxygen consumption was complete, the reaction was initiated with the addition of 6-methylheptan-2-one.

RESULTS

Metabolism of 19-HCA (I). A batch of 120 flasks containing 19-HCA (0.05%) was inoculated with *Moraxella* cells, and at the end of the incubation period, the contents were pooled and processed as described above. The crude neutral fraction (4.8 g) was subjected to column chromatography on silica gel (140 g), and the unmetabolized 19-HCA (I; 3.4 g) was eluted with 5% ethyl acetate in hexane. Elution of the column with 7% ethyl acetate in hexane yielded the major metabolite (II; 712 mg, R_f 0.67, system I) whose IR, NMR, and MS corresponded well with an authentic sample of estrone (II). Subsequently, the column was eluted with methanol to collect all the polar metabolites. This fraction (fraction I; 370 mg) upon TLC analysis (system I) showed the presence of seven metabolites (R_f 0.52, 0.4, 0.38, 0.29, 0.26, 0.18, and 0.1) four of which are present at very low levels. Fraction I (370 mg) was further subjected to column chromatography on silica gel (7.0 g), and the 15% ethyl acetate-in-hexane eluate (fraction II; 5 mg) contained one major (R_f 0.38, system I) and a few minor metabolites. Subsequently, the column was eluted with 20% ethyl acetate in hexane, and the early eluate contained a polar metabolite (33 mg, R_f 0.26, system I) in the pure form (compound A). From the latter fractions, another polar metabolite (58 mg, R_f 0.18, system I) was isolated (compound B). Finally, the column was eluted with 100% ethyl acetate from which the most polar metabolite (12 mg, R_f 0.1, system I) was obtained (compound C). Fraction II was again subjected to column chromatography over silica gel (1 g), and the compound with R_f 0.38 (system I) was eluted with 15% ethyl acetate in benzene (1 mg; compound D). All the metabolites isolated (A, B, C, and D) were subjected to various spectral analyses. The other minor neutral metabolites could not be isolated in the pure form and hence could not be characterized.

Compound A. Recrystallization of this compound (from CH_2Cl_2 -hexane) gave white crystals (33 mg), melting point (mp) 182 to 183°C; $[\alpha]_D^{24} + 15^\circ$ (c 0.6 in methanol [MeOH]); UV (MeOH)/nm 228; IR (Nujol)/ cm^{-1} 3,440 (–OH), 1,730 (–C=O), and 1,680 (α,β -unsaturated ketone); NMR (270 MHz, CDCl_3) δ_{H} : 0.92 (3H, s, 18- CH_3), 3.83 (1H, d, $J = 11.25$ Hz, 19- CH_2), 4.1 (1H, d, $J = 11.25$ Hz, 19- CH_2), 6.13 (1H, d, $J_{\text{cis}} = 10.13$ Hz, 2-H); 7.06 (1H, d, $J_{\text{cis}} = 10.13$ Hz, 1-H), NMR (90 MHz, CDCl_3) δ_{C} : 14.18, 21.34, 21.86, 27.45, 30.58, 32.0, 35.78, 36.04, 41.90, 43.98, 44.50, 48.14, 50.36, 52.05, 61.42, 131.03 (1-C), 153.66 (2-C), 201.16 (3-C), and 221.06 (17-C); MS: m/z 302 (M^+ , 42%), 284 ($\text{M}^+ - \text{H}_2\text{O}$, 9%), 272 (base peak, $\text{M}^+ - \text{CH}_2\text{O}$, 100%) and 108 ($\text{M}^+ - \text{C}_{12}\text{H}_{18}\text{O}_2$, 81%); high resolution mass spectra (HRMS): found M^+ 302.1884 $\text{C}_{19}\text{H}_{26}\text{O}_3$ requires M^+ 302.1882. On the basis of spectral analyses, compound A was identified as androst-1-en-19-ol-3,17-dione (III). The ring junction between A and B was established as *trans* by X-ray analysis (X-ray data will be published elsewhere). Thus the compound was identified as 5 α -androst-1-en-19-ol-3,17-dione (Fig. 1, III). Compound III appears to be hitherto unknown.

Compound B. Recrystallization of this compound (from MeOH) gave white crystals (58 mg) with an mp of 168 to 169°C. This was identified as androst-4-en-19-ol-3,17-dione

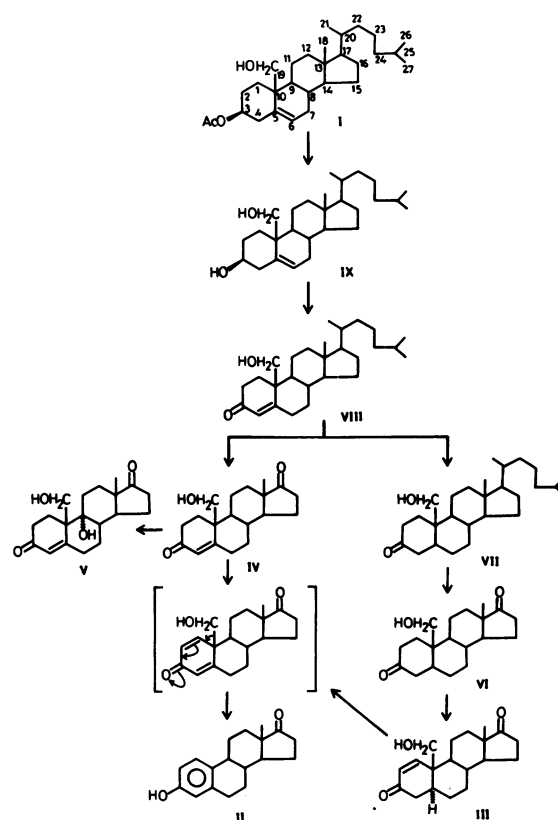


FIG. 1. Proposed pathway for the metabolism of 3 β -acetoxy-19-hydroxycholest-5-ene (I) by *Moraxella* sp.

(Fig. 1, IV) by comparison (IR, NMR, MS, and HPLC) with the authentic sample. The melting point of IV is the same as that reported for androst-4-en-19-ol-3,17-dione (3).

Compound C. Recrystallization of this compound (from MeOH) gave white crystals (12 mg) with an mp of 252 to 253°C. UV (MeOH)/nm 242; IR (Nujol)/ cm^{-1} 3,502, 3,376 (–OH), 1,728 (–C=O), 1,647 (α,β -unsaturated ketone), and 1,614 (C=C); NMR (270 MHz, CDCl_3) δ_{H} : 0.92 (3H, s, 18- CH_3), 3.92 (1H, d, $J = 11.25$ Hz, 19- CH_2), 4.14 (1H, d, $J = 11.25$ Hz, 19- CH_2) and 6.09 (1H, s, 4-H); MS: m/z 318 (M^+ , 10%), 300 ($\text{M}^+ - \text{H}_2\text{O}$, 55%), 288 ($\text{M}^+ - \text{CH}_2\text{O}$, 32%), 282 ($\text{M}^+ - 2\text{H}_2\text{O}$, 15%), 270 (base peak, $\text{M}^+ - [\text{CH}_2\text{O} + \text{H}_2\text{O}]$, 100%); HRMS: found M^+ 318.182 $\text{C}_{19}\text{H}_{26}\text{O}_4$ requires M^+ 318.1831. The ^1H NMR spectrum of this compound is similar to that of androst-4-en-19-ol-3,17-dione (IV). However, both IR spectral and MS analyses indicated the presence of one more hydroxyl than in compound IV. The proton NMR pattern suggests that the second hydroxyl is tertiary in nature and the possible positions are 8 β , 9 α , and 14 α . The absence of deshielding of 18- CH_3 protons rules out the possibility of a hydroxyl group at position 14 α . So, the logical conclusion is that the hydroxyl group may be at either the 8 β or 9 α position. X-ray analysis of compound C conclusively established that the position of the tertiary hydroxyl is at 9 α (5). Hence, metabolite C was identified as androst-4-en-9 α ,19-diol-3,17-dione (Fig. 1, V).

Compound D. Recrystallization of this compound gave a white residue (1 mg), NMR (90 MHz, CDCl_3) δ_{H} : 0.86 (3H, s, 18- CH_3), 3.84 to 4.28 (2H, m, 19- CH_2); MS: m/z 304 (60%) and 286 ($\text{M}^+ - \text{H}_2\text{O}$, 10%) 273 ($\text{M}^+ - \text{CH}_2\text{OH}$, 15%); HRMS: found M^+ 304.2039 $\text{C}_{19}\text{H}_{28}\text{O}_3$ requires M^+ 304.2039.

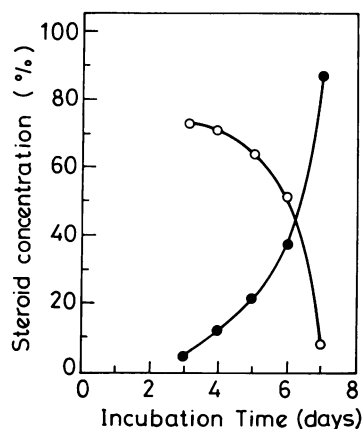


FIG. 2. Time course experiment: metabolism of 3 β -acetoxy-19-hydroxycholest-5-ene (I) by *Moraxella* sp. ○, 19-hydroxyandrost-4-en-3,17-dione; ●, estrone.

The ^1H NMR spectrum of this compound showed the absence of an olefinic proton (no signal between 5 and 7.5 δ). The MS analysis suggested that the molecular weight is two units higher than that of compound IV. On the basis of these observations, compound D was tentatively identified as androstan-19-ol-3,17-dione (Fig. 1, VI). Because of the paucity of the material, further analysis could not be carried out to confirm the structure assigned.

The time course plot (Fig. 2) on the fermentation of 19-HCA (I) carried out for a period of 7 days indicated that during the early stages of incubation (2 days), very little 19-HCA was metabolized (ca. 10%) and among the metabolites formed only androst-4-en-19-ol-3,17-dione (IV) was present in detectable amounts. However, prolonging the incubation period to 3 days resulted in the accumulation of IV (ca. 80% of the total metabolites formed, as judged by HPLC analyses) and low levels of estrone (II). When the fermentation was continued beyond 3 days and up to 7 days, the level of IV dropped gradually (Fig. 2), with a concomitant increase in II. In fact, at the end of 7 days, estrone (II) was the major metabolite formed and it was noticed that nearly 40% of the added 19-HCA (I) was utilized during this period (determined by the amount of unreacted 19-HCA recovered at the end of 7 days). The presence of metabolites III and V could be seen only after 3 days in small quantities (together constituting nearly 10% of the total metabolites formed; data not shown).

Metabolism of 19-HCA (I) in the presence of inhibitors. Fermentation was carried out in the presence of inhibitors as described above. TLC analysis of the neutral fractions obtained when α,α' -D and *n*-propanol were used as inhibitors revealed the presence of not only estrone (II) and androst-4-en-19-ol-3,17-dione (IV) but also three additional metabolites, E (R_f 0.33, system II), F (R_f 0.19, system II), and G (R_f 0.07, system II), which were not noticed when the fermentation was carried out without the inhibitors. Acidic metabolites were not formed even in the presence of inhibitors. The HPLC profile of the neutral fraction indicated that α,α' -D inhibited the formation of androst-4-en-9 α ,19-diol-3,17-dione (V), whereas its presence was seen at low levels when *n*-propanol was used as the inhibitor. It is interesting that higher levels of metabolites E, F, and G were formed when resting cells were incubated with 19-HCA in the presence of α,α' -D. The HPLC analysis showed the accumulation of one compound (later isolated and identified as compound F). Hence, a large-scale resting-cell

experiment was carried out to isolate and characterize metabolites E, F, and G.

A large-scale resting-cell experiment carried out in the presence of α,α' -D as described above yielded 145 mg of crude neutral fraction, which was subjected to chromatography on a column of silica gel (4 g). The unmetabolized 19-HCA (109 mg) and estrone (II) were eluted from the column with 5 and 15% ethyl acetate in hexane, respectively. Further elution of the column with 20, 25, and 30% ethyl acetate in hexane yielded compounds E (2 mg, R_f 0.33, system II), F (10 mg, R_f 0.19, system II), and G (12 mg, R_f 0.07, system II), respectively, in the pure form. Compounds E, F, and G were subjected to various spectral analyses.

Compound E. Recrystallization of this compound (from MeOH) gave a white residue (2 mg), IR (CHCl_3)/ cm^{-1} 1,707 ($-\text{C}=\text{O}$); NMR (90 MHz, CDCl_3) δ_{H} : 0.62 (3H, s, 18- CH_3), 0.74 to 0.96 (9H, m, 21, 26, and 27- CH_3), 3.8 to 4.2 (2H, m, 19- CH_2), and the ring methylene and methine protons appear as a multiplet in the region 1-2.5 δ ; MS: m/z 402 (M^+ , 20%) and 371 ($\text{M}^+-\text{CH}_2\text{OH}$, 10%); HRMS: found M^+ 402.3517 $\text{C}_{27}\text{H}_{46}\text{O}_2$ requires M^+ 402.3498. On the basis of these spectral analyses, compound E was identified as cholestan-19-ol-3-one (Fig. 1, VII).

Compound F. Recrystallization of this compound (from MeOH) gave a white residue (10 mg), UV (MeOH)/nm 256; IR (CHCl_3)/ cm^{-1} 3,388 ($-\text{OH}$), 1,662 (α,β -unsaturated ketone); NMR (90 MHz, CDCl_3) δ_{H} : 0.69 (3H, s, 18- CH_3), 0.76 to 1.0 (9H, m, 21, 26, and 27- CH_3), 3.97 (2H, ABq, $J_{\text{AB}} = 11.25$ Hz, $\Delta\nu_{\text{AB}} = 19.2$ Hz, 19- CH_2), 5.94 (1H, s, 4-H), and the ring methylene and methine protons appear as a multiplet in the region 1-2.5 δ ; MS: m/z 400 (M^+ , 20%) and 370 ($\text{M}^+-\text{CH}_2\text{O}$, 70%); HRMS: found M^+ 400.3417 $\text{C}_{27}\text{H}_{44}\text{O}_2$ requires M^+ 400.3341. On the basis of these spectral data, compound F was assigned the structure cholest-4-en-19-ol-3-one (Fig. 1, VIII).

Compound G. Recrystallization of this compound (from MeOH) gave a white residue (12 mg), IR (CHCl_3)/ cm^{-1} 3,580, 3,406 ($-\text{OH}$); NMR (90 MHz, CDCl_3) δ_{H} : 0.73 (3H, s, 18- CH_3), 0.76 to 1.0 (9H, m, 21, 26, and 27- CH_3), 3.4 to 3.9 (3H, m, 3-H and 19- CH_2), 5.73 (1H, broad singlet, 6-H), and the ring methylene and methine protons appear as a multiplet in the region 1-2.5 δ ; MS: m/z 402 (M^+ , 3%), 384 ($\text{M}^+-\text{H}_2\text{O}$, 30%), 372 ($\text{M}^+-\text{CH}_2\text{O}$, 20%), and 354 (base peak, ($\text{M}^+-[\text{H}_2\text{O}+\text{CH}_2\text{O}]$, 100%); HRMS: found M^+ 402.3366 $\text{C}_{27}\text{H}_{46}\text{O}_2$ requires M^+ 402.3498. On the basis of these spectral data, compound G was identified as cholest-5-en-3 β ,19-diol (Fig. 1, IX). The spectral data corresponded well with those of cholest-5-en-3 β -19-diol prepared chemically by alkaline hydrolysis of 19-HCA (I).

Time course of transformation of 19-HCA (I) by resting cells in the presence and absence of inhibitors. The HPLC profiles of the neutral metabolites formed at the end of 12, 24, and 48 h, when 19-HCA (I) was incubated with resting cells in the presence and absence of inhibitors, are shown in Fig. 3. Incubation carried out in the absence of inhibitors clearly indicated (Fig. 3a) that during the early stages (12 h), cholest-4-en-19-ol-3-one (VIII) was the major metabolite formed, whereas the levels of estrone (II) and androst-4-en-19-ol-3,17-dione (IV) were comparatively lower. However, by prolonging the incubation period to 24 and 48 h, the levels of VIII dropped significantly with a concomitant increase in II and IV. During this period, a considerable amount of androst-4-en-9 α ,19-diol-3,17-dione (V) was formed (Fig. 3a). When the experiment was carried out in the presence of α,α' -D, the level of VIII increased steadily with time, and at the end of 48 h, it was present at significantly high levels. During this period, androst-4-en-9 α ,19-diol-3,17-dione (V) was not formed at all

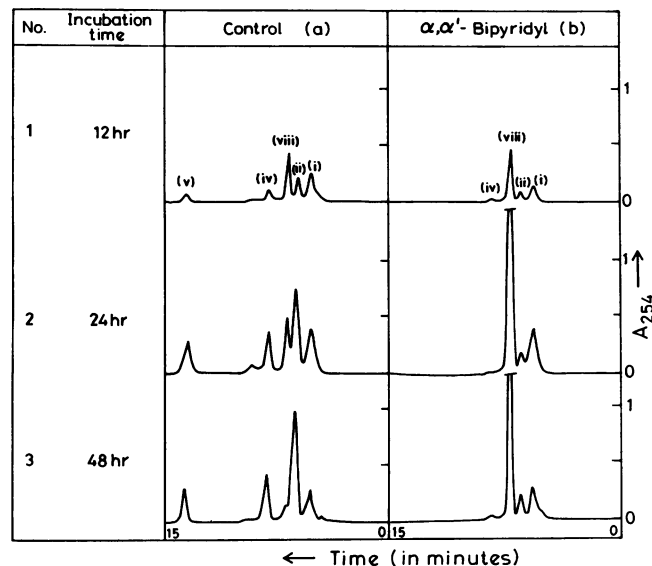


FIG. 3. Transformation of 3β -acetoxy-19-hydroxycholest-5-ene (I) by resting cells in control (a) and α,α' -D (b) reactions. (i), endogenous material from the cell ($R_t = 6.5$); (ii), estrone ($R_t = 7.0$); (viii), 19-hydroxycholest-4-en-3-one ($R_t = 7.75$); (iv) 19-hydroxyandrost-4-en-3,17-dione ($R_t = 9.0$); (v) $9\alpha,19$ -dihydroxyandrost-4-en-3,17-dione ($R_t = 14.5$).

and II and IV were present at extremely low levels (Fig. 3b). Similar results were also noticed when *n*-propanol was used instead of α,α' -D, although cholest-4-en-19-ol-3-one (VIII) was present at comparatively lower levels. Besides, *n*-propanol did not completely inhibit the formation of V, and levels of II and IV were higher than those in the experiment with α,α' -D. Hence, a large-scale experiment was carried out with α,α' -D to isolate VIII.

Transformation of androst-4-en-19-ol-3,17-dione (IV) with resting cells. Transformation products formed at different time intervals (12, 24, and 48 h) were analyzed by HPLC (Fig. 4a). It was noticed that the level of androst-4-en-19-ol-3,17-dione (IV) dropped gradually, with a concomitant increase in the level of estrone, and at the end of 24 h, nearly 80% of IV was converted into estrone (II). During this period, androst-4-en- $9\alpha,19$ -diol-3,19-dione (V) was formed at low levels (10 to 15% of the total product formed). A similar experiment carried out using androst-1-en-19-ol-3,17-dione (III) as the substrate also indicated its conversion to estrone (II) (Fig. 4b). However, the rate of conversion of III to II was considerably slower than the rate of conversion of IV to II. In fact, after 24 h, nearly 40% of III was converted into estrone (II).

Metabolism of androst-4-en-19-ol-3,17-dione (IV) in vitro. Partially purified 1,2-dehydrogenase was incubated with androst-4-en-19-ol-3,17-dione (IV) in the presence of phenazine methosulfate, as described above. One half of the assay mixture when extracted with methylene chloride yielded an enzymatic product (R_f 0.67, system I), purified by preparative thin-layer chromatography (system I) and identified as estrone (II) on the basis of NMR, MS, and HPLC analyses. To the other half of the assay mixture, 2,4-dinitrophenylhydrazine reagent was added and the hydrazone derivative formed was purified and identified as that of formaldehyde by comparison of the mass spectrum with that of the chemically prepared sample.

Manometric studies. Manometric studies using 6-methyl-

heptan-2-one were carried out as described above. The amount of O_2 consumed at $30^\circ C$ is 19.8 nmol/min/mg (dry wt).

DISCUSSION

The data reported herein suggest that *Moraxella* sp. transforms 19-HCA (I) into estrone (II) by following a pathway (Fig. 1) different from that established earlier (22–24). The degradation of the hydrocarbon side chain of 19-HCA (I) does not appear to proceed via C_{22} acid intermediates. cursory examination of all the metabolites formed from 19-HCA (I) and time course experiments provide information regarding the sequence of reactions taking place during the formation of estrone (II) and suggest that aromatization of the A ring in II takes place only after the cleavage of the C_{17} side chain. The time course study on the fermentation of 19-HCA (I) (Fig. 2) and resting-cell experiments carried out with androst-4-en-19-ol-3,17-dione (IV) (Fig. 4a) clearly indicate the precursor-product relationship between metabolites IV and II. 5α -Androst-1-en-19-ol-3,17-dione (III) and androst-4-en- $9\alpha,19$ -diol-3,17-dione (V) were formed only in small quantities, and at no time did they constitute more than 10% of the total metabolites formed. The cell extract prepared from steroid-induced cells contained higher levels of steroid 1,2-dehydrogenase than 4,5-dehydrogenase. The specific activities of the 1,2-dehydrogenase and 4,5-dehydrogenase were 3.143 and 0.667 nmol/min/mg, respectively. So, it appears that the major

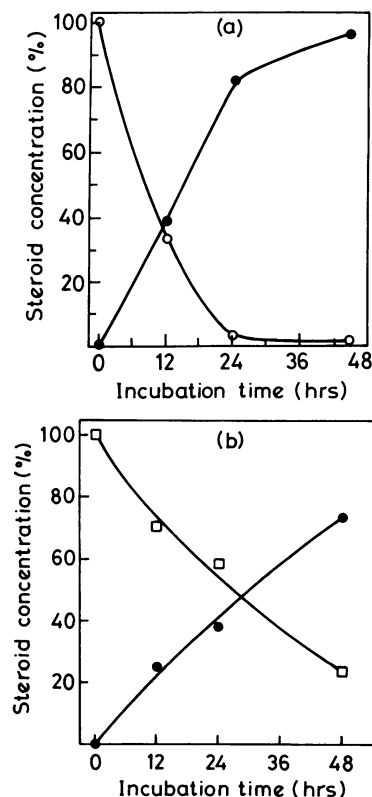


FIG. 4. Time course experiments. (a) Transformation of 19-hydroxyandrost-4-en-3,17-dione (IV) by resting cells. \circ , 19-hydroxyandrost-4-en-3,17-dione (IV); \bullet , estrone (II). (b) Transformation of 19-hydroxyandrost-1-en-3,17-dione (III) by resting cells. \square , 19-hydroxyandrost-1-en-3,17-dione (III); \bullet , estrone (II).

metabolite estrone (II) is formed mostly from androst-4-en-19-ol-3,17-dione (IV) through the action of 1,2-dehydrogenase. The introduction of a 1,2-double bond in a steroid molecule has been reported for several microbial species (4, 8, 9, 13–15) but not previously for *Moraxella* sp. We have clearly demonstrated by using partially purified enzyme that introduction of a 1,2-double bond in androst-4-en-19-ol-3,17-dione (IV) facilitates a spontaneous nonenzymatic retroaldol-type reaction to yield estrone (II) and formaldehyde. One would also expect a similar rearrangement to take place by introducing a 4,5-double bond in metabolite III. In accordance with that, we have shown that the resting cells transform III into estrone (II).

Our studies carried out with *Moraxella* sp. appear to differ from those reported earlier (24), where it was demonstrated that the microbial degradation of cholest-4-en-19-ol-3-one (VIII) into estrone (II) proceeds via C₂₂ phenolic acid intermediates. In that case, the three-carbon side chain of the C₂₂ phenolic acid is cleaved to estrone (II) and propionic acid, suggesting that the complete removal of the C₁₇ side chain takes place only after the aromatization of the A ring (24). Similar observations were also made by Arima et al. (1), noting that a species of *Nocardia* produces pregna-1,4-dien-3-one-20-carboxylic acid during the fermentation of cholesterol in the presence of α,α' -D. Further, it was established that the degradation of the side chain involves carbon-carbon bond fission at C₂₄-C₂₅, C₂₂-C₂₃, and C₁₇-C₂₀, resulting in the formation of 17-keto steroid (18). The mode of degradation of the hydrocarbon side chain of cholesterol in the microbial system differs from that of the mammalian system, which involves the cleavage of the C₂₀-C₂₂ and C₁₇-C₂₀ bonds (21). Our results appear to be different from either of these two modes of fission. We were not able to demonstrate the formation of acidic metabolites from 19-HCA (I) when the transformation was carried out in the presence of specific inhibitors or for a shorter duration, suggesting that acidic intermediates are not involved in the degradative sequence. So, it is quite possible that *Moraxella* sp. carries out the direct cleavage of the side chain of cholest-4-en-19-ol-3-one (VIII) between C₁₇ and C₂₀ to yield androst-4-en-19-ol-3,17-dione (IV) and 6-methylheptan-2-one. Our efforts to isolate 6-methylheptan-2-one from the incubation mixture consisting of 19-HCA (I) and resting cells were not successful. Interestingly, we have noticed that cells adapted to 19-HCA readily metabolize 6-methylheptan-2-one and the manometric studies with 6-methylheptan-2-one confirmed this observation. Earlier, Horvath and Kramli reported the isolation of 6-methylheptan-2-one after incubating cholesterol with *Azotobacter* sp., suggesting the fission of the side chain is between C₁₇ and C₂₀ (7).

The hitherto unknown 5 α -androst-1-en-19-ol-3,17-dione (III) could have been formed from cholestan-19-ol-3-one (VII). Earlier, workers from our laboratory showed that side-chain cleavage takes place independently even when the A ring is blocked (2). Hence, side-chain cleavage can take place even in the absence of a 3-keto-4-en system. It is reasonable to assume that the fission of the side chain in VII takes place between C₁₇ and C₂₀, in a manner analogous to the side-chain cleavage of cholest-4-en-19-ol-3-one (VIII), yielding androstan-19-ol-3,17-dione (VI). 1,2-Dehydrogenation of metabolite VI results in the formation of neutral metabolite III (Fig. 1). In fact, earlier it was demonstrated that 5 α -androst-3,17-dione was 1,2-dehydrogenated by *Corynebacterium simplex*, indicating that the dehydrogenase does not have any rigid requirement for a 4-en-3-one structure (27).

Many investigators have used inhibitors to enable detection of intermediary metabolites (18, 19). The addition of chelating

agents such as α,α' -D to cholesterol-decomposing microorganisms not only inhibits the 9 α -hydroxylation reaction, but also induces the accumulation of acidic intermediates. In this study, it was observed that incubation carried out in the presence of α,α' -D or *n*-propanol did not result in the formation of acidic metabolites. However, α,α' -D completely inhibited 9 α -hydroxylation when resting cells were incubated with 19-HCA (I) (Fig. 3). Besides, metabolite (VIII) was formed in appreciable amounts (Fig. 3). Similarly, the accumulation of cholest-4-en-3-one in the medium was observed during the microbial degradation of cholesterol in the presence of α,α' -D or *n*-propanol (18).

It appears that the pathway (Fig. 1) involved in the biotransformation of 19-HCA (I) into estrone (II) is initiated by the hydrolysis of the 3 β -acetate moiety followed by the oxidation of the 3 β -hydroxyl group, with concomitant isomerization of the 5,6-double bond to a 4-en-3-one structure prior to side-chain cleavage. Resting-cell experiments carried out in the presence of specific inhibitors clearly demonstrated the formation of cholest-4-en-19-ol-3-one (VIII). Once metabolite VIII is formed, the organism cleaves the side chain between C₁₇ and C₂₀ to yield metabolite IV and methylheptanone. The conversion of IV into II is initiated by the enzyme 1,2-dehydrogenase. Thus, we have established that the degradation of the hydrocarbon side chain of 19-HCA (I) does not appear to proceed via C₂₂ acid intermediates, but instead neutral metabolites play a key role in the conversion of 19-HCA (I) into estrone (II). Besides, complete removal of the C₁₇ side chain takes place prior to the aromatization of the A ring in estrone. The study also for the first time demonstrated the transformation of the hitherto unknown compound, 5 α -androst-1-en-19-ol-3,17-dione (III) into estrone.

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